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ORIGINAL RESEARCH

Evaluation of Methylation Status of the eNOS Promoter at Birth in Relation to Childhood Bone Mineral Content

Nicholas C. Harvey · Karen A. Lillycrop · Emma Garratt · Allan Sheppard · Cameron McLean · Graham Burdge · Jo Slater-Jefferies · Joanne Rodford · Sarah Crozier · Hazel Inskip · Bright Starling Emerald · Catharine R. Gale · Mark Hanson · Peter Gluckman · Keith Godfrey · Cyrus Cooper

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Abstract Our previous work has shown associations between childhood adiposity and perinatal methylation status of several genes in umbilical cord tissue, including endothelial nitric oxide synthase (eNOS). There is increasing evidence that eNOS is important in bone metabolism; we therefore related the methylation status of the eNOS gene promoter in stored umbilical cord to childhood bone size and density in a group of 9-year-old children. We used Sequenom MassARRAY to assess the methylation status of two CpGs in the eNOS promoter, identified from our previous study, in stored umbilical cords of 66 children who formed part of a Southampton birth cohort and who had measurements of bone size and density at age 9 years (Lunar DPXL DXA instrument). Percentage methylation varied greatly between subjects. For one of the two CpGs, eNOS chr7:150315553 + , after taking account of age and sex, there were strong positive associations between methylation status and the child's whole-body bone area ($r = 0.28$, $P = 0.02$), bone mineral content ($r = 0.34$, $P = 0.005$), and areal bone mineral density ($r = 0.34$, $P = 0.005$) at age 9 years. These associations were independent of previously documented maternal determinants of offspring bone mass. Our findings suggest an association between methylation status at birth of a specific CpG within the eNOS promoter and bone mineral content in childhood. This supports a role for eNOS in bone growth and metabolism and implies that its contribution may at least in part occur during early skeletal development.

Keywords Epigenesis · Methylation · Umbilical cord · eNOS · DXA

Nicholas C. Harvey and Karen A. Lillycrop are joint first author.

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N. C. Harvey · S. Crozier · H. Inskip · C. R. Gale · K. Godfrey · C. Cooper (✉)
MRC Lifecourse Epidemiology Unit, University of Southampton, Southampton, UK
e-mail: cc@mrc.soton.ac.uk

K. A. Lillycrop · E. Garratt · G. Burdge · J. Slater-Jefferies · J. Rodford · M. Hanson · K. Godfrey
Southampton Institute of Developmental Sciences, University of Southampton, Southampton, UK

A. Sheppard · C. McLean · P. Gluckman
Liggins Institute, University of Auckland, Auckland, New Zealand

A. Sheppard · C. McLean
AgResearch, Ruakura Research Centre, Hamilton, New Zealand

B. S. Emerald
Department of Anatomy, Faculty of Medicine and Health Sciences, UAE University, Al ain, United Arab Emirates

P. Gluckman
Singapore Institute for Clinical Sciences, Singapore, Singapore

K. Godfrey
Southampton NIHR Biomedical Research Unit in Nutrition, Diet and Lifestyle, University of Southampton, Southampton, UK

Osteoporosis is a major public health problem, due to the morbidity, mortality, and economic cost associated with the consequent fragility fractures [1]. Evidence is accruing that poor growth in fetal and early postnatal life is a risk factor for osteoporosis and fractures in older age [2]. Although there appears to be a significant genetic contribution to bone development, quantification of this fixed genetic component in several genomewide association studies has accounted for only a small proportion of the overall variance in adult bone

mineral density (BMD) [3, 4]. Experimental manipulation of maternal diet in pregnant animals may lead to changes in bone development in the offspring [5, 6], and recent work has suggested that alterations in epigenetic marking might explain these observations mechanistically [7, 8]. The concept of one genotype giving rise to several potential phenotypes in response to environmental cues is termed "developmental plasticity"; this phenomenon is ubiquitous in the natural world and, in mammals, provides a mechanism by which developmental cues before birth allow the next generation to adjust aspects of their phenotype to promote fitness in their expected later environment [9]. There is thus currently much interest in the role of epigenetic processes in developmental plasticity via graded control of expression of specific nonimprinted genes [9]. We have recently demonstrated associations between childhood body composition and perinatal epigenetic marking of several genes in the umbilical cord [10], including endothelial nitric oxide synthase (eNOS). eNOS has been shown to play a mechanistic role in the function of osteocytes [11], osteoblasts [12], and osteoclasts [13] there is evidence of a positive effect of nitrate use on bone density in clinical populations [14]. We therefore examined whether there were specific relationships between methylation at eNOS sites in the umbilical cord and bone size and density in childhood.

Methods

Subjects

In 1991–1992, Caucasian women at least 16 years old with singleton pregnancies of less than 17 weeks' gestation were recruited at the Princess Anne Maternity Hospital in Southampton, UK [15]; diabetics and those who had undergone hormonal treatment to conceive were excluded. In early (15 weeks' gestation) and late (32 weeks' gestation) pregnancy, a lifestyle questionnaire was administered to the women. Gestational age was estimated from menstrual history and scan data. When the children approached age 9 years, those still living in Southampton were invited to participate in another study. Of 461 invited, 216 (47%) agreed to attend a clinic [16, 17]. In 66 of these subjects genomic DNA was available from umbilical cord samples stored at −80°C and processed using a classical proteinase K digestion and phenol:chloroform extraction. Collection and analysis of umbilical cord samples and follow-up of the children was carried out with written informed consent from all subjects. Investigations were conducted according to the principles expressed in the Declaration of Helsinki, and institutional review board approval was given by the Southampton and South West Hampshire Joint Research Ethics Committee.

Assessment of Bone Size and Density

At age 9 years height was measured using a stadiometer and weight using digital scales (model 835; Seca, Birmingham, UK). The children underwent measurements of whole-body bone mass by DXA (Lunar DPX-L instrument using specific pediatric software, version 4.7c; General Electric, Madison, WI) [16]. The instrument was calibrated every day, and all scans were done with the children wearing light clothing. The short-term and long-term coefficients of variation of the instrument were 0.8% and 1.4%, respectively.

Quantitative DNA Methylation Analysis

Quantitative analysis of DNA methylation was carried out using the Sequenom (San Diego, CA) MassARRAY Compact System (http://www.sequenom.com) at the two sites (chr7:150315553+ and chr7:150315604+) within the eNOS promoter identified in the previous study [10]. Chromosomal coordinates are based on UCSC, human genome March 2006 assembly (hg18).

Sequenom Analysis

Briefly, this involves the gene-specific amplification of bisulfite-treated DNA, followed by in vitro transcription and analysis by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry [18]. DNA (1 μg) was bisulfite-converted using the EZ DNA Methylation kit (Zymo Research, Irvine, CA) per the manufacturer's protocol with Sequenom recommendations (alternative cycling protocol, 100 μl elution volume). PCR primers specific for bisulfite-converted DNA were designed using Methprimer [19]. Each reverse primer contained a T7-promoter tag for in vitro transcription (5′-cagtaatacgactcactatagggagaaggct-3′), and the forward primer was tagged with a 10mer to balance Tm (5′-aggaagagag-3′).

Bisulfite-treated DNA (1 μl) was PCR-amplified in a 5-μl reaction using Qiagen (Valencia City, CA) HotStar Taq Polymerase and 200 nM final primer concentration per Sequenom recommendations. PCR conditions consisted of 94°C for 15 min, followed by 45 cycles of 94°C for 20 s, 52–62°C for 30 s, and 72°C for 1 min. The final PCR step consisted of a 3-min extension at 72°C. No-template controls were included for each amplicon to monitor PCR specificity. Following PCR amplification, the reaction mixture was treated with Shrimp Alkaline Phosphatase (Sequenom) and heat-inactivated, and 2 μl was used as template in a 7-μl simultaneous in vitro transcription/T-cleavage reaction per the manufacturer's instructions (Sequenom). Transcription cleavage products were

desalted by the addition of 20 μl H_2O and 6 mg of CLEAN Resin (Sequenom) and spotted on a 384-pad SpectroCHIP (Sequenom) using a MassARRAY nanodispenser (Samsung, Seoul, South Korea). Mass spectra were acquired using a MassARRAY MALDI-TOF MS (Bruker-Sequenom); and peak detection, signal-to-noise calculations, and quantitative CpG site methylation were performed using proprietary EpiTyper software v1.0 (Sequenom). We excluded from analysis samples that failed to give a reliable PCR product or produced spectra with low confidence scores (<2.9 in EpiTyper). DNA methylation state was calculated by the ratio of methylated to unmethylated fragments. Nonquantifiable or ambiguous CpG units were excluded from analysis.

Statistical Analysis

Birth weight was adjusted for gestational age at birth. The percentage methylation at the CpG sites was measured across the whole tissue sample: at the individual-gene level a site is either methylated or not (i.e., 0 or 100%), but at the whole-sample level the proportion of cells in which an individual site is methylated varies between individuals. Thus, the overall level of methylation may vary in a continuous fashion from 0 to 100%. The methylation values were transformed using a Fisher-Yates transformation to satisfy statistical assumptions of normality, and outcome variables were transformed where necessary using logarithms. Pearson correlation and linear regression were used to examine the relation between epigenetic measurements and child characteristics, adjusting for age at examination where appropriate, using Stata V11 (Statacorp, College Station, TX). The bone outcomes were whole-body bone area (BA), bone mineral content (BMC), areal BMD (aBMD), and size-corrected BMD (estimated volumetric BMD [vBMD]: BMC adjusted for BA, height, and weight to minimize the effect of body size).

Results

Characteristics of Mothers and Children

There were 66 mother–baby pairs with Sequenom and DXA data, among whom 39 (59%) of the offspring were boys. Table 1 summarizes the characteristics of the mothers. These mothers were very similar to mothers of children in the study population as a whole who did not take part in the 9-year follow-up, with no statistically significant differences found for any characteristic. The boys had greater bone mass than the girls (see Table 2), and the outcomes were adjusted for gender of the child.

Table 1 Characteristics of the mothers ($n = 66$)

	Median	IQR
Age at delivery of the baby (years)	28.3	23.5–30.0
Pre-pregnancy weight (kg)	61	55.0–67.0
Mid-upper arm circumference at 32 weeks (cm)	26.9	25.4–29.0
Alcohol intake at 32 weeks (units/day)	0	0.0–0.5
	Mean	SD
Height (cm)	162.9	6.3
	n	%
Smoking at 32-week visit		
No	54	81.8
Yes	12	18.2
Number of times exercise taken at 32 weeks		
None	51	77.3
Once	4	6.1
2–6	9	13.6
7+	2	3
Previous pregnancy		
No	36	54.6
Yes	30	45.5

Methylation Status at eNOS Promoter and Offspring Bone Size and Density at 9 Years

Sequenom analysis revealed a wide range of methylation at the eNOS chr7:150315553 + site, skewed toward higher levels of methylation (median 93%, interquartile range 83–97%, range 56–100%), before transformation to normality using the Fisher-Yates method. After adjusting for the child's gender and age at the DXA scan, there were statistically significant positive relationships between percentage methylation of the eNOS chr7:150315553 + site in umbilical cord and offspring whole-body BA ($r = 0.28$, $P = 0.02$), BMC ($r = 0.34$, $P = 0.005$), and aBMD ($r = 0.34$, $P = 0.005$) at age 9 years. There was a trend toward a positive association between percentage methylation at eNOS chr7:150315553 + and offspring size-corrected BMD, but this did not attain statistical significance ($r = 0.19$, $P = 0.1$). Figure 1 summarizes these relationships, with eNOS methylation represented as quartiles of the distribution. Percentage methylation at eNOS chr7:150315553 + negatively predicted %BMC, but this did not achieve statistical significance ($r = -0.21$, $P = 0.09$). Those children who had been in the highest quartile of umbilical cord eNOS chr7:150315553 + methylation had 94.6 g (equivalent to 0.68 SD) greater BMC and 0.034 g/cm^2 (equivalent to 0.71 SD) greater aBMD than those who had been in the lowest quartile.

Table 2 Characteristics of the children

	All ($n = 66$)		Boys ($n = 39$)		Girls ($n = 27$)		P for gender difference
	Mean	SD	Mean	SD	Mean	SD	
Age at 9-year-old DXA (years)	8.8	0.3	8.9	0.3	8.8	0.3	0.553
WBMH BA (cm^2)	1,070.6	140.0	1,088.8	128.9	1,044.3	153.2	0.207
WBMH BMC (g)	786.4	148.2	813.9	138.5	746.7	155.2	0.07
WBMH aBMD (g/cm^2)	0.73	0.052	0.744	0.051	0.71	0.047	0.008
WBMH vBMD (units)	792.2	38.8	803.0	43.2	776.6	24.9	0.006
WBMH %BMC	3.1	0.3	3.2	0.3	2.9	0.3	<0.001
Proportion eNOS methylation[a]	0.9	0.8–1.0	0.9	0.8–1.0	0.9	0.9–1.0	0.794

[a] Median and IQR

WBMH whole-body minus head, *BA* bone area, *BMC* bone mineral content, *aBMD* areal bone mineral density, *vBMD* estimated volumetric bone mineral density

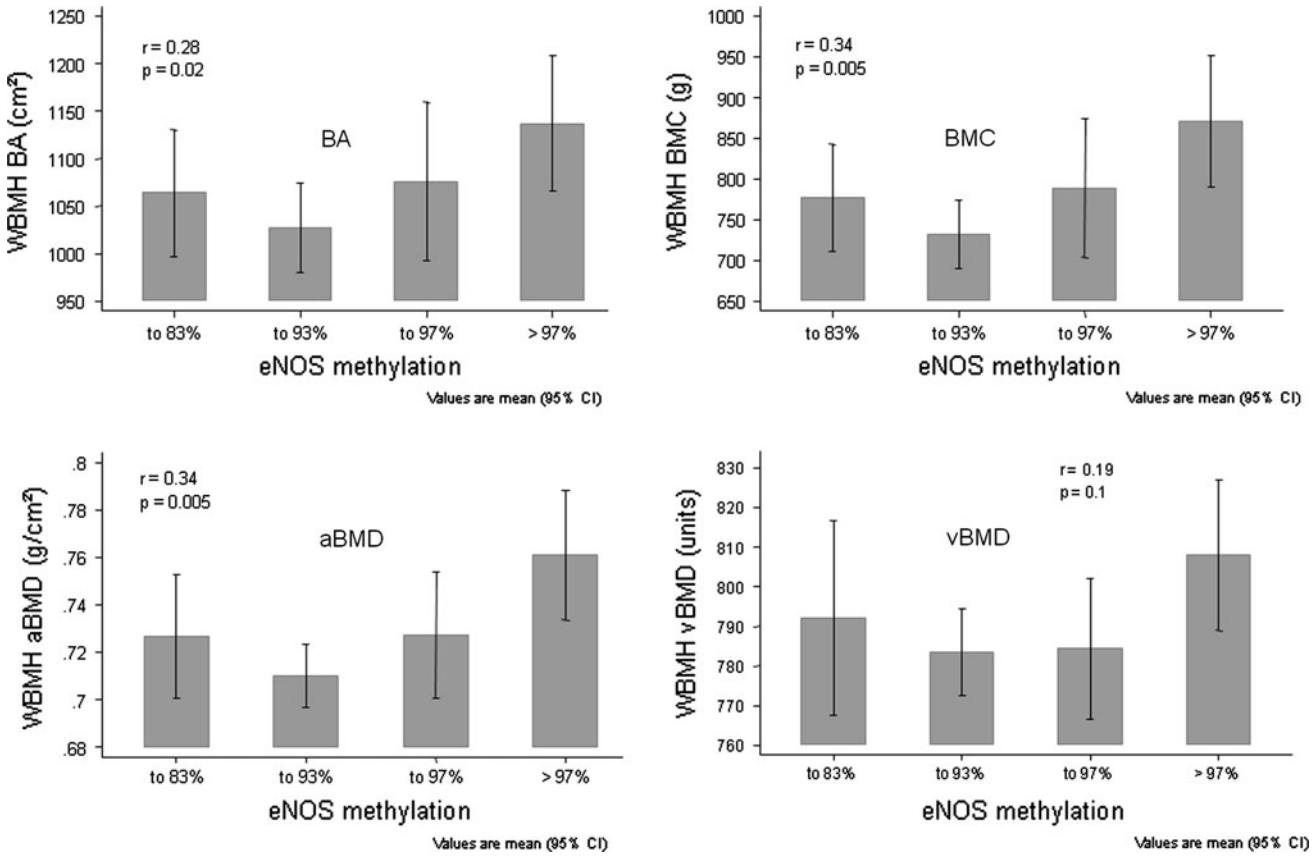

Fig. 1 Percent eNOS promoter methylation (chr7:150315553 +) in umbilical cord and offspring whole-body minus head (WBMH) DXA at 9 years (quarters of the distribution)

To check for consistency, analyses were repeated using Spearman correlation with the untransformed data. The results were very similar to those from the Pearson analyses of Fisher-Yates-transformed variables. Methylation at the other eNOS promoter site measured was not associated with offspring bone size or density. Associations between methylation at the same genomic locations in the eNOS promoter in umbilical cord and childhood bone mass were explored in another cohort of children, drawn from the Southampton Women's Survey. The methods have been previously published [10]. In this group of 6-year-old children, the relationships were weaker and did not achieve statistical significance for either eNOS promoter site.

Methylation Status at eNOS Promoter and Offspring Bone Measurements by Child's Gender

The associations between percent methylation and bone outcomes appeared to be rather stronger in boys than girls (Table 3); however, the formal interaction terms (eNOS methylation * sex) with bone outcomes were not

Table 3 Associations between percent methylation at eNOS chr7:150315553 + and childhood whole-body minus head bone measurements by child's sex: Pearson correlation coefficient and *P* value

	BA		aBMC		BMD		vBMD		%BMC	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
eNOS methylation (all)	0.28	0.02	0.34	0.005	0.34	0.005	0.19	0.1	–0.21	0.09
eNOS methylation (boys)	0.38	0.02	0.45	0.005	0.4	0.01	0.24	0.15	–0.14	0.4
eNOS methylation (girls)	0.15	0.46	0.19	0.34	0.25	0.21	0.07	0.74	–0.33	0.1

BA bone area, *BMC* bone mineral content, *aBMD* areal bone mineral density, *vBMD* estimated volumetric bone mineral density

statistically significant (BA $P = 0.61$, BMC $P = 0.54$, aBMD $P = 0.65$, vBMD $P = 0.45$, %BMC $P = 0.32$).

Maternal Influences

Maternal height, pre-pregnancy weight, mid-upper arm circumference, smoking, alcohol intake, and strenuous exercise in late pregnancy, associated with offspring bone indices in previous studies [20, 21], did not predict eNOS methylation (all $P > 0.05$). Inclusion of these variables in the regression models including eNOS methylation and bone indices did not appreciably alter the observed relationships.

eNOS Methylation, Placental Weight, and Birth Weight

To investigate whether the relationships might be mediated through an effect on overall size, we examined the relationships between eNOS methylation and placental weight and birth weight, both adjusted for gestational age at delivery. Neither of these relationships achieved statistical significance ($r = –0.06$, $P = 0.66$ and $r = 0.09$, $P = 0.48$, respectively). Inclusion of placental weight and birth weight in multivariate regression models did not substantially alter the associations between eNOS methylation and bone indices (Table 4).

Discussion

We have demonstrated for the first time that alteration of epigenetic marking of a specific region of the eNOS promoter in the umbilical cord predicts bone size and density in the offspring in childhood. These associations were present for only one of the two CpG sites measured, suggesting possible site specificity of methylation.

We used a prospective cohort with detailed characterization of mothers and children, using the gold-standard technique to assess bone mass. There are, however, several limitations to our study. Methylation analysis was carried out on samples that had been stored for 9 years, but our local data suggest that DNA methylation is likely to be stable in tissue stored at −80°C, consistent with findings from another study [22]. It remains possible that the associations we have observed are partly due to decay in methylation over time; however, this would require a systematically increased methylation decay in samples of those children at the lower end of the bone mineral distribution, and there is no reason to suppose that this would be the case. Random degradation would be much more likely and would result in a bias toward the null hypothesis. It is possible that our findings may have arisen by chance, but this is always a risk, particularly with an observational study. Indeed in a second mother–offspring cohort in Southampton the relationships between eNOS methylation and bone measures at 6 years were weaker and did not achieve statistical significance. Potential further explanations for these results include the younger age of the children (6 years compared with 9 years) and differences in lifestyle and nutritional factors between the mothers. We view this work as hypothesis-generating, and our findings will need to be tested in more controlled conditions and in larger studies. The CpG site we assessed was 3.5 kb

Table 4 Associations between eNOS methylation (chr7:150315553 +) and whole-body minus head bone outcomes adjusted for birth weight or placental weight, or birthweight and placental weight by inclusion of these variables in the regression models: Pearson correlation coefficient and *P* value

	BA		aBMC		BMD		vBMD		%BMC	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
eNOS methylation, adjusted for sex, DXA age and birth weight	0.27	0.03	0.33	0.007	0.33	0.007	0.19	0.14	–0.21	0.09
eNOS methylation, adjusted for sex, DXA age and placental weight	0.30	0.02	0.36	0.003	0.36	0.003	0.20	0.12	–0.21	0.1
eNOS methylation, adjusted for sex, DXA age, placental weight and birth weight	0.27	0.03	0.33	0.007	0.34	0.006	0.21	0.1	–0.19	0.13

BA bone area, *BMC* bone mineral content, *aBMD* areal bone mineral density, *vBMD* estimated volumetric bone mineral density

upstream of the promoter region, but several studies have reported promoter regulation by sites at this distance [23, 24]. Although the range of methylation associated with differences in childhood bone mineral was relatively narrow, other studies have demonstrated biological effects of a similar methylation range [25]. We analyzed methylation in cells from whole umbilical cord, and thus it is possible that the differential methylation we observed arose from variation in the proportions of different component cells (e.g., fibroblasts, epithelial cells) in individual samples. Measurement of bone mineral in children is hampered by their low absolute BMC. However, we used specific pediatric software, and studies of DXA indices compared to ashed mineral content in piglets have confirmed the accuracy of the technique [26]. The study cohort was a subset of the original mother–offspring group, but mothers whose children underwent DXA scanning and those whose children did not were very similar, with no statistical differences found for any maternal characteristics. Finally, the use of DXA does not allow measurement of true volumetric bone density, thus making it difficult to be certain about differential determinants of skeletal size and volumetric density.

eNOS has been shown to be expressed in umbilical cord vessels [27, 28] and upregulated in response to reduced umbilical blood flow [28], e.g., with intrauterine growth retardation. A few studies have suggested possible epigenetic regulation of eNOS in umbilical cord [29–31]. eNOS is expressed in osteocytes [11], osteoblasts [12], osteoclasts [13], and bone vasculature [32–34] use of nitrate medications has been correlated positively with BMD in humans [14]. Thus, there are several possible biological mechanisms which could underlie an association between perinatal eNOS methylation and later bone indices.

First, our observations could represent an effect of modulation of umbilical cord blood flow on the development of fetal body composition. Thus, a reduced placental perfusion might lead to a compensatory increase in eNOS expression [28] through alteration of methylation at its promoter, acting to minimize the adverse effect on fetal development. The direction of the effect would depend on the extent to which the compensatory mechanisms could offset the consequences of reduced nutrient supply. This mechanism should influence offspring bone mass through an effect on overall body size or composition. However, we did not find any association between eNOS promoter methylation and birth weight or placental weight; indeed, inclusion of birth weight or placental weight into the models did not alter the results. These data, taken together with our finding that eNOS promoter methylation was not statistically significantly related to percent BMC, therefore make this mechanism unlikely.

Given the very short half-life of NO in the circulation [35], a distant bone action for NO produced in umbilical cord seems highly unlikely, but there could be coregulation of eNOS expression within umbilical cord and bone cells. In this case the changes seen within umbilical cord would simply be markers of changes in osteoblasts, osteoclasts, or osteocytes, or more widely in the body. Although there is evidence that eNOS is expressed within all these cell types, it is very unclear what the overall effect of NO synthesis within bone cells is. Thus, BMD is increased in mice only when all three forms of NOS are knocked out [12], and NO has been implicated in osteoclast fusion [13]. NO may be involved in the osteocyte signaling in response to physical strain [11]. Additionally, NO has been implicated in chondrocyte development in the growth plate [36, 37]. Thus, the consequences of increased NO seem to differ depending on cell type and situation. It is unclear whether particular epigenetic changes represent a global increase in methylation or are tissue- and/or site-specific, but our findings of association between only one of two eNOS promoter CpG sites and other current data suggest the latter [31, 38], making any direct extrapolation to other tissue types speculative. Additionally, it is difficult to think of reasons why eNOS (i.e., the endothelial form) in umbilical cord vasculature should be coregulated with NO production in bone stromal cells.

A third, and potentially the most likely, possibility is that eNOS in umbilical cord vasculature is coregulated with eNOS in the vasculature within the epiphysis of the growing long bones. The distal parts of long bones are highly vascular, to enable sufficient nutrition for bone development. There are very few data relating to eNOS or NO specifically within bone vasculature, but the available studies indicate that this NO does play a role in regulation of bone blood flow [32–34, 39]. To our knowledge no studies have examined possible altered epigenetic marking of eNOS within blood vessel cells in bone, but the idea of coregulation of NO production in blood vessels in two areas of the body seems intuitively reasonable.

We previously demonstrated that maternal adiposity, smoking, physical activity, and parity all predict offspring bone size and geometry [20, 21]. However, none of these factors were associated with methylation of the eNOS promoter in the current study, although altered methylation of the eNOS promoter did seem to correlate better with markers of bone size (BA, BMC) than with the estimate of volumetric density. Clearly, further work will be needed to elucidate the direction of any effect on eNOS expression of methylation at this site in the eNOS promoter and to clarify whether these changes are site-specific or markers of changes in bone cells or vasculature.

Those children in the highest quartile of eNOS methylation had 0.66 SD greater BMC and aBMD than those in the lowest, a difference which, if maintained until peak bone mass is achieved, would equate to around a 50% difference in fracture risk in older age [40]. Thus, whatever

the underlying mechanisms, our results are likely to be biologically relevant. They clearly demonstrate that alteration of epigenetic marking in utero is associated with bone outcomes in the offspring, confirming a role for epigenetic regulation of the genome in influencing this aspect of development in addition to that of fixed genetic variation.

In conclusion, we have demonstrated that perinatal alteration of epigenetic marking within the promoter region of eNOS in umbilical cord was associated with bone size and, to a lesser extent, volumetric density of the offspring at 9 years old and that these associations were independent of previously identified determinants of offspring bone mass. The results may be potentially informative in the development of early markers of risk of later disease and might suggest avenues for future interventions.

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