

**Meta-Analysis of Genome-Wide Studies Identifies MEF2C
SNPs Associated with Bone Mineral Density at Forearm**

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1 **Meta-Analysis of Genome-Wide Studies Identifies *MEF2C* SNPs Associated with**
2 **Bone Mineral Density at Forearm**

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40 Running title: *MEF2C* is associated with forearm BMD

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3 1 ABSTRACT
4

5 2 **Background:** Forearm fractures affect 1.7 million individuals worldwide each year and most
6
7 3 occur earlier in life than hip fractures. While the heritability of forearm bone mineral density
8
9 4 (BMD) and fracture is high, their genetic determinants are largely unknown.

10 5 **Aim:** To identify genetic variants associated with forearm BMD and forearm fractures.

11 6 **Methods:** BMD at distal radius measured by dual-energy X-ray absorptiometry was tested for
12 7 association with common genetic variants. We conducted a meta-analysis of genome-wide
13 8 association studies for BMD in 5,866 subjects of European descent and then selected variants for
14 9 replication in 715 Mexican American samples. Gene-based association was carried out to
15 10 supplement the single-SNP test. We then tested the BMD-associated SNPs for association with
16 11 forearm fracture in 2,023 cases and 3,740 controls.

17 12 **Results:** We found that five SNPs in the introns of *MEF2C* were associated with forearm BMD at
18 13 a genome-wide significance level ($P < 5 \times 10^{-8}$) in meta-analysis (lead SNP, rs11951031[T] -0.20
19 14 standard deviations per allele, $P = 9.01 \times 10^{-9}$). The gene-based association test suggested an
20 15 association between *MEF2C* and forearm BMD ($P = 0.003$). The association between *MEF2C*
21 16 variants and risk of fracture did not achieve statistical significance (SNP rs12521522[A]: odds
22 17 ratio = 1.14 [95% CI: 0.92-1.35], $P = 0.14$). Meta analysis also revealed two genome-wide
23 18 suggestive loci at *CTNNA2* and 6q23.2.

24 19 **Conclusion:** These findings demonstrate that variants at *MEF2C* were associated with forearm
25 20 BMD thereby implicating this gene in the determination of bone mineral density at forearm.
26 21

27 22 **Keywords:** Genome-wide association study; Osteoporosis; Bone mineral density; Forearm;
28 23 Fracture; Meta-analysis; Gene-base; Conditional analysis.
29 24

1 INTRODUCTION

2 Osteoporosis is a common disease characterized by low bone mineral density (BMD), resulting in
3 an increased risk of fragility fracture[1]. BMD, the best clinical indicator of fracture risk, is a highly
4 heritable trait, with heritability estimates of 60%–85%[2]. Forearm fractures are among the most
5 common fractures, affecting 1.7 million individuals per year[3], and have heritability of 54%[4].

6
7 Genome-wide association studies (GWAS) have identified more than 10 genes associated with
8 BMD from the Wnt signaling pathway, which is crucial to bone biology[5, 6]. We recently
9 conducted two separate GWAS meta-analyses for cortical bone thickness and forearm BMD, and
10 reported *WNT16*, which encodes an important Wnt factor, to be associated with BMD, cortical
11 bone thickness, bone strength and osteoporotic fracture risk[7]. In the current study, we extended
12 our study on forearm BMD by adding an additional GWAS cohort with BMD data, increasing our
13 meta-analysis sample size for six GWAS cohorts to 5,866 European-descended samples. In this
14 new analysis we detected an additional locus associated with forearm BMD and then replicated
15 the association in an independent cohort comprising 715 Mexican American samples. We
16 additionally conducted a gene-based association test to more fully characterize association
17 signals from the meta-analysis. Finally, we selected the most compelling SNPs from these
18 analyses and genotyped them in three cohorts comprising 2,023 forearm fracture cases and
19 3,740 controls to test their effects on the risk of forearm fracture.

21 MATERIALS AND METHODS

22 The GWAS and fracture samples have been described previously[7]. Briefly, the six GWAS
23 cohorts include the Amish Family Osteoporosis Study (AFOS), the Gothenburg Osteoporosis and
24 Obesity Determinants (GOOD) study, the Anglo-Australasian Osteoporosis Genetics Consortium
25 (AOGC) study, TwinsUK1, TwinsUK23 and TwinsUK4, comprising a total of 5,866 European-
26 descended samples. The TwinsUK4 cohort, which includes 194 subjects phenotyped for forearm
27 BMD, was not included in our previous GWAS [7], nor was the Mexican American replication
28 sample (see below). Genotyping of the TwinUK4 was done on the Illumina HumanHap650K

1 platform. The quality control criteria are similar to TwinsUK23 described in Zheng et al [7].
2 Imputation was performed using the IMPUTE2 [8] based on HapMap2, release 22. BMD at distal
3 radius was measured in all cohorts by dual-energy X-ray absorptiometry following standard
4 manufacturer protocols. The fracture cohorts include AOGC, the Umea Fracture and
5 Osteoporosis (UFO) study, the Canadian Multi-Centre Osteoporosis study (CaMos) and the
6 Manitoba-McGill (ManMc) fracture study, comprising 2,023 forearm fracture cases and 3,740
7 controls. Forearm fracture was defined as fractures resulting from low trauma (such as a fall from
8 standing height) occurring at the wrist, ulna, radius, and forearm, as well as Colles' fractures.
9 There are no overlapping samples between BMD and fracture. De-novo genotyping of SNP
10 rs12521522 in fracture cases and controls was undertaken at Kbiosciences (England). All study
11 participants provided informed written consent. Approval by local institutional review boards was
12 obtained in all studies.

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15 The replication cohort is from the San Antonio Family Osteoporosis Study (SAFOS), which was
16 designed as a study of cardiovascular and bone health in a representative sample of
17 multigenerational Mexican American families[9]. Proband aged 40-60 years of age were
18 recruited from low-income neighborhoods in San Antonio, Texas regardless of health status. The
19 SAFOS samples were genotyped using the Illumina 550 HumanHap Beadchip by the Texas
20 Biomedical Research Institute as part of the San Antonio Family Heart Study. Association
21 analysis was conducted using the SOLAR software program[10] to account for family structure.
22 To minimize the risk of false associations due to stratification in this admixed sample, we
23 performed a principal component analysis using ~ one million genotypes to capture the total
24 genetic variation in the sample as previously described [11]. We then included as covariates into
25 the association analysis the first four principal components. A total of 715 samples with forearm
26 BMD data were analyzed in the current study; the mean age, height and weight of these study
27 subjects was 42 ± 14.7 (year), 161.9 ± 9.2 (centimeter) and 81.6 ± 21.5 (kilogram), respectively.
28
29 Statistical methods for the meta-analysis were similar to those used in the previous analysis[7].

1 Briefly, all cohorts independently conducted the association analysis of SNP allele dosage with
2 standardized BMD residuals, while adjusting for age, age², gender, height, weight and population
3 substructure where applicable, for center of recruitment (AOGC), and for family structure in
4 cohorts with family members. A meta-analysis of the GWAS results was conducted using the
5 GWAMA software (Genome-Wide Association Meta Analysis)
6 (<http://www.well.ox.ac.uk/gwama/>)[12] with fixed-effects inverse variance meta-analysis[13].

7
8 We next performed a gene-based association test following the procedure proposed by Liu *et al*
9 as implemented in the software VEGAS[14], a computationally feasible method for analyzing
10 meta-analytic results. We included all SNPs within genes (including ± 50 kb from the 5' and 3'
11 UTR) with a maximum of 1×10^6 simulations to account for the linkage disequilibrium (LD)
12 structure among SNPs within a gene. Conditional analysis was conducted using GCTA
13 0.93.9[15], an approximate conditional analysis method using summary-level statistics from the
14 meta-analysis and LD corrections between SNPs estimated from a reference sample[16]. We
15 used TwinsUK23 as the reference sample to calculate the LD information of SNPs, due to its
16 size.

17
18 SNPs that were associated with BMD were assessed for association with fracture risk using
19 logistic regression models adjusted for age, gender, height and weight. We used CatS[17] for
20 power calculation.

21 22 **RESULTS**

23 GWAS analyses were performed in the six cohorts for forearm BMD applying cohort-specific
24 genomic controls. The cohort-specific results were meta-analyzed using fixed effects meta-
25 analysis, again applying the overall meta-analytic genomic control (Overall $\lambda = 1.012$, and 1.051
26 for AFOS; 0.990 for AOGC; 1.014 for GOOD; 1.0089 for TwinsUK1; 1.0037 for TwinsUK23; 1.170
27 for TwinsUK4). A quantile-quantile plot of the observed P values showed a clear deviation at the
28 tail of the distribution from the null distribution (the distribution expected if there were no

1 association) even after 648 SNPs were removed from the *WNT16* region, which was reported
2 previously [7]. This suggests that the observed P values, particularly the ones within the tail of the
3 distribution, are smaller than those expected by chance and probably reflect true genetic
4 association (**Supplementary Figure S1**).

5
6 Genome-wide associations with forearm BMD were observed at two loci, *WNT16* (7q31) and
7 *MEF2C* (5q14.3). At *WNT16*, significant associations were observed with 30 SNPs (3.26×10^{-8}
8 $>P > 1.87 \times 10^{-13}$), replicating an association we have previously observed [7] (**Supplementary**
9 **Figure S2**). At *MEF2C*, five of eight SNPs were significantly associated with forearm BMD, with
10 the other three SNPs showing suggestive levels of association ($4.55 \times 10^{-7} >P > 3.15 \times 10^{-8}$) on meta-
11 analysis (**Supplementary Figure S2** and **Figure 1**). The most significantly associated SNP was
12 rs12521522 (-0.20 standard deviations [SD] per A allele, $P = 3.15 \times 10^{-8}$) (**Table 1**). These eight
13 SNPs were highly correlated with each other (HapMap CEU LD calculation: $1 > R^2 > 0.85$). Using
14 association results from the GWAS meta-analysis we next sought to determine if there were any
15 gene-based signals arising when GWAS summary statistics were collapsed across the genes
16 [14]; The gene-based test results support the single-SNP findings of the meta-analysis, with
17 collapsing P-values of 0.003 for gene *MEF2C*.

18
19 Meta analysis also revealed two genome-wide suggestive loci at *CTNNA2* and 6q23.2, including
20 34 genome-wide suggestive SNPs in the region of *CTNNA2* ($1.73 \times 10^{-6} <P < 5 \times 10^{-6}$) and 10
21 genome-wide suggestive SNPs at 6q23.2 ($5.52 \times 10^{-7} <P < 3.76 \times 10^{-6}$) (**Supplementary Figure S2**).

22
23 We attempted an *in silico* replication on the eight SNPs associated with forearm BMD at *MEF2C*
24 in the Mexican American population. Four of the eight SNPs were monomorphic in the replication
25 population. Of the remaining four polymorphic SNPs, three had effect sizes in the same direction
26 as, and even slightly larger than, those observed in the meta-analysis, including two SNPs for
27 which the associations in Mexican Americans achieved statistical significance at the 0.05
28 threshold (rs12522630 and rs17494872) (Table 1). In the joint analysis of discovery and

1 replication populations, evidence of association improved for the three SNPs, with the most
2 significant association at rs11951031 (-0.20 SD per T allele, $P = 9.01 \times 10^{-9}$) (Table 1 and Figure
3 2).

4
5 In order to investigate whether the variants showing association with forearm BMD also have an
6 effect on the risk of forearm fracture, we tested SNP rs12521522 for *de novo* genotyping in
7 samples with forearm fracture and their controls. In the meta-analysis for fracture, comprising
8 2,023 forearm fracture cases and 3,740 controls, from 3 cohorts. The association between
9 rs12521522 and risk of fracture did not achieve statistical significance (Odds Ratio [OR] = 1.14
10 [95% CI: 0.92-1.35], $P = 0.14$) (Table 1). The fracture associations for the other 7 SNPs at the
11 *MEF2C* locus were tested *in silico* in the much smaller AOGC fracture GWAS cohort in 155 cases
12 and 1672 controls and the results showed no evidence of association (Table 1).

13
14 Because SNP rs1366594, which locates upstream of *MEF2C* gene (Figure 1), has been
15 previously reported to be associated with femoral neck (FN) BMD [18], we evaluated whether this
16 SNP or signals from this region could explain the observed association with forearm BMD. First,
17 the minor allele frequency (MAF) of forearm BMD-associated SNP in our study (rs11951031,
18 MAF=0.06) was considerably lower than that of FN BMD-associated SNP (rs1366594,
19 MAF=0.45), and the effect size of rs11951031 (-0.20 SD per T allele) was much larger than
20 rs1366594 (-0.085 SD per C allele). Second, these two SNPs are only very weakly correlated
21 with each other (HapMap CEU LD calculation: R-square =0.087). Third, after conditioning on the
22 effect of rs1366594, the effect size for rs11951031 on forearm BMD decreased from -0.20 SD per
23 T allele (4.16×10^{-8}) to -0.18 SD per T allele (1.35×10^{-6}). Therefore, the SNPs we have found to be
24 associated with forearm BMD are distinct from those found previously [18].

25 26 DISCUSSION

1 We identified gene *MEF2C*, a member of the Wnt signaling pathway, to be associated with
2 forearm BMD in meta-analysis in a collection of 6,584 individuals. In addition, we observed a non-
3 significant trend towards risk of fracture at this locus.

4
5 The Wnt/ β -catenin signaling pathway is known to play an important role in the regulation of bone
6 mass and bone turnover[19]. *MEF2C* is an important member of this pathway[5, 6], and, in fact, in
7 the large GEFOS Consortium, a SNP (rs1366594) located upstream from this gene, was
8 associated with FN BMD, although not with lumbar spine BMD[18]. We report in this study that
9 intronic variants in *MEF2C* are associated with forearm BMD, a clinically distinct phenotype from
10 that at femoral neck.

11
12 Our finding adds three novel pieces to the genetics of BMD puzzle. First, bone at the forearm is
13 structurally different than bone at the femoral neck insofar as forearm bone contains a much
14 higher proportion of cortical bone. BMD at both sites predicts fracture at their respective
15 anatomical sites better than at other sites. Second, the associated variants for forearm BMD
16 appear to be quite distinct from the variants associated with FN BMD. Not only are they located
17 over ~237kb from each other (Figure 1), but they have very different allele frequencies (0.06 vs
18 0.45) and very different effect sizes (-0.20 SD vs -0.06 SD), and they are not correlated.
19 Moreover, conditional analyses reveal that the effect of rs11951031 on forearm BMD are largely
20 independent of any effect of rs1366594. We postulate that these common variants are likely
21 independent signals that have different independent effects on the two BMD phenotypes. It is
22 also possible that both associations arise from several rare causal variants on the same
23 haplotype background [20], however, this hypothesis will likely be tested as more sequencing
24 studies emerge for BMD. These observations also suggest that the same variants have
25 differential effects on different types of bone.

26
27 We did not observe a statistically significant association of *MEF2C* SNP (rs12521522) with
28 osteoporotic fracture in the current study. Our sample size (2,023 cases and 3,740 controls)

1 provided 44% power to detect an odds ratio of 1.14 for a risk allele having a frequency of 0.06.
2 However, the direction of effect of the alleles that decreased BMD was associated with in
3 increase in fracture risk across the study cohorts. Given the sample size for fracture in this study,
4 these results should be interpreted cautiously and require further replication. Additionally,
5 rs1366594, which was reported in Rivadeneira *et al* [18] showed no evidence of association with
6 forearm fracture neither in the AOGC *in silico* analysis (155 cases and 1672 controls, P=0.27).

7
8
9 In summary, our data provides first evidence that intronic variants at the *MEF2C* locus, a member
10 of the Wnt pathway, are associated with forearm BMD. These findings expand our understanding
11 of the genetic determinants of forearm BMD, a clinically relevant skeletal site.

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17 18 **Contributors**

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23
24 **Competing interests** None

25 **Patient Consent** Written informed consent was obtained from all study participants.

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1 Figure legend:

2 Figure 1. Scatter plots of the observed association with forearm BMD in the 800kb wide region around
3 rs12521522 in *MEF2C* locus. The P values of SNPs (shown as $-\log_{10}$ values in y-axis, from the genome-
4 wide single-marker association analysis using the linear regression model) are plotted against their map
5 position (b36) (x-axis). The color of each SNP spot reflects its r^2 with rs12521522. SNPs rs11951031 and
6 rs12521522 are in perfect LD, and rs1366594 is ~ 237 kb away from rs11951031.

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8 Figure 2. Forest plot of association of rs11951031 (effect allele T) with forearm BMD.
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Table 1: Association results of forearm BMD meta-analysis and fracture of the top SNPs.

9CHR	SNP	POSITION	GWAS Meta analysis						SAFOS			Joint analysis*			Fracture analysis#		
			EA	NEA	EAF	BETA	SE	P	EAF	BETA	SE	P	BETA	SE	P	OR	P
10 ⁵	rs17558256	88119209	C	T	0.06	-0.20	0.04	3.15x10⁻⁸	0	NA	NA	NA	NA	NA	NA	1.20 (0.82-1.43)	0.33
11 ⁵	rs11958689	88142609	G	C	0.06	-0.20	0.04	4.16x10⁻⁸	0.08	0.04	0.11	0.72	-0.18	0.03	3.35x10 ⁻⁷	1.21 (0.83-1.45)	0.31
12 ⁵	rs12521522	88148517	A	T	0.06	-0.20	0.04	3.15x10⁻⁸	0	NA	NA	NA	NA	NA	NA	1.14 (0.92-1.35)	0.14
13 ⁵	rs12521522	88148517	A	T	0.06	-0.20	0.04	3.15x10⁻⁸	0	NA	NA	NA	NA	NA	NA	1.14 (0.92-1.35)	0.14
14 ⁵	rs11955542	88148984	T	C	0.06	-0.20	0.04	3.15x10⁻⁸	0	NA	NA	NA	NA	NA	NA	1.20 (0.82-1.43)	0.33
15 ⁵	rs11951031	88174487	T	C	0.06	-0.20	0.04	4.16x10⁻⁸	0.04	-0.23	0.14	0.10	-0.20	0.04	9.01x10⁻⁹	1.20 (0.81-1.45)	0.33
16 ⁵	rs12515983	88189831	A	T	0.06	-0.20	0.04	6.12x10 ⁻⁸	0	NA	NA	NA	NA	NA	NA	1.19 (0.81-1.44)	0.34
17 ⁵	rs12515983	88189831	A	T	0.06	-0.20	0.04	6.12x10 ⁻⁸	0	NA	NA	NA	NA	NA	NA	1.19 (0.81-1.44)	0.34
18 ⁵	rs12522630	88224123	A	G	0.07	-0.18	0.04	3.35x10 ⁻⁷	0.04	-0.29	0.14	0.04	-0.19	0.03	5.19x10 ⁻⁸	1.25 (0.83-1.49)	0.22
19 ⁵	rs17494872	88228915	A	G	0.07	-0.18	0.04	4.55x10 ⁻⁷	0.04	-0.29	0.14	0.04	-0.19	0.03	6.82x10 ⁻⁸	1.25 (0.83-1.49)	0.23
20 ⁵	rs17494872	88228915	A	G	0.07	-0.18	0.04	4.55x10 ⁻⁷	0.04	-0.29	0.14	0.04	-0.19	0.03	6.82x10 ⁻⁸	1.25 (0.83-1.49)	0.23

EA: effect allele; NEA: non-effect allele; EAF: effect allele frequency;

NA: not applicable; these SNPs were not polymorphic in Mexican Americans (rs17558256, rs12521522, rs11955542 and rs12515983).

* Combined results of GWAS meta-analysis and SAFOS replication study.

SNP rs12521522 was tested in 2,023 cases and 3,740 controls; the other 7 SNPs were tested in 155 cases and 1672 controls.

Boldface indicated the genome wide significant SNPs.

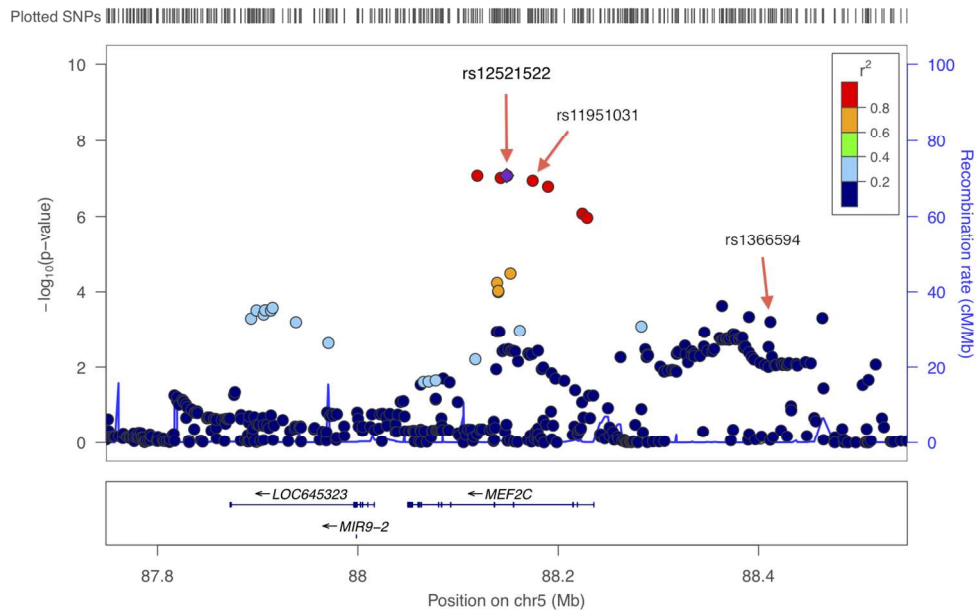


Figure 1. Scatter plots of the observed association with forearm BMD in the 800kb wide region around rs12521522 in MEF2C locus. The P values of SNPs (shown as $-\log_{10}$ values in y-axis, from the genome-wide single-marker association analysis using the linear regression model) are plotted against their map position (b36) (x-axis). The color of each SNP spot reflects its r^2 with rs12521522. SNPs rs11951031 and rs12521522 are in perfect LD, and rs1366594 is ~ 237 kb away from rs11951031.

254x177mm (150 x 150 DPI)

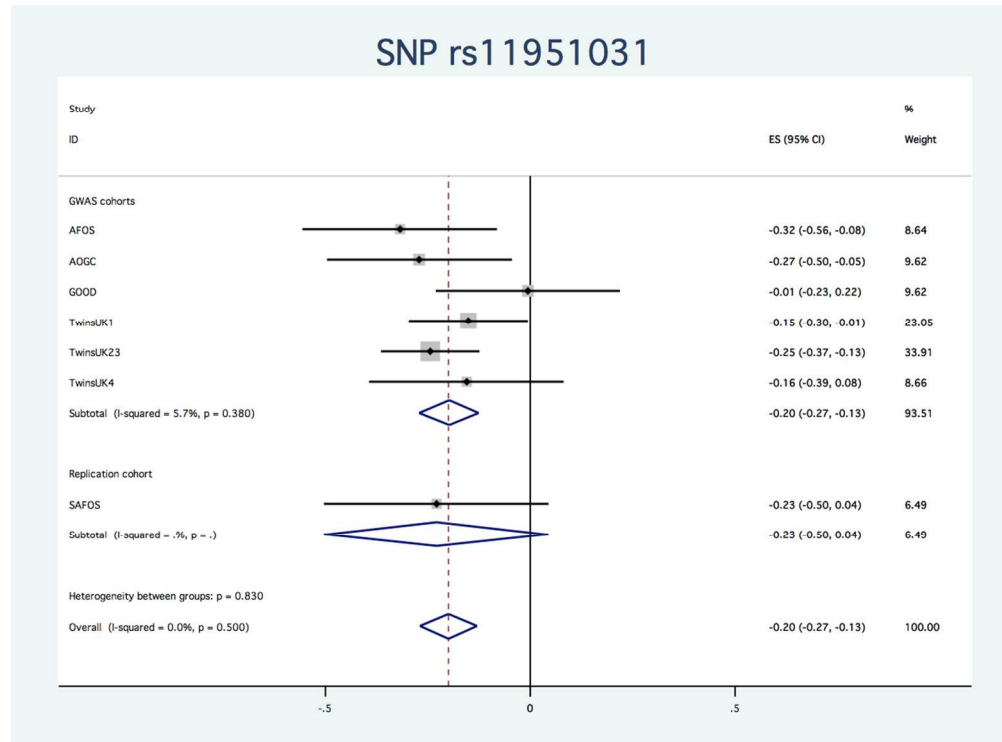
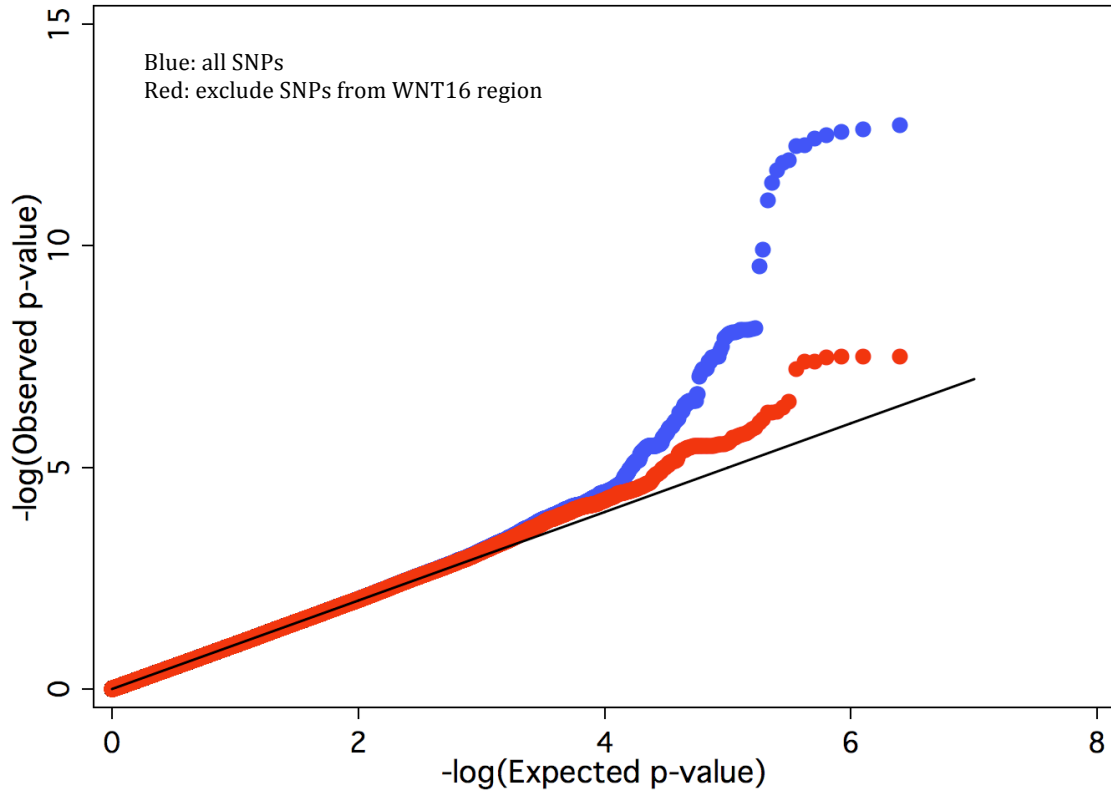


Figure 2. Forest plot of association of rs11951031 (effect allele T) with forearm BMD.
444x329mm (72 x 72 DPI)

Supplementary Figure S1

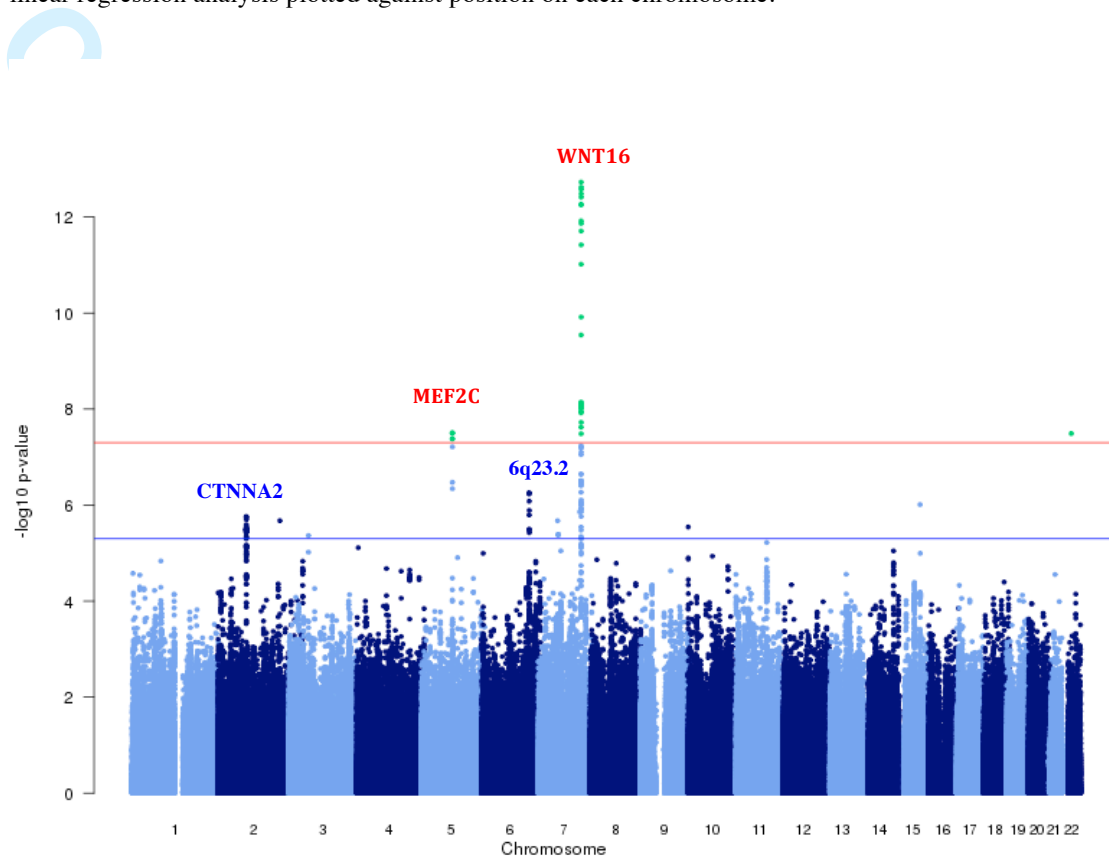
Quantile-quantile plots of the observed P values versus the expected P values for association. The dots in blue were plotted on the entire set of SNPs, whereas the dots in red were obtained after removing WNT16 region SNPs (+/- 400KB either side of rs2908004). The black line was the distribution expected if there were no association.



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Supplementary Figure S2

Manhattan plot for GWAS Meta-Analysis of Forearm BMD. Genome-wide P values ($-\log_{10} P$) of the linear regression analysis plotted against position on each chromosome.



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