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**Early life dietary and epigenetic influences on childhood musculoskeletal health: Update on the UK component of the *ALPHABET* project**

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**Abstract**

The *ALPHABET* project, funded through the European Research Area Healthy Diet for a Healthy Life (ERA-HDHL) Biomarkers call, aims to expand the knowledge base regarding interactions between diet, epigenetics and offspring health, characterising biomarkers that may inform future health strategies. This review focuses on the UK Biotechnology and Biological Sciences Research Council (BBSRC) -funded component in which the aim is to 1) generate and collate early life epigenetic data; and 2) investigate early diet and epigenetic marks as predictors of later bone health. The project builds on a wealth of evidence implicating environmental factors, such as maternal diet and body composition, as influences on the long-term health and development of the offspring, and that these relationships might be mediated at least in part through epigenetic signals. Experimental studies in animal models have demonstrated that manipulation of maternal diet during pregnancy leads to altered offspring epigenetic marking and phenotype. Human studies convincingly demonstrate associations between early environment and later health and disease for outcomes across musculoskeletal, respiratory, neurodevelopmental and cardiometabolic health. The priority now is to find ways in which such observations can be translated into improved lifelong health. A key approach is to identify early biomarkers of adverse health outcomes and then to test these, and subsequent interventions, in trials aimed at identifying strategies to optimise health throughout the life course. The *ALPHABET* project will inform this process for musculoskeletal outcomes, and the project as a whole should help elucidate not just novel mechanisms, but also potential strategies to reduce the burden of musculoskeletal, respiratory, neurodevelopmental and cardiometabolic disease in future generations.

***Keywords***

Maternal, nutrition, epigenetics, developmental, programming, bone

**Introduction**

The prevalence of obesity, cardiometabolic disease, asthma, osteoporosis and neurodevelopmental disorders have risen over recent decades (Vos *et al.* 2015). However, such increases in these non-communicable diseases cannot be fully explained by genetic or adult lifestyle factors – indeed there is increasing evidence to suggest that early life exposure to environmental factors, even before conception, influences health in older age (Godfrey *et al.* 2015a). The Developmental Origins of Health and Disease (DOHaD) hypothesis suggests that transient environmental exposures during critical periods of development (such as poor nutrition during fetal and early infant phases of life) can elicit lifelong effects on offspring health, through changes in gene expression both *in utero* and throughout life (Barker 1995; Godfrey & Barker 2000).

It is widely recognised that genes provide a library of information that can be read (expressed) differently, in different cells and at different times, according to function and need. In a single organism, although the genetic code contained in every somatic cell is the same, the genes expressed will vary widely from organ to organ, and even from cell to cell, often in response to environmental cues (Gluckman *et al.* 2008). This regulation of gene expression involves a range of epigenetic processes whereby the DNA is modified to influence its expression, without changes in the genetic code itself. Epigenetic adaptations to suboptimal nutrition during pregnancy or early childhood may perpetuate changes in the health of the offspring many years after the exposure, and the consequences may even be seen in future generations (Burdge *et al.* 2011). Therefore, early life is a window in which interventions may help to prevent obesity, related cardiometabolic conditions and other chronic non-communicable diseases such as osteoporosis and asthma.

Modifications to maternal diet may be one such change in behaviour, which could play an important role in maternal, neonatal and child health outcomes. The urgent need to optimise health during early life, for example preconception/during pregnancy and in the subsequent infancy of the offspring, has been emphasised by the World Health Organization (WHO) in its comprehensive implementation plan on maternal, infant and child nutrition (WHO 2014b) and more locally by the WHO European Food and Nutrition Action Plan 2015-2020 (WHO 2014a). The WHO aims to achieve a 30% reduction in low birthweight babies and no increase in the prevalence of children who are overweight. Both low and high birthweight are associated with fetal and neonatal mortality and increased risk of later life non-communicable disease (Godfrey *et al.* 2015a).

Funded through the European Research Area Healthy Diet for a Healthy Life (ERA-HDHL) Biomarkers call, the overarching aim of the *Early life programming of childhood health: a nutritional and epigenetic investigation of* ***a****diposity and* ***b****one,* ***c****ardiometabolic, neuro****d****evelopmental and respiratory health* (*ALPHABET)* project (led by Dr Catherine Phillips, University College Dublin) is to understand the specific dietary nutrients needed during pregnancy for optimal fetal growth and development, and to identify epigenetic processes responsible for linking maternal and childhood diet with future health and ageing. Whilst the full project will use data from a number of cohorts across Europe (see Appendix), and collaboration to generate novel dietary indices, this review will focus on the UK Biotechnology and Biological Sciences Research Council (BBSRC) -funded component of the overall project, the aim being to use the state-of-the-art in early life origins of bone health as an exemplar of the approach and the potential health benefits for the project as a whole.

**Epigenetic mechanisms and developmental plasticity**

Developmental plasticity, by which a single genotype may give rise to several different phenotypes in response to the prevailing environmental conditions, is found throughout the natural world. This process allows the next generation to be born appropriately adapted to the expected external environment, using cues from the organism’s surroundings acting during critical periods of development (Hanson & Gluckman 2014; Curtis *et al.* 2018b). A widely reported example is the meadow vole *(Microtus pennsylvanicus)*, in which the thickness of the coat in the offspring is determined by the number of hours of light and dark experienced by the mother during gestation. Pups born in autumn have a thicker coat than those born in spring (Lee & Zucker 1988), therefore adopting a developmental trajectory that is appropriate to the postnatal environment to which the meadow vole is likely to be exposed after birth. Maternal melatonin levels during pregnancy are the signal to the pup of the prevailing environmental conditions (Lee *et al.* 1989). However, it is easy to imagine how a mismatch between the expected postnatal environment and that to which the pup has been developmentally programmed would lead to a survival disadvantage (*e.g.* due to a change in the postnatal environment or inappropriate maternal cues) (Godfrey *et al.* 2007).

Various experimental studies have shown that alterations to maternal diet during pregnancy may lead to changes in offspring phenotype and gene expression (Lillycrop *et al.* 2005b; Burdge *et al.* 2007; Keleher *et al.* 2018; Zhang *et al.* 2017), and such effects may be underpinned by epigenetic mechanisms. They can be conserved across multiple generations but also can be reinstated *de novo* in each generation (Hanson & Skinner 2016; Burdge *et al.* 2011). There is some evidence of associations between nutritional challenges in pregnancy and phenotypic effects on the grandchildren, though this does not prove a transgenerational effect as epigenetic effects can be induced in the primordial germ cells of the F1 during F0 pregnancy and produce effects in the F2 generation (Jaenisch & Bird 2003; Grossniklaus *et al.* 2013). However, the epigenome can be regarded as a molecular record of life events, accumulating throughout a lifetime. Monozygotic twins are epigenetically most similar at birth, diverging with age, with the degree of divergence determined by the commonality of their environments (Fraga *et al.* 2005). It is clear that an understanding of these epigenetic processes has the potential to enable early intervention strategies to improve child development and later health; as a consequence, the study of epigenetic biomarkers is a rapidly advancing field (Godfrey *et al.* 2015b).

The three main epigenetic mechanisms are DNA methylation, histone modifications and non-coding RNAs, as summarised in Figure 1 (Jones *et al.* 2008; Gicquel *et al.* 2008; Tang & Ho 2007; Gluckman *et al.* 2008).



**Figure 1: The main types of epigenetic modifications.**

Epigenetic modifications are superimposed on the base sequence of DNA in multiple layers, which differ according to cell and tissue type. These include: 1) modification of nucleosides in DNA by methylation and hydroxymethylation, usually at cytosine adjacent to guanine bases (CpG sites); 2) post-translational modification of histone proteins, such as by methylation and acetylation can contribute information to chromatin remodelling machinery (this determines how the chromatin is packaged, leading to ravelling and unravelling of DNA, therefore genes and loci encoding non-coding RNAs become susceptible to transcription); and 3) small non-coding RNAs (such as micro RNAs) regulate gene expression by prompting mRNA degradation or modulating protein translation. Figure reproduced with permission from Jones *et al.* (2008)

**DNA methylation**

This review will focus on DNA methylation as it is the most widely studied of epigenetic modifications and is the target of investigation in the *ALPHABET* project though, of course, the epigenetic processes of histone modifications and micro RNAs work in concert with DNA methylation to control gene expression (Curtis *et al.* 2018b). DNA methylation involves the transfer of a methyl group to the 5′ carbon position of cytosine, creating 5-methylcytosine (5-mC) (Kumar *et al.* 1994). Though methyl marks can be added and removed throughout the life course, it is a relatively stable epigenetic mark that can be transmitted through DNA replication during mitosis (Bird 2002). Methylation of the cytosine base usually occurs within the dinucleotide sequence CpG, where a cytosine is immediately 5′ to a guanine, with a phosphate group between them denoted by ‘p’, although non-CpG methylation is also prevalent in embryonic stem cells (Ramsahoye *et al.* 2000). A CpG site can either be methylated or unmethylated in an individual cell; however, methylation is not completely uniform, even within a tissue or cell type. According to the level of methylation across a whole tissue where a particular site may be methylated or unmethylated in a large number of cells, a range of graded gene expression from 0% to 100% is possible (Gluckman *et al.* 2008).

Given their importance in transcriptional regulation, as would be expected, CpG dinucleotides are not distributed at random throughout the genome. They are clustered at the 5′ end of genes in regions known as CpG islands, with hypomethylation generally associated with gene activation and hypermethylation with gene silencing (Song *et al.* 2005). Transcriptional repression in hypermethylated regions occurs through blocking of transcription factors binding to the DNA, or through recruitment of a myriad of other repressive factors, such as methyl CpG binding protein 2 (MeCP2), which in turn mediate local chromatin changes to impair transcription factor binding (Fuks *et al.* 2003).

CpG methylation patterns are largely established during embryogenesis, fetal and perinatal life. DNA methylation marks on the maternal and paternal genomes are largely erased on fertilisation (with the exception of the imprinted genes and other specific genomic regions), followed by a wave of de novo methylation within the inner cell mass just prior to blastocyst implantation (Okano *et al.* 1999; Santos *et al.* 2002). DNA methyltransferases (DNMT) 3a and 3b (Santos *et al.* 2002) catalyse *de novo* DNA methylation, and methylation patterns are maintained through mitosis in differentiated tissues by methylation of hemi-methylated DNA by DNA methyltransferase 1 (DNMT1) (Bacolla *et al.* 1999). However, DNA methylation patterns are not necessarily maintained throughout life as was initially thought: in 2009 the existence of another epigenetic modification, 5-hydroxymethylcytosine (5hmC), was described as present in high levels in neurons and embryonic stem (ES) cells (Tahiliani *et al.* 2009). 5-mC may be oxidised to 5hmC by the enzymes of the TET (Ten-Eleven-Translocation) family (Ito *et al.* 2011) and has been proposed to act as a specific epigenetic mark opposing DNA methylation, as well as a passive intermediate in the demethylation pathway (Guibert & Weber 2013; Wen & Tang 2014).

**Modifications to the epigenome through early life nutrition**

*Animal studies*

Evidence is accruing that DNA methylation is modifiable, and a number of environmental factors such as nutrition, stress, placental insufficiency, endocrine disruptors and pollution, especially in early life, can alter the epigenome leading to long-term phenotypic changes in the offspring (Feil & Fraga 2011). A classic example of nutrition altering phenotype from the animal kingdom is that of the honeybee. Though genetically identical, female honeybee larvae incubated in the presence of royal jelly predominantly develop into queen bees, while those incubated in the absence of royal jelly develop into sterile worker bees (Maleszka 2008; Kucharski *et al.* 2008). Knockdown of DNA methyltransferase 3 (DNMT3), the major DNA methyl transferase in bees, was shown to increase the proportion of larvae developing into queen bees in comparison with sterile workers, indicating the role of DNA methylation in this process (Kucharski *et al.* 2008).

In mammals, a variety of studies have been carried out demonstrating the role of diet on phenotype, such as that of the agouti (Avy) mouse in which the coat colour is determined by the methylation status of an intracisternal-A particle (IAP) in the 5’ upstream region of the agouti gene. When the pregnant female mice were fed a diet supplemented with folic acid, cobalamin, choline and betaine, a graded shift in coat colour in the offspring occurred from mainly yellow (agouti) to brown (pseudo-agouti) (Waterland & Jirtle 2003). A change in methylation was detected as a result of this change in diet, with hypermethylation of seven CpG dinucleotides 600 bp downstream of the Avy IAP insertion site. Diet-induced changes in the offspring epigenome and metabolic health have been demonstrated in rats; feeding pregnant rats a protein rich diet was shown to induce hypomethylation of the glucocorticoid receptor and peroxisome proliferator activated receptor (PPAR)α promoters in the livers of juvenile and adult offspring, and this was accompanied by an increase in glucocorticoid receptor and PPARα expression and in the metabolic processes that they control (Lillycrop *et al.* 2005a; Lillycrop *et al.* 2007; Burdge *et al.* 2004). In comparison, the offspring of nutritionally restricted pregnant rats were shown to have increased levels of DNA methylation of PPARα and the glucocorticoid receptor in the liver (Gluckman *et al.* 2007), suggesting that the effects of maternal nutrition on the epigenome of the offspring depend upon the nature of the maternal nutrient challenge, and that they may provide a means of adapting to an adverse environment (Gluckman *et al.* 2005). High fat diets versus low fat diets in mice have similarly been shown to induce different methylation patterns – for example, hypomethylation of the μ-opioid receptor (MOR) and preproenkephalin (PENK) in the nucleus accumbens, prefrontal cortex and hypothalamus of offspring mice from dams that consumed a high fat diet during pregnancy (Vucetic *et al.* 2010). Various other examples can be found in mice, with sex-specific differences in terms of both exposure on the male or female parent and effects on the offspring. For example, a paternal high fat diet impacted glucose tolerance, pancreatic islet gene expression and hypomethylation of the anti inflammatory gene IL-13 receptor subunit alpha-2 *(Il13ra2*) in female offspring (Ng *et al.* 2010; Barres & Zierath 2016).

*Human studies*

Evidently, studies in animal models, where genetic variation in the subjects and diet pre- and post-pregnancy can be tightly controlled, have provided evidence of long-term effects of maternal nutrition on the offspring epigenome. Work in humans is more limited, though epidemiological studies on populations following periods of famine have provided important data. The study of the Dutch famine, or ‘Hunger Winter’, 1944, demonstrated alterations in the methylation of a number of genes in DNA isolated in whole blood from individuals whose mothers were exposed to famine. The timing of the nutritional constraint appeared to be important, as exposure to famine around the time of conception and in early gestation was associated with a small decrease in offspring CpG methylation of the imprinted IGF2 gene and an increase in methylation of leptin, IL-10, MEG3 and ABCA4 (Tobi *et al.* 2009; Heijmans *et al.* 2008), while late gestation famine exposure had no effect on methylation (Tobi *et al.* 2015). *In utero* experience of famine at any period in gestation led to an elevation in glucose levels and impaired glucose tolerance in adulthood, measured some 60 years after the famine exposure (Ravelli *et al.* 1998). Further links between type 2 diabetes risk and food restriction have been demonstrated in people with *in utero* famine exposure during the Chinese famine (1959-1961) (Li *et al.* 2017), the Ukranian famine (1932-1933) (Lumey *et al.* 2015) and Austrian famines (1918-1919, 1938 and 1946-1947) (Thurner *et al.* 2013). DNA methylation analysis in whole blood of individuals exposed to the Dutch famine demonstrated that DNA methylation mediated the association between famine exposure, adult body mass index (BMI) and serum triglycerides, but not with glucose (Tobi *et al.* 2018). The specific mechanisms behind this await elucidation.

In healthy human populations, studies of dietary maternal supplementation have been shown to have long lasting effects in the offspring. For example, supplementation with 400 µg per day of folic acid around the time of conception has been shown to alter methylation of specific CpG sites in the IGF2 gene in the peripheral blood cells of children (Steegers-Theunissen *et al.* 2009). Evidence also points towards plasticity in the human epigenome persisting into adulthood, as, for example, short-term high fat overfeeding in healthy young men was shown to induce methylation changes in over 6000 skeletal muscle genes, with only partial reversal after 6-8 weeks of a normocaloric diet (Jacobsen *et al.* 2012).

**DNA methylation and bone development: potential roles in later life osteoporosis**

*Early growth and bone development*

Osteoporosis is a common skeletal disorder characterised by low bone mass and loss of the normal bone microarchitecture, leading to increased bone fragility and therefore susceptibility to fracture (Consensus Report 1993). With the globally ageing population, the burden of osteoporosis is increasing, with a consequent increase in fragility fractures worldwide (Oden *et al.* 2015). Such fractures typically occur at the hip, spine, wrist, humerus, pelvis, scapula and ribs. Most major osteoporotic fractures are associated with substantial morbidity and mortality, particularly for hip fractures, with an excess mortality of 10%-20% in the first year after fracture, and a similar proportion requiring institutional care in the same period (Harvey *et al.* 2010a). Such fractures are very common - currently, the remaining lifetime risk of a fragility fracture in a UK woman aged 50 years is estimated at around 50%, and around 20% for a UK man (Harvey *et al.* 2010a; Curtis *et al.* 2016).

A strong predictor of later risk of osteoporotic fracture is an individual’s peak bone mass, the maximum total skeletal mass accrued at the completion of skeletal development (Harvey *et al.* 2014a). Bone mass increases throughout fetal, infant, childhood and early adult life reaching a peak in the third to fourth decade, and this peak bone mass has been shown in mathematical modelling studies to be a more powerful predictor of the age of osteoporosis development than age at menopause or rate of subsequent age-related bone loss (Hernandez *et al.* 2003). Peak bone mass is of course partly explained by genetic factors as demonstrated by various genome wide association studies (Duncan *et al.* 2011; Mullin *et al.* 2016). Known genetic loci account for a small percentage of the overall variance, but next generation sequencing approaches may permit further characterisation of this ‘missing heritability’ such as the identification of rare genetic variants of stronger effect. Other factors that may contribute to links between early development and later health and disease include epigenetic effects and environmental influences.

Seminal work by David Barker and Clive Osmond in the 1980s, demonstrating that low birthweight is associated with increased risk of cardiovascular disease later in life (Barker & Osmond 1986), was later applied to the field of osteoporosis by Cyrus Cooper. Weight in infancy was shown to correlate with adult bone mineral content in a variety of UK studies based in Bath, Sheffield and Hertfordshire (Cooper *et al.* 1995; Cooper *et al.* 1997; Gale *et al.* 2001). These findings, and those of several subsequent studies, were confirmed in a meta-analysis showing that overall each 1 kg increase in birthweight is associated with a 1.49 g increase in bone mineral content (BMC) at the lumbar spine and 1.41 g at the hip in adulthood. This effect was shown to be independent of adult weight and BMI (Baird *et al.* 2010). Furthermore, poor childhood growth and weight gain was associated with a greater risk of hip fracture in adulthood, further providing evidence that early nutrition is important for future skeletal development (Cooper *et al.* 2001; Javaid *et al.* 2011).

Mother-offspring cohorts have provided an opportunity for more detailed investigation into patterns of early growth (Harvey *et al.* 2014a; Harvey *et al.* 2012c; Harvey *et al.* 2010c) and specific maternal factors which might influence offspring development. The *Southampton Women’s Survey* (*SWS*) (Inskip *et al.* 2005), a British prospective cohort of 12 583 initially non-pregnant women aged 20-34 years and their subsequent offspring (*n*=3156), is an example of a cohort which has provided important insights into the relationships between maternal factors and offspring bone mass. Low maternal fat stores, first pregnancy, smoking and high levels of physical activity during late pregnancy were all associated with reduced whole body BMC at birth (Harvey *et al.* 2010b), confirming findings from an earlier smaller study (Godfrey *et al.* 2001).

*Epigenetic predictors of childhood bone health*

Using an established discovery pathway, from array to candidate, epigenetic predictors of bone development have been discovered (Curtis *et al.* 2018a). Analyses of DNA methylation in umbilical cord samples from the *Princess Anne Hospital Cohort* and the *SWS* (Godfrey *et al.* 2011) have identified two key loci linked to bone outcomes: *CDKN2A* (Godfrey *et al.* 2011; Lillycrop KA 2013; Murray *et al.* 2016; Curtis *et al.* 2017) and retinoid X receptor-alpha (*RXRA)* (Harvey *et al.* 2014d).

The *CDKN2A* locus encodes two cell cycle inhibitors: p14ARF and P16INK4a, which play roles in cellular senescence and ageing. The *CDKN2A* locus also encodes the long non-coding RNA ANRIL (antisense non-coding RNA in the *INK4* locus), a 3834bp transcript which can negatively regulate *p16INK4a*. Single nucleotide polymorphisms (SNPs) within the CDKN2A locus, particularly those located within *ANRIL,* have been associated with cardiovascular disease, type 2 diabetes and frailty (Congrains *et al.* 2012), and DNA methylation at this locus has recently been demonstrated to vary with age (Bell *et al.* 2016). Previous studies have demonstrated links between perinatal *CDKN2A* methylation and later adiposity (Lillycrop *et al.* 2017); the functional relationships between fat and bone are well characterised, and mediated via both mechanical and endocrine pathways (Johansson *et al.* 2014). More recently, supported by the BBSRC project, we have shown that DNA methylation at CpG sites within the *CDKN2A* gene was associated with offspring bone mass at age 4 and 6 years (Curtis *et al.* 2017) (Figure 2), with greater levels of methylation at the CDKN2A locus negatively associated with whole body minus head bone area, bone mineral content and areal bone-mineral density. This was confirmed in replication and combined data sets (all *p*<0.01), with each 10% increase in methylation being associated with a decrease in BMC of 4-9 g at age 4 years (*p*≤0.001). Relationships were similar with bone mass at 6 years, and functional investigations in a cell line demonstrated that methylation in this region could be important for transcription factor binding.



**Figure 2**

Perinatal methylation at CpG sites within the *CDKN2A* locus is associated with offspring bone area (BA), bone mineral content (BMC) and bone mineral density (BMD) at age 4 years in the *Southampton Women’s Survey*, *n*=555. Reproduced with permission (Curtis *et al.* 2017)

Secondly, in the *SWS*, methylation at several CpG sites around 2kb upstream from the promoter region of the *RXRA* gene in umbilical cord was correlated with lower offspring bone mineral content corrected for body size at 4 years old (β= -2.1 to -3.4 g/SD, *p*=0.002 to 0.047), with the results supported by findings from a second independent cohort, the *Princess Anne Hospital Study* (Harvey *et al.* 2014d). Intriguingly, methylation at one CpG site was related to an estimate of free 25(OH)-vitamin D [25(OH)D] (Figure 3). *RXRA* is of particular interest in the context of bone health as it is an essential part of vitamin D signalling, forming a heterodimer with the vitamin D receptor in the nuclear action of 1,25(OH)2-vitamin D, as well as with other bone-active nuclear hormones. Vitamin D plays a central role in calcium and phosphate homeostasis, and severe vitamin D deficiency can result in rickets, osteomalacia and neonatal hypocalcaemia*.* There is increasing evidence of a link between maternal gestational 25(OH)D status and offspring bone mass (Harvey *et al.* 2014b; Harvey *et al.* 2013; Harvey *et al.* 2006; Sayers & Tobias 2009; Viljakainen *et al.* 2010; Zhu *et al.* 2014; Javaid *et al.* 2006), although not in all studies (Lawlor *et al.* 2013; Garcia *et al.* 2017), and that this association may be mediated partly through umbilical cord calcium concentrations (Javaid *et al.* 2006). Expression of a particular active placental calcium transporter was positively associated with neonatal bone mass (Martin *et al.* 2007), with regulation by 1,25(OH)2-vitamin D implicated in experimental studies (Kip & Strehler 2004). More recently, the hypothesis that maternal gestational supplementation with vitamin D would lead to increased offspring bone mass was tested in the *Maternal Vitamin D Osteoporosis Study* (*MAVIDOS*), a randomised, placebo-controlled, double-blind trial of 1000 IU vitamin D versus placebo daily from 14 weeks gestation until delivery of the infant (Cooper *et al.* 2016; Harvey *et al.* 2012b). The results of this trial suggest that such a supplementary approach leads to improved offspring bone mineral content (of around 0.5 SD) compared with placebo, for neonates born in the winter months, when background 25(OH)D concentrations are lowest (although not in the population overall) (Cooper *et al.* 2016). Subsequent work has identified interactions with both environmental and genetic factors in the achieved 25(OH)D in response to treatment (Moon *et al.* 2016; Moon *et al.* 2017); mechanistic investigations using placental samples have identified novel relationships between vitamin D and placental nutrient transport (Cleal *et al.* 2015).

(b)

(a)

**Figure 3**

1. Percent DNA methylation at *RXRA* and offspring size corrected bone mineral content (scBMC), *n*=230.
2. Maternal free 25(OH)D index and *RXRA* DNA methylation.

Adapted with permission from (Harvey *et al.* 2014d)

These candidate focused studies contrast with findings from a recent investigation using the Illumina HumanMethylation450 BeadChip, an array covering methylation marks at over 450 000 CpG sites across the genome. Amongst 819 mother-offspring pairs in the *Norwegian Mother and Child Cohort* and 597 mother-offspring pairs in the *Avon Longitudinal Study of Parents and Children* (*ALSPAC*), there were no convincing associations between maternal mid-pregnancy 25(OH)D status and methylation profile in cord blood DNA. Additionally, use of the same array technology in blood DNA from 5515 adults of European descent identified only one CpG site associated with femoral neck bone mineral density. However, these studies do not specifically address links between perinatal epigenetic marks and offspring bone development and there are a number of important considerations here. These include 1) the tissue specificity of epigenetic marks and the use of blood DNA compared with perinatal tissues, such as umbilical cord, which contain mesenchymal stem cells that have potential to develop into target tissues such as bone, muscle and fat; 2) despite coverage of >450 000 CpG sites, the array targets a tiny percentage of the number of potentially methylated CpG sites across the genome; and 3) although the variance explained by molecular phenotype is generally greater than that explained by fixed genetic variation, the sample sizes of these consortium-based studies have been relatively small compared with what has recently been possible with genetic analysis. For example, a genome-wide association study for heel bone mineral density in the UK *Biobank* *cohort*, using the then available subset of 142 487 individuals, yielded 307 loci, including 153 previously unreported sites (Kemp *et al.* 2017). This represents a step change in the number of sites identified compared with previous studies, for example comprising combined cohorts of around 50 000 individuals, identifying 56 loci (Estrada *et al.* 2012).

**Planned work**

Through the BBSRC-funded component of the *ALPHABET* project, the aim is to investigate dietary and epigenetic predictors of later bone health, and to address questions raised by the evidence base to date. The BBSRC-funded component comprises three parts**.**

1. The generation of the epigenetic data, led by Professor Caroline Relton at the Medical Research Council (MRC) Integrative Epidemiology Unit, University of Bristol.
2. The analysis of links between early diet, perinatal epigenetic marking and offspring bone health, led by Professor Nicholas Harvey at the MRC Lifecourse Epidemiology Unit, University of Southampton.
3. The application of causal analysis methods to improve the evidence base for causal pathways rather than merely observed associations.

The following briefly summarises the planned work.

*Generation and collation of methylation data*

DNA methylation profiles generated by the Illumina Infinium HumanMethylation450 BeadChip are available for *ALSPAC* (1000 mother-child pairs, for mothers during pregnancy and 18 years after delivery, and for children at birth and at 7 years and 15-17 years, and for a subset of around 500 fathers) (Relton *et al.* 2015), *Polish Mother and Child Cohort* (*Repro\_PL)* (150 mother-child pairs, for mothers during pregnancy and birth) and *Generation R* (1500 birth, 500 each at 6 years and 9 years). Offspring DNA from birth are from cord blood; follow-up and maternal DNA are from peripheral blood. Led by the University of Bristol, framework and scripts are being designed and distributed for analysing DNA methylation profiles from individual datasets to allow meta-analyses of summary statistics from each ‘discovery’ dataset. A series of diet-methylation and methylation-phenotype analyses are being performed. Mediation analysis, and two-sample Mendelian randomisation where possible, of identified ‘hits’ will test whether methylation may be on the causal pathway. Currently unprofiled cohorts for which DNA is available may be used for replication of specific CpGs identified in the discovery set, and in further analyses epigenetic age of mothers and children will be investigated.

*Early diet, epigenetic marks and offspring bone health*

Using the *ALSPAC*, *SWS* and *Randomised cOntrol trial of LOw glycaemic index diet versus no dietary intervention to prevent recurrence of fetal macrosomia* (*ROLO*) cohorts, and led from the University of Southampton, this work package explores: 1) relationships between maternal and childhood dietary indices [Dietary Approaches to Stop Hypertension (DASH) and Dietary Inflammation Index (DII) (Fung *et al.* 2008; Shivappa *et al.* 2014)] and child bone indices by dual energy X-ray absorptiometry (DXA); and 2) perinatal epigenetic marks from Illumina 450K/850K array analysis (maternal blood and cord blood) and DXA bone outcomes, controlling for potential covariates. Where offspring biological samples are available later in childhood, differences in epigenetic profile will be related to maternal and perinatal profiles, and whether they relate to current bone mass or can be explained by offspring body composition, physical activity and diet will be explored. Adjunctive data may be obtained from the *MAVIDOS* trial, in which Illumina 850K analysis has been undertaken on umbilical cord tissue, together with specific validation of candidates using techniques such as Pyrosequencing on *SWS* and *MAVIDOS* cohorts.

**Conclusion**

The BBRSC-funded musculoskeletal work forms just a part of the multinational collaborative ERA-HDHL Biomarkers *ALPHABET* project on maternal nutrition, child health and epigenetics, and which provides a unique opportunity to improve maternal and offspring health for the future through state-of-the-art science. With the prevalence of many chronic non-communicable diseases increasing worldwide, population level strategies are needed to reduce the future burden of these conditions. In *ALPHABET,* the developmental origins of disease paradigm is being investigated through the study of DNA methylation, to understand interactions between maternal nutrition, offspring phenotype and future health, across thousands of mother-child pairs throughout Europe. In addition to informing mechanism understanding, the findings from the *ALPHABET* study should inform potential novel dietary interventions, identify epigenetic signals that may constitute biomarkers of future disease risk, and thus targeted and population-wide strategies to optimise periconception and early postnatal environment, and consequently reduce the burden of chronic non-communicable conditions such as osteoporosis and cardiometabolic disease in future generations.

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**Conflicts of Interest**

No conflicts of interest have been declared.

**Appendix: Details of the cohorts contributing data to the ERA-HDHL *ALPHABET* project**

A number of European longitudinal birth cohorts currently collaborate as part of the *ALPHABET* project, providing important insights into the influence of maternal exposures on offspring health. Here we summarise the cohorts and a selection of relevant findings leading up to the *ALPHABET* project.

**Avon Longitudinal Study of Parents and Children (ALSPAC)**

*ALSPAC* (Boyd *et al.* 2013; Fraser *et al.* 2013) is a prospective pregnancy cohort, recruiting 13 761 women resident in England during 1991-1992. It has tested a large number of associations between early life dietary exposures and later health outcomes (Emmett & Jones 2015; Emmett *et al.* 2015). DNA methylation profiles are available for 1000 mother-children pairs during pregnancy, at birth and up to 18 years follow-up, as part of the *Accessible Resource for Integrated Epigenomic Studies* (*ARIES*) (Relton *et al.* 2015). Relevant investigations include those of associations of DNA methylation with birthweight and gestational age (Simpkin *et al.* 2016; Simpkin *et al.* 2015), maternal pre-pregnancy BMI, gestational weight gain (Sharp *et al.* 2015), serum vitamin D (Suderman *et al.* 2016) and prenatal smoking (Richmond *et al.* 2015) and investigation of DNA methylation as a causal mechanism for these associations (Richmond *et al.* 2016; Kupers *et al.* 2015).

**The Southampton Women’s Survey (SWS)**

The *SWS* (Inskip 1999), recruited 12 583 women aged 20-34 years between 1998-2002 in Southampton, UK; of these 3158 children were born between 1998-2007 and followed-up to age 10-13 years. Importantly, maternal data are available pre-conception. The main aims of the study are to characterise maternal diet, lifestyle and intrauterine effects on offspring fetal growth and pathways leading to poor health outcomes. Dietary factors during pregnancy have been associated with childhood adiposity, bone, muscle and cognitive development (Okubo *et al.* 2014; Gale *et al.* 2009; Harvey *et al.* 2012a; Harvey *et al.* 2014c), and between maternal adiposity (Pike *et al.* 2013) or vitamin D status (de Jongh *et al.* 2014) and offspring respiratory health. DNA methylation analysis demonstrated associations with later bone mineral content (Harvey *et al.* 2014d; Curtis *et al.* 2017), adiposity (Lillycrop *et al.* 2017) and neurocognitive function and behaviour (Lillycrop *et al.* 2015), providing support for a role of epigenetic processes in mediating the long-term consequences of early life environment on health.

**The Generation R Study**

The *Generation R* Study (Jaddoe *et al.* 2006; Kooijman *et al.* 2016) is a population-based prospective cohort study of ~10 000 pregnant women and their children in the Netherlands. Its aims are to identify both genetic and early environmental causal pathways relating to growth, development and health from fetal life to young adulthood. Relevant findings include associations between maternal diet during early pregnancy and childhood body composition, bone mass, and risk of wheezing and eczema at 4-6 years (van der Valk *et al.* 2013; Braun *et al.* 2015). Higher plasma folate and vitamin B12 concentrations, or high folic acid intake of mother during pregnancy was associated with lower offspring bodyweight and BMI and increased risk of eczema at 4-6 years (van der Valk *et al.* 2013; Braun *et al.* 2015; Kiefte-de Jong *et al.* 2012). DNA methylation studies (subgroup *n*=1232), through either candidate gene or epigenome-wide association studies, showed that maternal plasma folate concentrations and smoking during pregnancy impacts DNA methylation in newborns (Bouwland-Both *et al.* 2015; Joubert *et al.* 2016a; Joubert *et al.* 2016b).

**The EDEN mother-child cohort study**

Based in France, the *EDEN* mother-child cohort study of 2002 pregnant women investigates prenatal and early postnatal determinants of fetal and postnatal growth, adiposity development, respiratory and bone health and neurodevelopment, with methylation profiles available from umbilical cord and in peripheral blood at age 6 years (Heude *et al.* 2016). Lines of investigation include diet, environmental pollutants, socioeconomic and psycho-emotional factors. In the study, detailed questionnaire and clinical data on phenotypes and exposures and biological samples were collected from pregnancy until the child was 8 years old. Relevant findings from the *ALPHABET* project include tracking of dietary patterns from infancy to pre-school age (Lioret *et al.* 2015), associations between maternal dietary factors (caffeine and fatty acids) with offspring IQ (Galera *et al.* 2016), neurodevelopment (Bernard *et al.* 2013) and DNA methylation (Azzi *et al.* 2014), and between gestational weight change and offspring adiposity (Diouf *et al.* 2014; Jacota *et al.* 2017).

**The Polish Mother and Child Cohort (Repro\_PL)**

The *Repro\_PL* is a multicentre prospective cohort study of 1800 mother-child pairs, in which the children have been followed up to age 7 years. It was established in 2007 with the aim of evaluating the contribution of environmental factors to pregnancy outcomes, children’s health and neurodevelopment (Polanska *et al.* 2011). Findings to date suggest that maternal lifestyle, micronutrient and vitamin D status during pregnancy and child environment after birth have significant impacts on child health and psychomotor development (Polanska *et al.* 2016; Stelmach *et al.* 2014; Polanska *et al.* 2015; Stelmach *et al.* 2015).

**The Lifeways Cross-Generation Cohort Study**

Based in the Republic of Ireland, the *Lifeways Cross-Generation Cohort Study* recruited 1100 pregnant women in 2001 and is one of very few worldwide containing data on grandparents of both lineages (Kelleher *et al.* 2014). The children have been followed up at age 5 and 9 years, with physical examinations and linkage to hospital data and general practice records. Consistent familial and cross-generational associations, particularly along the maternal line, have been reported between parental and grandparental health status and child outcomes, including BMI and asthma (Kelly *et al.* 2014; Murrin *et al.* 2012).

**The ROLO Study**

The *ROLO* study, also based in the Republic of Ireland, recruited 800 women. In the low glycaemic index diet intervention group, reduced gestational weight gain and improved maternal glucose intolerance was seen. However, no difference in offspring weight or BMI at birth or at 6 month follow-up was found between groups (Horan *et al.* 2016; Walsh *et al.* 2012). Five year follow up of the mothers and children is currently underway.

Secondary analysis of the *ROLO* cohort examined the effect of dietary calcium, dietary vitamin D and seasonal variation in serum 25(OH)D on a marker of bone resorption [urine cross-linked N-telopeptides of type I collagen (uNTX) during pregnancy] (O'Brien *et al.* 2017). In late pregnancy, during winter months when 25(OH)D is inadequate, intakes of dietary calcium <1000 mg/day were associated with significantly increased bone resorption. Additional dietary calcium is associated with reduced bone resorption in late pregnancy, with greater effect observed in winter pregnancies.

**The PEARS study**

Building on findings from the *ROLO* study, the *Pregnancy, Exercise and Nutrition Research Study* (*ROLO*) (with smart phone App support) of 500 women in the Republic of Ireland will assess the impact of a 'healthy lifestyle package' involving targeted, low glycaemic index nutritional advice plus daily physical activity delivered before 18 week’s gestation, together with a smart phone App to provide ongoing healthy lifestyle advice and support throughout pregnancy. Biological samples from early and late pregnancy as well as cord blood will be collected and a wide range of maternal and fetal health outcomes measured (Kennelly *et al.* 2016). The study aims to evaluate the effectiveness of a smart phone App intervention, grounded in behaviour change theories and techniques, with the aim of preventing gestational diabetes mellitus in an overweight or obese pregnant population.

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