Title:
Distinct DNA methylation profiles in bone and blood of osteoporotic and healthy postmenopausal women

One sentence summary:
DNA methylations in bone and blood correlate to the osteoporotic phenotype and show complex associations with transcript levels

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ABSTRACT

DNA methylation affects expression of associated genes and may contribute to the missing genetic effects from genome-wide association studies of osteoporosis. To improve insight into the mechanisms of postmenopausal osteoporosis, we combined transcript profiling with DNA methylation analyses in bone. RNA and DNA were isolated from 84 bone biopsies of post-menopausal donors varying markedly in bone mineral density (BMD). In all, 2529 CpGs in the top 100 genes most significantly associated with BMD were analyzed. The methylation levels at 63 CpGs differed significantly between healthy and osteoporotic women at 10% FDR. Five of these CpGs at 5% FDR could explain 14% of BMD variation. To test whether blood DNA methylations reflect the situation in bone (as shown for other tissues), an independent cohort were selected and BMD association was demonstrated in blood for 13 of the 63 CpGs. Four transcripts, representing inhibitors of bone metabolism: *MEPE*, *SOST*, *WIF1* and *DKK1* showed correlation to a high number of CpG methylations at 5% FDR. Our results link DNA methylation to the genetic influence modifying the skeleton, and the data suggest a complex interaction between CpG-methylation and gene regulation. This is the first study in the hitherto largest number of post-menopausal women to demonstrate a strong association between bone CpG methylations, transcript levels and BMD/fracture. This new insight may have implications for evaluation of osteoporosis stage and susceptibility.

INTRODUCTION

DNA methylation is essential for normal development and is associated with key processes like genomic imprinting, X-chromosome inactivation, suppression of repetitive elements, and carcinogenesis.[1] In mammals, the cytosine nucleotide is methylated in 60-90% of all CpG dinucleotides, and this methylation of DNA is an important regulator of transcription. In promoter regions, levels of DNA methylation appear to be steadily acquired during the course of life, while global genome DNA methylation is reduced.[2] Furthermore, the epigenetic variation in the population also increases gradually with age due to stochastic processes and environmental exposures.[3] Increased methylation in promoter regions tends to reduce transcription, probably by inhibition of transcription factor binding, while increased methylation within the transcribed part of DNA often increase transcription, probably due to reduced use of intragenic spurious promoters.[4] We recently showed that bone DNA methylation in the *SOST* promoter, bone *SOST* transcripts as well as serum sclerostin levels, correlated strongly with fracture risk in postmenopausal women.[5] Postmenopausal osteoporosis is a polygenetic disease, and the heritability (h^2) of bone mineral density (BMD) estimated by twins and mother-daughter familiar studies is 60 – 80%, dependent on skeletal sites.[6, 7] However, the BMD associated loci discovered by genome-wide association studies (GWAS) only explain up to 6% of the BMD variation in Caucasian populations.[8] DNA methylation has been indicated in a few studies[9, 10] to be partly heritable and has emerged as a candidate to explain part of the missing genetic effects in several diseases associated with complex traits. In mice, it was shown that a mutation in a folate metabolism gene, *Mtrr*, resulted in developmental defects and altered DNA methylation levels in wild-type descendants for up to four generations.[11] Recently, Grundberg et al., studying DNA-methylation in adipose tissue in twins, estimated a heredity of 19% genome wide and as much as 37% (median h^2=0.34) in topmost varied sites.[12] Furthermore, they showed that shared environment contributed on average only 0.2% to the methylation variation, while 10.5% of CpGs were associated with nearby SNPs (± 100 kb).

We earlier described 142 transcripts significantly associated to BMD in postmenopausal women at 10% FDR,[13] and study in this paper the methylation profiles of the corresponding genes. We restricted the analysis to the top 100 of these genes in order to maintain statistical power. In total we have analyzed 2729 CpG sites in the 100 genes, and postulate that the methylation levels could be strongly associated to bone metabolism and thus may vary between osteoporotic and healthy women. The bone DNA methylation profiles were

successfully confirmed in peripheral blood samples in an independent cohort of postmenopausal women and men. Finally, we investigated which DNA methylations levels in the human bone tissues were associated with expression levels of the 100 genes looking at possible cis and trans-correlations representing transcripts from the same or different genes, respectively.

RESULTS

Experimental design

Two cohorts were studied: 1. The Oslo study group in which global transcript profiling and DNA methylation analysis were performed on 84 bone biopsies from postmenopausal women. 2. The Framingham cohort of 210 men and 250 women in which global blood DNA methylation levels, fractures and BMD had been mapped. To increase power of the study we focused on methylation levels in the 2529 CpGs present on the Illumina array from the 100 genes (Table S1) whose bone transcripts associated most significantly with BMD. We first identified bone DNA methylations associated with the osteoporotic phenotype in the Oslo study group, and then checked if the same associations could be found in blood in the Framingham cohort. Finally, we looked at DNA methylation vs transcript relationships by correlating DNA methylation levels with transcript levels from the 100 studied genes across 80 bone donors, in which both had been mapped within the same biopsy.

Postmenopausal healthy and osteoporotic women show differentially methylated CpG sites in bone

The osteoporotic (n=26) and the healthy women (n=40) (described in Table S2) were compared to find differentially methylated CpGs using the method stratifying on age, as presented in Chen et al.[14] (see Materials and methods under Statistical analysis). Table 1 shows the 63 significant CpG sites, using FDR<0.1. In 53 of the 63 CpGs, the methylation levels are higher among osteoporotic women compared to healthy (Table S3). Furthermore, distinct methylation was most pronounced comparing the older women with OP against the older healthy women (Fig. S1). To further validate the significance of these 63 discoveries, we reshuffled the healthy versus OP labels, and counted the number of discoveries (FDR<0.1) for the reshuffled data. Performing 100 randomized runs, 1 run found

2 significant CpGs, 4 runs found 1 significant CpG, and 95 runs found no significant CpGs. Thus the validation test strengthened markedly the significance of the 63 CpGs.

The top 5 most significantly differentially methylated sites can explain 14% of BMD variation

Using a more strict discovery rate, namely FDR<0.05 (instead of FDR<0.1), we detected 5 significant CpGs which differentiated between osteoporotic and healthy women. These 5 CpGs are the top 5 CpGs in Table 1. Next, we calculated adjusted R square as explained in materials and method (under Statistical analysis). The result showed that these 5 CpGs explained as much as 14% of the variation in BMD. Random R squares, based on a random set of 5 CpGs, explained on average only 5%. The methylation levels of these 5 CpGs are plotted in Fig. S2.

Supporting evidence for differentially methylated CpGs, between healthy and patients using an intermediate group

The 63 CpGs which showed distinct methylation levels between osteoporotic and healthy (FDR=0.1) were tested in two separate ways in an intermediate group of postmenopausal women (n=18).

Firstly, we simply looked at the average methylation levels in each of the three groups (healthy, intermediate and osteoporotic. The 18 women had an intermediate mean methylation level in a majority (53 out of 63) of the CpGs (Table S1), suggesting a dose-response relationship between BMD and degree of methylation.

Secondly, we evaluated whether or not we had a significant association between methylation and BMD in all 84 donors adjusted for variation in age. For each CpG site we fitted the model as explained in Materials and methods (Statistical analysis). In as much as 57

out of the 63 methylation sites, we found BMD to be significantly associated to methylation, in a linear fashion. Raw and adjusted p-values (FDR) are shown in Table S4.

Verification by pyrosequencing

In order to confirm methylation differences, four CpG sites that differed with 10 or more methylation percentage points were investigated in 20 subjects (10 osteoporotic and 10 healthy controls, subsets of the original 84 subjects). These four CpG sites were *TNXB* (cg26266427), *UGP2* (cg00074365), *MEPE* (cg07651189) and *BRE (*cg11906781). The *TNXB* CpG site was 48% methylated in controls versus 57% methylated in patients (P=0.17). For *MEPE*, the methylation difference between controls and patients was much less (71% versus 74%, P=0.38), but in the same direction as observed by Illumina 450k. However, the DNA methylation levels of the *TNXB* and *MEPE* CpG sites both correlated significantly with the results obtained with Illumina 450k (R^2=0.75, P<0,0001 and R^2=0.2, P=0.04, respectively) across the 20 samples (Fig. S3). The methylation differences in *BRE* and *UGP2* could not be confirmed by pyrosequencing, probably due to the lower absolute methylation levels observed by the pyrosequencing method, low number of samples tested and the higher technical variability of pyrosequencing, about 2-10 percentage points.[15] The relatively low number of samples being part of the reason for missing replication by pyrosequencing is underscored by finding that the *BRE* CpG also did not reach significance when comparing Illumina results between controls and patients in the same 20 samples (p value=0.06 in a two tailed students t-test).

CpG methylations in blood from an independent cohort partly reflect bone methylations

Since several studies[16-19] indicate that blood methylations at least partly reflect DNA methylation profiles in other tissues, we decided to test the 63 differentially methylated CpGs in blood of an independent cohort from the Framingham study (Materials and methods, Statistical analysis).

Among the 63 CpGs associated with osteoporotic status, 13 were reproduced in blood in subjects from the Framingham cohort (Table 2). Both femur neck (FN) and lumbar spine (LS) skeletal sites were represented among the 13 CpGs reproduced in blood as BMD associated. Among the 5 CpG sites with FDR < 0.05 (Table 1), 3 were replicated, but not CpG sites cg26617611 (*SLC25A37*) or cg08105005 (PACS2). Two other blood CpG sites in PACS2 were however associated with LS or FN BMD in men. In Table 2, we list all the blood cell CpG sites that were significantly associated with BMD at either LS or FN. The effect directions can be observed by the beta coefficient of the residuals of the CpG methylation level. The most significant association was found for CpG site cg14170597 (*PEX14*), in which the methylation degree was associated with lower BMD at both LS and FN in women. Most statistical significant associations were observed for LS BMD. To evaluate whether the methylation levels of the 63 CpG sites were also associated with osteoporotic fracture, we performed association analyses using generalized estimating equations (GEE) with osteoporotic fractures as the dichotomous outcome. A permutation (10,000 times) with minimum statistics was used to correct for multiple testing ($P_{corrected}$). Among 250 available women, we found 31 with osteoporotic fractures. Due to low incidence of osteoporotic fractures in men, we only performed association analyses in women. We found methylation level at the CpG sites cg04997235 (*LRP8*, $P_{corrected}$=0.006), cg01279843 (*USP31*, $P_{corrected}$=0.01) and cg17513789 (*XIST*, $P_{corrected}$=0.02) to be associated with a higher risk of having osteoporotic fractures; and methylation level at the CpG site cg11906781 (*BRE*, $P_{corrected}$=0.02) was associated with a lower risk of having osteoporotic fractures. Finally, we

used multiple linear regression to calculate the degree of BMD variation explained by the blood CpGs. For calculation of R2, we included age + height + weight with or without the relevant blood methylation probes for comparison. When age + height + weight were included with the significant blood methylations, R2 showed only a small increase of 0.008 or 0.015 units for FN BMD in women or men, respectively (Table S5). When all 63 significant bone CpGs from Table 1 were included, R2 increased with 0.071 or 0.122 units for FN BMD in women or men, respectively (Table S5). Similar results were observed for LS BMD.

Results from the correlation analysis between transcript levels and DNA-methylations in bone

To get a deeper insight into the possible relationships between transcript levels and methylation levels we calculated the correlations between expression levels of all 100 genes and the 2529 CpGs as illustrated in Fig. 1 and described in Materials and methods. Results are shown in the matrix (Fig. S4) with red or blue shadings, where red indicates significant positive correlation, and blue indicates significant negative correlation (FDR<0.05). The total number of significant correlations at 5% FDR was 1470 (mainly corresponding to correlations $r_{ij} > 0.4$ or $r_{ij} < -0.4$). In forty-two of these (<3%) the CpGs and transcripts originated from the same chromosome, and only five represented cis correlations (CpGs and transcripts from the same gene). The 5 significant cis correlating CpGs are cg13255629 (*MEPE*, r=0.58); cg04789420 (RASA4P, r=0.55); cg10904972 (USP2, r=0.44); cg08533336 (USP2, r=0.49); cg10065957 (WIF1, r=-0.46). The characteristics of these CpGs are presented in Table S6. The histograms of the raw p-values for cis and trans, separately and jointly, are shown in Fig. S5. Cis and trans-correlations were combined before correcting for multiple testing.

Selected transcripts are significantly correlated with a high number of CpG methylations

Figure 2A presents the 15 transcripts with the highest number of significant correlations with the CpG methylation levels (5% FDR) across all genes. For comparison, the average number of correlated CpGs for the 100 transcripts is shown to the right in the figure. Most of the correlations (70%) showed an inverse relationship.

MEPE (Matrix Extracellular Phosphoglycoprotein1) encodes a secreted calcium-binding phosphoprotein that belongs to the small integrin-binding ligand, N-linked glycoprotein (SIBLING) family of proteins, and correlated to DNA methylation levels in >200 CpGs, more than 20 fold the average gene in Fig. 2A. The C terminus of MEPE contains an acidic, serine- and aspartic acid-rich motif (ASARM) acting as an inhibitor of mineralization.[20] Interestingly, also three of the other transcripts encode inhibitors of bone formation, via the Wnt signaling pathway (*SOST, WIF1, DKK1*). *DLEU2* encode no protein, but two micro RNAs (*miR-15a* and *miR-16*) while *MIR17HG* is the host gene for the *MIR17-92* cluster, encoding a group of at least six micro RNAs (*Mir-17, Mir-18a, Mir-19a, Mir-19b-1, Mir-20a* and *Mir-92a-1*) that may regulate osteoblast proliferation and differentiation[21] in addition to being involved in cell survival, proliferation, differentiation, and angiogenesis.[2222] *SLC20A2* (solute carrier family 20 (phosphate transporter), member 2) is regulated by parathyroid hormone (PTH) a principle conductor of bone remodeling, but has not yet been assigned a specific function in bone.[23] Functions and names of the genes in Fig. 2A are detailed in Table 3. Note that single CpGs within one CpG island tended to show similar DNA methylation levels. To adjust for this possible skewing of the results, we also counted the number of CpG islands significantly correlated to each transcript. A CpG island was treated as significant if one or more CpG sites within that island were significantly correlated to the transcript. As shown in Fig. S6, this resulted in a very similar pattern to that shown in

Fig. 2A, except for a moderate reduction in the number of CpGs correlating to MEPE (from 236 to 176). Of the 15 transcripts associated with the highest number of CpG methylations, 8 are annotated with a defined role in bone biology (Table 3).

Methylations at selected CpGs correlate significantly to a high number of transcripts

We also identified the number of transcripts significantly correlated to each CpG methylation site. The 14 CpGs correlated to the highest number of transcripts (5% FDR) are shown in Fig. 2B along with the average number of correlated transcripts for all 2529 CpGs. While the average gene in the Illumina 450k array covered 17 CpGs, the array measured 577 CpGs within *TNXB,* and 6 of these are among the top 10, meaning that they correlated to the highest number of transcripts. These 6 CpGs are located in an enhancer region within 150 bp in the gene body (Table S7). *TNXB* encodes the extracellular matrix glycoprotein tenascin-X, which is not well described in bone, but deficiency causes Ehlers Danlos syndrome,[2, 24] characterized by reduced amounts of collagen fibers in the skin, in tendons and other connective tissues.[25] The CpG correlating to the highest number of transcripts (5% FDR) lies within an enhancer in the 3` end of *SOX6,* which is well known to have a significant function in cartilage and bone.[26] Ubiquitin Specific Peptidase 2 (*USP2*) (6^{th} and 7^{th} from top) is induced by the osteotropic agents PTH, PTHrP, and PGE2[27], but its specific function in bone is not yet known.

Several bone CpGs differentially methylated between healthy and osteoporotic women correlate to many transcripts.

Several of the 63 CpG sites, which differentiated between healthy and osteoporotic women,

were among those whose methylation degree was correlated to most transcripts (Fig. 2, *TNXB*, *SOX6*) or present in a gene encoding one of the transcripts which again correlated to the highest number of CpG methylation sites (Fig. 2, *DLEU2*). All CpGs from *TNXB* are located in introns of the gene, and there is a potential enhancer in the same genomic region (Table S7). Most of the CpGs that correlated to 5 or more transcripts at 5% FDR were found in the introns/exons of genes, and many also had enhancers located in the same genomic regions (Table S7).

DISCUSSION

In this study, we performed DNA methylation profiling in bone biopsies from 84 postmenopausal women, and analyzed in depth 2529 CpG sites corresponding to the 100 genes whose expression levels (transcripts) were most significantly associated with BMD.[13] Among these CpG sites, 63 CpG methylation levels were statistically different between osteoporotic and healthy women. The results were corroborated by showing that the intermediate group of postmenopausal women, with BMD levels and clinical features between the osteoporotic and healthy also showed intermediate DNA methylation levels for 84% of the 63 significant methylations. Furthermore, DNA pyrosequencing confirmed the distinct *MEPE* and *TNXB* CpG profiles between osteoporotic and healthy women, while two other CpG sites showed p-values close to significance, probably due to few samples analyzed. Most CpG methylation differences were small on a percentage scale, as the case with *BRE* and *UGP2*, possibly explaining why pyrosequencing was not able to confirm all the Illumina results.

Interestingly, 13 of the 63 CpGs also showed association with BMD in blood in an independent cohort. Twelve of these (all but cg12876517 in *UGP2*) were changed in the same direction as found in bone when comparing high vs low BMD (Table 2), thus indicating similar regulatory mechanisms in bone and blood.

It was necessary and important to take age into account as a variable since it could influence the results of the statistical analyses.[2, 14] We therefore stratified the healthy (n=40) and osteoporotic (n=26) based on age. By combining the p-values for each age strata, using the Fisher's method,[28, 29] we increased the overall power, because the combined p-value becomes significant if only one (or more) of the age strata p-values were significant. In relation to the typical issue connected to power and sample size, we recognize that we could potentially underreport, so that there may be significant CpGs we did not detect. On the other

hand, we emphasize that there are few false findings among the 63 differentially methylated CpGs (using FDR=0.1). A false discovery rate on 0.1 indicates 6.3 false findings and 56.7 true findings in our list of totally 63 significant CpGs. Furthermore, the low number of significant CpGs after reshuffling of OP vs healthy labels strengthen the value of our findings.

In our data (Table 1) standard deviations tended to be lower in the patient than in the healthy group. This may be explained by the fact that a majority of OP bone donors have methylation values closer to the extremes (0% or 100% methylation) than healthy, and this group then is given "less room" for variation. Although several of the highly statistically significant differences in methylation were percentwise moderate (Table 1), it is of interest that *in vitro* demethylation by AzadC in the osteoblastic cell line MG-63, reducing *SOST* promoter methylation with 29%, resulted in ~2000 fold increase in *SOST* mRNA expression.[30] Results from chemical demethylation of osteoblastic cells in culture are not directly applicable to this study, but certainly indicate that a small difference in methylation may have inproportionally strong effects on transcription. Furthermore the Illumina array only covers a small fraction of all CpGs. Thus, neighboring CpGs not covered by our arrays but showing similar degree of methylation may have an additive or synergistic effect on gene transcription.

It is noteworthy, but maybe not surprising, that the difference in methylations was most pronounced between the elderly osteoporotic and age matched healthy women. Osteoporosis is a progressive disease caused by advancing molecular pathology, probably also resulting inincreasing DNA methylation alterations with age. Thus, elderly patients will become even more distinct from healthy age matched controls as indicated by the present data. The identified 63 significant CpGs were most distinct in the oldest and largest group (age $\geq$ 68 years, Fig. S1), and we therefore focused on this group in the extended analysis in blood.

In general, DNA methylation patterns have been considered to be tissue specific,[31] however, recent reports indicate that tissue methylation patterns are partly replicated in blood.[16-19] In line with this, we found our results obtained in bone to be reflected in an independent cohort with CpG measurements from whole blood in 210 Caucasian men and 250 Caucasian women aged ≥ 68 years. Among the 63 CpGs associated with osteoporotic status, 13 were found significantly associated with BMD at either FN or LS. In addition, we found that methylation levels among the 63 CpG sites were also associated with either a higher risk of having osteoporosis (cg04997235 in *LRP8*, cg01279843 in *USP31* and cg17513789 in *XIST*) as well as associated with a lower risk of having osteoporotic fractures (CpG site cg11906781 in *BRE*).

Multiple linear regression for the thirteen significant blood CpGs (Table 2) increased the adjusted R2 values obtained by age+height+weight with 0,007 to 0,015 units (male LS or FN, respectively) (Table S5). These results are in concordance with the impact of SNPs for BMD as identified by several GWAS.[8, 32, 33] Since we here analyzed CpGs from only 100 genes, and few blood CpGs provided this contribution, the total contribution from global CpG methylations may explain a significant part of BMD variation.

To identify how DNA-methylation and BMD are interconnected is beyond the scope of this study, but since the elderly women showed the greatest variance in methylation, bone related genes can evidently be subject to acquired epigenetic changes. However, twin studies of methylation in adipose tissue have shown that DNA methylation variation is highly heritable (h(2)median=0.34)[12] and gene heterogeneities like SNPs may guide methylations. Grundberg et al.[12] showed that common variants explained 19% of the total genetic variance of DNA methylation. The present analyses measured acquired and inherited methylation changes, but the acquired ones seem to contribute most to the differences between patients and healthy.

We correlated the 100 transcripts most significantly correlated with BMD with all accessible DNA methylation levels around these genes across 80 bone donors to get further insight into their relationship. Interestingly, out of 1470 significant correlations, only 5 were correlated in cis (correlation to transcripts from the same gene in which it is located). The small number of CpGs that show cis correlations was somewhat surprising, but the cis CpG methylations may affect more than one gene indirectly and thus be of functional importance for multiple genes and their transcriptional activity. This is also indicated by the high number of significant trans-correlated transcripts to these 5 CpGs, on average 13. This finding exemplifies the complexity of gene regulation exerted at the epigenetic level.

The top four transcripts (*MEPE, SOST, DKK1, WIF1*) correlating to the highest number of CpG methylations in bone biopsies are all inhibitors of bone formation. Recently, we presented data to strongly support that *SOST* promoter DNA methylation had functional implications in the same population.[5] *SOST* promoter methylations were increased at low BMD, concomitantly with reduced bone *SOST* mRNA and serum sclerostin levels.[5] The overall effect would be a reduced inhibition of Wnt signaling and thereby reduced bone formation. A similar mechanism may exist for the three other inhibitors (MEPE, DKK1 and WIF1). *MEPE* and *DKK1* both contains CpGs within an upstream enhancer region with DNA methylation levels inversely correlated to their transcript levels, while *WIF1* contains a CpG in its gene body that are positively correlated to its transcript levels. It may also be relevant that these four transcripts are highly correlated to each other in bone, with r values ranging from 0.94 (*SOST* vs *MEPE*) to 0.63 (*MEPE* vs *WIF1*), as calculated using our mRNA correlation browser (http://app.uio.no/med/klinmed/correlation-browser/iliac-v1.1/).[34] These results indicate a concerted regulation of these transcripts, of which *SOST*, *WIF1* and *DKK1* encoded proteins are all targeting the Wnt pathway. Furthermore, MEPE is a direct target of canonical Wnt signaling,[35] and like the three Wnt inhibitors, MEPE is a potent inhibitor of

bone formation.[36] It should also be noted that 5 of the transcripts in Fig. 2A (*SOST, DKK1, DLEU2, ABCA8, NIPSNAP3B*) are among 8 that were found to explain >40% of the variation in BMD after correction for variation in age and body mass index.[13]

The functional effect of several neighboring CpGs being methylated is unknown, but one may assume that they have additive or synergistic effects. As an example, it is striking that the CpGs in *TNXB* correlating to most transcripts lie within an enhancer of *TNXB* (Table S5), and these may thus affect association of DNA binding proteins cooperatively. Furthermore, the CpGs correlating to the highest number of transcripts also tended to have the highest correlation values e.g. the ten topmost correlations values were represented by CpGs correlating to an average of 24 transcripts (not shown). The highest correlation value was between cg11823230 in *TNXB* and the *ALDOA* transcript (r=0.655) and this CpG correlated significantly to 42 different transcripts.

It should be noted that some genes contain CpGs correlating in cis to transcript level just above 5% FDR e.g. *SOX6 (*cg09749669) (r=0.395, p=0.0003, FDR= 0.053), and other transcripts may be regulated by methylation sites in cis that are not covered by the Illumina 450k beads. The methylation level of cg09749669 correlated to 40 out of the 100 transcripts and is located in an enhancer in the 3' UTR of *SOX6,* and showed reduced methylation in osteoporotic women (8.3% FDR, Table 1), indicating SOX6 to be a more central player in bone metabolism than previously appreciated.

ChIP-Seq assay (Chromatin-immunoprecipitation (ChIP) followed by sequencing of the immuno-precipitated DNA), performed on mouse fetal myotubes identified SOX6 binding sites in 867 refseq genes,[37] including *Sox6* and 3 genes among the 40 (*Hspa9*, *Cln8* and *Rad23*). Another ChiP-Seq assay on Rat chondrosarcoma (RCS) cells identified SOX6 binding sites on *Sox6* and 9 of the 40 genes (*Bre*, *Ccni*, *Cmbl*, *Pacs2*, *Pbx3*, Pim3, *Slc20a2*, *Tfdp2* and *Usp2*).[38] Binding sites for more of the 40 genes might be found in bone, as other

DNA regions would be accessible for SOX6 binding. It is also of interest that SOX6 was shown to bind to nearly all superenhancers in addition to many regular enhancers in the RCS cells.[38] Super-enhancers designate very active enhancers or clusters of enhancers that are used as hubs by master transcription factors to govern the expression of major cell identity genes in various cell types.[39, 40]

Importance of SOX6 in bone metabolism is supported by identification of 5 fold reduced *SOX6* expression in primary cultured osteoblasts from a person with high bone mass.[41] Furthermore, SOX6 has recently been found to bind to a *RUNX2* enhancer, increasing its expression in *in vitro* studies.[42] In the bone biopsies, however, *RUNX2* mRNA is inversely correlated to *SOX6* mRNA, (r=-0.41, FDR=0.0006) as identified using our bone mRNA correlation browser (http://app.uio.no/med/klinmed/correlation-browser/iliac-v1.1/).[34] Also, 19 SNPs within or near *SOX6* have been shown to be significantly associated with hip BMD after false discovery rate adjustment.[43] The *SOX6* CpG cg09749669 overlaps the SNP rs75382723, such that presence of the minimal allele would prohibit DNA methylation at this site. However, the minimal A allele has only been observed in African and Southern Asian populations at very low frequency and is thus probably of minor importance to influence BMD of the general population. Using the ECR Browser (http://ecrbrowser.dcode.org/ecrInstructions/ecrInstructions.html), 2 transcription factor binding sites were found within the sequence flanking 20 bp to each side of the SNP, namely the vitamin D (1,25- dihydroxyvitamin D3) receptor (VDR) and spermatogenic leucine zipper 1 (SPZ1).

Most previous studies of bone DNA methylations in osteoporosis have used tibial or femoral bone from osteoarthritic patients as surrogate controls,[44-46] thus using stressed/loaded bone, whereas this study used unloaded iliac bone from bona fide healthy controls. The difference in study materials and our strategy of only analysing methylation in a limited set of

genes may explain why only few common genes is found among the current and other studies. The most extensive study performed to date by Delgado-Calle et al.[44] identified >200 CpGs with differential methylation level between OP and OA femoral biopsies. However, only LY9 (Lymphocyte Antigen 9) was a common finding between the two studies. Lhaneche et al.[46] showed that DNA methylations in tibial bone *SOST* exon 1 was positively correlated with *SOST* expression levels, while we found an inverse relationship between CpGs in the promoter region and *SOST* trancripts in iliac bone.[5] The different findings may be due to difference between the bone source (loaded tibia vs unloaded iliac) as well as different gene regions (exon 1 vs promoter). A recent blood epigenome wide study (EWAS) of >5500 individuals identified only one BMD associated CpG methylation using the same platform as the current study.[47] The CpG located to the gene *CES*, which was not among those assessed in our study. Also their different statistical approach (no candidate CpGs) may explain why none of our blood associated CpGs were identified.

The study has a limitation in being based on trans-iliacal bone biopsies with a heterogeneous cell population containing, in addition to bone cells, marrow cells of the hematopoietic and immunological lineages, comprising a minor part of the total cell population, ca. 20% fraction as shown by RNA sequencing of the same samples (unpublished). Also, minor contribution of adipocytes, endothelial and stromal cells need to be considered. However, to use primary osteoblast/osteocyte cell cultures would not be representative of the in vivo situation, since cells in culture tend to dedifferentiate, probably including epigenetic and gene expressional changes. Established bone cell lines are also not a physiological model to study bone biology. The strong aspect of utilizing fresh bone tissue, as in the present paper, gives the instant picture of a physiologically in vivo representative bone and marrow functions. Furthermore, we have studied a very limited number of genes representing the topmost BMD associated transcripts, of which many are typical for bone cells. Also, we have performed Affymetrix microchip analysis

of blood from an overlapping set of the bone donors, but the blood transcripts from the 100 analyzed genes did not correlate significantly to BMD (not shown). Thus it is not likely that our results are much influenced by blood contamination of the bone biopsies. Furthermore, reproduction of DNA methylation patterns in blood from the Framingham cohort is valid and independent of the amount of non-bone cells in the biopsies.

It is also a limitation that the Illumina 450 k microchips cover just a fraction of all CpGs, thus more CpGs with larger differences in methylation of unknown impact may exist in the same area. E.g. we detected 20 CpGs at 10% FDR for *TNXB*, all with increased methylation in osteoporotic women, but ranging from 1.7 to 19.9%. This could be the case for other genes as well if more CpGs were analyzed. Strength: The study involves the largest number of human bone biopsies from a clinically well described cohort of postmenopausal women where global DNA methylation mapping has been performed and correlated with functional genetics. We conclude that gene-specific DNA methylation levels are associated with BMD and osteoporotic fractures, and the results represent the first fundament to elucidate the impact of epigenetics as cause or risk for osteoporosis.

MATERIALS AND METHODS

Ethics statement

The study was approved by the Norwegian Regional Ethical Committee (REK no: 2010/2539) and conducted according to the Declaration of Helsinki (2000). The blood sample replication study was approved by the Hebrew SeniorLife Institute for Aging Research Ethical Committee.

The Oslo study group

The women in this group (50–86 years) showed varying bone mineral densities (BMDs) and were free of primary diseases known to affect the skeleton. Most of the osteoporotic women were recruited at the Out-patient clinic, Lovisenberg Diakonale hospital in Oslo, while most of the healthy were recruited through announcements in local newspapers. The participants were motivated by a total health checkup and wanted to make a contribution to an important field of research aiming to understand the mechanisms of the most prevalent disease in Europe and the western world above 50 years of age. Except two sister pairs, none of the participants were related.

Methylation and gene expression analyses were performed in 84 postmenopausal women subjected to bone biopsies. The included participants had normal endocrine, clinical, biochemical and nutritional status, and had been postmenopausal and without estrogen medication for at least 2 years. Patients on bisphosphonates, corticosteroids or other drugs known to affect bone turnover, were not included. All participants were on standard Norwegian diet and had similar nutritional supplements and life style factors as previously described.[13] Osteoporotic patients were defined, according to the World Health Organization's operational definition (i.e., bone density of ≤2.5 deviations below the mean of

a 30-year-old man/woman (T-score ≤2.5)), whereas T-scores between −2.5 and −1.0 were defined as osteopenia, and T-scores greater than −1.0 were considered normal. The 84 participants subjected to DNA methylation analysis comprised a set of 26 patients with generalized OP and at least one low-energy fracture in the spine, rib, wrist, or hip and 40 healthy controls, as well as a third cohort consisting of 18 women who presented clinical features and laboratory characteristics between the OP and control groups. This intermediate group were age matched, had normal or slightly osteopenic hip BMD, vertebral osteoporosis, minor height reduction, no fragility fractures, and showed intermediate bone metabolism parameters.[48] As presented in Table S2, this group of postmenopausal females represented a cohort different from the healthy and osteoporotic with intermediate biochemical parameters as well as clinical characteristics. Bone biopsies from 80 women were subjected to both global DNA methylation and global transcriptional analysis.

Bone mineral density Bone densitometry of total hip, femoral neck (FN), lumbar spine (LS) L1, lumbar spine L2-L4, total body and trochanter were performed by dual-energy X-ray absorptiometry (DXA), using a Lunar Prodigy densitometer (Lunar Corporation, Documentation version 3/95; DPX version 4.6c).

Bone RNA analysis Trans-iliacal bone biopsies (0.8 x 1.5 cm), containing cortical and trabecular bone, were collected and used for RNA isolation, as described.[13] Microarray analysis was performed as described[13] by use of GeneChip® Expression 3′ Amplification One-Cycle Target Labeling Kit (Affymetrix) and hybridization of cRNA to HG-U133 plus 2.0 chips (Affymetrix). All gene expressions signal values were used at $\log_2$ scale.

Bone CpG methylation analysis Bone DNA was isolated from the same samples that were used to isolate bone RNA. Eighty of the biopsies were subjected to both transcriptional and DNA methylation profiling. Upon RNA isolation with TRIzol® (Invitrogen, 15596026), an interphase containing DNA is generated between the aquatic upper and organic lower phase. DNA was isolated from the interphase according to the manufacturer's instructions. Methylation mapping was performed using the Infinium HumanMethylation450 BeadChip, according to the manufacturer's instructions (Illumina). These arrays enabled detection of the methylation status of 485,000 individual CpGs, with an average of 17 CpG sites per gene region distributed from 1500 bp upstream of the transcription start site, across the promoter, 5'UTR, first exon, gene body, and 3'UTR. Illumina provides a set of annotations for each CpG, and the Illumina annotated genes for the relevant CpGs were used when assessing whether transcript vs methylation correlations were cis or trans. The fluorescence data were analyzed in BeadStudio (Illumina) to determine the beta values (quantitative measurement of the methylation) for each CpG. Quality control checks followed in BeadStudio and minfi.[49] Data were preprocessed using minfi[49] and normalized using BMIQ.[50] Lastly, beta values were exported from BMIQ for further analyses.

Pyrosequencing The degree of methylation of four CpG sites was verified by bisulphite-PCR followed by pyrosequencing. DNA from bone biopsies (approximately 100 ng) was bisulfite-treated and purified using EpiTect Bisulfite kit (Qiagen, 59104). PCR- and sequencing primers were designed using PyroMark Assay design 2.0 (Qiagen) (Table S8). Amplicons were amplified from ~10 ng bisulfite-converted DNA in 25µl reactions using 1 x PyroMark PCR Master Mix (Qiagen, 978703), 1 x CoralLoad Concentrate (Qiagen, 201203) and 0,2 µM primers. Cycling conditions were an initial denaturation step at 95°C for 15 min followed by 50 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and a final extension period at

72°C for 10 min. Sequencing was performed in a PyroMark Q24 instrument with PyroGold reagents (Qiagen, 970802). Results were analyzed using PyroMark Q24 2.0.6 software. The methylation analysis was performed twice for each CpG site.

The Framingham cohort

Blood CpG methylation analysis The CpG methylation profiles in bone from the Oslo study were tested in peripheral blood cells from a subgroup of the Framingham study described in Results. To avoid the confounding effect of differences in blood cell distribution, blood was only collected from healthy individuals free of infection and blood diseases and having normal blood cell parameters. In this study leg, participants had BMD analyzed with DXA and DNA methylations studied in blood cells.[51]

We used Infinium HumanMethylation450 BeadChip, according to the manufacturer's instructions (Illumina), and the detailed information is given at dbGaP NCBI (http://www.ncbi.nlm.nih.gov/gap; dbGaP Study Accession: phs000724.v3.p10). In brief, genome-wide DNA methylation were assessed from peripheral blood samples (N=2,846) collected during Framingham offspring cohort examination cycle 8 (2005-2008). Genomic DNA was extracted from buffy coat preparations using the Gentra Puregene DNA extraction kit (Qiagen, 158667), and bisulfite treatment used the EZ DNA Methylation kit (Zymo Research, D5002). Bisulfite-converted DNA was hybridized to the Illumina Infinium Human Methylation450 BeadChip. Methylation data were normalized within laboratory batches using a DASEN method.[50]

Statistical analysis

Data analysis in bone of 100 BMD correlated genes and their methylation levels

Earlier we found 142 transcripts correlating to total hip T-score at 10% FDR.[13] The top 100 of these genes were selected for analysis in this study. When more than one transcript represented a gene, only the transcript with the highest Affymetrix signal value was used. We selected the 2529 CpGs covered by the Illumina microchip in these genes for analysis. See Fig. S7 for summary of the number of methylation sites per gene.

Detection of 63 differentially methylated CpGs between healthy and osteoporotic women in bone

We used the method described by Chen et al.[14] which accounts for age when detecting CpG methylations that differed significantly between the 40 bone donors classified as healthy and the 26 patients with established osteoporosis (see section "The Oslo study group" above). Methylation may be affected by age but not necessarily in a linear fashion.[2] Therefore, we performed a stratified analysis by dividing the participants into equally sized age groups; resulting in young ($\leq$57.50 years), middle ($>$57.50, $\leq$68.75 years) and old ($>$68.75 years). Within each age group we tested the null hypothesis that the degree of methylation is equal between healthy and osteoporotic bone donors, against the two-sided alternative, that the methylation levels are different. Due to the somewhat complicated distribution of methylation-values, we did not want to assume any distribution, and therefore used the non-parametric Wilcoxon rank sum test. To increase power, the three p-values from each age group were combined to one p-value, by the established method called Fisher's combined probability test.[28, 29] We corrected for multiple testing by use of the Benjamini and Hochberg procedure,[52] which controls the false discovery rate (FDR), i.e. the expected proportion of false discoveries in a list of rejected hypotheses.

Calculation of adjusted R square

We used adjusted R square to quantify the amount of variation in BMD explained by a set of CpGs. In more detail, we fitted a linear regression having BMD T-scores as outcomes (a continuous variable) and the set of methylation sites as covariates. Then the adjusted R square gives a value between 0 and 1, which can be interpreted as a percentage. To see whether or not it can be regarded as interestingly high, we compared it to a large number (10,000) of random R squares. The random R square is found using a random set of CpGs, where the size of the set is equal to the original set.

Calculations on the intermediate group of bone donors to support the analysis of differentially methylated CpGs between healthy and patients

In addition to the patients and healthy, an intermediate group included data on 18 women with clinical and laboratory features between the two other groups. For a selected CpG site, we investigated whether or not the methylation levels of this intermediate group positioned between the methylation levels of the two other groups, either increasingly or decreasingly, suggesting a potential dose-response relationship. Using all 84 bone donors (healthy, intermediate, osteoporotic), we fitted a continuous linear regression with methylation as response and tested if BMD was a significant covariate. A model with BMD was compared to a model without BMD using a likelihood ratio test. In both models we included age and the interaction between BMD and age to account for possible differences in the age groups.

Testing on selected bone CpG methylations in blood cells from an independent cohort

The current analyses were limited to donors ≥ 68 years old from whom blood was obtained for DNA methylation analyses. A total of 460 participants (210 men and 250 women) were included in the final statistical analyses, since we excluded 250 samples with low quality of the methylation measurements. A total of 63 CpG methylation sites (listed in Table 1) were

selected for testing, representing both sex-specific and sex-combined association analyses. We first generated residuals for each CpG methylation sites by mixed-effect models to adjust for potential confounders including age, sex (combined sex models), batch effects, technical covariates and cell counts. The residuals of CpG methylation were then used to perform association analyses between methylation levels and BMD phenotypes via a linear mixed-effect model implemented in R Kinship package to take into account the potential within family correlations among samples. The BMD phenotypes in the statistical analyses included LS and FN BMD as well as the T-scores for both LS and FN BMD. To correct for multiple testing, we performed a permutation test (10,000 permutations) with the maximum test statistics approach, and the permutation p-values were reported considering p-values < 0.05 as statistically significant.

Correlations of bone transcript and methylation levels in bone

We investigated all possible correlations between the 100 gene transcripts and 2529 CpGs across 80 donors, both cis and trans, as illustrated in Fig. 1. The Pearson correlation ρ_{ij} between transcript level gene *i*, and methylation level site *j*, resulted in 100x2529 empirical correlations r_{ij}. We tested furthermore if each correlation was equal to zero ($\rho_{ij} = 0$), against the alternative hypothesis that the correlation was not equal to zero ($\rho_{ij} \neq 0$), by use of a permutation test. For every pair of gene and methylation site we therefore permuted the 80 samples, and calculated the randomized correlation. A p-value was calculated based on the amount of randomized correlations that were larger than the observed correlation.[53] 100 000 permutations were performed for each pair. We corrected for multiple testing by use of the Benjamini and Hochberg procedure,[52] which controlled the false discovery rate (FDR). Note that the Benjamini and Hochberg procedure is valid for positively correlated tests,[54] and can therefore be used in our study.

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FIGURE LEGENDS

Figure 1.

Strategy for correlating transcript levels and DNA-methylations in trans-iliacal bone biopsies in postmenopausal women.

DNA and RNAs from individual participants were extracted from the same bone biopsies. Genes encoding the 100 mRNAs most associated to BMD (matrix A) and the associated CpGs (matrix B) were analyzed. These two sets of data were then correlated giving the matrix C. A permutation analysis identified the CpGs, which were significantly correlated to transcript levels at 5% FDR. An enlarged correlation matrix (C) is presented in Fig. S4.

Figure 2.

Transcript levels significantly correlated to CpGs methylation levels (A) and vice versa (B) at 5% FDR.

A. The 15 transcripts with the highest number of significant correlations with CpG methylations (5% FDR) compared with average number of correlations with CpG methylation levels among all 100 transcripts (see "AVERAGE"). **B.** The 14 CpGs with the highest number of significant correlations with transcripts (5% FDR), and average number of significant correlations to transcripts for all 2529 CpGs (see AVERAGE). In cases with more than one CpG in one gene, only the CpG with the highest number of significant correlations is included. The label *MIRHG17* represents a cluster of 6 genes, namely *MIR17, MIR18A, MIR19A, MIR19B1, MIR92A1, MIR20A*. The black bars represent positive correlation ($r>0$) and the white bars represent negative correlation ($r<0$). Fig. S8 shows the 30 CpGs correlated to most transcripts, and vice versa, and includes multiple CpGs for some of the genes.

Data access

Data have been submitted to the European Bioinformatics Institute (EMBL-EBI) ArrayExpress repository, ID: E-MEXP-1618.

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Disclosures

The authors report no conflict of interest.

Authors' contributions:

KMG, SR, TGL and KG designed the study; YHH designed replication study; SR, KMG, YHH, RY, VTG, OKO, MK, HB and RL collected data; RL, SR, YHH, RY, OKO and TGL prepared data; TGL YHH, RY and IKG did statistical analysis; SR drafted manuscript; all authors critically revised manuscript and approved final version.

Table 1. Sixty-three Bone CpGs showing significant distinct methylation levels between healthy and osteoporotic postmenopausal women (FDR=0.1)

CpG ID	Osteoporotic (n=26) mean methylation (%) ±SD	Healthy (n=40) mean methylation (%) ±SD	Difference	Change (%)	p-value	FDR-corrected p-value	Gene symbol	Gene name
cg14919562	2.80 ± 0.57	3.32 ± 0.68	**-0,52**	**-15.7**	2.50E-05	3.16E-02	*RAD23B*	RAD23 homolog B (*S. cerevisiae*)
cg14170597	90.57 ± 2.57	86.79 ± 3.18	**3,78**	**4.4**	2.35E-05	3.16E-02	*PEX14*	peroxisomal biogenesis factor 14
cg03822479	92.55 ± 1.73	89.34 ± 6.75	**3,21**	**3.6**	5.98E-05	3.78E-02	*TNXB*	tenascin XB
cg26617611	98.21 ± 0.85	96.90 ± 1.26	**1,31**	**1.3**	5.42E-05	3.78E-02	*SLC25A37*	solute carrier family 25 (mitochondrial iron transporter), member 37
cg08105005	96.04 ± 1.39	94.05 ± 1.75	**1,99**	**2.1**	7.92E-05	4.00E-02	*PACS2*	phosphofurin acidic cluster sorting protein 2
cg17948986	96.40 ± 3.70	91.58 ± 9.89	**4,82**	**5.3**	1.27E-04	5.36E-02	*KCNRG/DLEU2**	potassium channel regulator/deleted in lymphocytic leukemia 2 (non-protein coding)
cg08946014	47.24 ± 2.21	50.63 ± 4.88	**-3,39**	**-6.7**	1.80E-04	6.52E-02	*PEBP1*	phosphatidylethanolamine binding protein 1
cg11906781	13.54 ± 5.58	27.77 ± 16.03	**-14,23**	**-51.3**	4.59E-04	7.43E-02	*BRE*	brain and reproductive organ-expressed (TNFRSF1A modulator)
cg12409083	75.10 ± 9.44	69.67 ± 8.13	**5,43**	**7.8**	3.87E-04	7.43E-02	*TNXB*	tenascin XB
cg00123333	96.48 ± 1.19	94.82 ± 1.65	**1,66**	**1.7**	4.95E-04	7.43E-02	*CMBL*	carboxymethylenebutenolidase homolog (Pseudomonas)
cg09230442	29.82 ± 3.49	23.61 ± 6.10	**6,21**	**26.3**	3.51E-04	7.43E-02	*DPP8*	dipeptidyl-peptidase 8
cg04348080	93.67 ± 1.93	91.37 ± 2.23	**2,3**	**2.5**	4.57E-04	7.43E-02	*MDH2*	malate dehydrogenase 2, NAD (mitochondrial)
cg15111386	96.27 ± 1.58	94.35 ± 1.83	**1,92**	**2.0**	2.68E-04	7.43E-02	*TNXB*	tenascin XB
cg07609381	96.92 ± 1.31	95.22 ± 1.45	**1,7**	**1.8**	4.32E-04	7.43E-02	*TNXB*	tenascin XB
cg17513789	88.89 ± 3.37	80.85 ± 12.77	**8,04**	**9.9**	4.99E-04	7.43E-02	*XIST*	X inactive specific transcript (non-protein coding)
cg06988099	95.95 ± 1.67	94.01 ± 1.69	**1,94**	**2.1**	4.73E-04	7.43E-02	*USP31*	ubiquitin specific peptidase 31
cg16485952	93.03 ± 2.18	90.65 ± 2.22	**2,38**	**2.6**	2.72E-04	7.43E-02	*TNXB*	tenascin XB
cg01279843	98.20 ± 0.79	96.99 ± 1.24	**1,21**	**1.3**	5.54E-04	7.53E-02	*USP31*	ubiquitin specific peptidase 31
cg21593986	95.30 ± 2.04	92.65 ± 2.92	**2,65**	**2.9**	5.66E-04	7.53E-02	*TFDP2*	transcription factor Dp-2 (E2F dimerization partner 2)
cg01188191	38.02 ± 2.43	35.54 ± 2.25	**2,48**	**7.0**	8.44E-04	8.26E-02	*TNXB*	tenascin XB
cg20370839	98.74 ± 0.76	97.49 ± 1.37	**1,25**	**1.3**	1.02E-03	8.26E-02	*SLC20A2*	solute carrier family 20 (phosphate transporter) member 2
cg26871717	92.10 ± 1.58	90.40 ± 2.19	**1,7**	**1.9**	1.01E-03	8.26E-02	*MUM1*	melanoma associated antigen (mutated) 1
cg24506088	95.86 ± 1.59	93.82 ± 2.17	**2,04**	**2.2**	1.04E-03	8.26E-02	*TNXB*	tenascin XB
cg09749669	83.97 ± 3.72	88.06 ± 2.85	**-4,09**	**-4.6**	1.02E-03	8.26E-02	*SOX6*	SRY (sex determining region Y)-box 6
cg20117260	55.73 ± 2.70	53.14 ± 4.14	**2,59**	**4.9**	6.62E-04	8.26E-02	*ATP5SL*	ATP5S-like
cg12112719	91.09 ± 5.07	89.60 ± 1.84	**1,49**	**1.7**	7.23E-04	8.26E-02	*TNXB*	tenascin XB
cg25751172	16.91 ± 4.70	15.25 ± 3.03	**1,66**	**10.9**	8.17E-04	8.26E-02	*DKK1*	dickkopf WNT signaling pathway inhibitor 1
cg02668819	93.50 ± 2.01	91.15 ± 2.21	**2,35**	**2.6**	7.53E-04	8.26E-02	*TNXB*	tenascin XB
cg24054898	78.50 ± 3.75	83.11 ± 4.33	**-4,61**	**-5.6**	1.03E-03	8.26E-02	*GYG1*	glycogenin 1
cg10793101	94.48 ± 2.08	92.24 ± 2.09	**2,24**	**2.4**	9.74E-04	8.26E-02	*RNF216*	ring finger protein 216
cg23371655	95.26 ± 2.54	90.97 ± 6.62	**4,29**	**4.7**	1.01E-03	8.26E-02	*UBE2G1*	ubiquitin-conjugating enzyme E2G 1
cg14256699	85.11 ± 3.37	81.56 ± 3.17	**3,55**	**4.4**	9.82E-04	8.26E-02	*SOST*	Sclerostin
cg16590552	97.03 ± 1.19	95.41 ± 1.64	**1,62**	**1.7**	1.12E-03	8.57E-02	*USP31*	ubiquitin specific peptidase 31
cg07910408	94.72 ± 1.73	92.77 ± 1.92	**1,95**	**2.1**	1.16E-03	8.66E-02	*TNXB*	tenascin XB
cg24475062	91.20 ± 3.98	89.19 ± 3.94	**2,01**	**2.3**	1.35E-03	8.84E-02	*TNXB*	tenascin XB
cg17979173	98.63 ± 0.57	97.44 ± 1.37	**1,19**	**1.2**	1.40E-03	8.84E-02	*TNXB*	tenascin XB
cg00074365	29.32 ± 4.14	40.60 ± 12.69	**-11,28**	**-27.8**	1.26E-03	8.84E-02	*UGP2*	UDP-glucose pyrophosphorylase 2
cg04483460	96.74 ± 1.34	95.00 ± 1.83	**1,74**	**1.8**	1.38E-03	8.84E-02	*LRP8*	tenascin XB
cg04997235	87.72 ± 1.92	85.89 ± 2.12	**1,83**	**2.1**	1.39E-03	8.84E-02	*LRP8*	tenascin XB
cg14924781	91.93 ± 2.67	88.94 ± 2.83	**2,99**	**3.4**	1.26E-03	8.84E-02	*TNXB*	tenascin XB
cg07641807	8.84 ± 3.61	6.23 ± 3.41	**2,61**	**41.9**	1.69E-03	8.85E-02	*MIR17HG***	miR-17-92 cluster host gene (non-protein coding)
cg14011611	96.47 ± 1.46	94.59 ± 1.92	**1,88**	**2.0**	1.87E-03	8.85E-02	*TNXB*	tenascin XB
cg26266427	63.93 ± 9.19	53.34 ± 12.09	**10,59**	**19.9**	1.53E-03	8.85E-02	*TNXB*	tenascin XB
cg22740866	96.97 ± 1.34	95.12 ± 2.15	**1,85**	**1.9**	1.77E-03	8.85E-02	*GYS1*	glycogen synthase 1 (muscle)
cg12876517	95.94 ± 2.21	89.77 ± 8.75	**6,17**	**6.9**	1.89E-03	8.85E-02	*UGP2*	UDP-glucose pyrophosphorylase 2
cg17004212	25.94 ± 6.94	17.12 ± 7.89	**8,82**	**51.5**	1.82E-03	8.85E-02	*DLEU2*	deleted in lymphocytic leukemia 2 (non-protein coding)
cg26379707	77.76 ± 2.75	75.50 ± 2.77	**2,26**	**3.0**	1.86E-03	8.85E-02	*PACS2*	phosphofurin acidic cluster sorting protein 2
cg20319604	87.26 ± 3.24	90.69 ± 3.34	**-3,43**	**-3.8**	1.62E-03	8.85E-02	*PPM1B*	protein phosphatase, Mg2+/Mn2+ dependent, 1B
cg19109439	94.82 ± 1.86	93.00 ± 2.30	**1,82**	**2.0**	1.55E-03	8.85E-02	*TNXB*	tenascin XB
cg12158535	69.36 ± 4.65	65.69 ± 3.97	**3,67**	**5.6**	1.65E-03	8.85E-02	*PACS2*	phosphofurin acidic cluster sorting protein 2
cg07625630	93.95 ± 1.86	91.99 ± 2.00	**1,96**	**2.1**	1.81E-03	8.85E-02	*MICA*	MHC class I polypeptide-related sequence A
cg25369058	96.33 ± 1.44	94.58 ± 1.94	**1,75**	**1.8**	1.80E-03	8.85E-02	*TNXB*	tenascin XB
cg18319635	89.06 ± 2.88	85.75 ± 3.07	**3,31**	**3.9**	1.77E-03	8.85E-02	*TNXB*	tenascin XB
cg17690515	94.76 ± 1.36	93.15 ± 1.71	**1,61**	**1.7**	1.50E-03	8.85E-02	*MUM1*	melanoma associated antigen (mutated) 1
cg17284609	75.45 ± 5.77	82.58 ± 6.26	**-7,13**	**-8.6**	1.95E-03	8.95E-02	*SOX6*	SRY (sex determining region Y)-box 6
cg08250055	92.11 ± 2.74	89.97 ± 3.02	**2,14**	**2.4**	1.99E-03	8.97E-02	*TNXB*	tenascin XB
cg00583733	30.40 ± 2.11	29.01 ± 3.06	**1,39**	**4.8**	2.11E-03	9.38E-02	*ALDOA*	aldolase A, fructose-bisphosphate
cg19763210	97.21 ± 1.42	95.73 ± 2.15	**1,48**	**1.5**	2.28E-03	9.71E-02	*XPR1*	xenotropic and polytropic retrovirus receptor 1
cg22616343	76.93 ± 3.48	73.57 ± 4.61	**3,36**	**4.6**	2.26E-03	9.71E-02	*TNXB*	tenascin XB
cg05253005	1.73 ± 0.43	2.08 ± 0.65	**-0,35**	**-16.6**	2.30E-03	9.71E-02	*GYS1*	glycogen synthase 1 (muscle)
cg06860460	86.23 ± 1.84	88.26 ± 2.91	**-2,03**	**-2.3**	2.35E-03	9.72E-02	*MYL12B*	myosin, light chain 12B, regulatory
cg06671964	96.07 ± 1.63	94.71 ± 1.76	**1,36**	**1.4**	2.39E-03	9.76E-02	*TNXB*	tenascin XB
cg07651189	68.22 ± 7.96	57.89 ± 12.45	**10,33**	**17.8**	2.47E-03	9.93E-02	*MEPE*	matrix extracellular phosphoglycoprotein

The table shows the CpG ID and their mean and SD methylation levels in osteoporotic and healthy women. The p-values are calculated based on the Wilcoxon rank sum test, taking age into account, testing for difference in methylation between osteoporotic and healthy women. FDR are calculated by the Benjamini-Hochberg procedure.[52] Gene symbols and names in the rightmost columns are those containing the CpGs in the leftmost column.**KCNRG/DLEU2* are encoded on opposite DNA strands, **MIR17HG cluster is listed with 6 miRNA genes (MIR18A, *MIR19A*, *MIR19B1*, *MIR20A*, *MIR17*,*MIR92A1*)

Table 2. Associations between selected blood CpG methylation sites and BMD in Framingham participants

Lumbar spine BMD

		Women		Men	
CpG#	**Gene symbol**	**Beta**	**P**	**Beta**	**P**
cg25751172	DKK1	-0.30	**3.61E-02**	-0.42	3.48E-01
cg06860460	MYL12B	0.66	3.46E-01	1.82	9.27E-02
cg12158535	PACS2	-0.61	3.30E-01	-1.07	**1.08E-02**
cg26379707	PACS2	-0.24	8.52E-01	-4.01	**1.65E-02**
cg14170597	PEX14	-2.21	**3.46E-04**	-0.45	6.01E-01
cg14919562	RAD23B	1.16	**5.27E-02**	0.35	8.09E-01
cg10793101	RNF216	-1.07	**3.84E-03**	0.47	6.86E-01
cg03822479	TNXB	-0.88	**4.43E-02**	-0.53	5.31E-01
cg07609381	TNXB	-0.23	8.20E-01	-0.44	7.38E-01
cg08250055	TNXB	-0.59	3.13E-01	-1.72	**5.94E-03**
cg16485952	TNXB	-0.76	5.01E-01	-0.97	5.49E-01
cg24506088	TNXB	-1.65	**3.92E-03**	-0.55	4.41E-01
cg12876517	UGP2	0.92	**4.84E-02**	0.44	5.08E-01

Femoral neck BMD

		Women		Men	
CpG#	**Gene symbol**	**Beta**	**P**	**Beta**	**P**
cg25751172	DKK1	-0.03	8.65E-01	-0.40	1.32E-01
cg06860460	MYL12B	0.17	7.02E-01	0.60	**3.30E-02**
cg12158535	PACS2	-0.59	1.33E-01	-0.15	7.77E-01
cg26379707	PACS2	-0.44	5.90E-01	-1.91	**4.97E-02**
cg14170597	PEX14	-0.88	**2.49E-02**	-0.14	7.88E-01
cg14919562	RAD23B	0.10	9.04E-01	0.86	3.28E-01
cg10793101	RNF216	-0.15	7.53E-01	-0.87	2.11E-01
cg03822479	TNXB	-0.18	7.37E-01	-0.33	5.92E-01
cg07609381	TNXB	-0.64	3.04E-01	-1.52	**4.97E-02**
cg08250055	TNXB	-0.44	**1.97E-02**	-0.49	1.92E-01
cg16485952	TNXB	-0.05	9.41E-01	-2.39	**1.37E-02**
cg24506088	TNXB	-0.38	2.98E-01	0.19	6.61E-01
cg12876517	UGP2	0.36	2.36E-01	0.25	5.35E-01

Beta coefficients are from the linear mixed-effect model of the association between methylation levels and BMD, adjusting for potential confounders including age, sex, batch effects, technical covariates and cell counts. P-values: Permutation p-values corrected for multiple testing. $P < 0.05$ was considered as study-wide statistical significance. Significant values are shaded.

Table 3. Functional association of genes with transcripts associated with highest number of CpG methylations

Gene symbol	Name	Function	Possible relation to bone metabolism
MEPE	Matrix Extracellular Phosphoglycoprotein	uptake of Pi, mineralization	negative regulation of bone mineralization
SOST*	sclerostin	Wnt pathway inhibitor	negative regulation of ossification
WIF1	WNT inhibitory factor 1	Wnt pathway inhibitor	Inhibitor of bone formation
DKK1*	Dickkopf WNT Signaling Pathway Inhibitor 1	Wnt pathway inhibitor	negative regulation of ossification
MIR17HG	miR-17-92 cluster host gene	Encodes 6 miRNAs: miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1	regulates osteoblast proliferation and differentiation
NDNF	Neuron-Derived Neurotrophic Factor1	glycosaminoglycan binding and heparin binding	Promotes matrix assembly and cell adhesiveness
DLEU2*	deleted in lymphocytic leukemia 2	Encodes 2 miRNAs: miR-15a and miR16-1	tumor suppressor
CSRNP3	Cysteine/serine-rich nuclear protein 3	positive regulation of apoptotic process	negative regulation of phosphatase activity
ABCA8*	ATP-Binding Cassette, Sub-Family A (ABC1), Member 8	Lipid transporter	«may regulate lipid metabolism»
ACAT1	acetyl-CoA acetyltransferase 1	branched-chain amino acid catabolic process	
GBAS (NIPSNAP2)	glioblastoma amplified sequence	oxidative phosphorylation	
NIPSNAP3B*	Nipsnap Homolog 3B (C. Elegans)		"putative roles in vesicular trafficking"
SLC20A2	Solute Carrier Family 20 (Phosphate Transporter), Member 2	Phosphate transporter	
RAD23B	RAD23 homolog B (*S. cerevisiae*)	nucleotide-excision repair, DNA damage recognition	
BBX	bobby sox homolog (*Drosophila*)	hmg-box transcription factor	bone development

*Belong to 8 genes explaining >40% of variation in BMI and age adjusted BMD[13]
"Function" and "possible relation to bone metabolism" were obtained from Gene Ontology (http://geneontology.org/)