Published in final edited form as:

Calcif Tissue Int. 2012 February ; 90(2): 120–127. doi:10.1007/s00223-011-9554-5.

Evaluation of methylation status of the eNOS promoter at birth in relation to childhood bone mineral content

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Abstract

Aim—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.

Methods—We used Sequenom MassARRAY to assess the methylation status of 2 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).

Results—Percentage methylation varied greatly between subjects. For one of the two CpGs, eNOS chr7:150315553+, after taking account of age and sex there was a strong positive association 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.

Conclusions—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

Epigenetic; methylation; umbilical cord; eNOS; DXA

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Disclosure of financial conflict of interest

All authors report no disclosures

INTRODUCTION

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 genome wide association studies has only accounted for a small proportion of the overall variance in adult bone mineral density[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 non-imprinted genes[9]. We have recently demonstrated associations between childhood body composition and perinatal epigenetic marking of several genes in 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] and there is evidence of a positive effect of nitrate use on bone density in clinical populations[14]. We therefore aimed to examine whether there were specific relationships between methylation at eNOS sites in 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 wk 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 the 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 (SECA model no. 835). The children underwent measurements of whole body bone mass by DXA (Lunar DPX-L instrument using specific pediatric software, version 4.7c; GE Corp., 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 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 co-ordinates 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]. 1µg of DNA was bisulfite converted using EZ DNA Methylation kit (Zymo Research) as per the manufacture's protocol with Sequenom recommendations (alternative cycling protocol, 100µL 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'-cagtaatacgcactcactataggagaaggct-3'), and the forward primer was tagged with a 10mer to balance Tm (5'-aggaagagag-3'). 1µL of bisulfite-treated DNA was PCR amplified in a 5µL reaction using Qiagen HotStar Taq Polymerase and 200nM final primer concentration as per Sequenom recommendations. PCR conditions consisted of 94°C for 15mins followed by 45 cycles of 94°C for 20 seconds, 52-62°C for 30 seconds and 72°C for 1 min. The final PCR step consisted of a 3min 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), heat inactivated, and 2µL was used as template in a 7µL simultaneous *in vitro* transcription/T-cleavage reaction as per manufacturer's instructions (Sequenom). Transcription cleavage products were desalted by the addition of 20µL H₂O and 6mg of CLEAN Resin (Sequenom) and spotted on a 384-pad SpectroCHIP (Sequenom) using a MassARRAY nanodispenser (Samsung). Mass spectra were acquired using a MassARRAY MALDI-TOF MS (Bruker-Sequenom) and peak detection, signal-to-noise calculations and quantitative CpG site methylation was 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. Non-quantifiable 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 (ie 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, Texas, USA). The bone outcomes were whole body bone area (BA), bone mineral content (BMC), areal bone mineral density (aBMD) and size-corrected bone mineral density (estimated volumetric BMD (vBMD): BMC adjusted for BA, height and weight to minimise the effect of body size).

RESULTS

Characteristics of mothers and children

There were 66 mother-baby pairs with Sequenom and DXA data, among which 39 (59%) of the offspring were boys. Table 1 summarises 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.

Methylation status at eNOS promoter and offspring bone size and density at 9 years

The Sequenom analysis revealed a wide range of methylation at the eNOS chr7:150315553+ site, skewed towards 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 bone area ($r=0.28$, $p=0.02$), bone mineral content ($r=0.34$, $p=0.005$), areal bone mineral density ($r=0.34$, $p=0.005$) at age 9 years. There was a trend towards 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 summarises 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 94.6g (equivalent to 0.68 SD) greater BMC and 0.034g/cm² (equivalent to 0.71 SD) greater aBMD than those who had been in the lowest quartile.

To check for consistency the 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 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, and 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 birthweight

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 birthweight, 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 birthweight 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 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 characterisation 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 towards 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 upstream of the promoter region, but there are several studies reporting promoter regulation by sites at this distance[23, 24]. Although the range of methylation associated with differences in childhood bone mineral was relatively narrow, there are other studies demonstrating biological effects of a similar methylation range[25]. We analysed 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 (for example fibroblasts, epithelial cells) in individual samples. Measurement of bone mineral in children is hampered by their low absolute BMC. However, we used specific paediatric 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 to be upregulated in response to reduced umbilical blood flow[28], for example 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] and osteoclasts[13], and bone vasculature[32-34], and use of nitrate medications have been

shown to correlate 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 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 birthweight or placental weight; indeed inclusion of birthweight 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, secondly, there could be co-regulation 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 only increased in mice 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 co-regulated with NO production in bone stromal cells.

A third, and potentially most likely, possibility is that eNOS in umbilical cord vasculature is co-regulated 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 there have been no studies which have examined possible altered epigenetic marking of eNOS within blood vessel cells in bone, but the idea of co-regulation of NO production in blood vessels in two areas of the body seems intuitively reasonable.

We have 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 till peak bone mass were 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.

Acknowledgments

NCH and KAL are joint first authors. We thank the mothers and their children who gave us their time; and a team of dedicated research nurses and ancillary staff for their assistance. This work was supported by grants from the Medical Research Council, British Heart Foundation, Arthritis Research Campaign, National Osteoporosis Society, International Osteoporosis Foundation, Cohen Trust, Southampton NIHR Biomedical Research Unit in Nutrition, Diet and Lifestyle, and NIHR Musculoskeletal Biomedical Research Unit, University of Oxford. Participants were drawn from a cohort study funded by the Medical Research Council and the Dunhill Medical Trust. We thank Mrs G Strange for helping to prepare the manuscript.

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Figure 1.
Evaluation of methylation.

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 per day)	0	0.0-0.5
	Mean	SD
Height (cm)	162.9 n	6.3 %
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

Table 2

Characteristics of the children.

	<i>All (n=66)</i>		<i>Boys (n=39)</i>		<i>Girls (n=27)</i>		<i>P for gender difference</i>
	<i>Mean</i>	<i>SD</i>	<i>Mean</i>	<i>SD</i>	<i>Mean</i>	<i>SD</i>	
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)	1070.6	140.0	1088.8	128.9	1044.3	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*	0.9	0.8-1.0	0.9	0.8-1.0	0.9	0.9-1.0	0.794

WBMH=Whole body minus head; BA= Bone area; BMC=Bone mineral content; aBMD=Areal bone mineral density; vBMD=Estimated volumetric bone mineral density.

* median and IQR

Table 3

Associations between percent methylation at eNOS chr7:150315553+ and childhood whole body minus head bone measurements by child's sex. Table shows the 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.

Table 4

Associations between eNOS methylation (chr7:150315553+) and whole body minus head bone outcomes adjusted for birthweight, placental weight or birthweight and placental weight by inclusion of these variables in the regression models. Table shows the 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.