PGC1α Promoter Methylation in Blood at 5–7 Years Predicts Adiposity From 9 to 14 Years (EarlyBird 50)

The early environment, acting via epigenetic processes, is associated with differential risk of cardiometabolic disease (CMD), which can be predicted by epigenetic marks in proxy tissues. However, such measurements at time points disparate from the health outcome or the environmental exposure may be confounded by intervening stochastic and environmental variation. To address this, we analyzed DNA methylation in the peroxisome proliferator–activated receptor γ coactivator 1α promoter in blood from 40 children (20 boys) collected annually between 5 and 14 years of age by pyrosequencing. Body composition was measured annually by dual X-ray absorptiometry, physical activity by accelerometry, and pubertal timing by age at peak high velocity. The effect of methylation on transcription factor binding was investigated by electrophoretic mobility shift assays. Seven cytosine guanine dinucleotide (CpG) loci were identified that showed no significant temporal change or association with leukocyte populations. Modeling using generalized estimating equations showed that methylation of four loci predicted adiposity up to 14 years independent of sex, age, pubertal timing, and activity. Methylation of one predictive locus modified binding of the proadipogenic pre-B-cell leukemia homeobox-1/homeobox 9 complex. These findings suggest that temporally stable CpG loci measured in childhood may have utility in predicting CMD risk.

Genetic variations explain only part of the variability in risk of cardiometabolic disease (CMD) (1). Epidemiological studies suggest that the early life environment also makes an important contribution (1), and both experimental and observational studies have implicated variation in the epigenetic regulation of specific genes as a key mechanism (2). An epigenetic explanation is supported by altered patterns of DNA methylation in individuals exposed to nutritional constraint in utero and by predictive associations between epigenetic marks in samples collected early in life and subsequent assessment of risk factors for CMD (3–9). For example, exposure to famine in utero is associated with altered methylation status of specific cytosine guanine dinucleotide (CpG) loci in blood in imprinted (6) and nonimprinted (7) genes related to the CMD phenotype in elderly individuals. Furthermore, methylation of the retinoid-x-receptor-α in umbilical cord predicted >25% of the variation in age- and sex-adjusted fat mass in children at 6 and 9 years old (3). Such findings imply that epigenetic changes associated with the CMD phenotype are induced by environmental exposures early in life and persist beyond the period of challenge.

However, crucial evidence from studies in childhood and from longitudinal studies is lacking (10) and may yet be challenged by recent reports of marked variation in DNA methylation of specific CpG loci during childhood...
and of its response to acute exercise (12) and environmental pollutants (13). Such influences could confound the contribution of early epigenetic variation to future risk of CMD in relation to that of later environmental exposures. Mechanism is a key component of hypothesis testing, but none of the studies reporting association between promoter methylation and CMD risk have so far demonstrated that differential methylation alters gene function (3–9). This limits the plausibility that differentially methylated loci in proxy tissues reflect altered epigenetic regulation of genes in the tissue of interest.

To address these issues, we investigated the temporal stability of CpG loci in the promoter region of peroxisome proliferator–activated receptor γ coactivator (PGC) 1α in the blood of children obtained annually from 5–14 years. PGC1α is central to energy homeostasis through regulation of mitochondrial function, pancreatic β-cell function, and adipogenesis (14) and is, therefore, of potential relevance to obesity and CMD risk. We also investigated whether differential methylation of individual CpG loci was related to adiposity and whether differential methylation of any CpGs that were associated with body composition might alter transcription factor binding at that locus.

**RESEARCH DESIGN AND METHODS**

**Subjects and Study Design**

We conducted the study in accordance with the ethics guidelines of the Declaration of Helsinki II; ethics approval was granted by the Plymouth Local Research Ethics Committee (1999), and parents gave written consent and children verbal assent. The EarlyBird Diabetes Study incorporates a 1995/1996 birth cohort recruited in 2000/2001 when the children were 5 years old (307 children, 170 boys) (15). Here, we report findings for 40 subjects (20 boys) selected randomly, stratified by insulin resistance at 14 years. (Subject characteristics at 5 and 14 years are summarized in Table 1.) Insulin resistance was determined each year from fasting glucose (Cobas Integra 700 analyzer; Roche Diagnostics) and insulin (DPC IMMULITE) (cross-reactivity with pro-insulin <1%) using the homeostasis model assessment program (16), which has been validated in children (17). BMI was derived from direct measurement of height (Leicester Height Measure; Child Growth Foundation, London, U.K.) and weight (Tanita Solar 1632 electronic scales), performed in blind duplicate and averaged. BMI SD scores were calculated from the British 1990 standards (18). Whole-body dual-energy X-ray absorptiometry scans for body composition were performed annually from age 9 years using a Prodigy Advance fan beam densitometer and analyzed using enCORE 2004 software, version 8.10.027 (GE Healthcare). Physical activity was measured annually from 5 years by accelerometry (Actigraph [formerly MTI/CSA]) (19,20). Children were asked to wear the accelerometers for 7 consecutive days at each annual time point, and only recordings that captured at least 4 days were used. Pubertal stage was adjusted for by age at peak height velocity (APHV), determined as the tangential velocity at the middle time point of three consecutive rolling height measurements taken 6 months apart. Peripheral blood was collected annually into EDTA tubes after an overnight fast and stored at −80°C.

**Analysis of Methylation of Specific CpG Loci by Pyrosequencing**

Genomic DNA was prepared from 400 μL whole blood using the QiAamp DNA Blood Mini kit (Qiagen), according to the manufacturer’s instructions. Analysis of methylation of individual CpG loci within the PGC1α promoter was carried out by pyrosequencing (21). Briefly, genomic DNA (1 μg) was treated with sodium metabisulphite using the EZ DNA Methylation kit (Cambridge Bioscience). Modified DNA was amplified with hot start Taq DNA Polymerase (Qiagen) using the PCR primers in Table 2. PCR products (10 μL) were immobilized on streptavidin–sepharose beads (GE Healthcare), washed, denatured, and released into annealing buffer containing the sequencing primer instructions. Analysis of methylation of specific CpG loci by pyrosequencing.

**Table 1—Subject characteristics at 5 and 14 years**

<table>
<thead>
<tr>
<th></th>
<th>Boys</th>
<th>Girls</th>
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<tbody>
<tr>
<td></td>
<td>5 years</td>
<td>14 years</td>
</tr>
<tr>
<td>Age (years)</td>
<td>5.0 ± 0.4</td>
<td>14.0 ± 0.4</td>
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<tr>
<td>Adiposity (% fat)</td>
<td></td>
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<tr>
<td></td>
<td>22.1 ± 10.2</td>
<td>22.1 ± 10.2</td>
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<tr>
<td>BMI SDS</td>
<td>0.17 ± 0.85</td>
<td>0.68 ± 1.04</td>
</tr>
<tr>
<td>Moderate-vigorous physical activity (min/day)</td>
<td>51.2 ± 24.9</td>
<td>58.0 ± 36.1</td>
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<tr>
<td>Insulin resistance (HOMA-IR)</td>
<td>0.49 ± 0.52</td>
<td>1.21 ± 1.10</td>
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<tr>
<td>APHV</td>
<td>13.3 ± 0.58</td>
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Data are means ± SD except for insulin resistance (median ± interquartile range). Significant sex differences by one-way ANOVA were detected for APHV (P < 0.001) and adiposity (P = 0.03 at 5 years, P = 0.004 at 14 years). HOMA-IR, homeostasis model assessment of insulin resistance; SDS, SD scores standardized for age and sex.
Pyrosequencing was carried out using PyroMark Gold Q96 Reagents on a PyroMark Q96 MD pyrosequencer (Qiagen), and the percentage methylation was calculated using PyroMark CpG software 1.0.11 (Qiagen). Within-assay precision (coefficient of variation) was CpG 2 783, 0.3%; CpG 2 816, 1.6%; CpG 2 783, 1.9%; CpG 2 652, 2.6%; CpG 2 617, 2.7%; CpG 2 521, 1.8%; and CpG 2 515, 1.8%. Between-assay precision was CpG 2 841, 2.0%; CpG 2 816, 2.4%; CpG 2 783, 3.4%; CpG 2 652, 4.4%; CpG 2 617, 2.8%; CpG 2 521, 2.7%; and CpG 2 515, 3.7%.

**Analysis of Transcription Factor Binding by Electromobility Shift Assay**

Electrophoretic mobility shift assays were performed as described (22) using a LightShift Chemiluminescent EMSA kit (Thermo Scientific). Oligonucleotide probes are detailed in Table 2. Biotin-labeled double-stranded oligonucleotide probes (10 fmol) corresponding to a region of the PGC1α promoter from −2 797 to −2 764 relative to the transcription start site containing either an unmethylated or methylated cytosine base at −2 783 were incubated with 5 μg nuclear extracts derived from the SW-872 liposarcoma cells. For testing of specificity of binding, a 50-, 100-, or 500-fold excess of either the unlabeled probe, a nonspecific competitor, a homeobox (HOXB)9/pre–B-cell leukemia homeobox (PBX)-1 consensus sequence, or 1 μg anti-HOXB9 monoclonal antibody (AbCam) or anti–PBX-1 polyclonal antibody (Abcam) was incubated with the nuclear extract prior to addition of 10 fmol biotin-labeled probe and a further 20-min incubation on ice. Complexes were resolved on a prerun 5% nondenaturing polyacrylamide gel in 0.5Tris-borate-EDTA for 50 min at 100 V and 4°C, followed by semidry transfer (200 mA for 1h) to a positive nylon membrane and ultraviolet cross-linking. After incubation in blocking buffer, the membrane was incubated with streptavidin–horseradish peroxidase conjugate and visualized with a chemiluminescent substrate according to the manufacturer’s instructions (Thermo Scientific).

**Transfection and Luciferase Reporter Gene Assays**

SW-872 cells were transfected using with FuGENE HD (SwitchGear Genomics) according to the manufacturer’s instructions. Transfections were carried out with 5 × 10³ cells in a 96-well plate and transfected with 100 ng pGL3PGC1α promoter (−909 to 92 bp) and 1.25 ng pCMV Renilla (Promega), together with 0, 250, 500, 750, and 1,000 ng expression vector containing the full-length cDNA of PBX-1 (IRQLp5017E064D; Source BioScience LifeSciences) for 48 h. The amount of transfected DNA for all transfections was equalized by the addition of empty expression vector. Transfections were carried out in triplicate. Luciferase and Renilla activity were determined using the dual luciferase assay (Promega) according to the manufacturer’s instructions. Data were normalized by dividing luciferase activity by Renilla activity.
Statistical Analysis and Modeling
Statistical analyses were performed using IBM SPSS Statistics, version 19.0. Pearson correlation was used to determine the association between percent methylation at baseline (5–7 years) and percent methylation at subsequent ages. A longitudinal tracking coefficient was computed for each CpG, where the measurement at baseline (5–7 years) was regressed on all other measurements from 9 to 14 years simultaneously. In order to take into account the correlations between repeated measurements on the same children, we used generalized estimating equations to model the association between methylation at 5–7 years and adiposity (percent body fat) measured annually by dual-energy X-ray absorptiometry from 9 to 14 years of age. Age, sex, APHV, and physical activity and all relevant two-way interactions were included in this model. As expected, there were significant interactions between adiposity and age and sex ($P < 0.001$) and between age and APHV ($P < 0.001$) and physical activity ($P < 0.005$). Methylation of specific CpG loci in the PCG1α promoter at 5–7 years was significantly associated with future adiposity (Fig. 3). For each 10% difference in methylation at 5–7 years, percent body fat differed by 12.5 (95% CI 4.7–20.3; adjusted 95% CI 2.2–22.7) at CpG locus $-841$ ($P = 0.002$), 7.5 (2.6–12.4; 1.1–13.9) at CpG $-816$ ($P = 0.003$), 6.3 (0.9–11.7; −0.8 to 13.4) at CpG $-783$ ($P = 0.02$), and 7.6 (1.0–14.0; −2.1 to 16.1) at CpG $-521$ ($P = 0.03$). There were no significant interactions between methylation at any of these loci and sex ($P \geq 0.52$), APHV ($P \geq 0.06$), physical activity ($P \geq 0.23$), or age (all $P \geq 0.21$, except for CpG $-521$, which showed a significant interaction with age ($P < 0.001$). There were no significant associations between adiposity and methylation at CpG loci $-652$ ($\beta = 0.7$ [95% CI $-3.3$ to 4.6, $P = 0.75$]), $-617$ (2.9 [−1.0 to 6.8], $P = 0.14$), or $-515$ (1.3 [−1.8 to 4.3], $P = 0.42$) bases.

Exemplification of the Effect of Differential Methylation on Transcription Factor Binding
We used electrophoretic mobility shift assays to determine whether differential methylation at CpG $-783$, which is located within a putative HoxB9/PBX-1 response element (Fig. 1), altered transcription factor binding. A biotin-labeled double-stranded oligonucleotide probe corresponding to a region of the PCG1α promoter from −797 to −764 containing either an unmethylated or methylated cytosine base at −783 was incubated with nuclear extracts from the liposarcoma cell line SW-872. Two complexes of similar mobility were seen binding to both the unmethylated and methylated probes, the binding of which (Fig. 3A) was reduced markedly by coinubcation with 500-fold excess of the unlabeled probe (specific competitor) but not with a 500-fold excess of an unlabeled nonspecific competitor indicating specific protein binding to this region of the PCG1α promoter. For determination of whether methylation at CpG $-783$ affected binding to this sequence, the unmethylated biotin-labeled probe was incubated with nuclear extracts from the liposarcoma cells with a 50-, 100-, and 500-fold excess of either the unmethylated or methylated specific competitor. While binding to the biotin-labeled unmethylated probe was markedly reduced in the presence of a 500-fold excess of the unmethylated that were measured (all $P > 0.1$; neutrophils $r = -0.002$ to 0.34, lymphocytes 0.001–0.23).

Methylation of Specific CpG Loci in the PCG1α Promoter at 5–7 Years and Predicted Percent Body Fat
Having established that methylation of these CpG loci tracked from 5–7 to 14 years of age, we next used generalized estimating equations to model the association between methylation at 5–7 years and adiposity (percent body fat) measured annually by dual-energy X-ray absorptiometry from 9 to 14 years of age. Age, sex, APHV, and physical activity and all relevant two-way interactions were included in this model. As expected, there were significant interactions between adiposity and age and sex ($P < 0.001$) and between age and APHV ($P < 0.001$) and physical activity ($P < 0.005$). Methylation of specific CpG loci in the PCG1α promoter at 5–7 years was significantly associated with future adiposity (Fig. 3). For each 10% difference in methylation at 5–7 years, percent body fat differed by 12.5 (95% CI 4.7–20.3; adjusted 95% CI 2.2–22.7) at CpG locus $-841$ ($P = 0.002$), 7.5 (2.6–12.4; 1.1–13.9) at CpG $-816$ ($P = 0.003$), 6.3 (0.9–11.7; −0.8 to 13.4) at CpG $-783$ ($P = 0.02$), and 7.6 (1.0–14.0; −2.1 to 16.1) at CpG $-521$ ($P = 0.03$). There were no significant interactions between methylation at any of these loci and sex ($P \geq 0.52$), APHV ($P \geq 0.06$), physical activity ($P \geq 0.23$), or age (all $P \geq 0.21$, except for CpG $-521$, which showed a significant interaction with age ($P < 0.001$). There were no significant associations between adiposity and methylation at CpG loci $-652$ ($\beta = 0.7$ [95% CI $-3.3$ to 4.6, $P = 0.75$]), $-617$ (2.9 [−1.0 to 6.8], $P = 0.14$), or $-515$ (1.3 [−1.8 to 4.3], $P = 0.42$) bases.

RESULTS
Temporal Stability of the Methylation of CpG Loci in the PCG1α Promoter Between 5 and 14 Years
Forty subjects (20 boys, 20 girls) were selected from the EarlyBird cohort (15) (Table 1). Data collected at 5–7 years and 8–9 years were combined for statistical analysis in order to ensure complete longitudinal data sets. Seven CpG loci were sequenced (located between $-841$ to $-515$ bases relative to the transcription start site) (Fig. 1), which have been shown previously to be hypermethylated and associated with decreased PGC1α expression in pancreatic islet cells from overweight subjects with type 2 diabetes compared with normal-weight control subjects (26). Methylation of individual CpG loci in the PCG1α promoter varied from $20.4 \pm 3.5\%$ (CpG $-521$) to $64.9 \pm 2.8\%$ (CpG $-841$) across all subjects and years (Fig. 2) and did not differ between sexes ($P \approx 0.05$). Longitudinal tracking coefficients were high for all CpG loci (CpG $-515$, 0.93; $-521$, 0.83; $-617$, 0.97; $-652$, 0.99; $-783$, 0.95; $-816$, 0.96; and $-841$, 0.91; all $P < 0.001$) such that methylation at 5–7 years predicted between 77 and 88% of the variation at 14 years. There were no significant associations at any age between the proportions of neutrophils or lymphocytes in the blood samples from which the DNA was isolated and the level of methylation of any of the CpG loci
specific competitor, only a 50-fold excess of the methylated specific competitor was required to effectively compete out binding (Fig. 4B). This finding suggests that methylation at Cpg -783 enhances binding to the PGC1α promoter sequence. Increased binding to the methylated sequence was also observed when the labeled methylated probe was incubated with increasing concentrations of the non-biotin-labeled unmethylated or methylated competitors (data not shown).

Binding to the biotin-labeled unmethylated or methylated probe was markedly diminished by coincubation with 100- and 500-fold excess of an unlabeled oligonucleotide containing the consensus sequence for the HoxB9/PBX-1 heterodimer (Fig. 4C). For confirmation of the identity of the proteins binding to this sequence, antibodies specific to PBX-1 and HOXB9 were incubated with the protein extract prior the addition of the probe. While the addition of the antibody against PBX-1 substantially reduced binding of both complexes to the unmethylated probe (Fig. 5A), an antibody against HOXB9 probe had little effect on protein binding to the unmethylated probe (Fig. 5A). In contrast, antibodies against PBX-1 or HOXB9 both substantially reduced protein binding to the methylated probe (Fig. 5A). To confirm that the transcription factor PBX-1 can regulate PGC1α promoter activity, we transfected the PGC1α promoter construct together with increasing concentrations of an expression vector containing PBX-1. We found that the addition of PBX-1 led to a marked decrease in PGC1α promoter activity in liposarcoma cells, thus confirming that PBX-1 can regulate PGC1α expression (Fig. 5B).

**DISCUSSION**

In order to contribute causally to disease risk and have utility as biomarkers of disease risk, differentially methylated loci...
induced early in life must be stable in the face of subsequent environmental change and have clinical relevance. Our findings provide the first direct evidence that specific epigenetic marks measured in childhood can satisfy these criteria in relation to obesity.

The level of methylation of the seven CpG loci that were measured in the PCG1α promoter in leukocyte DNA showed significant year-on-year associations between 5–7 and 14 years of age. Heterogeneity in blood cell populations has been identified as a potential confounder for measuring DNA methylation marks in blood (27), although the proportion of variation in methylation explained by leukocyte heterogeneity appears to differ between genes. For example, the proportion of neutrophils accounted for 50% of the variation in interleukin-10 methylation in blood samples collected from adults 11–20 years apart (28). However, the variation in methylation explained by the proportion of neutrophils was less for imprinted (IGF2, 3.5%; GNASAS, 0.8) and nonimprinted (FTO, 0.1%; LEP, 7.4%) metabolism-associated genes (28). Corresponding differential leukocyte counts were available for all the samples that we analyzed for DNA methylation. There were no significant associations between the proportions of the major circulating leukocyte populations, neutrophils, and lymphocytes and the methylation of the seven CpG loci measured in the PCG1α promoter. Together, our findings with those of Talens et al. (28) suggest that the impact of variations in leukocyte populations on the methylation status of individual CpG loci measured in whole blood is related to the nature of the gene of interest both in children and in adults and is not a primary concern for the interpretation of this study.

The DNA methylome differs markedly between centenarians and infants (29), which implies either continuous...
plasticity or periods of increased variation in DNA methylation across the life course. Little is known about the plasticity of DNA methylation marks during childhood and puberty, although infancy and puberty have been proposed to be periods of increased epigenetic change (30). Recent studies in mono- and dizygotic twins showed that the methylation of specific CpG loci in genes associated with neurological function in buccal cells differed by up to 40% between 5 and 10 years of age (11). This is in marked contrast to our findings where there was no significant change in the methylation of the PGC1α promoter during childhood and puberty. This suggests that, at least for some loci, methylation patterns are established by early childhood, in this case by 5 years, and are not subsequently influenced significantly by environmental factors such as exercise (12), pollution (13), or puberty and is consistent with the paradigm that epigenetics thus is a causal process that links the early life environment to the future phenotype.

Four of the CpG loci that were measured in the PGC1α promoter predicted adiposity irrespective of sex, pubertal timing, or levels of physical activity, even though the percent body fat of the boys fell sharply and girls diverged in temporal trends in fat mass in the peri-pubertal period. The magnitude of the association suggested that 10%
difference in methylation at these loci predicted up to 12% difference in fat mass. Since body fat is associated causally with insulin resistance in children (31), a major underlying risk factor for CMD (32), our findings imply that differential methylation in mid-childhood of CpG loci that exhibit relatively strong temporal stability may be able to predict differences in patterns of adiposity that have longer-term clinical relevance. Thus, these findings are in agreement with, but also extend substantially, previous findings (3,5,9) by providing direct evidence that supports an assumption on which these studies were based: that methylation of specific CpG loci that are induced early in life can be maintained essentially independent of lifestyle, growth, and development during childhood and predicts health-related outcomes.

Because of the tissue-specific nature of the level of methylation at many CpG loci (33), it is not possible to infer directly from the present findings that the level of methylation of CpG loci in whole blood reflects differential methylation of those CpGs in adipose tissue or that they are involved in the regulation of fat mass by PCG1α in adipose. However, significant associations have been reported between individual CpG loci in human cells from different embryonic tissue lineages (28). Furthermore, three CpG loci with stable methylation status predictive of future adiposity at positions −783, −816, and −841 have been shown previously to be hypermethylated in pancreatic islet cells and associated with variation in mRNA levels in overweight subjects (26). Because it was not possible to collect adipose tissue from children, we have cautiously sought to exemplify the plausibility that differential methylation of CpG dinucleotide sequences but blocked binding to the unmethylated sequence. Because the antibodies were added prior to the addition of the probe, this suggests that the failure of the HOXB9 antibody to block binding to the unmethylated probe does not reflect a difference in conformation of the PBX-1/HOXB9 heterodimer bound to the DNA and a masking of the epitope. Instead, these data suggest that while both PBX-1 and HOXB9 bound to the methylated sequence, PBX-1 may be bound to the unmethylated sequence with a different partner.

Methylation of DNA has generally been associated with a reduction in transcription factor binding (36–39); however, there is increasing evidence that mCpG-binding activity is widespread among specific transcription factor families, such as homeodomain transcription factors (40), of which PBX1 and HOXB9 are members. Our data provide the first evidence that suggests methylation of a specific CpG locus may induce the binding of alternative PBX-1–containing heterodimers to the EMSA probes, potentially adding a further layer of complexity to regulation by PBX-1. However, whether this occurs in vivo at the PCG1α promoter requires confirmation in intact cells. Consistent with a role of PBX-1 in the regulation of PCG1α expression, overexpression of PBX-1 led to a marked decrease in PCG1α transcription in the luciferase reporter construct in liposarcoma cells, although whether this occurs in intact cells remains to be determined. Thus, it is possible that methylation at this locus within the promoter of PCG1α may alter PCG1α transcription and exert downstream effects on adipocyte development that are consistent with, but do not demonstrate directly, greater methylation leading to increased adiposity, although further experiments are required to determine whether PBX1 binds to the PCG1α promoter and regulates endogenous PCG1α expression in vivo to demonstrate this conclusively. Thus, our findings provide evidence to support the view that the methylation status of specific CpG loci in blood may mark the level of methylation of these CpG dinucleotides in adipocytes. If so, these findings suggest that methylation at this locus may have been induced during early development before the divergence of mesodermal stem cells into adipocyte and hematopoietic lineages. However, these findings cannot rule out the possibility that differential adiposity between children was driven primarily by differences in caloric intake or another environmental factor—or genetic predisposition. Furthermore, differential methylation of PCG1α is a consequence rather than a determining factor of adiposity in children. Nevertheless, whether such associations reflect cause or consequence of variation in adiposity, these epigenetic loci retain utility as potential predictive biomarkers of risk of inappropriate weight gain in children.

Overall, these findings support the view that epigenetic marks measured in childhood that exhibit temporal stability may have utility in predicting future disease risk. Identification of such marks in blood may increase the number of individuals in whom such associations can...
be tested beyond those for whom fetal tissue is available (3) and provide opportunities for further investigation of longitudinal associations and the impact of therapeutic interventions.

Acknowledgments. The authors thank Val Morgan (Plymouth Hospital National Health Service [NHS] Trust, Plymouth, U.K.) and Karen Brookes (Plymouth Hospital NHS Trust) for their technical skills and the EarlyBird children and their parents for their remarkable commitment.

Funding. The EarlyBird Diabetes Study received financial support from the Bright Future Trust, Bupa Health Foundation, Kirby Laing Foundation, Peninsula Foundation, and EarlyBird Diabetes Trust. The laboratory analyses were supported by the National Institute for Health Research Southampton Biomedical Research Centre in Nutrition, which also provided salary support for R.C.-H. and K.M.G. R.C.-H., J.H., and K.A.L. conceived and designed the laboratory experiments and contributed to the first draft of the manuscript. J.H. performed the experiments, and contributed to the first draft of the manuscript. K.A.L. conceived and designed the laboratory experiments and provided substantial input for the first draft of the manuscript. J.P. and K.M.G. contributed to discussions and to editing the manuscript. L.D.V. conceived and designed the cohort study, performed the experiments, and contributed to the first draft of the manuscript. K.A.L. conceived and designed the laboratory experiments and provided substantial input for the first draft of the manuscript. L.D.V. conceived of and designed the cohort study, performed the experiments, and contributed to the first draft of the manuscript. J.H. analyzed data, carried out the mathematical modeling, and provided substantial input into the first draft of the manuscript. J.P. and K.M.G. contributed to discussions and to editing the manuscript and contributed to the first draft of the manuscript. L.D.V. conceived of and designed the cohort study, performed the experiments, and contributed to the first draft of the manuscript. K.A.L. conceived and designed the laboratory experiments and provided substantial input for the first draft of the manuscript. G.C.B. conceived and designed the laboratory experiments and wrote the first draft of the manuscript. K.A.L. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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