**Title:** Genetic variation, intrauterine growth, and adverse pregnancy conditions predict leptin gene DNA methylation in blood at birth and 12 months of age

**Working Title:** Genetic variation, growth, and leptin methylation

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# Abstract

**Background:** Leptin regulates satiety and energy homeostasis, and plays a key role in placentation in pregnancy. Previous studies have demonstrated regulation of leptin gene (*LEP*) expression and/or methylation in placenta and cord blood in association with early life exposures, but most have been small and have not considered the influence of genetic variation. Here, we investigated the relationship between maternal factors in pregnancy, infant anthropometry and *LEP* genetic variation with *LEP* promoter methylation at birth and 12 months of age.

**Methods:** *LEP* methylation was measured in cord (n=877) and 12-month (n=734) blood in the Barwon Infant Study, a population-based pre-birth cohort. Infant adiposity at birth and 12-months was measured as triceps and subscapular skinfold thickness. Cross-sectional regression tested associations of methylation with pregnancy and anthropometry measures, while longitudinal regression tested if birth anthropometry predicted 12-month *LEP* methylation levels.

**Results:** Male infants had lower *LEP* methylation in cord blood (-2.07% average methylation, 95% CI (-2.92, -1.22), p<0.001). Genetic variation strongly influenced DNA methylation at a single CpG site, which was also negatively associated with birth weight (r=-0.10, p=0.003). Pre-eclampsia was associated with lower cord blood methylation at another CpG site (-6.06%, 95% CI (-10.70, -1.42), p=0.01). Gestational diabetes was more modestly associated with methylation at two other CpG units. Adiposity at birth was associated with 12-month *LEP* methylation, modified by rs41457646 genotype. There was no association of *LEP* methylation with 12-month anthropometric measures.

**Conclusions:** Infant sex, weight, genetic variation, and exposure to pre-eclampsia and gestational diabetes, are associated with *LEP* methylation in cord blood. Infant adiposity at birth predicts 12-month blood *LEP* methylation in a genotype-dependent manner. These findings are consistent with genetics and anthropometry driving altered *LEP* epigenetic profile and expression in infancy. Further work is required to confirm this and to determine the long-term impact of altered *LEP* methylation on health.

# Introduction

*In utero* exposures such as maternal obesity and gestational diabetes mellitus (GDM) are linked to increased offspring cardiometabolic risks including childhood obesity (1) and increased blood pressure and adverse cardiometabolic markers (2). Pre-eclampsia is associated with increased blood pressure and BMI (3). These risk are mediated, at least in part, by specific epigenetic variation including DNA methylation (reviewed in 4).

Leptin is a hormone strongly related to cardiometabolic health and energy homeostasis (5). Leptin levels increase during pregnancy (6), potentially regulating maternal-fetal nutrient exchange and placental function (7). Leptin resistance, which has been linked to obesity (reviewed in 8), becomes apparent in pregnancy (6), presumably to enable increased maternal nutrient intake. Animal models also suggest leptin plays a role in early organ maturation (reviewed in 9).

The leptin gene (*LEP*) is expressed predominantly in adipose tissue, but also across other several tissues including placenta (10, 11). Expression *LEP* is modulated by promoter methylation status (12-14) and higher maternal pre-pregnancy body mass index (BMI) and hyperglycaemia have been linked to decreased cord blood *LEP* methylation levels (15-17) and increased leptin levels (17). Maternal obesity and GDM are associated with increased *LEP* methylation in the placenta (18), though the direction of effect of impaired maternal glucose tolerance on placental *LEP* methylation differs on the maternal and fetal sides of the placenta (19). Pre-eclampsia is associated with decreased *LEP* methylation (20, 21) and increased *LEP* expression in the placenta (22), as well as increased cord plasma leptin levels in infants (23). Though differential *LEP* methylation in association with pre-eclampsia has yet to be determined in cord blood, corticotropin-releasing hormone, which is elevated in pre-eclampsia (24), has been linked to increased cord blood *LEP* methylation (25). Infants born small for gestational age (GA) show increased cord blood *LEP* methylation (15), and

macrosomia is associated with decreased *LEP* methylation in cord blood (26) but not placenta tissue (27). Exposure to famine around the periconceptional period or late gestation is associated with increased *LEP* methylation in the blood of offspring in adulthood (28), while low birth weight has been linked to increased adipose *LEP* methylation in adulthood (29). Genetic variation in *LEP* may influence promoter methylation levels in cord blood (15), as well as leptin levels and cardiometabolic health measures later in life (30-34), but data are limited and conflicting. In addition, at present there is limited information about the longitudinal relationship between early life *LEP* methylation and infant growth measures.

In this study, we investigated the relationship between maternal factors, GDM, pre-eclampsia, fetal growth, and offspring *LEP* genetic variation with *LEP* methylation in cord blood. In addition, we investigated the longitudinal relationship between *LEP* methylation and infant weight and adiposity over the first postnatal year, and how this relationship may be influenced by other maternal or infant factors.

## Materials and Methods

*Study participants*

The Barwon Infant Study (BIS) is a population-based pre-birth cohort based in south-eastern Australia (35). Women were recruited prior to 32 completed weeks of pregnancy from two hospitals in the Barwon region (Victoria) between 2010 and 2013, and provided written informed consent. Offspring inclusion criteria were a GA of at least 32 weeks, and no diagnosis of genetic disease, congenital malformation, or serious illness. The Barwon Health Human Research Ethics Committee approved the study protocol. Of 1 064 eligible mothers and 1 074 infants in the study, 877 with both cord blood samples and genetic data were included in analysis at birth, and 734 with data at birth and 12 months were included in the longitudinal analysis.

*Maternal and infant measures*

Maternal health measures were (i) maternal age at conception, (ii) pre-pregnancy BMI, (iii) early pregnancy weight gain, (iv) GDM status during pregnancy, and (v) diagnosis of preeclampsia. Pre-pregnancy BMI was calculated from self-reported weight and directly measured height. Pregnancy weight gain was estimated from pre-pregnancy weight compared to weight at 28-weeks pregnancy. GDM was defined using International Association of Diabetes and Pregnancy Study Groups (IADPSG) criteria (36). Diagnosis of pre-eclampsia was made from medical records according to the International Society for the Study of Hypertension in Pregnancy (ISSHP) criteria (37). Infant health measures included sex, weight and GA recorded at birth, which was used to calculate weight z-score (weight adjusted for sex and either gestational age at birth or postnatal age at 12-month follow-up), and mean triceps/subscapular skinfold thickness (a measure of adiposity in infancy (38)) measured using Holtain callipers (39) within two days of birth and at the 12-month follow-up. Weight z-scores were based on revised British UK-WHO growth charts (40) and WHO child growth standards (41) for birth and 12-month weight, respectively. The change in infant weight z-score between birth and 12 months was considered as a 12-month measure.

LEP *methylation measurement*

Genomic DNA was extracted from cord blood and infant 12-month whole blood using the QIAamp DNA QIAcube HT Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol, and underwent bisulphite conversion using the MagPrep Lightning Conversion Kit (Zymo Research, Irvine, California, United States). DNA methylation was quantified in all available blood samples to maximise study power. A region of the *LEP* promoter (145bp, hg19:chr7:127 881 231-127 881 375) examined in other studies (15, 19, 20, 27, 28) was targeted. For amplification, the forward primer

was 5'-CGGGGCGGGAGTTGGCGTTAGA-3' with a balance tag, and the reverse primer was 5'-CGGCGCGTTTTTTTTTTTTTTTTTGTT-3' with a T7 promoter tag. Based on *in silico* prediction, the region covered 10 measurable CpG units containing a total of 14 CpG sites (**Supp. Figure 1**). A single product of the expected sequence was amplified from bisulphite converted human genomic DNA (not shown). Methylation was quantified using the locus-specific SEQUENOM MassARRAY EpiTYPER platform (Agena Bioscience, San Diego, California, United States) as described previously (42, 43). Samples were quantified in triplicate, and replicates that differed by more than 10% methylation excluded. The intra-individual mean of the remaining replicates was used in analysis. CpG units missing data for more than 20% of participants at either timepoint were excluded.

To consider the potential influence of cellular heterogeneity of the blood samples, flow cytometry (FACsCalibur) was used to assess the proportions of lymphocytes, monocytes and granulocytes (44), presented as a percentage of total white blood cells. These measures were used in sensitivity analysis.

LEP *genetic variation*

Genotyping was performed on the Infinium Global Screening Array-24 v1.0 BeadChip (Illumina, San Diego, California, United States) by the Erasmus MC University Medical Center. The Sanger Imputation Service (Wellcome Sanger Institute, Hinxton, United Kingdom), with the Haplotype Reference Consortium reference population (45), was used to impute genotypes for a total of 7 582 152 single nucleotide polymorphisms (SNPs) after quality control. Participants were excluded if their initial genotyping failed at more than 5% of SNPs (n=4) or had a heterozygosity rate of greater than 4 standard deviations (SDs) from the cohort mean (n=15). SNPs were excluded if they failed in more than 5% of participants (3% of SNPs), were rare (minor allele frequency (MAF) < 0.01; 24% of SNPs pre-imputation and 80% of SNPs post-imputation), not in Hardy-Weinberg Equilibrium ($p<1x10^{-6}$; 2.3% of SNPs pre-imputation and 1% of SNPs post-imputation), or had a MAF more than 0.2 different

to the reference population (0.01% of SNPs post-imputation) (46). Imputed SNPs were also dropped if they had an imputation information score less than 0.8. SNPs in or within 20kb of the *LEP* gene (56,352bp in total, hg19:chr7:127 861 331-127 917 682) were extracted for this study. The HaploView software was used to select tag SNPs to act as proxies for clusters of SNPs with $R^2$>0.1, after mandating the inclusion of two of three SNPs previously associated with BMI, leptin expression or *LEP* methylation: rs7799039 (31, 47) and rs2167270 (15, 30, 48). While rs10249476 is also associated with the specified outcomes (34), it was excluded in this analysis due to complete linkage disequilibrium with rs2167270. This resulted in 16 tag SNPs (see **Supp. Table 5**) for inclusion in analysis.

*Statistical analysis*

Initial analysis was performed to test the strength of association between methylation at each *LEP* CpG unit and maternal, infant or genetic measures (using Student t-test, Pearson's correlation test, or one-way ANOVA, as appropriate). All analysis was adjusted for EpiTYPER batch. For comparisons of methylation between groups (Student t-test or one-way ANOVA), equal variance was a reasonable assumption (Bartlett's test for equal variances). To determine the independence of signals identified in the initial analysis, multivariable linear regression models predicting methylation were used; first to determine which SNPs were independently associated with methylation, and then with additional inclusion of maternal/infant measures with evidence of association stronger than $p<0.05$ in the initial analysis. All linear regression models with 12-month measures were adjusted for infant age in months at the time of the 12-month review. Potential sex- or genotype-specific relationships between maternal/infant measures and *LEP* methylation were investigated by adding sex*measure and SNP*measure product terms sequentially to the regression models. Continuous measures were centred for interaction effect analysis by subtracting the sample mean. For sensitivity analysis, blood cellular composition, delivery mode, and bisulphite conversion plate were included as variables in

models, with breastfeeding (months exclusively breastfed and months with any breastfeeding) included to determine if it modified associations with *LEP* methylation, as has previously been reported (49). Sensitivity analysis for longitudinal models (birth methylation predicting 12-month anthropometry or birth anthropometry predicting 12-month methylation) included adjusting the models for the corresponding 12-month measure of the predictor. Interaction terms and variables considered in sensitivity analysis were only included in the final model if they substantially improved the fit of the model (likelihood ratio test $p<0.05$) and modified the effect sizes of the main exposures of interest by more than 15%. All analysis was performed in Stata 15 IC (StataCorp, College Station, Texas, United States).

*Data and code availability*

The data and code used in this analysis are available upon reasonable request.

# Results

*Cohort characteristics and* LEP *methylation*

The details of maternal and infant characteristics are shown in **Table 1**. Following quality control, of 10 analytical CpG units (**Supp. Table 1**), CpG1.2 and CpG12 were excluded due to excessive missing data. Methylation levels were approximately normally distributed for all CpG units at both timepoints and generally increased from birth to 12 months of age (**Figure 1**). The largest increase was at CpG11, which showed a mean methylation of 44.7% (SD 12.2%) at 12-months, compared to 22.9% (SD 7.3%) at birth. The methylation levels at each CpG unit between the two timepoints were correlated for some but not all units ($r = 0.02$ to $0.27$, **Supp. Table 2**). At each timepoint, methylation between pairs of CpG units was correlated ($r = 0.11$ to $0.57$ at birth, **Supp. Table 3**; $r = 0.14$ to $0.63$ at 12-months, **Supp. Table 4**).

*Early development and genetic determinants of* LEP *cord blood DNA methylation*

At birth, male sex was associated with lower average *LEP* methylation across the region and at most individual CpG units (**Table 2**). Exposure to pre-eclampsia was associated with lower CpG14 methylation (6.06% decrease, 95% CI -10.70% to -1.42%, p=0.01), while the evidence for an association of LEP methylation with GDM was more modest with higher CpG4.5.6 (cg00840332) (1.59% increase, 95% CI 0.20% to 2.99%, p=0.03) and lower CpG3 methylation (1.53% decrease, 95% CI -3.12% to 0.07%, p=0.06). Of the infant anthropometry measures, birth weight z-score was negatively associated with CpG7 (cg19594666) methylation (r -0.10, p=0.003). There was modest evidence for negative relationship between GA and methylation at CpG3 (r -0.08, p=0.02) and CpG4.5.6 (r -0.07, p=0.05). No signals were observed for maternal age, pre-pregnancy BMI, pregnancy weight gain or infant mean of skinfold thickness with any *LEP* methylation measures at birth.

Genotypes at three SNPs (rs2167270, rs7799039, rs2071045) were associated with DNA methylation at most CpG units tested, with the greatest magnitude of effect seen at CpG7. The minor allele of rs2167270 was associated with lower CpG7 methylation, while rs7799039 and rs2071045 minor alleles were associated with higher CpG7 methylation. Minor alleles at several other SNPs (rs112440054, rs76129516, rs4731424, rs28954111, rs41457646) were only associated with CpG7 methylation (**Supp. Table 5**). Effect sizes ranged from 4 to 10% change in CpG7 methylation per additional minor allele, with rs2167270 having the largest effect size (-13.20% for one minor allele, and an additional decrease (-7.08%) for homozygote minor allele).

The negative relationship between birth weight z-score and CpG7 methylation persisted after adjustment for rs2167270 minor allele count, though the magnitude was diminished (**Table 3**). In this combined model, genotype predicted a much larger proportion of CpG7 methylation variation than sex or birth weight z-score ($R^2$ of 40.11%, 0.60% and 0.45%, respectively).

The relationship between pre-eclampsia and CpG14 methylation was substantially altered by inclusion of pre-eclampsia*rs7799039 genotype interaction terms in the model. While rs7799039 genotype was not associated with CpG14 methylation, pre-eclampsia predicted a 16.64% decrease in CpG14 methylation in infants with G/G genotype (4 of 129 infants exposed to pre-eclampsia, 95% CI -25.49 to -7.78, p<0.001), but only a 2.45% decrease in infants with G/A genotype (6 of 346 infants exposed) and 1.34% in infants with A/A genotype (4 of 140 infants exposed). Pre-eclampsia and the pre-eclampsia*rs7799039 genotype interaction effect explained 0.98% and 1.14% of CpG14 methylation, respectively. The association of GDM and GA with methylation did not vary by infant sex or genotype. The final models for birth CpG3 and CpG4.5.6 methylation each included GDM status, infant sex and rs2167270 genotype (**Supp. Table 6**).

*12-month* LEP *blood methylation analysis*

At 12 months of age, the association remained between male infants and lower average *LEP* methylation, although the effect was of smaller magnitude (average difference of -0.86%, 95% CI -1.57% to -0.13%, p=0.02). None of the weight or adiposity measures at 12 months were cross-sectionally related to average *LEP* methylation nor individual CpG unit methylation (**Supp. Table 7**). As at birth, genetic variants were associated with average and unit-specific *LEP* methylation levels at 12-months, albeit slightly attenuated. In contrast to birth, there was no evidence of an association between exposure to pre-eclampsia and CpG14 methylation at 12 months. Similarly, there was no evidence for exposure to GDM influencing CpG3 or CpG4.5.6 methylation at 12 months.

*Longitudinal associations between infant anthropometry and* LEP *methylation*

We found evidence that mean triceps/subscapular skinfold thickness at birth predicted decreased average methylation at 12 months (r -0.09, p=0.02) (**Table 4a**), even after accounting for

triceps/subscapular skinfold thickness at 12 months. There was evidence for an interaction between birth triceps/subscapular skinfold thickness and rs41457646 genotype, with skinfold thickness predicting decreased average methylation at 12 months specifically in infants with G/G genotype, with increased methylation in those with a G/A genotype (**Table 4b**). Inclusion of interaction terms improved the fit of the model (p=0.009) and explained an additional 1.31% of variation for average 12-month methylation. In combination, sex, genotype and skinfold thickness measures explained a total of 3.91% of the variance in average 12-month methylation levels. There was no evidence that average or unit-specific *LEP* methylation predicted 12-month weight or adiposity measures (**Supp. Table 8**).

*Additional sensitivity analyses*

Adjusting for cellular composition of cord blood samples did not alter any of the reported relationships. Cellular composition of 12-month blood samples was associated with average methylation across the region at 12 months, with proportion of lymphocytes positively associated with methylation (r 0.24, p<0.001) and granulocytes inversely associated with methylation (r -0.21, p<0.001). Inclusion of proportion of lymphocytes and labour exposure variables to the longitudinal model of birth mean triceps/subscapular skinfold thickness and rs41457646 genotype predicting average 12-month methylation explained an additional 1.79% of 12-month methylation variance, but did not alter the effect sizes of skinfold thickness, rs41457646 genotype or the gene-skinfold thickness interaction. Adjusting for bisulphite conversion batch or breastfeeding duration in models containing 12-month measures did not alter any of the above findings.

# Discussion

In this large pre-birth cohort, we report evidence for relationships between genetic variation, adverse pregnancy conditions, and fetal growth with *LEP* methylation in cord blood. We also found evidence that birth adiposity measures predict *LEP* methylation in peripheral blood at 12 months of age in a genotype-dependent manner (**Figure 2**).

The negative relationship between fetal growth and *LEP* methylation is consistent with increased *in utero* nutrition negatively influencing *LEP* promoter methylation. Based on previous studies, this decreased methylation would be anticipated to increase *LEP* expression (12, 13). Genotype of the *LEP* gene may play a role in influencing the established link between increased *in utero* nutrition and increased circulating leptin levels (17, 50). The direction of effect for infant sex and adiposity was consistent across multiple CpG units, while specific genetic variants, health during pregnancy, and fetal growth showed evidence of associations with methylation variation only at specific CpG units. Our findings highlight the importance of considering both genetics and environment as potential drivers of early life methylation levels.

## LEP *methylation is influenced by genetic variation and sex*

At birth, only genetic variation and infant sex were associated with average *LEP* methylation across the assayed region. The minor alleles of several SNPs were independently associated with *LEP* methylation, with rs2167270 genotype showing the strongest effect. This is the only SNP within the assayed *LEP* region (**Supp. Figure 1**), located in the first exon (hg19:chr7:127,881,349). The rs2167270 genotype has also previously been reported to influence *LEP* cord blood methylation (15), circulating leptin levels, BMI, and other cardiometabolic measures, however findings have been inconsistent (30-33). The rs10249476 genotype (in complete linkage disequilibrium with rs2167270) has also been linked to adult leptin levels following adjustment for BMI (34).

Infant sex was associated with *LEP* methylation across the region. Lower *LEP* methylation in males is consistent with previous findings in both infant (49) and adult (28) whole blood. A previous study found a stronger effect of prenatal famine on *LEP* methylation in adult whole blood in males (28). Another found male-specific associations between placental *LEP* methylation, *LEP* expression and infant neurological outcomes (51). However, in this study, we did not find evidence of male-specific associations between exposures and *LEP* methylation in blood.

LEP *methylation and birth weight*

CpG7 (cg19594666), negatively related with birth weight adjusted for age and sex in this study, is located in a predicted CEBPB transcription factor binding site. CEBPB binding increases *LEP* expression and higher CEBPB expression in adult adipose tissue predicts elevated adipose *LEP* expression (52). Cord blood leptin levels are strongly positively associated with birth weight, birth length and ponderal index, and negatively associated with weight gain over the first 4 postnatal months (53). The negative direction of the relationship between birth CpG7 methylation and birth weight z-score is in agreement with two previous studies of *LEP* cord blood methylation that reported an association between small for GA infants and increased *LEP* methylation (15), and an association between lower *LEP* methylation and macrosomia (26). It is also consistent with increased CpG7 methylation reducing CEBPB binding and consequently leptin expression, though an *in vitro* study reported that the oxidation state of methylation determined its effect on CEBPB binding (54).

LEP *methylation and pre-eclampsia*

We have found evidence of an association between pre-eclampsia and lower cord blood methylation at CpG14. This is consistent with a previous study investigating *LEP* methylation in placental tissue, that found early-onset pre-eclampsia, but not late-onset, was associated with decreased

methylation at CpG14 but not CpG sites upstream (20). However, this previous study reported that other CpGs proximal to CpG14 were also hypomethylated in association with pre-eclampsia, which we did not observe in our study. Pre-eclampsia has been linked to both elevated *LEP* mRNA in placenta (55), and increased leptin levels in cord plasma (23). In this study, we found that the effects of pre-eclampsia of CpG14 methylation differed by genotype at rs7799039, a SNP that has previously been linked to BMI and gestational weight gain (31, 47).

LEP *methylation and maternal metabolic health*

Previous studies have reported associations of maternal BMI and GDM with offspring *LEP* methylation. Two studies, one investigating maternal BMI and GDM (n=535) (18) and the other investigating maternal glycemia (n=48) (19), reported that these measures are positively associated with *LEP* methylation in placental tissue. In studies of cord blood, two studies (n=81 and 114) reported negative associations between maternal BMI and *LEP* methylation (15, 16), while another investigating the effects of GDM on cord blood methylation (n=251) reported there was no association (56). We found modest evidence of a relationship between GDM and *LEP* methylation at CpG3 and CpG4.5.6 (albeit in in opposite directions). As such, we are the first to report evidence of an association between GDM and *LEP* methylation in cord blood. However, in contrast with previous studies (15, 16), we observed no signals for maternal pre-pregnancy BMI influencing *LEP* methylation in infants. This could potentially be influenced by reporting bias as pre-pregnancy BMI was derived from self-reported weight in this study, rather than clinical records (15).

LEP *methylation and growth over time*

Average methylation levels increased across most CpG units from birth to 12 months. However, the increase in CpG11 methylation was substantially greater at 12 months (**Figure 1**) and subsequently

may have specific and temporal biological significance. The mean triceps and subscapular skinfold thickness at birth predicted lower 12-month methylation most strongly at CpG7, CpG10 and CpG11.

The potential for genetics and infant anthropometric measures to predict *LEP* promoter methylation appears to be greater at birth. Excluding technical variables, the regression model for CpG7 methylation levels explained 41.26% of variation in methylation in total, with the rs2167270 genotype explaining substantially more variation than infant sex and weight z-score at birth. In contrast, rs41457646 genotype and mean skinfold thickness at birth explained only 2.93% in the model for average 12-month methylation across the *LEP* region. This may reflect the broader range of potential influences on *LEP* methylation in the postnatal environment, or a dynamic change in *LEP* regulation during postnatal development. While breastfeeding has been linked to altered *LEP* methylation later in childhood (57), adjusting for breastfeeding did not modify any of our findings.

*Strengths, limitations and implications*

This study is the first to investigate white blood cell *LEP* methylation across two early life time points, and is larger than previous *LEP* methylation studies. In combination with the maternal health measures, infant anthropometry and *LEP* genotyping, it has allowed for the investigation of the independence of influences on the longitudinal relationship between *LEP* methylation and infant anthropometry at 12 months. A limitation of this study is the lack of comparable *LEP* expression levels or serum leptin data to support a downstream functional role of altered methylation. Although decreased *LEP* promoter methylation has been linked to increased *LEP* expression in cancer cell lines (12), adipocytes (13), and chondrocytes (14) *in vitro*, the functional consequences of altered *LEP* methylation in cord blood are yet to be established. Findings from studies investigating the relationship between *LEP* methylation and mRNA expression in the placenta have been contrasting (19, 27), though there is evidence for cord blood *LEP* methylation influencing cord blood leptin levels (17). As this study analysed maternal and infant measures previously linked to leptin or

*LEP* methylation, we did not perform correction for multiple comparisons. As such, this increases the probability that at least one of the reported associations are due to chance, though our core findings have persisted in multivariable and sensitivity analysis. Our study may have had limited capacity to investigate GDM and pre-eclampsia due to the low number of cases in our cohort (**Table 1**), and the analysis of pre-pregnancy BMI is potentially influenced by reporting bias. There may also be possible confounding due to unknown and unmeasured factors. While we have focused on metabolic and growth-related factors as part of our analysis, other environmental exposures have previously been linked to altered *LEP* epigenetic regulation or expression, including air pollution (variation in *LEP* methylation in placenta (58) or serum leptin levels in adults (59)) and endocrine disruptors (variation in *LEP* expression in adult adipose tissue (60) or cultured adipocytes (61)).

As infant anthropometric measures showed stronger evidence of relationships with cord blood *LEP* methylation than maternal factors, it is plausible that the observed variations in *LEP* methylation primarily reflect the nutritional status of the neonate, rather than influences of sub-optimal *in utero* exposures. Similarly, our longitudinal analysis showed that infant adiposity at birth was longitudinally associated with *LEP* methylation at 12 months, rather than cord blood *LEP* methylation playing a role in predicting infant weight at 12 months. The impact of this early-development shaping of *LEP* methylation levels on health throughout life is currently unknown.

*Conclusion*

We report a large increase in the methylation level of a specific CpG site in the promoter of *LEP* between birth and 12 months. Birth adiposity predicts decreased average *LEP* methylation across the assayed region in 12-month whole blood, interacting with genetic variation. Infant sex and *cis*-genetic variation influences methylation across the region, with genetic effects strongest at a predicted CEBPB transcription factor binding site, and having a stronger effect on methylation at birth. At a predicted CERPB site, methylation at birth was negatively associated with infant weight z-

score at birth. There was also evidence of pre-eclampsia predicting decreased cord *LEP* methylation at a specific CpG site, and more modest evidence of GDM predicting methylation at other CpG sites in different directions in the final regression models. Further studies considering both genetics and environmental factors are warranted to replicate these findings and to identify other exposures potentially impacting *LEP* promoter methylation in blood, and also assess any functional effects of variation in LEP methylation in blood on circulating leptin levels, growth and/or cardiometabolic health.

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## Barwon Infant Study Investigator Team

The members of the Barwon Infant Study Investigator Team are the following: Peter Vuillermin, Barwon Health, Deakin University, the Murdoch Children's Research Institute, and University of Melbourne; Anne-Louise Ponsonby, John Carlin, Katie Allen, Mimi Tang, Richard Saffery, Sarath Ranganathan, and David Burgner, the Murdoch Children's Research Institute, University of Melbourne; Terry Dwyer, the Murdoch Children's Research Institute and the George Institute for Global Health; Kim Jachno, the Murdoch Children's Research Institute; and Peter Sly, University of Queensland, Queensland Children's Medical Research Institute.

## Competing Interests

The authors declare that they have no competing interests.

## Supplementary Information

Supplementary information is available at International Journal of Obesity's website

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# Figure Legends

**Figure 1.** Distribution and mean differences between *LEP* methylation at birth (open circles) and 12 months (filled circles) by CpG unit. Mean differences (Δ) and p-values are adjusted for actual age at 12 months and EpiTYPER batch. Error bars are mean $\pm$ standard deviation.

**Figure 2.** Summary of relationships observed in this study between genetic, pregnancy, infant anthropometry, and *LEP* methylation measures. β, p and $R^2$ values are from final linear regression models.

# Table Legends

**Table 1.** Maternal, birth and 12-month infant measures of participants included in study.

**Table 2.** Associations of pregnancy exposures and birth phenotypes with cord blood *LEP* methylation levels.

**Table 3.** Multivariable linear regression full model predicting birth CpG7 methylation (%) (n=754).

**Tables 4a and 4b.** Longitudinal analysis of birth weight and adiposity predicting 12-month *LEP* methylation.

**Table 4a.** Associations between birth weight/adiposity and 12-month *LEP* methylation.

**Table 4b.** Final linear regression model predicting 12-month average *LEP* methylation (%) across the assayed region (n=395).