Epigenetic Gene Promoter Methylation at Birth Is Associated With Child’s Later Adiposity

Keith M. Godfrey,1,2,3 Allan Sheppard,4,5 Peter D. Gluckman,4,6 Karen A. Lillycrop,1 Graham C. Burdge,1 Cameron McLean,4,5 Joanne Rodford,1,3 Joanne L. Slater-Jefferies,1 Emma Garratt,1,3 Sarah R. Crozier,2 B. Starling Emerald,6 Catharine R. Gale,2 Hazel M. Inskip,2 Cyrus Cooper,2,3 and Mark A. Hanson1,3

OBJECTIVE—Fixed genomic variation explains only a small proportion of the risk of adiposity. In animal models, maternal diet alters offspring body composition, accompanied by epigenetic changes in metabolic control genes. Little is known about whether such processes operate in humans.

RESEARCH DESIGN AND METHODS—Using Sequenom MassARRAY we measured the methylation status of 68 CpGs 5’ from five candidate genes in umbilical cord tissue DNA from healthy neonates. Methylation varied greatly at particular CpGs: for 31 CpGs with median methylation ≥5% and a 5–95% range ≥10% we related methylation status to maternal pregnancy diet and to child’s adiposity at age 9 years. Replication was sought in a second independent cohort.

RESULTS—In cohort 1, retinoid X receptor-α (RXRA) chr9:136355885+ and endothelial nitric oxide synthase (eNOS) chr7:150315553+ methylation had independent associations with sex–adjusted childhood fat mass (exponentiated regression coefficient [β] 17% per SD change in methylation [95%CI 4–31], \( P = 0.009, n = 64, \) and \( P = 0.001, n = 66, \) respectively) and %fat mass (\( P = 0.02, n = 64 \) and \( P = 0.002, n = 66, \) respectively). Regression analyses including sex and neonatal epigenetic marks explained >25% of the variance in childhood adiposity. Higher methylation of RXRA chr9:136355885+, but not of eNOS chr7:150315553+, was associated with lower maternal carbohydrate intake in early pregnancy, previously linked with higher neonatal adiposity in this population. In cohort 2, cord eNOS chr7:150315553+ methylation showed no association with adiposity, but RXRA chr9:136355885+ methylation showed similar associations with fat mass and %fat mass (\( \beta = 6\% \) [2–10] and \( \beta = 4\% \) [1–7], respectively, both \( P = 0.002, n = 239, \)

CONCLUSIONS—Our findings suggest a substantial component of metabolic disease risk has a prenatal developmental basis. Perinatal epigenetic analysis may have utility in identifying individual vulnerability to later obesity and metabolic disease.

From the 1Institute of Developmental Sciences, University of Southampton, Southampton University Hospitals NHS Trust, U.K.; the 2MRC Lifecourse Epidemiology Unit, University of Southampton, Southampton University Hospitals NHS Trust, U.K.; the 3NIHR Nutrition, Diet and Lifestyle Biomedical Research Unit, University of Southampton, Southampton University Hospitals NHS Trust, U.K.; the 4Liggins Institute, University of Auckland, Auckland, New Zealand; 5AgResearch, New Zealand; and the 6Singapore Institute of Clinical Sciences, Singapore, Singapore.

Corresponding author: Keith M. Godfrey, kmg@mrclBSITE.ac.uk.

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with later phenotype, would provide an approach to demonstrating the role of prenatal environment in pre-
disposition to adiposity.

Here we first measured the methylation status of CpGs
in the promoters of candidate genes in DNA extracted
from umbilical cord tissue obtained at birth in children
who were later assessed for adiposity at age 9 years (the
Princess Anne Hospital [PAH] study) (21). Measurements
of perinatal DNA methylation were related to adiposity in
later childhood and to information on mother’s diet during
pregnancy. Because of the strong associations found, we
then sought to replicate associations between umbilical
cord CpG methylation and child’s adiposity in a second
independent group of children from the Southampton
Women’s Survey (SWS) (22).

RESEARCH DESIGN AND METHODS

We studied two prospective cohorts recruited antenatally in Southampton, U.K.
In the PAH study, Caucasian women >16 years old with singleton pregnancies
<17 weeks’ gestation were recruited and a validated food frequency ques-
tionnaire (23) was administered at 15 weeks’ gestation; patients with diabetes
and hormonally induced conceptions were excluded. When the children
approached 9 years, we wrote to 461 still living locally. Another 216 (47%)
attended a clinic and adiposity was measured using dual energy X-ray
absorptiometry (DEXA) with a Lunar DPX-L scanner (Lunar). DNA was extracted
from an umbilical cord sample collected at birth and stored at −80°C. None of the mothers were administered
glucocorticoids during pregnancy.

In the SWS, women aged 20–34 years were recruited when not pregnant;
who subsequently conceived were followed through pregnancy and the
offspring followed up (22). To replicate associations with adiposity found in
the PAH cohort, we studied 230 children selected as having both umbilical
cord DNA available and childhood adiposity measurements.

Both groups underwent adiposity measurement by DEXA (PAH study, age 9
years; Lunar DPX-L, pediatric software, version 4.7c; GE Corporation, Madison,
WI [6]; SWS, age 6 years: Hologic Discovery, pediatric scan mode; Hologic,
Bedford, MA). Instruments were calibrated daily; coefficients of variation were
<1%. Follow-up of the children and sample collection/analysis was carried out
under Institutional Review Board approval (Southampton and SW Hampshire
Research Ethics Committee) with written informed consent. Investigations
were conducted according to the principles expressed in the Declaration of
Helsinki.

Selection of candidate genes. DNA from 15 randomly selected PAH study
umbilical cords underwent methylation-specific chromatin precipitation fol-
lowed by contribution to a commercial tiled oligomer microarray ( NimbleGen
Systems HG17_min, promoter array [24]), which uses 50-mer oligonucleotides
positioned on average every 100 bp from −3,750 bp to +500 bp relative to the
transcription start site of 24,134 human genes. We focused our analysis ini-
tially on a panel of 78 candidate genes, 28 selected on the basis of animal data
from our and other laboratories (25,26) and 50 selected from the array as
having the highest SD between subjects relative to the SD of within-subject
replicates. For this panel we identified those with correlations between overall
gene methylation status and DEXA measurements of adiposity at age 9 years
and from these genes chose five genes for further study based on individual
oligomers showing evidence of correlation with DEXA measurements at age 9
years, biological plausibility and feasibility of designing amplicons suitable for
Sequenom analysis; genes selected were retinoid X receptor-α (RXRA), en-
dothelial nitric oxide synthase (eNOS), superoxide dismutase-1 (SOD1), in-
terleukin-8 (IL8), and phosphoinositide-3-kinase, catalytic, δ-polypeptide
(PI3KCD). These genes all had associations of comparable strength between
childhood body composition and both overall gene promoter methylation and the
methylation of individual oligomers. All chromosomal coordinates are
based on UCSC, human genome March 2006 assembly (hg18).

Sequenom and single nucleotide polymorphism analysis. Genomic DNA
was purified from frozen cords by proteinase K digestion and phenol/chloro-
form extraction. DNA methylation was measured with the Sequenom Mas-
sARRAY Compact System (www.sequenom.com/). Briefly, gene-specific
amplification of bisulfite-treated DNA was followed by in vitro transcription
and analysis by matrix-assisted laser desorption ionization time-of-flight (MALDI-
TOF) mass spectrometry (27,28). Sequenom assay design and methods were as
per the manufacturer’s protocol. DNA (1 μg) was bisulfite converted using EZ
DNA Methylation Kit (Zymo Research). PCR primers specific for bisulfite-con-
verted DNA were designed (Methprimer). Each reverse primer contained a T7-
promoter tag for in vitro transcription (5′-cattatagacgctactcattaggaggaagct-3′).

and the forward primer was tagged with a 10mer to balance Tm (5′-aggaagagag-
3′). Supplementary Table 1 lists amplicons, primer sequences, PCR annealing
temperature, and genomic coordinates for the extended promoter regions
measured. Bisulfite-treated DNA was PCR amplified (Qiagen HotStar Taq Poly-
merase) in 5 μL reactions and treated with shrimp alkaline phosphatase
(Sequenom) (20 min at 37°C), heat inactivated (85°C for 5 min), and simulta-
neously in vitro transcription/uracil-cleavage reaction was carried out (7 μL
reactions) using Sequenom T-cleavage reagent mix. Transcript cleavage
products were desalted with 6 mg of Clean-Resin and 20 nL spotted on a 384-pL
SpectroCHIP (Sequenom) using a MassARRAY nanodispenser (Sasung). Mass
spectrum were acquired using a MassARRAY MALDI-TOP MS (Bruker-Sequenom)
and peak detection, signal-to-noise calculations, and quantitative CpG site
methylation performed using proprietary EpiTyper software v1.0 (Sequenom).
Consistent with the manufacturer’s specification (www.sequenom.com) agree-
ment between technical replicates was good, duplicate measures differing by
<10% methylation in almost all cases. We excluded from analysis samples that
failed to give a reliable PCR product or produced spectra with low confidence
scores (<2.9 in EpiTyper). For fragments containing a single CpG site, DNA
methylation was calculated by the ratio of methylated to unmethylated frag-
ments. Limitations imposed by Sequenom analysis treat cleavage products
containing multiple CpG sites as single units, and the methylation values
reported are weighted averages across the unit (referred to as a CpG group).
DNA quality and no-template controls, 0%, and 100% methylated DNA were in-
corporated in all assays. Supplementary Table 2 shows the distributions of methyl-
ation values for CpGs and CpG groups and numbers of subjects with
measurements available.

Pyrosequencing analysis (PyroMark Q66MD; Qiagen) was undertaken to
exclude single nucleotide polymorphisms (SNPs) involving CpG dinucleotides,
which would result in loss of potential for methylation. Briefly, DNA was PCR
amplified, and the product annealed to streptavidin-coated sepharose beads
and denatured (0.2M NaOEt) to a single-stranded product. Nucleotides were
incorporated to the open 3′ DNA strand in which pyrophosphate is released
and used in a luciferase re-action, and analyzed using PyroMark MD v1.0 software (Qiagen) (29).

Statistical analysis. Using Stata 11 (StataCorp) study variables were
analyzed using Fisher-Yates (30) for methylation measurements) or logarithmic
transformations where necessary to satisfy statistical assumptions of nor-
mality/normalcy and when restricted to binary outcomes were restricted to
correlation coefficient ≥5% and a 5–90% range ≥10%. In PAH subjects we
first used Pearson correlation (r) and linear regression to examine CpG methylation in relation to
child’s adiposity. Mutually adjusted regression models were built including
CpG sites significantly associated with each outcome in univariate analyses
(P < 0.05). Because the measurements of childhood adiposity are on the log
scale and the CpG measurements are interpreted as percent change in childhood adiposity measurements
per SD change in methylation. To allow assessment of the influence of taking account
of the child’s sex, exponentiated regression coefficients are first presented
excluding and including adjustment for child’s sex. We then preadjusted all
adiposity measurements for sex and took account of the mother’s age, adi-
posity (as continuous variables), and smoking during pregnancy (as a binary
variable) to adjust the outcome variables as covariates in the regression model; we
next related mother’s diet to CpG methylation at birth. Finally, we sought
replication of the associations between CpG methylation and child’s adiposity
in the SWS cohort. SWS measurements, taken over a wider age range than
those in PAH subjects, were preadjusted for age and sex.

RESULTS

Median (interquartile range) birth weight, age, fat mass,
and percent body fat at follow-up values for the PAH
subjects were 3,330 g (3,010–3,790), 8.66 years (8.52–8.73),
5.41 kg (3.97–8.41), and 18.3% (14.4–27.0), respectively.
Comparable values for the 239 SWS children were 3,485 g
(3,205–3,773), 6.59 years (6.41–6.78), 4.60 kg (3.66–5.69),
and 23.4% (19.7–27.6), respectively. Similar percentages of
mothers smoked in the PAH and SWS cohorts (21 vs. 24%,
respectively); median maternal age and prepregnancy BMI
were lower in the PAH mothers (28 vs. 31 years and 22.3
27.6), respectively. Similar percentages of
mothers smoked in the PAH and SWS cohorts (21 vs. 24%,
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were lower in the PAH mothers (28 vs. 31 years and 22.3
kg/m2, respectively). The above characteristics are
similar to the overall values for participants in the two
cohorts.

PAH cohort methylation studies. Supplementary Table
2 shows the distribution of percent methylation of the 68
CpGs and CpG groups among the 78 PAH subjects; 31
CpGs/CpG groups met a priori criteria for further analysis. Particularly marked interindividual variation in percent methylation was seen at some sites (for example, RXRA chr9:136355885+ had a median of 50% with 5–95th centiles 4–99%; eNOS chr7:150315553+ had a median of 93%, with 5–95th centiles 64–100%). Correlations between methylation of different CpGs were generally low (for example, \( r_p = -0.03, P = 0.81, n = 55 \) between the above two CpGs).

Of the 31 CpGs with variable methylation above the a priori threshold, seven had significant associations with the child’s adiposity at age 9 years (Supplementary Table 3). Table 1 shows the results of univariate and multivariate analyses of CpG sites with significant independent associations with the child’s adiposity. Comparison of the regression coefficients for the univariate and multivariate associations of RXRA chr9:136355885+ and eNOS chr7:150315553+ methylation with child’s fat mass, percent fat, and ratio of trunk to limb fat shows that mutual adjustment for each other and sex has little effect on the coefficients; adiposity measurements were therefore pre-adjusted for sex in further analyses. RXRA chr9:136355885+ methylation had positive associations with childhood fat mass, percent fat mass (scatterplots shown in Supplementary Fig. 1), and ratio of trunk to limb fat, with similar positive associations for eNOS chr7:150315553+ methylation with child adiposity. Comparison of the standardized coefficients and \( P \) values for eNOS chr7:150315553+ methylation were 20% (95% CI) per SD change in methylation (\( \beta = 0.36, P = 0.028, n = 37 \)), and SOD1 chr 21:31853827+ (\( \beta = -0.27, P = 0.029, n = 65 \)). RXRA chr9:136355885+ methylation showed a weak association with the subjects’ sex and age adjusted height (\( r_p = 0.24, P = 0.054 \)) but was not associated with the infant’s weight or ponderal index at birth.

**Methylation analysis in the SWS cohort.** We next sought to replicate the stronger associations found in the first cohort (eNOS chr7:150315553+ and RXRA chr9:136355885+ CpG methylation and child’s adiposity) in the SWS cohort (20). In these 239 SWS children, eNOS chr7:150315553+ showed no association with adiposity, but RXRA chr9:136355885+ showed remarkably similar and statistically significant associations (fat mass, \( \beta = 6\%\) [2–10], \( P = 0.002 \); %fat mass, \( \beta = 4\%\) [1–7], \( P = 0.002 \), both \( n = 239 \)) to those in PAH children. As in the PAH cohort, these associations were little changed after taking account of birth weight and maternal age, smoking, and BMI. Figures 2 and 3 show the graded associations between cord RXRA chr9:136355885+ methylation and child’s fat mass and percent fat mass in the two cohorts.

Pyrosequencing across RXRA chr9:136355885+ in SWS subjects showed no C allele SNPs that could account for differences in methylation observed between individuals.

### Table 1

|                     | eNOS chr7:150315553+ (%) | P     | RXRA chr9:136355885+ (%) | P     | Sex (%) | P     | Variance explained (%) |
|---------------------|--------------------------|-------|--------------------------|-------|---------|-------|------------------------|
| Total fat mass (ln kg) |                          |       |                          |       |         |       |                        |
| Univariate analysis  | 21                       | 0.001 | 21                       | 0.003 | 39      | 0.001 |                        |
| Multivariate analysis| 19                       | 0.002 | 23                       | 0.001 | 47      | 0.001 | 44                     |
| Percentage fat (ln %) |                          |       |                          |       |         |       |                        |
| Univariate analysis  | 13                       | 0.006 | 14                       | 0.006 | 42      | <0.001|                        |
| Multivariate analysis| 11                       | 0.009 | 14                       | 0.005 | 48      | <0.001| 47                     |
| Ratio trunk to limb (ln) |                         |       |                          |       |         |       |                        |
| Univariate analysis  | 7                        | 0.008 | 7                        | 0.016 | 12      | 0.009 |                        |
| Multivariate analysis| 8                        | 0.004 | 7                        | 0.021 | 11      | 0.059 | 28                     |

Values are the percent change in outcome variable per SD change in CpG methylation and \( P \) value. Univariate analysis rows show the regression coefficients and \( P \) values relating each of eNOS chr7:150315553+, RXRA chr9:136355885+, and sex to outcomes individually, whereas multivariate analysis rows show regression coefficients and \( P \) values for a combined analysis of eNOS chr7:150315553+, RXRA chr9:136355885+, and sex in relation to outcomes, together with the variance explained by the multivariate model. For variances explained, \( n = 55 \).
DISCUSSION

This study provides novel evidence for the importance of the developmental contribution to later adiposity. We found that greater methylation of RXRA chr9:136355885+ measured at birth was strongly correlated with greater adiposity in later childhood in two independent cohorts. Although we studied a subset of children in both cohorts these were selected on the basis of subject and specimen availability, so it is unlikely that selection bias could explain the relationships observed unless the association between RXRA methylation and adiposity was different in the remainder of the cohort. The data build on animal experiments suggesting that the developmental environment acts through epigenetic processes to exert a strong influence on postnatal body composition and metabolic function (7–11).

Our study shows that specific components of the epigenetic state at birth predict later childhood adiposity. The associations with adiposity were linked to specific CpGs 5′ to the start site of the selected candidate genes. Although some of the CpGs studied were either within the proximal promoter or close to it, others were more distal and may be exerting effects through the regulation of other genes. Nevertheless the data indicate possible mechanistic pathways, suggesting avenues for future study. Our observation that adjacent or nearby CpGs within the same promoter showed differences in the strength of association with child’s adiposity suggests highly specific changes in the transcriptional regulation of these genes induced by the developmental environment, rather than generalized changes in promoter methylation. Both CpG hyper- (eNOS chr7:150315553+ and RXRA chr9:136355885+) and hypomethylation (SOD1 chr21:31853660/63+) at different sites were associated with body fat distribution, again indicating complexity in transcriptional control. The specificity of the associations between methylation of an individual CpG and both maternal diet and child’s phenotype endorses the concept of a fine control of development by environmental factors via epigenetic processes.

Our observation indicates one potential mechanistic pathway involved, because induction of transcription by RXRA is dependent on its binding to ligands including the peroxisome proliferator–activated receptors, involved in insulin sensitivity, adipogenesis, and fat metabolism (31,32). Moreover, RXRA chr9:136355885+ is located in a region considered to contain positive regulatory elements of transcription (33). Figure 4 shows the proximity of RXRA chr9:136355885+ to proposed binding sites for RXR, MAF, NF-κB, and AP1. Retinoid receptor biology is complex, and increased RXRA methylation might be acting through a variety of pathways (34); however, an association between increased RXRA methylation and adiposity is consistent with the observation of strongly diminished RXRA expression in visceral white adipose tissue from obese mice (35). Moreover, a role for retinoid receptor methylation in developmental influences on later metabolic risk is supported by recent experimental data showing an influence of maternal diet during pregnancy on methylation of LXRA, a heterodimeric partner of RXRA (36).
Genome-wide association studies suggest that fixed genetic variation makes a relatively small contribution to risk of obesity, heart disease, and diabetes (1,2); our findings raise the possibility that the developmental environment component may be equally or more important. We excluded the presence of a SNP at RXRA chr9:136355885+ by sequencing, but without genome-wide analysis it is not possible to exclude a genetic effect of distant SNPs, which could influence both DNA methylation of a particular sequence and child’s phenotype. However, even if this were the case, our data clearly indicate that epigenetic measures at birth may have prognostic value. Although epigenetic changes can be dynamic, experimental studies have shown that environmental factors acting on the genotype during development relate to epigenetic profile in adulthood (7,15), and there are longitudinal human studies showing that DNA methylation is often stable over time (37). Such changes can be tissue specific, and in this respect the umbilical cord may be advantageous because it contains a high proportion of fetal vascular tissue and mesenchymal cells, which may be relevant to later adiposity. Furthermore, unlike the placenta it is a tissue in which consistency of sampling between individuals is more likely. Although experimental work in the rat suggests that methylation changes induced by maternal diet can be similar in the umbilical cord and liver (38), further

![Schematic diagram of the RXRA promoter region](image)

**FIG. 4.** Schematic diagram of the RXRA promoter region, showing the position of the CpG group at RXRA chr9: 136355885+ (underlined) and of neighboring transcription factor binding sites.

**FIG. 3.** In a second independent cohort, child’s percent fat mass and fat mass at age 6 years increases with higher umbilical cord RXRA chr9:136355885+ methylation in SWS subjects. Values are means ± SEM.
work is needed to determine the relevance of epigenetic changes in human umbilical cord tissue. Recent data show that for some genomic regions methylation appears largely independent of tissue of origin, whereas for others there is a clear tissue-specific dependence (39).

Many epidemiological studies have shown associations between fetal development, through the proxy measure of birth size, and later adiposity and metabolic function (3,4), but the developmental contribution to such phenotypic characteristics has remained uncertain and controversial. This study provides the first estimate of the developmental contribution to phenotype associated with human disease risk based on measures of the underpinning biology: our data for RXRA chr9:136355885+ suggest that a substantial proportion of the variation in adiposity in prepubertal children can be explained by epigenetic measurements made at birth. Although our data are correlative and thus can only imply an association between DNA methylation at birth and later phenotype, the importance of the observation stands irrespective of whether the RXRA methylation is causally related to the development of adiposity. Even if it is simply a noncausal association, the changed epigenetic status provides an objective marker of altered developmental trajectory by the time of birth. Despite the limitations of dietary intake assessment tools, the instrument we used is both validated and provides information that can be used to rank the nutrient intakes of individuals (23).

Methylation of eNOS chr7:150315553+ was associated with later adiposity in the initial cohort only. This may reflect a chance finding, or different maternal characteristics in the two cohorts, such as the greater maternal adiposity and maternal folate supplementation in the SWS cohort (22,40,41). Alternatively, adipocyte proliferation is high during the first year of life but then remains low until a second proliferative phase from age 9–14 years (42). Thus the processes that determine adiposity at age 9 years might differ from those at age 6 years. Nitric oxide synthesis by eNOS promotes preadipocyte differentiation (43). Therefore, a further possible explanation is that eNOS methylation in umbilical cord marks capacity for adipogenesis which has a greater net contribution to adiposity at age 9 years than age 6 years.

Our findings show strong associations between epigenetic markers and childhood total and central body fat. Beyond these simple associations, multivariate analysis indicates that the associations explain substantial proportions of the variances in outcomes, emphasizing an important developmental contribution to phenotypes associated with metabolic dysfunction and disease risk. It is noteworthy that the genes for which we report effects are not imprinted. In vitro fertilization increases risk of imprinting disorders (44), and methylation effects on imprinted genes have been reported in offspring of mothers exposed to famine during various periods of pregnancy (45,46), but with no associations with phenotype reported. The current study implicates the human prenatal environment with epigenetic changes in nonimprinted genes and is the first to link epigenetic status at birth with clinically relevant later phenotypic variation.

Variation in the degree of methylation of nonimprinted genes and in later cardiovascular and metabolic physiology can be induced experimentally by manipulation of the developmental environment, for example by altering maternal nutrition or administering glucocorticoids during pregnancy (8,9), often without necessarily affecting the birth size of the offspring. Although we found associations between some epigenetic markers and birth weight, these were weaker associations than those with later phenotype and were for different markers; moreover, previous reports on the same cohort showed only modest associations between birth weight and later body composition (6). Because birth weight in humans is influenced by multiple factors including the mother’s own birth weight and height and by gestational length (40), epigenetic changes may provide a more sensitive index than birth weight of environmentally induced effects on fetal development.

There are potentially important implications of the strong and replicated association between RXRA chr9:136355885+ methylation and later adiposity. First, the effect is considerably greater than that of factors such as birth weight or maternal body composition, suggesting that epigenetic measurements made in the neonate may be useful predictors of later obesity and other phenotypic outcomes. Second, the association between CpG methylation and child’s adiposity operates within the normal ranges of maternal nutritional state and birth size; this supports the argument that developmental programming is the consequence of an evolved and potentially adaptive process involving the mechanisms of developmental plasticity (10). Indeed the data provide strong evidence supporting a role for developmental plasticity in determining individual risk of metabolic disease. Third, the data suggest that developmental factors may be more significant in contributing to phenotypic variation and disease risk than generally considered. Fourth, the association between RXRA chr9:136355885+ methylation and mother’s carbohydrate intake raises the possibility that conditions in early pregnancy could affect child’s adiposity through this pathway. This provides additional support for the argument that all women of reproductive age should have appropriate nutritional, education, and lifestyle support to improve the health of the next generation. Finally, our data suggest that epigenetic measures at birth may have prognostic value and potential utility for monitoring programs to optimize maternal health and nutrition for long-term benefits to the offspring; however, evaluation of this possibility will require further research correlating methylation measurements in early life with those in later life.

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K.M.G. conceived of the study, undertook the statistical analysis, wrote the first draft of the manuscript, and reviewed and edited the manuscript. A.S. undertook the molecular biology and reviewed and edited the manuscript. P.D.G. conceived of the study, wrote the first draft of the manuscript, and reviewed and edited the manuscript. K.A.L. conceived of the study, undertook the molecular biology and reviewed and edited the manuscript. G.C.B. conceived of the study and reviewed and edited the manuscript. C.M., J.R., J.L.S.-J., and E.G. undertook the molecular biology and reviewed and edited the manuscript. S.R.C. undertook the statistical analysis and...
reviewed and edited the manuscript. B.S.E. undertook the molecular biology and reviewed and edited the manuscript. C.R.G. coordinated the phenotyping of the children and reviewed and edited the manuscript. H.M.I. coordinated the phenotyping of the children, assisted in the statistical analysis, and reviewed and edited the manuscript. C.C. assisted in the statistical analysis and reviewed and edited the manuscript. M.A.H. conceived of the study, wrote the first draft of the study, and reviewed and edited the manuscript.

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