Best Practice Research Clinical Endocrinology and Metabolism

Epigenetic Regulation of Bone Mass

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**Practice points**

* **BMD and fracture risk have both genetic and environmental determinants, with gene-environment interactions playing an important role. Epigenetic processes regulate gene expression through a variety of mechanisms - DNA methylation, histone modifications and non-coding RNAs.**
* **Histone acetylation, deacetylation and methylation have all been shown to play a role in regulation of bone mass.**
* **Micro-RNAs (mi-RNAs), the best studied of the long non-coding RNAs play important roles in osteogenic differentiation and osteoporosis development through mRNA repression or translational repression of target genes.**
* **DNA methylation has been shown to be important in a variety of pathways involved in the regulation of bone mass - from vitamin D supplementation in pregnancy inducing changes in *RXRA* gene methylation, to differential methylation in key target genes such as *OPG* and *RANKL* being associated with osteoporosis.**

**Research agenda**

* **An understanding of epigenetic processes is relevant to many fields of medicine, including bone health. Epigenetic changes can be used as biomarkers for bone health, and may highlight interventions to improve early development and later health.**
* **Histone acetyltransferases, deacetylases and methylases are being considered as therapeutic targets in osteoporosis but require further research.**
* **miRNAs have been considered to be a promising class of biomarkers for the diagnosis and prognosis of osteoporosis, but further work in larger cohorts is required.**
* **When examining the association between DNA methylation and bone health, tissue specific relationships are observed. Methylation array analyses (epigenome-wide association studies) have yielded different results between bone and blood; whole blood may not be a helpful tissue in which to detect associations. Targeted studies in cells with a clear role in bone biology (e.g. monocytes due to their role in osteoclastogenesis) may be more informative.**

**Abstract**

Osteoporosis, characterised by low bone mass, poor bone structure, and an increased risk of fracture, is a major public health problem. There is increasing evidence that the influence of the environment on gene expression, through epigenetic processes, contributes to variation in BMD and fracture risk across the lifecourse. Such epigenetic processes include DNA methylation, histone and chromatin modifications and non-coding RNAs. Examples of associations with phenotype include DNA methylation in utero linked to maternal vitamin D status, and to methylation of target genes such as OPG and RANKL being associated with osteoporosis in later life. Epigenome-wide association studies and multi-omics technologies have further revealed susceptibility loci, and histone acetyltransferases, deacetylases and methylases are being considered as therapeutic targets. This review encompasses recent advances in our understanding of epigenetic mechanisms in the regulation of bone mass and osteoporosis development, and outlines possible diagnostic and prognostic biomarker applications.

## Key words

Epigenetics, Osteoporosis, Epidemiology, Bone, Development, DNA methylation, Histone modification, ncRNA

## Introduction to gene-environment interactions and bone mass

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[1]. With the globally ageing population, the burden of osteoporosis is increasing, with a consequent increase in fragility fractures worldwide[2]. 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[3]. Such fractures are very common – in many countries, the remaining lifetime risk of a fragility fracture in a woman aged 50 years is estimated at around 50%, and around 20% for a UK man[3-6].

An individual’s peak bone mass, the maximum total skeletal mass accrued at the completion of skeletal development is a key determinant of later bone health[7]. Bone mass increases throughout fetal, infant, childhood and early adult life reaching a peak in the third to fourth decade. Peak bone mass is estimated 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[8]. Twin and parent-offspring studies demonstrate evidence of genetic heritability of bone mass[9, 10], with various genome-wide association studies identifying specific genetic loci. The most recent, and largest studies in the UK Biobank cohort has identified further loci, cumulatively explaining 20% of the variance in estimated heel BMD (eBMD)[11, 12]. Known genetic loci account for only part of the overall suggested genetic 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 and large numbers of loci with very small effects. Importantly, genetic heritability simply refers to the proportion of the variance in the aBMD distribution within a population which is explained by genetics[13]. The overall mean of the distribution can therefore be moved, for example by population-wide secular changes in diet quality and socioeconomics, without changing the spread of the distribution. The secular changes in height in several populations over recent decades provides a case in point[14-16]. The same genetic effect in a population with a low prevalence of a key environmental factor, compared with a population with a high prevalence of that factor will lead to different estimates of genetic heritability. Importantly, it is well established that environmental, as well as genetic, factors may influence gene expression and that the interaction between gene and environment plays an important role in phenotypic expression[17, 18]. In this article, we review the molecular mechanisms underlying the genetic and environmental influences on gene expression, and how understanding of these mechanisms has added to our knowledge of the evolution of both disease in older age and of the development of skeletal phenotypes even as early as conception. Using a narrative review approach, we searched PubMed for terms including “bone”, “osteoporosis”, “bone mineral density”, “epigenetic”, “methylation”, “micro RNA”, “histone”, “regulation” together with derived and related terms. Articles were chosen on the basis of relevance, being full-text and being written in the English language. No constraints were put upon date of publication and where appropriate reference lists were checked for additional publications.

## Introduction to epigenetic mechanisms

In order to properly consider epigenetic mechanisms, one must first consider the processes of developmental plasticity, i.e. that a single genotype may give rise to several different phenotypes in response to the prevailing environmental milieu, ubiquitous in the natural world[19]. This process allows the next generation to be born appropriately adapted to the expected external environment, using cues from the prevailing environmental conditions acting during critical periods of development[20]. A range of experimental studies have shown that alterations to maternal diet during pregnancy may lead to changes in offspring phenotype and gene expression [21, 22]. These effects are likely to be underpinned by epigenetic mechanisms, processes by which gene expression is modified but without changes in the DNA code itself (the term epigenetics was coined by Conrad Waddington in 1942, as a bridge between genotype and environment interactions). Such epigenetic signals are essential in determining when and where genes are expressed. In humans most epigenetic effects have only been shown to pass to the grandchildren, which 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[23, 24]. The epigenome can, however, be regarded as a molecular record of life events, accumulating throughout a lifetime. For example, monozygotic twins have been shown to be epigenetically most similar at birth but their epigenomes diverge with age at a rate that is lessened if they share a common environment[25]. An understanding of these epigenetic processes has the potential to enable early intervention strategies to improve early development and later health; consequently the study of epigenetic biomarkers is a rapidly advancing field, including in the field of osteoporosis[26].

Epigenetic mechanisms include histone modifications, non-coding RNAs(ncRNAs) and DNA methylation[17, 27]. Whilst studies have demonstrated involvement in all three processes (and others) in the regulation of bone mass and osteoporosis development, we will briefly discuss histone modifications and ncRNAs (recently thoroughly reviewed by Xu et al[28] and Bellavia et al[29]) before focusing more closely on DNA methylation.

## Post translational histone modifications

Post-translational histone modifications and the accompanying histone-modifying enzymes form a major part of the epigenetic regulation of genes. DNA is wrapped around an octamer of four different histone molecules (H2A, H2B, H3 and H4) to form a nucleosome, the basic unit of chromatin. The flexible N-terminal tails of core histones that protrude from the nucleosome undergo various post-translational modifications, e.g. acetylation, methylation, phosphorylation, ubiquitination[30]. The patterns of histone modifications alter the transcriptional accessibility of the chromatin. It has been shown that euchromatin, a more relaxed, actively transcribed state of DNA, is characterized by high levels of acetylation and trimethylated (H3) lysine residues (K-number) on specific histones H3K4, H3K36 and H3K79, while low levels of acetylation and high levels of H3K9, H3K27 and H4K20 methylation are indicative of a more condensed, transcriptionally inactive heterochromatin[31]. The majority of histone post-translational modifications are dynamic and regulated by families of enzymes that promote or reverse specific modifications, e.g. histone acetyltransferases (HATs) add acetylation marks, whereas histone deacetylases (HDACs) remove them. Many transcriptional co-activators or co-repressors either possess HAT or HDAC activity or associate with these enzymes, so the balance between histone methylation and acetylation and demethylation/deacetylation is important in modifying expression of target genes. Chromatin immunoprecipitation sequencing can be undertaken to identify DNA segments bound by a histone with specific modifications and is the main method for detecting histone modifications[32].

### Histone modifications and osteoporosis

There is evidence to support the role of histone acetylation in the development of osteoporosis. This has been established in a model of glucocorticoid-induced osteoporosis (GIOP), in which there is reduced H3K9/K14 and H4K12 acetylation in the regulatory regions of *Runx2* and *Osx*. In addition, enhanced H3K9/K14 and H4K12 hyper-acetylation in the *PPARγ2* regulatory region in osteoporotic bone marrow-derived mesenchymal stem cells (BMSCs), enhances their adipogenic rather than their osteogenic potential[33]. In another model of osteoporosis, in OVX-induced mice, a decline in GCN5 and PCAF has been found - these are two other histone H3K9 acetyltransferases that enhance osteogenic differentiation and bone formation via the acetylation of H3K9 loci in the promoters of *Wnt* genes and *BMP* genes, respectively[34, 35]. Histone deacetylases also may play a role - for example the deacetylase HDAC1 promotes osteogenesis of MSCs via the deacetylation of *FOXO3a* and (via mechanical loading) also inhibits osteogenic differentiation in human BMSCs via the deacetylation of *JAGGED1 (JAG1)*[36, 37]. Consequently, histone acetyltransferases and deacetylases are being considered as possible therapeutic targets for osteoporosis (for example, the histone deacetylase inhibitor suberoylanilide hydroxamic acid (SAHA; vorinostat)[38].

Histone methylation is also important in regulating bone mass. In the GIOP model, there is hyper-tri-methylation in *Runx2* and *Osx* genes and H3K9 hypo-di-methylation on the *PPARγ2* regulatory region in osteoporotic BMSCs[33, 39]. Enhancer of zeste homologue 2 (EZH2) is perhaps the best-studied deregulated master chromatin remodeler implicated in osteoporosis. EZH2 catalyzes H3K27me3 deposition and in osteoporotic mesenchymal stem cells (MSCs) the elevated EZH2 level enhances H3K27me3 levels at the promoters of *Wnt1*, *Wnt6*, and *Wnt10a*. In osteoporotic mice (compared with controls), delivery of an EZH2 inhibitor or an H3K27me3 inhibitor results in osteoblast differentiation of MC3T3-E1 cells, leading to a marked increase in bone formation and reduction in bone loss, raising its potential as an anabolic therapy[40-42].

## Non-coding RNAs

Recent studies have shown that up to 90% of the eukaryotic genome is transcribed, but only 1–2 % of the genome encodes proteins[43]. It has become increasingly apparent that the non-protein coding portion of the genome plays a crucial role in the control of biological processes such as proliferation, differentiation, and apoptosis. Micro RNAs (miRNAs) are the best characterised ncRNAs, these are approximately 21 nucleotide long non-coding RNA molecules. miRNAs are incorporated into an RNA induced silencing complex (RISC) in order to facilitate binding to the target messenger RNA (mRNA), usually within its 3′ untranslated region. If the miRNA and the target mRNA match, this results in the degradation of the targeted transcript. More commonly, miRNAs bind imperfectly to their targets, causing translational repression only, without the destruction of mRNA. In general, miRNAs are considered predominantly as negative regulators of gene expression involved in physiological and pathological processes, however some have been shown to activate translation under certain conditions[44].

miRNAs are, however, part of a larger family of ncRNAs, which includes small nucleolar RNAs and long ncRNAs (lncRNAs, which are more than 200 nucleotides in length). The lncRNAs make up the largest portion of the mammalian non-coding transcriptome, but their mechanism of action is still not fully understood. Studies have shown they can regulate gene expression either by acting as antisense transcripts or as scaffolds for the recruitment of histone modifiers[45].

### miRNAs and osteoporosis

In terms of their function, miRNAS are the best characterised of the ncRNAs and play important roles in osteogenic differentiation and the development of osteoporosis through mRNA degradation or translational repression of target genes[29]. Evidence for the effects of miRNAs on bone come from animal models *in vivo* and studies of bone cell differentiation *in vitro*. One example is miR-188, which is elevated in BMSCs from aged versus young mice and aged versus young humans. Transgenic mice with miR-188 overexpression have low bone mass, whereas miR-188 deletion protects against age-associated bone loss[46]. Another example comes from studies of Rictor, a specific component of the mechanistic target of rapamycin complex 2, which is downregulated with aging in osteoblasts, in association with a reciprocal upregulation of miR-218 and miR-188. miR-218 has been shown in *in vivo* studies to have a regulatory function, associated with a reduction in the number of functional osteoblasts and accelerated age-related bone loss[47]. Thus, miR-188 and miR-218 gave been proposed to be therapeutic targets in the treatment of osteoporosis[28].

Micro RNAs have also been shown to play a role in the coupling of angiogenesis and osteogenesis in a specific bone vessel subtype, strongly positive for CD31 and endomucin (CD31hiEmcnhi). In this blood vessel endothelium, expression of a miR cluster, miR-497-195 is high, but gradually decreases during aging in mice. Mice with depletion of miR-497-195 have fewer CD31hiEmcnhi blood vessels and have lower bone mass. Transgenic mice overexpressing this miR cluster show reversal of the age-related depletion of CD31hiEmcnhi blood vessels and bone loss. An endothelium-specific activator of MiR-195, aptamer-agomiR-195, was injected intravenously into aged mice, and stimulated CD31hiEmcnhi vessel and bone formation, possibly representing a new therapeutic target for age-related osteoporosis[48].

 Furthermore, miRNAs have been considered to be a promising class of biomarkers for the diagnosis and prognosis of various diseases, including osteoporosis[49]. A recent study of 126 postmenopausal women with vertebral fractures has examined the association of candidate miRNAs in patients with vertebral fractures compared to two control groups: patients without vertebral fractures with normal BMD, and those with low BMD. Seven up-regulated (p < 0.05) miRNAs (miR-375, miR-532-3p, miR-19b-3p, miR-152-3p, miR-23a-3p, miR-335-5p, miR-21-5p) were identified in patients with vertebral fractures and low BMD, compared to the control group individuals. Correlations between miRNAs and bone turnover markers (BTMs), CTX, P1NP, OC and bone ALP were also detected. However, no significant differences existed between low BMD and healthy controls. The authors proposed that whilst these miRNAs are unlikely to reflect low BMD, they may indicate changes in bone quality and fracture healing[50]. In the French OFELY cohort, in which serum levels of 32 preselected miRNAs were examined in 682 women (99 premenopausal and 583 postmenopausal), there were strong associations between the miRNA levels and age, which accounted for any associations observed with bone mineral density, bone turnover markers, bone microarchitecture or fragility fractures[51]. An earlier study compared women with type-II diabetes with or without prior fragility fracture and secondly, nondiabetic postmenopausal women with a prior fragility fracture versus a control group of fracture free postmenopausal women (20 per each of the 4 groups). It was found that 48 miRNAs differentiated fracture status in women with type-II diabetes and that 4 miRNAs discriminated diabetes-related fractures with high specificity and sensitivity (area under the receiver-operating characteristic curve values [AUCs], 0.92 to 0.96; 95% CI, 0.88 to 0.98). Further in vitro studies suggested links between particular miRNAs and osteogenic/adipogenic differentiation[52]. Whilst these findings are intriguing, the participant groups are very small and there appears to be no matching or adjustment for confounders in the analysis. Given the role of age in the OFELY analyses, it is clear that further work on larger cohorts is required to fully establish whether miRNA measurements could offer a novel approach to fracture prediction, independent of traditional and more easily obtained risk factors.

## DNA methylation

DNA methylation is the most widely studied of epigenetic modifications and will be the focus of the remainder of this review. DNA methylation is a common modification in eukaryotic organisms, and involves the transfer of a methyl group to the 5′ carbon position of cytosine, creating 5-methylcytosine (5-mC)[53]. It is a relatively stable epigenetic mark that can be transmitted through DNA replication during mitosis[54], although methyl marks can be added and removed throughout the lifecourse. Cytosine methylation mainly 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[55]. A CpG site can either be methylated or unmethylated in an individual cell; however, 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[17].

CpG dinucleotides are not randomly distributed throughout the genome but are clustered at the 5′ end of genes in regions known as CpG islands, with hypermethylation of CpG islands generally associated with gene silencing and hypomethylation with activation[56]. DNA methylation can act directly to block binding of transcription factors to the DNA or by recruiting 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[57].The pattern of CpG methylation is 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[58, 59]. The de novo methylation of DNA is catalysed by DNA methyltransferases (DNMT) 3a and 3b[59] and is maintained through mitosis by methylation of hemi-methylated DNA by DNA methyltransferase 1 (DNMT1)[60]. This enables lineage-specific methylation patterns to be maintained in differentiated tissues. DNA methylation was initially thought to be relatively stable and generally maintained throughout life, but this concept has now been challenged. 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[61]. 5hmC has been shown to arise from the oxidation of 5-mC by the enzymes of the TET (Ten-Eleven-Translocation) family[62] and has been proposed to act as a specific epigenetic mark opposing DNA methylation, as well as a passive intermediate in the demethylation pathway[63, 64]. The high levels found in the brain and neurons indicate a role in the control of neuronal differentiation and neuronal plasticity[65].

## Age related changes in DNA methylation

Age related changes in DNA methylation are well documented[19]. Various studies have worked towards an understanding of epigenetic predictors of ageing and mortality, using methylation measures from multiple CpG sites across the genome to predict chronological age in humans[66, 67]. Hannum et al. created an age predictor based on a single cohort in which DNA methylation was measured in whole blood[66], whilst Horvath developed an age predictor using DNA methylation data from multiple studies (including the Hannum dataset) and multiple tissues[67]. In both studies, the difference between methylation-predicted age and chronological age (that is, Δage) was proposed as an index of disproportionate ‘biological’ aging and was hypothesised to be associated with risk for age-related diseases and mortality. Other studies have shown that this Δage, or marker of accelerated ageing, can be used to predict mortality independently of health status, lifestyle factors and known genetic factors[68, 69].

## Methylation mechanisms in bone in older age

### Investigation of candidate genes

The relevance of DNA methylation has been demonstrated in the pathogenesis of osteoporosis, initially through the study of a few candidate genes with known relevance to bone biology[70]. For example, in a study of postmenopausal women (27 with osteoporosis and 36 without osteoporosis), methylation of *SOST* (the sclerostin gene, a known inhibitor of bone formation) in iliac bone samples (measured using the Infinium HumanMethylation450 BeadChip (Illumina)) was increased in osteoporotic patients, and SOST mRNA in bone cells decreased. The authors observed strong positive associations between SOST mRNA and serum levels of sclerostin with age and BMI-adjusted total hip BMD, and inversely to serum bone turnover markers. They suggested that the increased *SOST* promoter methylation observed in osteoporosis is a compensatory mechanism, lowering serum sclerostin concentrations and reducing the inhibition of Wnt signalling in an attempt to promote bone formation[71]. Conflicting results were reported, however, in a 2019 femoral bone biopsy study of 16 Chinese patients with osteoporosis and hip fractures and 16 controls (with traumatic fractures and normal BMD). *SOST* gene expression was significantly increased and the *SOST* promoter was slightly hypomethylated in the patients with osteoporosis, indicating that this is an area which warrants further investigation[72]. Differential methylation at the estrogen receptor alpha (*ERa*)[73], *BMP2*[74], (*OPG*) and *RANKL*[75] gene promoters have also been shown to be associated with menopause (in the case of *ERa*) or osteoporosis.

It has further been demonstrated that *Alu* elements, (the major interspersed repetitive DNA elements) show loss of methylation in adulthood, from the age of approximately 40 years. *Alu* hypomethylation has been shown to be are associated with both advanced patient age and lower BMD in analysis of blood from 323 postmenopausal women[76].

### Genome-wide methylation studies in older people

Genome-wide methylation profiling studies in older patients comparing individuals with low versus normal BMD have also suggested early life influences on bone quality in older age. One study, comparing bone biopsies of older people with hip fractures (n=27) to osteoarthritis patients (n=26) using a methylation array covering 23,367 CpG sites, identified differentially methylated regions (DMRs) enriched in genes associated with cell differentiation and skeletal embryogenesis, including those in the *homeobox* superfamily. The authors suggested that this indicates the existence of a developmental component in the predisposition to osteoporosis[77]. Further evidence for such a developmental component was presented by a multi-omics analysis incorporating gene expression, DNA methylation and miRNA data in high BMD versus low BMD women. The authors identified four potential regulatory patterns of gene expression to influence BMD status, two of which, the mTOR and insulin signalling pathway, have been linked to bone cell differentiation and postnatal bone growth[78].

A study investigating the differential methylation of human mesenchymal stem cells acquired from the femoral neck of those undergoing hip replacement for hip fracture and those undergoing hip replacement for osteoarthritis found differential methylation in gene pathways enriched for hMSC growth and osteoblast differentiation[79]. However, it should be recognised that differential methylation signatures in bone tissue which has recently fractured may be influenced by the trauma itself and not purely representative of the osteoporotic disease process.

A Norwegian study examined associations between osteoporosis and methylation in bone biopsies collected in the absence of trauma at 2529 CpG sites in 100 genes previously identified to be associated with bone mineral density in post-menopausal women, via 450K array[80]. In 84 postmenopausal women, CpG methylation at 63 sites differed significantly between osteoporotic and non-osteoporotic post-menopausal women at a 10% false discovery rate (FDR). These included CpGs within the *tenascin XB* gene (a protein which supports the structure and maintenance of bone, muscle and connective tissues), glycogen synthase 1 in muscle. Five of these CpGs at a 5% FDR level explaining 14% of the BMD variation including *Matrix Extracellular Phosphoglycoprotein* (*MEPE*, responsible for phosphate uptake and bone mineralisation), *SOST*, *Wnt Inhibitory factor 1* (*WIF1*) and *Dickkopf Wnt Signaling pathway inhibitor 1* (*DKK1*), the latter three all being Wnt pathway inhibitors.

Peripheral blood offers a more accessible option for the study of DNA methylation and bone health. In a sample taken from the Canadian Multicenter Osteoporosis Study, methylation in whole blood DNA from 22 osteoporotic postmenopausal was were compared against 22 non-osteoporotic controls. The researchers found 1233 differentially methylated CpG sites (using 450k array) with an FDR of 5% in peripheral blood leukocytes. They then subdivided the osteoporotic group into early and advanced osteoporosis (using a T-score threshold of ≥3.0 for advanced disease) and selected a panel of 5 of the most significantly associated, biologically plausible genes to create a polygenic risk score to predict the development of early osteoporosis[81]. Interestingly the panel of genes included Programmed Cell Death Protein 1 (PDCD1), which plays a role in T- and B- cell regulation.

The largest, collaborative EWAS of peripheral blood leukocyte methylation and BMD to date was carried out by another Canadian research group, also using 450k array technology. This included a 4616 individual discovery dataset covering multiple population-based cohorts of European ancestry (mean age spanning 47 to 60 years across the cohorts). In the discovery dataset, one CpG site, cg23196985, was shown to be associated with BMD in females. However, this association was not reproduced in the 901 individual validation dataset. The authors stated that whilst whole blood methylation changes may not be the ideal tissue in which to test epigenetic influences on bone, the fact that osteoclasts and monocytes/macrophages originate from the same precursors mean that it has many links to bone biology. It may be the case that DNA methylation changes may not have a large influence on BMD, but the extent to which methylation changes are shared between bone and whole blood are not well known. Therefore, well-powered, longitudinal studies covering a wide range of time points and including methylation measurements in bone samples (or at least targeted EWAS in specific cell types in blood with a clear role in bone biology, e.g. monocytes due to their role in osteoclastogenesis) may be more informative[82].

Recently in 2020, researchers have sought to identify the role of DNA methylation modifications along the causal pathway to osteoporosis, either as the mediating factor contributing to, or as a consequence of the trait. Mendelian randomization (MR), a statistical method for dealing with this problem, usus genetic variants which are robustly associated with modifiable exposures as instrumental variables to infer causal relationships between exposure and outcome[83]. Using DNA from peripheral blood monocytes (PBMs) from 118 Caucasian women with divergent BMD values, a group of researchers implemented a MR framework to infer the causal pathways mediated by differentially methylated CpGs (DMCs). The team identified 2,188 DMCs between the low and high BMD groups and distinguished 30 DMCs that may mediate the genetic effects on BMD. They then mapped the 25 causal genetic variants most likely to affect the methylation levels at the mediator DMCs. These causal genetic variants, or methylation quantitative loci, and DMCs were found to occur in potential regulatory regions such as cell type-specific histone mark peaks, enhancers, promoters, promoter flanking regions and transcription factor binding sites[84]. This approach has helped to narrow down the causal paths that are likely to have a real biological effect on BMD, which can then be prioritised for follow-up biological studies, with the aim of validating whether they truly have functional or pathological significance.

## DNA methylation in bone development

Returning to development, evidence that epigenetic processes may be important understanding the link between early environmental exposures and long-term skeletal health is now emerging from unique mother-offspring cohorts[85]. Well-established discovery pipelines have identified methylation marks at genes implicated in vitamin D metabolism and cell senescence, which are associated with later bone outcomes[7].

### Vitamin D and DNA methylation

Vitamin D has been shown to be an environmental factor which may play an important role in bone development from the fetal period onwards, perhaps through regulation of DNA methylation[85]. In terms of a mechanistic link between maternal vitamin D status and offspring bone mass, data suggest that this may be mediated, at least in part, through placental calcium transport[86]. More recent research has suggested that placental amino acid transport might also be partly regulated by maternal 25(OH)-vitamin D [25(OH)D] status and vitamin D binding protein levels, presenting another complementary mechanism for this association[87]. In a UK-based mother-offspring cohort, mRNA expression of an active ATP-dependent placental calcium transporter, *PMCA3*, in placental tissue, was positively associated with offspring bone area and bone mineral content of the whole body site at birth[88]. The regulation of placental calcium transfer is poorly characterized in humans, and any mechanistic role of vitamin D remains to be elucidated, but members of the *PMCA* family appear to be regulated by 1,25(OH)2-vitamin D [1,25(OH)2D] in animal studies[89]. Further insights into vitamin D metabolism indicate that the *1α-hydroxylase* gene is regulated by 1,25(OH)2D, through VDR mediated transcriptional regulation. Furthermore, the ongoing regulation of vitamin D metabolism may involve methylation of sites in the *1α-hydroxylase* promoter region, with the 1,25(OH)2-vitamin D/VDR/RXR complex inducing DNA methylation at the *1α-hydroxylase* promoter, whilst PTH signalling leads to demethylation of this region through a different pathway. This suggests a role for epigenetic processes in the vitamin D-parathyroid hormone axis[90, 91].

Collection of umbilical cord samples from mother-offspring cohorts has allowed the elucidation of relationships between epigenetic marking at candidate sites, identified through array approaches[92], and offspring bone size, mineralization, and density[85].

In another mother-offspring cohort, the Southampton Women’s Survey, greater methylation at 4 out of 6 CpG sites in the promoter region of *retinoid X receptor-alpha* (*RXRA*) in umbilical cord was correlated with lower offspring BMC corrected for body size at four years of age, with the results supported by findings from a second independent cohort, the Princess Anne Hospital Study [93]. In this study, an estimate of maternal free vitamin D index was inversely related to *RXRA* methylation at CpG 4/5 (chromosome 9, 136355593, 600+), as shown in Figure 1 (left panel). As mentioned, RXRA forms a heterodimer with several nuclear hormones known to influence bone metabolism, including 1,25(OH)2-vitamin D, perhaps implying that maternal 25(OH)D status plays a permissive role in the transcriptional regulation of the *RXRA* gene.

Furthermore, in a randomised, double-blind, placebo-controlled trial of vitamin D supplementation in pregnancy, MAVIDOS, vitamin D supplementation was shown to lead to reduced *RXRA* DNA methylation in umbilical cord tissue at birth, compared to placebo, as shown in figure 1 (right panel). Overall, methylation levels were significantly lower in the umbilical cord from offspring of cholecalciferol-supplemented mothers, reaching statistical significance at four CpG sites, for example at the cluster of CpG sites represented by CpG5: mean difference in % methylation between the supplemented and placebo groups was −1.98% (95% CI, −3.65 to −0.32, *p* = 0.02. ENCODE (Encyclopedia of DNA Elements) evidence supports the functionality of this locus with strong DNase hypersensitivity and enhancer chromatin observed within bone cells, including osteoblasts. Enrichment of the enhancer-related H3K4me1 histone mark is also seen in this region, as are binding sites for a range of transcription factors with roles in cell proliferation, response to stress, and growth factors[94]. Such studies not only inform our understanding of the early life mechanisms underpinning maternal vitamin D status, epigenetic change and bone development, but may also indicate novel epigenetic biomarkers for a child’s future bone health.

**Figure 1:** (Left Panel) In the Southampton Women’s Survey, perinatal umbilical cord *RXRA* methylation was inversely associated with maternal late pregnancy estimated free 25(OH)-vitamin D index and with offspring bone mass in childhood[93]. (Right Panel) Subsequently, in the MAVIDOS trial, maternal pregnancy supplementation with cholecalciferol 1000 IU/day versus placebo led to a reduction in umbilical cord *RXRA* methylation at birth[94]. Adapted with permission from Harvey et al, 2014[93] and Curtis et al., 2018[94].



It is possible that a greater understanding of the actions of vitamin D on DNA methylation may come from EWAS studies. An EWAS analysis of DNA methylation in severely vitamin D deficient African Adolescents demonstrated altered methylation in several genes, including genes involved in vitamin D metabolism such as the 24 and 25-hydroxylase genes[95]. Other studies have assessed the DNA methylation in CYP enzymes which are part of the vitamin D metabolism pathway, and found a relationship between [methylation](http://www.sciencedirect.com/topics/page/Methylation) of the genes *CYP2R1 (25-hydroxylase)* and *CYP24A1 (24-hydroxylase)* and variations in circulating 25(OH)D levels[91]. Another study, using the ALSPAC cohort and the Norwegian Mother and Child Cohort (MoBa) demonstrated no convincing associations between maternal 25(OH)D status and DNA methylation in the umbilical cord blood of 1416 newborn babies using 450k array analysis, thereby covering 473,731CpG DNA methylation sites[96]. The authors suggested that to further identify associations, larger consortium studies, expanded genomic coverage, and investigation of alternative cell types or 25(OH)D status at different gestational time points was needed.

### DNA methylation and skeletal development and homeostasis

In bone development, DNA methylation has been shown to play an important role in osteoblast differentiation; studies have identified the importance of cyclin dependent kinases and their inhibitors in this process. In the osteogenic differentiation of adipose-derived mesenchymal stem cells, the promoters of *RUNX2, osteocalcin* and *osterix* genes are actively demethylated in a process dependent upon growth arrest and DNA-damage-inducible protein, GADD45, which is known to interact with both *CDK1* and *CDKN1A*[97]. Wnt 3a has also been shown to play a role in osteoblast differentiation, through stimulation of *bone morphogenetic protein 2 (BMP2)* and *alkaline phosphatase (ALP)* expression, in a process which appears to be regulated by *BMP2* and *ALP* promoter methylation[98].

In differentiated bone tissue of various cell types, the importance of DNA methylation marks has been demonstrated in bone remodelling and osteoclastogenesis, through regulation of the *receptor activator of nuclear factor NFκB ligand (RANKL)* gene and its soluble decoy receptor *osteoprotegerin (OPG)* [99]. Finally, DNA methylation has been shown play a role in the ultimate state of osteoblast differentiation to osteocytes, embedded in mineralised bone, through the regulation of various genes including *ALP* and *sclerostin (SOST)*[100], and through the transduction of mechanical stimuli[101].

At the level of the human fetal femur, DNA methylation studies using pyrosequencing and gene expression studies using qRT-PCR have demonstrated that DNA methylation inversely correlates with expression of genes including *iNOS* (*NOS2*) and *COL9A1,* but not catabolic genes including *MMP13* and *IL1B*. This study also showed that significant demethylation was evident in the *osteocalcin* promoter between the fetal and adult developmental stages, demonstrating the importance of DNA methylation at the tissue level[102]. This style of research clearly does not enable follow-up in later development, leading to the study of methylation in other tissues such as umbilical cord and peripheral blood.

Previous array analysis of umbilical cord samples from the Princess Anne Hospital Cohort and the Southampton Women’s Survey[92] identified an association between offspring fat mass and methylation at another locus, *CDKN2A*[92, 103, 104]. 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 3,834bp transcript which can negatively regulate *p16INK4a*. SNPs within the *CDKN2A* locus, particularly those located within *ANRIL* have been associated with cardiovascular disease, diabetes and frailty(31), and DNA methylation at this locus has recently been demonstrated to vary with age(32).

Studies have demonstrated links between perinatal *CDKN2A* methylation and offspring fat mass, demonstrating it is a marker for later adiposity[105]. The functional relationships between fat and bone are well characterised, and mediated via both mechanical and endocrine pathways. Furthermore, DNA methylation at CpG sites within the *CDKN2A* gene was associated with offspring bone mass at age 4 and 6 years. Functional investigation of the differentially methylated region in the SaOS-2 osteosarcoma cell line demonstrated that transcription factors were able to bind to the identified CpGs in a methylation-specific manner, and that mutating the CpGs modulated the expression of the long non-coding RNA, ANRIL[106]. Such findings further support the importance of DNA methylation in epigenetic processes in bone metabolism, particularly with regard to loci implicated in cellular differentiation, cell cycle regulation and bone cell function, from early in development to older age[85].

## Conclusion

From the studies described, it is apparent that epigenetic marking both in early and later life is associated with later phenotypic variation, including signals associated with bone health and fracture risk. However, given the potential tissue specificity of epigenetic signals, the variation of such marks over time, and the difficulty in differentiating cause from effect, the exact characterization of epigenetic mechanisms in disease etiology and pathology is a very complex process. Histone modifications, non-coding RNAs and DNA methylation changes identified in human cohorts through array and candidate investigation must be replicated in separate independent cohorts to robustly establish associations with later disease. Experimental work using cell culture and animal models is also required to document the detailed molecular processes, regulation and functional consequences. A combination of such fundamental investigation and linkage to disease development will be essential to fully understand the role of epigenetic mechanisms in the development of osteoporosis. In the meantime, whether the observed epigenetic marks are cause or consequence, if replicated, the signals identified may well present useful novel biomarkers for later adverse bone development.

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## Conflict of Interest

The authors report no conflicts of interest relevant to this work.

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