



Morris, J. A., Tsai, P-C., Joehanes, R., Zheng, J., Trajanoska, K., Soerensen, M., ... Bell, J. T. (2017). Epigenome-wide Association of DNA Methylation in Whole Blood With Bone Mineral Density. *Journal of Bone and Mineral Research*, 32(8), 1644-1650. <https://doi.org/10.1002/jbmr.3148>

Peer reviewed version

Link to published version (if available):
[10.1002/jbmr.3148](https://doi.org/10.1002/jbmr.3148)

[Link to publication record in Explore Bristol Research](#)
PDF-document

This is the author accepted manuscript (AAM). The final published version (version of record) is available online via Wiley at <https://onlinelibrary.wiley.com/doi/abs/10.1002/jbmr.3148> . Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available:
<http://www.bristol.ac.uk/pure/about/ebr-terms>

Journal for Bone and Mineral Research (Original Research)

Title: Epigenome-wide association of DNA methylation in whole blood with bone mineral density

Authors: John A Morris^{1,2} (BSc)*, Pei-Chien Tsai³ (PhD)*, Roby Joehanes^{4,5,6} (PhD)*, Jie Zheng⁷ (PhD)*, Katerina Trajanoska⁸ (MD)*, Mette Soerensen^{9,10} (PhD)*, Vincenzo Forgetta² (PhD), Morten Frost¹¹ (PhD), Tim D Spector³ (FRCP), Kaare Christensen^{9,10,12} (PhD), Lene Christiansen⁹ (PhD), Fernando Rivadeneira⁸ (MD), Jonathan H Tobias^{7,13} (PhD), David M Evans^{7,11} (PhD), Douglas P Kiel^{4,5,6} (MD), Yi-Hsiang Hsu^{4,5,6} (PhD)[§], J Brent Richards^{1,2,3,11} (MD)[§], Jordana T Bell³ (DPhil)[§]

Affiliations:

1. Department of Human Genetics, McGill University, Montreal, Canada
2. Lady Davis Institute for Medical Research, Department of Medicine, Jewish General Hospital, McGill University
3. Department of Twin Research and Genetic Epidemiology, King's College London, London, United Kingdom
4. Institute for Aging Research, Hebrew SeniorLife, Boston, MA, USA
5. Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, USA
6. Broad Institute of MIT and Harvard, Cambridge, MA, USA
7. MRC Integrative Epidemiology Unit, University of Bristol, Bristol, UK
8. Departments of Internal Medicine and Epidemiology, Erasmus Medical Center, Rotterdam, Netherlands
9. The Danish Twin Registry and The Danish Aging Research Center, Epidemiology, Biostatistics and Biodemography, Institute of Public Health, University of Southern Denmark, Odense, Denmark
10. Department of Clinical Genetics, Odense University Hospital, Odense, Denmark
11. Endocrine Research Unit, KMEB, University of Southern Denmark, Odense, Denmark
12. Department of Clinical Biochemistry and Pharmacology, Odense University Hospital, Odense, Denmark
13. Musculoskeletal Research Unit, University of Bristol, Bristol, UK
11. The University of Queensland Diamantina Institute, Translational Research Institute, Princess Alexandra Hospital, Brisbane, Australia
11. Departments of Epidemiology and Biostatistics, and Medicine, McGill University, Montreal, Canada

* Denotes equal contribution to the work

§ Correspondence to:

Jordana T. Bell
Department of Twins Research and Genetic Epidemiology
King's College London
Tel: +44 (0)207 188 1506
Email: jordana.bell@kcl.ac.uk

J. Brent Richards
Lady Davis Institute for Medical Research
Jewish General Hospital, McGill University
Tel: +1 (514) 340 8222
Email: brent.richards@mcgill.ca

Yi-Hsiang Hsu
Institute for Aging Research

Hebrew SeniorLife, Harvard Medical School
Tel: +1 (617) 971 5368
Email: yihsianghsu@hsl.harvard.edu

The authors report no disclosures.

Abstract

Genetic and environmental determinants of skeletal phenotypes such as bone mineral density (BMD) may converge through the epigenome, providing a tool to better understand osteoporosis pathophysiology. As the epigenetics of BMD have been largely unexplored in humans, we performed an epigenome wide association study (EWAS) of BMD. We undertook a large-scale BMD EWAS using the Infinium HumanMethylation450 array to measure site-specific DNA methylation in up to 5,515 European descent individuals ($N_{Discovery} = 4,614$, $N_{Validation} = 901$). We associated methylation at multiple cytosine-phosphate-guanine (CpG) sites with dual-energy X-ray absorptiometry derived femoral neck and lumbar spine BMD. We performed sex-combined and stratified analyses, controlling for age, weight, smoking status, estimated white blood cell proportions, and random effects for relatedness and batch effects. A 5% false-discovery rate was used to identify CpGs associated with BMD. We identified one CpG-site, cg23196985, significantly associated with femoral neck BMD in 3,444 females ($P = 7.9 \times 10^{-11}$) and 4,827 females and males ($P = 3.0 \times 10^{-8}$). cg23196985 was not associated with femoral neck BMD in an additional sample of 474 females ($P = 0.64$) and 901 males and females ($P = 0.60$). Lack of strong consistent association signal indicates that among the tested probes, no large-effect epigenetic changes in whole blood associated with BMD, suggesting future epigenomic studies of musculoskeletal traits measure DNA methylation in a different tissue with extended genome coverage.

Introduction

Osteoporosis is primarily an aging-related disease characterized by compromised bone strength that increases the risk of fracture. Due to population ageing worldwide the incidence of osteoporosis is increasing, exceeding \$17 billion per year in direct care costs within the United States of America⁽¹⁾ and costing upwards of €37 billion per year in the EU-27 member states.⁽²⁾ Identifying the causes of osteoporosis will improve the understanding of its pathology, leading to better or more efficient treatments of this common and costly disease. Low bone mineral density (BMD) is one of the major risk factors for fracture, and is largely used in clinical prediction tools for fracture and gauging response to treatment. Genome-wide association studies (GWAS) of BMD have been instrumental in identifying novel genetic loci influencing osteoporosis disease-risk.^(3,4) However, epigenetic variation in the genome, which can be influenced by both genetic and environmental factors,^(5,6) may also influence BMD, yet the epigenetic influences on BMD have largely been unexplored.

One of the most stable epigenetic processes is DNA methylation, or, the addition of a CH₃ methyl group to cytosine, typically in the context of cytosine paired sequentially to a guanine nucleotide, separated by a phosphate group (CpG). DNA methylation is known to play a role in gene expression and cell differentiation^(7,8) and differential DNA methylation has been linked to multiple human complex traits and disease phenotypes.^(5,9–12) Studies performed using bone samples have identified epigenetic alterations that influence bone cell function.^(13,14) We studied epigenetic variation in whole blood, as a proxy for difficult-to-acquire samples such as bone, in relation to BMD because epigenetic markers are often stable across multiple tissues, and immune cells within blood are known to influence bone homeostasis.⁽¹⁵⁾ Furthermore, osteoclasts are derived from the monocyte-macrophage lineage found in whole blood.⁽¹⁶⁾ Although epigenetic profiling has been performed previously in bone samples from osteoporotic and osteoarthritic patients⁽¹⁷⁾ and an epigenome-wide association study (EWAS) of BMD has been performed in mice,⁽¹⁸⁾ EWAS of BMD have not been reported in humans with validation of significant findings.

We, therefore, undertook a large-scale BMD EWAS, assessing the association of up to 473,882 CpGs quantified in whole-blood with BMD measured in up to 4,614 individuals across five cohorts from Europe and North America. To our knowledge, this study is the largest EWAS of a musculoskeletal trait performed to date. We used an additional 901 individuals as a validation cohort to increase the reliability of our results.

Methods

Individual cohorts

We performed our EWAS in cohorts comprised of European descent individuals. Cohorts used for the discovery analysis were the TwinsUK Registry (TUK), Framingham Study Offspring Cohort (FOS), Avon Longitudinal Study of Parents and Children (further information on the ALSPAC cohorts and ARIES project is included in the supplementary tables 1), Rotterdam Study (RS), and the Danish Twin Registry (DTR). The cohort used for validation of significant findings was the Framingham Study Generation 3 cohort (Gen3), a cohort including family members of FOS (Table 1; Supplementary Table 1).

Both, or one of, femoral neck (FN) and lumbar spine (LS) BMD were measured in each cohort by dual-energy X-ray absorptiometry (DXA) (Supplementary Table 2). All cohorts, except the ALSPAC and DTR, followed the same methods for extracting DNA from whole-blood tissue and quantifying DNA methylation. Whole-blood tissue DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, Inc.), followed by bisulfite conversion of 750 ng DNA using the EZ DNA Methylation Kit (Zymo Research) following manufacturer instructions. The ALSPAC cohort and the DTR cohort performed DNA extraction and conversion as described previously.^(19, 29) DNA methylation across the genome was quantified using the Infinium Human Methylation450 BeadChip (Illumina), assaying up to 482,421 CpGs throughout the human genome. Image intensities were extracted using GenomeStudio Methylation Module (v1.8) software. Cohort-specific criteria were applied in further quality control and normalization of probe intensities (Supplementary Table 3).

Statistical analyses

For discovery analyses, each cohort followed a pre-specified analysis plan. FN and LS BMD residuals were calculated by fitting a linear regression model, adjusting for age, weight, and sex. For sex-specific analyses, the term for sex was removed from the model. In addition, the DTR adjusted for birth weight discordance as historically, DTR samples were selected to address birth weight discordance in twins (Supplementary Table 1). To address the issue of cell heterogeneity in whole blood tissue, each cohort calculated the estimated white blood cell proportions of eosinophils, lymphocytes, monocytes, and neutrophils using a reference-free method.⁽²⁰⁾ DNA methylation for each probe was transformed to a standard normal distribution using quantile normalization. The association between DNA methylation and BMD was then calculated by fitting a linear mixed effects model for normalized DNA methylation, including BMD residuals, smoking (measured as smokers, non-smokers, or former-smokers), age, weight, sex, and estimated white blood cell proportions as fixed effects, and terms for family structure and batch effects as random effects, where relevant. Association testing was performed in male, female, and combined samples. Each cohort was assessed for epigenome-wide statistical inflation by calculating the genomic inflation factor λ and generating a quantile-quantile plot (QQ-plot). λ can be calculated to estimate the deviation of a distribution from a null expected distribution, whereas QQ-plots can be used to visualize the deviation of a distribution from a null expected distribution.

Fixed-effects meta-analyses were performed using METAL⁽²¹⁾ for FN and LS sex-combined and sex-stratified analyses. We used the I^2 statistic to quantify the variability in association effect estimates due to statistical heterogeneity, excluding probes with heterogeneous I^2 statistics ($P_{Het} < 0.05$). Statistical significance, when considering the multiple testing burden, was determined by calculating Benjamini-Hochberg (BH) adjusted P -values for each meta-analysis. Probes with significant BH-adjusted P -values ($P_{BH} < 0.05$) would, therefore, be significant at a 5% false-discovery rate (FDR).

Associated probes were assessed for the influence of single nucleotide polymorphisms (SNPs) that overlapped the probe body by mapping these probes to dbSNP 146.⁽²²⁾ This was assessed by adding a term for the dosage of each SNP to the discovery linear mixed effects models in cohorts

with genotype data, to observe if the association between DNA methylation and BMD was influenced by the genetic polymorphism at the probe.

Gen3 samples were assessed using the same methods as in the analysis of FOS samples to perform validation analyses of significantly associated probes. Probes were deemed robustly associated with BMD if they met a validation P -value of less than 0.05. These samples are not completely independent from the FOS samples because the Framingham Study is a family-based study with several cohorts, and, therefore, there is underlying family structure.

We used the R package “pwr” to calculate the variance explained, given our study parameters and setting the level of desired power at 80%.

Results

Meta-analysis

Meta-analyses of discovery cohorts identified CpG-site cg23196985 associated at a 5% FDR for FN sex-combined ($\beta = 0.66$, $SE = 0.19$, $P = 2.99 \times 10^{-8}$, $P_{BH} = 1.30 \times 10^{-2}$) and FN female ($\beta = 0.95$, $SE = 0.15$, $P = 7.86 \times 10^{-11}$, $P_{BH} = 3.41 \times 10^{-5}$; Figure 1; Figure 2; Table 2) analyses. CpG-site cg23196985 maps to the 5' untranslated region of the liver carboxylase 1 gene (*CESI*), which is expressed in the liver and whole-blood⁽²³⁾, yet with no currently reported associations with BMD by GWAS in the same chromosomal region (16q12.2) and with the nearest BMD associated SNP mapping approximately 4 mega base pairs upstream at the *SALL1* and *CYLD* locus⁽²⁴⁾. The calculated lambdas and QQ-plots for the meta-analyses of FN female and sex-combined analyses revealed no statistical inflation of the association P -values ($\lambda_{\text{female}} = 1.02$, $\lambda_{\text{sex-combined}} = 0.97$; Supplementary Table 7). We observed no significantly associated CpG-sites with LS BMD in sex-combined or sex-stratified analyses (Supplementary Figures).

We tested for influence of SNPs underlying cg23196985 in females from the FOS, RS, and ALSPAC cohorts, as the strength of the association was stronger in females than in the sex-combined analysis. Four SNPs were mapped to the cg23196985 50 base pair probe sequence, and these included rs144950224, rs12149371, rs12149373, and rs3815583, with rs144950224 mapping directly to the probe's target CpG-site. SNP rs144950224 was found to be rare within our cohorts, with a minor allele frequency (MAF) of approximately 0.5% in FOS samples, 0.1% in RS samples, and no carriers in ALSPAC samples. We observed no notable change in association P -values upon conditioning with each of the four SNPs (Supplementary Table 8).

In the validation sample, cg23196985 was not associated with FN in Gen3 female ($P = 0.64$) and sex-combined ($P = 0.60$) analyses. However, after meta-analyzing Gen3 validation data with discovery results, the probe remained strongly associated in female-only analyses ($\beta = 0.86$, $SE = 0.14$, $P = 3.7 \times 10^{-10}$; $N = 4,345$) but not in sex-combined analysis ($P = 0.68$, $N = 5,301$).

To calculate the minimum detectable variance explained given our study design, we used the R package “pwr” with the following parameters: a sample size of 5,000, a conservative Bonferroni threshold of 1.03×10^{-7} ($0.05/482,421$), and 80% power. We used a Bonferroni threshold adjusting for the total number of probes to be stringent in our power calculation and to obtain more conservative estimates for expected variance explained. We estimated that we would be

able to detect an association that has a correlation r of approximately 0.087, and therefore explains a variance r^2 of at least 0.8%.

Individual cohorts

Individual cohort analyses identified seven probes that were significantly associated in two cohorts with sex-combined or sex-stratified analyses (Supplementary Table 4), but there were no other cohort-specific significant associations. The DTR LS female analysis identified four significantly associated probes, two of which map to genes, cg04081651 (*MAP3K8*), cg09832237, cg14793931 (*ZFR2*), and cg24029028. The DTR LS male analysis identified one significantly associated probe, cg23214071 (*HLA-DQB1*). The TUK LS female analysis identified two significantly associated probes, cg24117468 (*P4HA2*) and cg02526790 (*TG*) (Supplementary Table 4). The calculated lambda and QQ-plot for the DTR LS female analysis revealed statistical inflation of the association P -values ($\lambda = 1.46$), but the lambda and QQ-plots for the remainder of the cohorts showed no large inflation or deflation of association P -values (Supplementary Table 7).

Discussion

In the first large-scale assessment of the contribution of epigenetic changes in whole blood to BMD we did not identify methylation changes reliably associated with this clinically relevant trait. CpG-site cg23196985 was found in the discovery meta-analysis to be strongly associated with FN BMD in females-only and in analyses combining males and females, but upon validation in an extended sample which included related individuals, the association was attenuated in the female analysis and completely absent in sex-combined analyses.

These findings provide important insights into the field of epigenetics. The first, is that using a precisely measured trait, BMD, which is highly heritable and for which genetic determinants have been identified through GWAS,^(25,3,4) there do not appear to be associations between methylation changes and BMD. While whole blood methylation changes may not be the ideal tissue within which to test epigenetic influences on bone, this conveniently accessible tissue has many links to bone biology, including the fact that osteoclasts and monocyte/macrophages originate from the same precursors.^(15,16) The extent to which methylation changes are shared between bone and whole blood is not well known. However, evidence shows that a significant proportion of methylation variation genome-wide can be conserved across tissues.⁽²⁶⁾ Additional explanations for our mostly null findings include the possibility that DNA methylation changes may not have a large influence on BMD.

Notwithstanding the general lack of consistent associations with BMD across the genome, we did generate evidence for suggestive association of cg23196985 with FN BMD in females. However, we caution that these findings require further replication. Since we are unaware of any available replication data to test this hypothesis, these findings will require replication in future studies.

There is limited evidence for the effects of DNA methylation on bone. A methylation profiling study that compared the differences between bone samples of 27 osteoporotic and 23 osteoarthritic patients was undertaken on an earlier DNA methylation platform, the

HumanMethylation27 BeadChip (assessing approximately 27,000 CpGs in the genome), and were able to identify bone genes following pathway analyses of over 200 differentially methylated CpGs, however, these to date results lack replication.⁽¹⁷⁾ Another study failed to demonstrate specific effects of DNA methylation, assessed by sequencing methods, on *RANKL* in the bones of patients with osteoporotic fractures.⁽²⁷⁾ The evidence from previous studies and our own suggests that if strong effects of DNA methylation on bone biology are to be identified, they may not be detectable with current analytical approaches.

A strength of our study was the sample size, and a conservative estimate of statistical power to identify epigenetic effects on BMD that account for 0.8% of its variance. The large sample size also allowed us to classify several cohort-level associations were likely to be false positives. For example, TUK female analyses identified two probes significantly associated with LS BMD, but this association was not observed in any other cohort, suggesting the associations were false positives (Supplementary Table 4; Supplementary Table 5).

One of the key limitations of cohort-based epigenetic studies is the lack of cell-sorted data for analysis. As discussed by Birney *et al.*,⁽²⁸⁾ optimal planning at the outset of a study is ideal, however, such coordination is difficult to implement in large cohorts and so bioinformatics methods must be applied *post hoc* to adjust for suboptimal study designs. We adjusted for cell heterogeneity within whole blood, and therefore the signal we tested for association with BMD would be ubiquitous within whole blood. Such ubiquitous signals within whole blood may only be detectable for extremely strong environmental modifiers of DNA methylation, such as cigarette smoking.^(5,6) Targeted EWAS of specific cell types within whole blood with clear roles in bone biology, such as monocytes due to their role in osteoclastogenesis, may be more fruitful. Furthermore, longitudinal studies can identify disease-risk biomarkers and provide mechanistic insights, however, these are generally underpowered when studying BMD due to the relatively small changes in BMD that occur over time. As we have shown that large-scale whole blood EWAS of BMD does not identify disease-risk biomarkers for osteoporosis risk, a well-powered longitudinal study with a wide-range of time points and bone samples may be more informative.

In the largest EWAS meta-analysis to date of BMD, we observed a probe near *CESI* to be associated with FN BMD in the discovery sample of up to 4,826 individuals, but not with the same phenotype in a semi-independent validation sample of 901 individuals. In conclusion, these findings suggest that there are no large effects of methylation changes on BMD in whole blood in the epigenome, which are common and well captured by the Infinium HumanMethylation450 BeadChip.

Acknowledgments

JAM, VF, and JBR were supported by the RMGA, FRQS, and CIHR. PCT, TDS, JTB were supported by the European Commission and the UK Economic and Social Research Council (ES/N000404/1). The Framingham Study was funded by NIH contract N01-HC-25195. DPK was supported by the NIH (R01AR41398). KT was supported by Erasmus Mundus Western Balkans. MS, MF, KC, and LC were supported by the European Union's Seventh Framework Programme (FP7/2007-2011) under grant agreement 259679 and the Danish National Program for Research Infrastructure 2007 (09-063256). The DTR was supported by the Velux Foundation. JZ, JHT and DME were supported by the Medical Research Council Integrative

Epidemiology Unit. We are extremely grateful to all the families who took part in this study, the midwives for their help in recruiting them, and the whole ALSPAC team, which includes interviewers, computer and laboratory technicians, clerical workers, research scientists, volunteers, managers, receptionists and nurses. The UK Medical Research Council and Wellcome (Grant ref: 102215/2/13/2) and the University of Bristol provide core support for ALSPAC. Methylation data in the ALSPAC cohort were generated as part of the UK BBSRC funded (BB/I025751/1 and BB/I025263/1) Accessible Resource for Integrated Epigenomic Studies (ARIES, <http://www.ariesepigenomics.org.uk>). JAM, P-CT, Y-HH, JBR, and JTB conceived of the study design. JAM, P-CT, Y-HH, RJ, JZ, KT, MS, and VF performed the analyses. P-CT, MF, KC and LC participated in acquisition of data. All authors have revised the manuscript and given their final approval.

References

1. Burge R, Dawson-Hughes B, Solomon DH, Wong JB, King A, Tosteson A. Incidence and economic burden of osteoporosis-related fractures in the United States, 2005-2025. *J. Bone Miner. Res.* 2007 Mar;22(3):465–75.
2. Hernlund E, Svedbom A, Ivergard M, Compston J, Cooper C, Stenmark J, McCloskey E V, Jonsson B, Kanis JA. Osteoporosis in the European Union: Medical management, epidemiology and economic burden: A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA). *Arch. Osteoporos.* 2013;8(1-2):136.
3. Estrada K, Styrkarsdottir U, Evangelou E, Hsu Y-H, Duncan EL, Ntzani EE, Oei L, Albagha OME, Amin N, Kemp JP, Koller DL, Li G, Liu C-T, Minster RL, Moayyeri A, Vandenput L, Willner D, Xiao S-M, Yerges-Armstrong LM, Zheng H-F, Alonso N, Eriksson J, Kammerer CM, Kaptoge SK, Leo PJ, Thorleifsson G, Wilson SG, Wilson JF, Aalto V, Alen M, Aragaki AK, Aspelund T, Center JR, Dailiana Z, Duggan DJ, Garcia M, Garcia-Giralt N, Giroux S, Hallmans G, Hocking LJ, Husted LB, Jameson KA, Khusainova R, Kim GS, Kooperberg C, Koromila T, Kruk M, Laaksonen M, Lacroix AZ, Lee SH, Leung PC, Lewis JR, Masi L, Mencej-Bedrac S, Nguyen T V, Nogue X, Patel MS, Prezelj J, Rose LM, Scollen S, Siggeirsdottir K, Smith A V, Svensson O, Trompet S, Trummer O, van Schoor NM, Woo J, Zhu K, Balcells S, Brandi ML, Buckley BM, Cheng S, Christiansen C, Cooper C, Dedoussis G, Ford I, Frost M, Goltzman D, González-Macías J, Kähönen M, Karlsson M, Khusnutdinova E, Koh J-M, Kollia P, Langdahl BL, Leslie WD, Lips P, Ljunggren Ö, Lorenc RS, Marc J, Mellström D, Obermayer-Pietsch B, Olmos JM, Pettersson-Kymmer U, Reid DM, Riancho JA, Ridker PM, Rousseau F, Slagboom PE, Tang NLS, Urreizti R, Van Hul W, Viikari J, Zarrabeitia MT, Aulchenko YS, Castano-Betancourt M, Grundberg E, Herrera L, Ingvarsson T, Johannsdottir H, Kwan T, Li R, Luben R, Medina-Gómez C, Palsson ST, Reppe S, Rotter JI, Sigurdsson G, van Meurs JBJ, Verlaan D, Williams FMK, Wood AR, Zhou Y, Gautvik KM, Pastinen T, Raychaudhuri S, Cauley JA, Chasman DI, Clark GR, Cummings SR, Danoy P, Dennison EM, Eastell R, Eisman JA, Gudnason V, Hofman A, Jackson RD, Jones G, Jukema JW, Khaw K-T, Lehtimäki T, Liu Y, Lorentzon M, McCloskey E, Mitchell BD, Nandakumar K, Nicholson GC, Oostra BA, Peacock M, Pols HAP, Prince RL, Raitakari O, Reid IR, Robbins J, Sambrook PN, Sham PC, Shuldiner AR, Tylavsky FA, van Duijn CM, Wareham NJ, Cupples LA, Econs MJ, Evans DM, Harris TB, Kung AWC, Psaty BM,

- Reeve J, Spector TD, Streeten EA, Zillikens MC, Thorsteinsdottir U, Ohlsson C, Karasik D, Richards JB, Brown MA, Stefansson K, Uitterlinden AG, Ralston SH, Ioannidis JPA, Kiel DP, Rivadeneira F. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. *Nat. Genet.* 2012 May;44(5):491–501.
4. Zheng H-F, Forgetta V, Hsu Y-H, Estrada K, Rosello-Diez A, Leo PJ, Dahia CL, Park-Min KH, Tobias JH, Kooperberg C, Kleinman A, Styrkarsdottir U, Liu C-T, Uggla C, Evans DS, Nielson CM, Walter K, Pettersson-Kymmer U, McCarthy S, Eriksson J, Kwan T, Jhamai M, Trajanoska K, Memari Y, Min J, Huang J, Danecek P, Wilmot B, Li R, Chou W-C, Mokry LE, Moayyeri A, Claussnitzer M, Cheng C-H, Cheung W, Medina-Gómez C, Ge B, Chen S-H, Choi K, Oei L, Fraser J, Kraaij R, Hibbs MA, Gregson CL, Paquette D, Hofman A, Wibom C, Tranah GJ, Marshall M, Gardiner BB, Cremin K, Auer P, Hsu L, Ring S, Tung JY, Thorleifsson G, Enneman AW, van Schoor NM, de Groot LCPGM, van der Velde N, Melin B, Kemp JP, Christiansen C, Sayers A, Zhou Y, Calderari S, van Rooij J, Carlson C, Peters U, Berlivet S, Dostie J, Uitterlinden AG, Williams SR, Farber C, Grinberg D, LaCroix AZ, Haessler J, Chasman DI, Giulianini F, Rose LM, Ridker PM, Eisman JA, Nguyen T V, Center JR, Nogues X, Garcia-Giralt N, Launer LL, Gudnason V, Mellström D, Vandenput L, Amin N, van Duijn CM, Karlsson MK, Ljunggren Ö, Svensson O, Hallmans G, Rousseau F, Giroux S, Bussière J, Arp PP, Koromani F, Prince RL, Lewis JR, Langdahl BL, Hermann AP, Jensen J-EB, Kaptoge S, Khaw K-T, Reeve J, Formosa MM, Xuereb-Anastasi A, Åkesson K, McGuigan FE, Garg G, Olmos JM, Zarrabeitia MT, Riancho JA, Ralston SH, Alonso N, Jiang X, Goltzman D, Pastinen T, Grundberg E, Gauguier D, Orwoll ES, Karasik D, Davey-Smith G, Smith A V, Siggeirsdottir K, Harris TB, Zillikens MC, van Meurs JBJ, Thorsteinsdottir U, Maurano MT, Timpson NJ, Soranzo N, Durbin R, Wilson SG, Ntzani EE, Brown MA, Stefansson K, Hinds DA, Spector T, Cupples LA, Ohlsson C, Greenwood CMT, Jackson RD, Rowe DW, Loomis CA, Evans DM, Ackert-Bicknell CL, Joyner AL, Duncan EL, Kiel DP, Rivadeneira F, Richards JB. Whole-genome sequencing identifies EN1 as a determinant of bone density and fracture. *Nature.* 2015 Oct 1;526(7571):112–7.
 5. Tsaprouni LG, Yang T-P, Bell J, Dick KJ, Kanoni S, Nisbet J, Viñuela A, Grundberg E, Nelson CP, Meduri E, Buil A, Cambien F, Hengstenberg C, Erdmann J, Schunkert H, Goodall AH, Ouwehand WH, Dermitzakis E, Spector TD, Samani NJ, Deloukas P. Cigarette smoking reduces DNA methylation levels at multiple genomic loci but the effect is partially reversible upon cessation. *Epigenetics.* 2014 Oct;9(10):1382–96.
 6. Joehanes R, Just AC, Marioni RE, Pilling LC, Reynolds LM, Mandaviya PR, Guan W, Xu T, Elks CE, Aslibekyan S, Moreno-Macias H, Smith JA, Brody JA, Dhingra R, Yousefi P, Pankow JS, Kunze S, Shah S, McRae AF, Lohman K, Sha J, Absher DM, Ferrucci L, Zhao W, Demerath EW, Bressler J, Grove ML, Huan T, Liu C, Mendelson MM, Yao C, Kiel DP, Peters A, Wang-Sattler R, Visscher PM, Wray NR, Starr JM, Ding J, Rodriguez CJ, Wareham NJ, Irvin MR, Zhi D, Barrdahl M, Vineis P, Ambatipudi S, Uitterlinden AG, Hofman A, Schwartz J, Colicino E, Hou L, Vokonas PS, Hernandez DG, Singleton AB, Bandinelli S, Turner ST, Ware EB, Smith AK, Klengel T, Binder EB, Psaty BM, Taylor KD, Gharib SA, Swenson BR, Liang L, DeMeo DL, O'Connor GT, Herceg Z, Ressler KJ, Conneely KN, Sotoodehnia N, Kardina SLR, Melzer D, Baccarelli AA, van Meurs JBJ, Romieu I, Arnett DK, Ong KK, Liu Y, Waldenberger M, Deary IJ, Fornage M, Levy D, London SJ. Epigenetic Signatures of Cigarette Smoking. *Circ. Cardiovasc. Genet.* 2016

- Sep 20;CIRCGENETICS.116.001506.
7. Robertson KD. DNA methylation and human disease. *Nat. Rev. Genet.* 2005 Aug;6(8):597–610.
 8. Suzuki MM, Bird A. DNA methylation landscapes: provocative insights from epigenomics. *Nat. Rev. Genet.* 2008 Jun;9(6):465–76.
 9. Grundberg E, Meduri E, Sandling JK, Hedman AK, Keildson S, Buil A, Busche S, Yuan W, Nisbet J, Sekowska M, Wilk A, Barrett A, Small KS, Ge B, Caron M, Shin S-Y, Consortium MThER, Lathrop M, Dermitzakis ET, McCarthy MI, Spector TD, Bell JT, Deloukas P, Multiple Tissue Human Expression Resource Consortium, Lathrop M, Dermitzakis ET, McCarthy MI, Spector TD, Bell JT, Deloukas P. Global analysis of DNA methylation variation in adipose tissue from twins reveals links to disease-associated variants in distal regulatory elements. *Am J Hum Genet.* 2013 Nov 7;93(5):876–90.
 10. Yuan W, Xia Y, Bell CG, Yet I, Ferreira T, Ward KJ, Gao F, Loomis AK, Hyde CL, Wu H, Lu H, Liu Y, Small KS, Viñuela A, Morris AP, Berdasco M, Esteller M, Brosnan MJ, Deloukas P, McCarthy MI, John SL, Bell JT, Wang J, Spector TD. An integrated epigenomic analysis for type 2 diabetes susceptibility loci in monozygotic twins. *Nat. Commun.* 2014 Jan;5:5719.
 11. Chambers JC, Loh M, Lehne B, Drong A, Kriebel J, Motta V, Wahl S, Elliott HR, Rota F, Scott WR, Zhang W, Tan S-T, Campanella G, Chadeau-Hyam M, Yengo L, Richmond RC, Adamowicz-Brice M, Afzal U, Bozaoglu K, Mok ZY, Ng HK, Pattou F, Prokisch H, Rozario MA, Tarantini L, Abbott J, Ala-Korpela M, Albetti B, Ammerpohl O, Bertazzi PA, Blancher C, Caiazzo R, Danesh J, Gaunt TR, de Lusignan S, Gieger C, Illig T, Jha S, Jones S, Jowett J, Kangas AJ, Kasturiratne A, Kato N, Kotea N, Kowlessur S, Pitkäniemi J, Punjabi P, Saleheen D, Schafmayer C, Soininen P, Tai E-S, Thorand B, Tuomilehto J, Wickremasinghe AR, Kyrtopoulos SA, Aitman TJ, Herder C, Hampe J, Cauchi S, Relton CL, Froguel P, Soong R, Vineis P, Jarvelin M-R, Scott J, Grallert H, Bollati V, Elliott P, McCarthy MI, Kooner JS. Epigenome-wide association of DNA methylation markers in peripheral blood from Indian Asians and Europeans with incident type 2 diabetes: a nested case-control study. *lancet. Diabetes Endocrinol. Europe PMC Funders;* 2015 Jul;3(7):526–34.
 12. Tsai P-C, Van Dongen J, Tan Q, Willemsen G, Christiansen L, Boomsma DI, Spector TD, Valdes AM, Bell JT. DNA Methylation Changes in the IGF1R Gene in Birth Weight Discordant Adult Monozygotic Twins. *Twin Res. Hum. Genet.* 2015 Dec;18(6):635–46.
 13. Reppe S, Noer A, Grimholt RM, Halldórsson B V, Medina-Gomez C, Gautvik VT, Olstad OK, Berg JP, Datta H, Estrada K, Hofman A, Uitterlinden AG, Rivadeneira F, Lyle R, Collas P, Gautvik KM. Methylation of bone SOST, its mRNA, and serum sclerostin levels correlate strongly with fracture risk in postmenopausal women. *J. Bone Miner. Res.* 2015 Feb;30(2):249–56.
 14. Delgado-Calle J, Sañudo C, Bolado A, Fernández AF, Arozamena J, Pascual-Carra MA, Rodriguez-Rey JC, Fraga MF, Bonewald L, Riancho JA. DNA methylation contributes to the regulation of sclerostin expression in human osteocytes. *J. Bone Miner. Res.* 2012 Apr;27(4):926–37.
 15. Greenblatt MB, Shim J-H. Osteoimmunology: a brief introduction. *Immune Netw.* 2013 Aug;13(4):111–5.
 16. Novack DV, Mbalaviele G. Osteoclasts-Key Players in Skeletal Health and Disease. *Microbiol. Spectr.* 2016 Jun;4(3).

17. Delgado-Calle J, Fernández AF, Sainz J, Zarrabeitia MT, Sañudo C, García-Renedo R, Pérez-Núñez MI, García-Ibarbia C, Fraga MF, Riancho JA. Genome-wide profiling of bone reveals differentially methylated regions in osteoporosis and osteoarthritis. *Arthritis Rheum.* 2013 Jan;65(1):197–205.
18. Orozco LD, Morselli M, Rubbi L, Guo W, Go J, Shi H, Lopez D, Furlotte NA, Bennett BJ, Farber CR, Ghazalpour A, Zhang MQ, Bahous R, Rozen R, Lusis AJ, Pellegrini M. Epigenome-wide association of liver methylation patterns and complex metabolic traits in mice. *Cell Metab.* NIH Public Access; 2015 Jun 2;21(6):905–17.
19. Christiansen L, Lenart A, Tan Q, Vaupel JW, Aviv A, McGue M, Christensen K. DNA methylation age is associated with mortality in a longitudinal Danish twin study. *Aging Cell.* 2016 Feb;15(1):149–54.
20. Houseman EA, Molitor J, Marsit CJ. Reference-free cell mixture adjustments in analysis of DNA methylation data. *Bioinformatics.* 2014 May 15;30(10):1431–9.
21. Willer CJ, Li Y, Abecasis GR. METAL: fast and efficient meta-analysis of genomewide association scans. *Bioinformatics.* 2010 Sep 1;26(17):2190–1.
22. Sherry ST, Ward MH, Kholodov M, Baker J, Phan L, Smigielski EM, Sirotkin K. dbSNP: the NCBI database of genetic variation. *Nucleic Acids Res.* 2001 Jan 1;29(1):308–11.
23. GTEx Consortium. Human genomics. The Genotype-Tissue Expression (GTEx) pilot analysis: multitissue gene regulation in humans. *Science.* 2015 May 8;348(6235):648–60.
24. Welter D, MacArthur J, Morales J, Burdett T, Hall P, Junkins H, Klemm A, Flicek P, Manolio T, Hindorff L, Parkinson H. The NHGRI GWAS Catalog, a curated resource of SNP-trait associations. *Nucleic Acids Res.* 2014 Jan;42(Database issue):D1001–6.
25. Richards JB, Zheng H-F, Spector TD. Genetics of osteoporosis from genome-wide association studies: advances and challenges. *Nat. Rev. Genet.* Nature Publishing Group; 2012 Jul 18;13(8):576–88.
26. Dick KJ, Nelson CP, Tsaprouni L, Sandling JK, Aïssi D, Wahl S, Meduri E, Morange P-E, Gagnon F, Grallert H, Waldenberger M, Peters A, Erdmann J, Hengstenberg C, Cambien F, Goodall AH, Ouwehand WH, Schunkert H, Thompson JR, Spector TD, Gieger C, Trégouët D-A, Deloukas P, Samani NJ. DNA methylation and body-mass index: a genome-wide analysis. *Lancet.* 2014;383(9933):1990–8.
27. Delgado-Calle J, Sañudo C, Fernández AF, García-Renedo R, Fraga MF, Riancho JA. Role of DNA methylation in the regulation of the RANKL-OPG system in human bone. *Epigenetics.* 2012 Jan 1;7(1):83–91.
28. Birney E, Smith GD, Greally JM, Bird A, Relton C, Davey SG, Michels K, Binder A, Dedeurwaerder S, Epstein C, Greally J, Gut I, Plongthongkum N, Diep D, Zhang K, Liu X, Gao Q, Li P, Zhao Q, Zhang J, Li J, Schübeler D, Rakyan V, Down T, Balding D, Beck S, Heijmans B, Mill J, Tsai P, Bell J, Horvath S, Richmond R, Simpkin A, Woodward G, Gaunt T, Lyttleton O, McArdle W, Smith G, Lawlor D, Harbord R, Timpson N, Day I, Ebrahim S, Sterne J, Davey SG, Smith G, Ebrahim S, Colquhoun D, Houseman E, Accomando W, Koestler D, Christensen B, Marsit C, Nelson H, Jaffe A, Irizarry R, Houseman E, Molitor J, Marsit C, Wijetunga N, Delahaye F, Zhao Y, Golden A, Mar J, Einstein F, Liu Y, Aryee M, Padyukov L, Fallin M, Hesselberg E, Runarsson A, Berko E, Suzuki M, Beren F, Lemetre C, Alaimo C, Calder R, Farlik M, Sheffield N, Nuzzo A, Datlinger P, Schönegger A, Klughammer J, Smallwood S, Lee H, Angermueller C, Krueger F, Saadeh H, Peat J, Ding Z, Ni Y, Timmer S, Lee B, Battenhouse A, Louzada S, Zilberman D, Gehring M, Tran R, Ballinger T, Henikoff S, Ball M, Li J, Gao Y, Lee J,

- LeProust E, Park I, Bell J, Pai A, Pickrell J, Gaffney D, Pique-Regi R, Degner J, Gertz J, Varley K, Reddy T, Bowling K, Pauli F, Parker S, Grundberg E, Meduri E, Sandling J, Hedman A, Keildson S, Buil A, Bell J, Tsai P, Yang T, Pidsley R, Nisbet J, Glass D, Richmond R, Sharp G, Ward M, Fraser A, Lyttleton O, McArdle W, Liu Y, Siegmund K, Laird P, Berman B, Gaujoux R, Seoighe C, Gibbs J, Brug M van der, Hernandez D, Traynor B, Nalls M, Lai S, Smith GD, Hemani G, Relton C, Davey SG. Epigenome-wide Association Studies and the Interpretation of Disease -Omics. Barsh GS, editor. PLOS Genet. Public Library of Science; 2016 Jun 23;12(6):e1006105.
29. Caroline L Relton, Tom Gaunt, Wendy McArdle, Karen Ho, Aparna Duggirala, Hashem Shihab, Geoff Woodward, Oliver Lyttleton, David M Evans, Wolf Reik, Yu-Lee Paul, Gabriella Ficz, Susan E Ozanne, Anil Wipat, Keith Flanagan, Allyson Lister, Bastiaan T Heijmans, Susan M Ring, and George Davey Smith. Data Resource Profile: Accessible Resource for Integrated Epigenomic Studies (ARIES) Int. J. Epidemiol. (2015) 44 (4): 1181-1190

Figure Legends

Figure 1. Quantile-quantile plots (QQ-plots) of the distribution of observed $-\log_{10}$ association P -values against the expected null distribution, for discovery meta-analyses of FN BMD in (a) females-only and (b) sex-combined analyses. Genomic inflation lambda scores are given in each QQ-plot, to quantify statistical inflation of P -values. No evidence for inflation was observed in the QQ-plots or as calculated by lambda scores.

Figure 2. Manhattan plots of $-\log_{10}$ association P -values for (a) females-only and (b) sex-combined analyses.

Tables

Table 1. Sample Sizes of Discovery and Replication Cohorts.

| Phase | Cohort | Sample Size | | | | | |
|------------|--|-------------|-------------|-------------|-------------|-------------|-------------|
| | | FN BMD | | | LS BMD | | |
| | | Pooled | Females | Males | Pooled | Females | Males |
| Discovery | Avon Longitudinal Study of Parents and Children (ALSPAC) | 715 | 715 | 0 | 0 | 0 | 0 |
| | Danish Twin Registry (DTR) | 267 | 132 | 135 | 260 | 132 | 128 |
| | Framingham Study (FS) | 2207 | 1254 | 953 | 2203 | 1259 | 953 |
| | Rotterdam Study (RS) | 650 | 356 | 294 | 633 | 346 | 287 |
| | TwinsUK (TUK) | 775 | 775 | 0 | 770 | 770 | 0 |
| | Discovery Total | 4614 | 3444 | 1382 | 3866 | 2507 | 1368 |
| Validation | FOS Gen3 | 901 | 448 | 453 | 0 | 0 | 0 |
| | Discovery + Validation Total | 5515 | 3892 | 1835 | 3866 | 2507 | 1368 |

Table 2. Meta-Analysis Results for Association of cg23196985 with FN BMD in Both Female (Top Row) and Sex-Pooled (Bottom Row) Analyses in Discovery phase, with Replication and Combined Discovery and Replication Analyses.

| Discovery | | | | | Validation | | | Combined | | | |
|-----------|------|---------|----------|-----------|------------|------|------|----------|------|---------|-----------|
| β | SE | P | P_{BH} | P_{Het} | β | SE | P | β | SE | P | P_{Het} |
| 0.95 | 0.15 | 7.9E-11 | 3.4E-05 | 0.81 | 0.19 | 0.39 | 0.64 | 0.86 | 0.14 | 3.8E-10 | 0.43 |
| 0.66 | 0.12 | 3.0E-08 | 1.3E-02 | 0.1 | -0.01 | 0.02 | 0.6 | 0.01 | 0.02 | 0.68 | 3.0E-07 |

β = effect size; SE = standard error; P_{BH} = Benjamini-Hochberg adjusted P -value; I^2 = heterogeneity measure; P_{Het} = heterogeneity P -value

