The $-839$(A/C) Polymorphism in the $ECE1$ Isoform b Promoter Associates With Osteoporosis and Fractures

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Context: We previously found that variation in a quantitative trait locus, including the gene-encoding endothelin-converting enzyme 1 ($Ece1$), accounted for 40% of the variance in bone biomechanics and bone mineral density (BMD) in an intercross of recombinant congenic mouse strains.

Objective: We hypothesized that single nucleotide polymorphisms (SNPs) within the human $ECE1$ isoform b promoters, at $ECE1$ b $-338$(G/T) and $ECE1$ b $-839$(A/C), would associate with osteoporosis in postmenopausal women.

Design: We genotyped DNA for the $ECE1$ $-338$(G/T) and $-839$(A/C) SNPs.

Setting: A community medical center.

Participants: Postmenopausal women (3564) with $\geq$1 dual-energy X-ray absorptiometry scan $\geq$60 years of age.

Main Outcome Measures: BMD, osteoporosis, and clinical fractures.

Results: In multivariate models controlling for age, weight, healthcare duration, and tobacco, the CC genotype reduced the odds of lifetime fracture (OR 0.33, 95% CI 0.12, 0.87) and fracture $\geq$50 years of age (OR 0.31, 95% CI 0.11, 0.87), whereas the AC genotype increased odds of osteoporosis (OR 1.34, 95% CI 1.02 1.78) relative to the AA genotype. However, when controlling the false-discovery rate, findings were no longer significant. We found no consistent relationship between the $ECE1$ b $-338$(G/T) and study outcomes.

Conclusions: The CC genotype was associated with fewer fractures, whereas the AC genotype was associated with osteoporosis. Our small sample size and few minorities are study limitations. Findings should be tested in another cohort to confirm a link between the $ECE1$ $-839$(A/C) SNPs and osteoporosis.

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Abbreviations: BMD, bone mineral density; BMI, body mass index; DXA, dual-energy X-ray absorptiometry; ECE1, endothelin-converting enzyme 1; ET1, endothelin 1; GWAS, genome-wide association study; ISCD, International Society for Clinical Densitometry; PMRP, Personalized Medicine Research Project; SNP, single nucleotide polymorphism.
Approximately two-thirds of peak bone mass is genetically determined [1]. Endothelin 1 (ET1) is a 21-amino acid, endothelium-derived, secreted, vasoactive peptide linked to hypertension and ischemic events [2, 3]. ET1 is secreted as a 39-amino acid inactive precursor, big ET1, which must be activated by proteolytic cleavage. Endothelin-converting enzyme 1 (ECE1) is a membrane-bound extracellular protease that catalyzes this reaction. ET1 promotes Wnt signaling to increase uncontrolled bone growth in osteoblastic bone metastases [4].

We previously demonstrated that ECE1-dependent ET1 signaling affects bone physiology in in vitro, ex vivo, and in vivo studies. Addition of exogenous, big ET1 to the culture media of immortalized mouse osteoblasts increased mineralization and decreased sclerostin production via upregulation of microRNA 126-3p [5, 6]. Addition of big ET1 to the media of human bone cores cultured ex vivo mimicked the response of bone to mechanical loading [7]. Congenic mice, harboring identical alleles at all loci except for the region surrounding Ece1, demonstrated variation in bone size and strength [8]. Furthermore, ablation of the endothelin receptor A in germline osteoblasts decreased bone mineral density (BMD) in 12-week-old mice [9].

Hu et al. [10] found that addition of ET1 to bone marrow-derived mesenchymal stem cells increased osteogenesis in vitro and in vivo (calvarium defect experiment). In postmenopausal women, Mestek et al. [11] found an inverse relationship between spine BMD and the vasodilating response to endothelin A receptor blockade (r = −0.44, P < 0.006). In genome-wide association studies (GWASs) for BMD and fracture, the single nucleotide polymorphism (SNP) rs7521902, located close to ECE1 on chromosome 1p36.12, was significantly associated with lumbar spine and femoral neck BMD and fracture in women [12]. These lines of evidence support a role for ET1 signaling in skeletal health.

Based on prior studies, we hypothesized that ECE1 isoform b promoter polymorphisms at ECE1 b −338(G/T) and ECE1 b −839(A/C) would associate with BMD, osteoporosis diagnosis, and clinical fractures in postmenopausal women. These SNPs exist at rs213045 and rs213046, respectively (Table 1). We used banked DNA and medical records’ data from postmenopausal women participating in the Marshfield Clinical Personalized Medicine Research Project (PMRP) to test our hypothesis.

1. Materials and Methods

We studied postmenopausal women, who are at the greatest risk of osteoporosis and therefore, often undergo dual-energy X-ray absorptiometry (DXA) tests to screen for the condition. We collected medical records’ data from all postmenopausal women with banked DNA in the Marshfield Clinical PMRP and at least one BMD test at age ≥60 years. The Marshfield Clinic began the PMRP in 2002, and it currently contains DNA samples from ~20,000 patients, along with links to their electronic health records [13]. Participants provided written consent to participate in the PMRP repository, including consent to bank DNA for future studies.

BMD scans were obtained, using DXA on Lunar Prodigy (GE Healthcare, Madison, WI) densitometers. From 1986 to 2001, all scans were performed at Ministry St. Joseph’s Hospital in Marshfield, Wisconsin. From 2002 to 2015, all scans were performed at the Marshfield Clinic and satellite centers. BMD data for lumbar spine, femoral neck, and total hip were extracted from densitometer databases. The earliest available scans that met inclusion criteria and satisfied technical validity checks were used for each subject. Before 2002, four of the eight Marshfield Clinic radiology technicians were International Society for Clinical Densitometry (ISCD) certified. From 2002 onward, all technicians were ISCD certified. Likewise, all physicians interpreting the scans were ISCD certified. The accuracy of the
densitometer measurements was monitored using daily quality-assurance tests and weekly phantom scans. Personnel repeated precision studies whenever there was a change in technician, a technician’s quality of work, or densitometer equipment.

Marshfield Clinic researchers de-identified all subjects’ data and then labeled samples and health records data with a unique identification number to preserve subject confidentiality. We recorded the duration of medical care at the Marshfield Clinic, because this could influence the number of observed clinical fractures per patient. We also recorded subjects’ age, race, ethnicity, body weight, height, spine, and hip BMD, T- and Z-score values, clinical fractures, diagnosis and treatment of osteoporosis, and use of tobacco and systemic glucocorticoid therapy.

Personnel shipped DNA samples from the Marshfield PMRP Biorepository to the University of Wisconsin-Madison Biotechnology Center for analysis. We genotyped all DNA samples for the $ECE1\, 2338(G/T)$ (rs213045) and $2839(A/C)$ (rs213046) SNPs by KASPar (KBiosciences, Hoddeston, United Kingdom) allele-specific amplification, using primer sets given in Table 1. Primer sets were validated before analysis of subjects’ DNA, using the Sigma Life Science Human Random Control DNA Panel. Subjects’ DNA concentration was verified using the Quant-iT™ PicoGreen® double-stranded DNA kit (Life Technologies, Grand Island, NY). For the KASPar PCR reaction, DNA samples were first standardized to 0.5 ng/µL using epMotion 5075 and pH 7.5 10 mM Ultrapure™ Tris-HCl (Life Technologies Carlsbad, CA). Each reaction contained 2 µL of 0.5 ng/µL DNA and 2 µL of KASPar reaction mix following standard procedures, with an annealing temperature of 56°C. Samples were stored at 10°C until read. Reactions were warmed in the dark at room temperature, and genotypes were assessed by fluorometry using a Synergy 2 (BioTek, Winooski, VT) plate reader and Gen5 software. Based on the 1000 Genomes Project data for populations of European ancestry [14], the two SNPs are in low linkage disequilibrium with each other ($r^2 = 0.262$). Additionally, the rs7521902 SNP is not in linkage disequilibrium with either $ECE1\, 2338(G/T)$ ($r^2 = 0.000$) or $ECE1\, 2839(A/C)$ ($r^2 = 0.002$). We evaluated the relationship between $ECE1$ polymorphisms and skeletal health, as reflected by BMD values and clinical fractures.

### A. Statistical Analysis

The 1000 Genome Project data found that in individuals of European ancestry, the frequency of the $−338(A)$ allele was 0.287. To calculate the potential power of the study, we estimated that at least 28% of postmenopausal women would have sustained a symptomatic fracture after age 50 years [15]. Assuming that we would identify 2880 eligible women with both a bone density test and banked DNA in the PMRP, we had 90% power to detect a 6% increase in fracture in the subset of 827 women with the $−338(A)$ allele using the $\chi^2$ test. We evaluated relationships between $ECE1$ isofrom b promotor polymorphisms and osteoporosis, clinical fractures, spine, and hip T-scores. A radiographic diagnosis of osteoporosis was noted when the T-score at the spine, femoral neck, or total hip was less than or equal to $−2.5$. Race was unknown for 47 subjects and non-white for 37 others (n = 84). Given our

| RSID   | Primer Name | Length | Sequence                      |
|--------|-------------|--------|-------------------------------|
| rs213045 | rs213045_G  | 42     | GAAGGTGACCAAGTTCATGCTCTTGGATTGCTGTCTGGCCAC |
|         | rs213045_T  | 44     | GAAGGTCCGGATCAAGGGATTTCTGCTGATTCATGCTGTCTGGCCAA |
| rs213046 | rs213046_A  | 45     | GAAGGTGACCAAGTTCATGCTAAATCTGCTGGGTAGACCTCTCT    |
|         | rs213046_C  | 43     | GAAGGTCCGGATCAAGGGATTTCTGCTGATTCATGCTGTCTGGCCAA |
|         | rs213046_C1 | 26     | CTCTTCGGATATGAGGTGTTGCAGTT |

Abbreviation: RSID, Reference SNP Identification.
study’s limited power to detect the impact of race on osteoporosis or fracture, we analyzed data with and without these subjects. Because genetic factors influence bone strength throughout life, we modeled fracture using two definitions: a clinical fracture at age ≥50 years and a clinical fracture at any age. As few women took systemic glucocorticoid therapy at the time of the bone density test (n = 3), we excluded this variable in our models. All models were performed with and without inclusion of two individuals with unusually high BMD values (one with a spine and hip T-score of +10.3 and +4.3 and the other with a spine and hip T-score of −1.5 and +5.6, respectively).

We modeled five separate study outcomes: spine T-score, lowest hip T-score, osteoporosis, clinical fracture ≥50 years of age, and lifetime clinical fracture. We fit single-variable models initially and then fit multiple-variable models, adjusting for covariates known to affect BMD and fracture risk, including age, weight, and tobacco use. We analyzed tobacco use as a three-level variable (current, prior, or never) and as a dichotomous variable (ever vs never). As rates of diagnosed clinical fracture would likely depend on duration of care at the Marshfield Clinic, we adjusted multiple-variable models for duration of care. For each modeled outcome, we explored the effects of the A/C polymorphism and of the G/T polymorphism. In the multiple-variable analyses, we fit models using both polymorphisms. In a sensitivity analysis, we reran models, including all subjects, regardless of race. Finally, we performed multiple-variable analyses in which we entered the two polymorphisms using an interaction term.

In a sensitivity analysis, we used the Benjamini-Hochberg procedure to control the false-discovery rate. The Benjamini-Hochberg procedure ranks the $P$ values within a model from smallest to largest and then determines the largest $P$ value that satisfies $p_k = (k/m) \times \alpha$. The $P$ value that equals this number and all smaller $P$ values are considered significant. In this formula, $k$ is each $P$ value’s rank, $m$ is the number of total $P$ values or comparisons, and $\alpha$ is set at a false-discovery rate of 0.05.

2. Results

Subjects were 3564 postmenopausal and predominantly non-Hispanic Caucasian (98%) women with a mean age of 66 ± 10 years and body mass index (BMI) of 29.6 ± 6.2 kg/m² (Table 2). Most women never smoked (63%) and more than one in five (22%) were deceased at the time of data collection. The entire cohort had a mean spine T-score of −0.6 ± 1.7 and lowest hip T-score of −1.5 ± 1.1. Nearly 49% sustained a fracture after age 50 years, and 20% had osteoporosis based on T-score values, yet only 10% had received osteoporosis treatment.

DNA testing was unsuccessful in 34 subjects for the G/T polymorphism and in 58 subjects for the A/C polymorphism. Among 3530 subjects with G/T genotype results, the GG (n = 1839, 52%), GT (n = 1433, 41%), and TT (n = 258, 7%) genotype frequencies were consistent with the Hardy-Weinberg equilibrium ($P = 0.35$). Among 3506 subjects with A/C genotype results, the AA (n = 2942, 84%), AC (n = 541, 15%), and CC (n = 23, <1%) genotypes also followed the Hardy-Weinberg equilibrium ($P = 0.72$).

Women with the CC genotype had fewer fractures ≥50 years of age compared with women with the AA and AC genotypes (22%, 51%, and 49%, respectively, $P < 0.001$). We found no differences in BMD or other study outcomes when comparing women with the GG, GT, and TT genotypes (Table 2). As expected, age was inversely associated with the spine and lowest hip T-scores, whereas weight and duration of care at the Marshfield Clinic were positively associated with spine and lowest hip T-scores (Table 3).

In single-variable logistic-regression models, predicting osteoporosis and fracture (Table 4), age increased the odds of osteoporosis (OR 1.08, 95% CI 1.07, 1.09) and the odds of lifetime fracture (OR 1.03, 95% CI 1.03, 1.04). Weight (OR 0.95, 95% CI 0.94, 0.95) and duration of care (OR 0.90, 95% CI 0.89, 0.92) both reduced the odds of osteoporosis based on a T-score diagnosis. Compared with the AA genotype, the CC genotype reduced the odds of lifetime fracture (OR 0.32, 95% CI 0.13, 0.82) and the odds of fracture ≥50 years of age (OR 0.31, 95% CI 0.11, 0.83).

We used multiple-variable logistic-regression models to explore the impact of the $ECE1$ b−839(A/C) and $ECE1$ −338(G/T) alleles on study outcomes. Our primary models excluded
Table 2. Demographic Characteristics and BMD, Osteoporosis, and Fracture Measures

| Demographic characteristics | All Subjects, n = 3564 | GG, n = 1839 | GT, n = 1433 | TT, n = 258 | P Value | AA, n = 2942 | AC, n = 541 | CC, n = 23 | P Value |
|-----------------------------|------------------------|-------------|-------------|-------------|---------|-------------|-------------|-----------|---------|
| Age, y                      | 66 ± 10                | 66 ± 10     | 66 ± 9      | 66 ± 10     | 0.521   | 66 ± 10     | 66 ± 9      | 67 ± 10   | 0.740   |
| Height, cm                  | 160 ± 6                | 160 ± 6     | 160 ± 6     | 160 ± 6     | 0.704   | 160 ± 6     | 160 ± 6     | 160 ± 6   | 0.336   |
| Weight, kg                  | 76.0 ± 16.4            | 75.9 ± 16.2 | 76.0 ± 16.5 | 76.0 ± 16.7 | 0.992   | 76.1 ± 16.4 | 75.5 ± 16.1 | 77.0 ± 18 | 0.769   |
| BMI, kg/m²                  | 29.6 ± 6.2             | 29.6 ± 6.2  | 29.5 ± 6.3  | 29.5 ± 6.2  | 0.990   | 29.6 ± 6.3  | 29.5 ± 6.0  | 30.4 ± 6.7 | 0.739   |
| Deceased (%)                | 258 (22)               | 394 (21)    | 332 (23)    | 57 (22)     | 0.492   | 653 (22)    | 119 (22)    | 4 (17)    | 0.855   |
| Tobacco use                 |                        |             |             |             |         |             |             |           |         |
| Current (%)                 | 271 (8)                | 154 (8)     | 103 (7)     | 14 (5)      | 0.504   | 228 (8)     | 38 (7)      | 1 (4)     | 0.964   |
| Prior (%)                   | 954 (27)               | 479 (26)    | 402 (28)    | 66 (26)     | 785 (27) | 149 (28)    | 5 (22)     |           |         |
| Never (%)                   | 2324 (63)              | 1152 (63)   | 885 (62)    | 170 (66)    | 1844 (62)| 337 (62)    | 16 (70)    |           |         |
| Unknown (%)                 | 105 (3)                | 54 (3)      | 45 (3)      | 8 (3)       | 85 (3)   | 17 (3)      | 1 (4)      |           |         |
| Race (%)                    | 3480 (98)              | 1797 (99)   | 1397 (99)   | 252 (99)    | 0.343   | 2879 (99)   | 523 (98)    | 22 (96)   | <0.001  |
| White (%)                   | 37 (1)                 | 17 (1)      | 17 (1)      | 3 (1)       | 23 (1)   | 11 (2)      | 1 (4)      |           |         |
| Other (%)                   | 3507 (98)              | 1810 (100)  | 1408 (100)  | 255 (100)   | 0.249   | 2897 (100)  | 530 (99)    | 23 (100)  | 0.025   |
| Ethnicity                   |                        |             |             |             |         |             |             |           |         |
| Not Hispanic/Latina (%)     | 9 (1)                  | 3 (1)       | 6 (1)       | 0 (0)       | 4 (1)   | 4 (1)       | 0           |           |         |
| Hispanic/Latina (%)         | 1 (1)                  | 3 (1)       | 3 (1)       | 0 (0)       | 4 (1)   | 4 (1)       | 0           |           |         |
| Unknown (%)                 | 47 Subjects            |             |             |             |         |             |             |           |         |
| BMI characteristics         |                        |             |             |             |         |             |             |           |         |
| Spine, g/cm²                | 1.107 ± 0.198          | 1.105 ± 0.199 | 1.108 ± 0.199 | 1.104 ± 0.182 | 0.904 | 1.108 ± 0.199 | 1.098 ± 0.194 | 1.081 ± 0.199 | 0.431 |
| Spine T-score               | −0.6 ± 1.7             | −0.7 ± 1.7  | −0.6 ± 1.7  | −0.7 ± 1.5  | 0.913   | −0.6 ± 1.7  | −0.7 ± 1.6  | −0.9 ± 1.6 | 0.411   |
| Spine Z-score               | 0.7 ± 1.7              | 0.7 ± 1.7   | 0.8 ± 1.7   | 0.7 ± 1.5   | 0.666   | 0.7 ± 1.7   | 0.7 ± 1.6   | 0.6 ± 1.6  | 0.639   |
| Lowest femoral neck BMD, g/cm² | 0.869 ± 0.141        | 0.872 ± 0.138 | 0.866 ± 0.146 | 0.865 ± 0.133 | 0.491 | 0.872 ± 0.140 | 0.856 ± 0.147 | 0.852 ± 0.117 | 0.067 |
| Lowest femoral neck T-score | −1.2 ± 1.0            | −1.2 ± 1.0  | −1.2 ± 1.1  | −1.2 ± 1.0  | 0.489   | −1.2 ± 1.0  | −1.3 ± 1.1  | −1.3 ± 0.9 | 0.067   |
| Lowest femoral neck Z-score | 0.3 ± 0.9              | 0.3 ± 0.9   | 0.3 ± 1.0   | 0.2 ± 0.9   | 0.514   | 0.3 ± 0.9   | 0.2 ± 1.0   | 0.2 ± 0.8  | 0.074   |
| Lowest hip T-score          | −1.5 ± 1.1             | −1.3 ± 1.0  | −1.4 ± 1.0  | −1.4 ± 0.9  | 0.534   | −1.3 ± 1.0  | −1.4 ± 1.1  | −1.5 ± 0.8 | 0.073   |
| Fractures and other characteristics |                      |             |             |             |         |             |             |           |         |
| Osteoporosis diagnosis (%)  | 696 (20)               | 353 (19)    | 287 (20)    | 48 (19)     | 0.781   | 561 (19)    | 121 (22)    | 3 (13)    | 0.152   |
| Fracture under 50 y old (%) | 353 (10)               | 195 (11)    | 119 (8)     | 30 (12)     | 0.051   | 291 (10)    | 57 (11)     | 2 (9)     | 0.882   |
| Fracture over 50 y old (%)  | 1751 (49)              | 907 (49)    | 712 (50)    | 120 (47)    | 0.642   | 1444 (49)   | 277 (51)    | 5 (22)    | 0.020   |
| Care duration, d            | 7992 (7077, 8154)      | 7997 (7111, 8163) | 7981 (6962, 8144) | 7997 (7181, 8162) | 0.168 | 7986 (7060, 8155) | 8014 (7099, 8152) | 8072 (6735, 8134) | 0.973 |
| Osteoporosis treatment (%)  | 366 (10)               | 188 (10)    | 142 (10)    | 31 (12)     | 0.590   | 298 (10)    | 60 (11)     | 2 (9)     | 0.772   |

The P values within the table reflect comparisons across the homozygous and heterozygous genotypes, using parametric or nonparametric tests as appropriate to the distribution of data. Where data were skewed, the median (interquartile range) was reported, and data were analyzed using the Kruskal-Wallis test. Data with a normal distribution were summarized using means ± SD and analyzed using ANOVA. Statistically significant findings are highlighted in bold text.

aDNA testing was unsuccessful in 34 subjects for the GT and in 58 subjects for the AC polymorphs. Height was not available for 1 woman, weight was not available for 35 women, and BMI was unknown for 36 women. All women had measures of hip BMD, but spine BMD was missing in 65 women.

bOnly three women were taking glucocorticoid therapy within 90 d of their bone density test.
non-Caucasian women, women with an unknown racial background, and two outliers with high BMD (86 subjects). The model predicting osteoporosis found that age, care duration, weight, and the AC genotype of \( ECE1 \) \( b \) – 839(A/C) were important predictors (Table 5). Age increased, whereas weight and duration of care reduced the odds of osteoporosis. Additionally, with the AA genotype as the referent, the AC genotype increased the odds of osteoporosis (OR 1.34, 95% CI 1.02, 1.78). The AC genotype was no longer significant when controlling analyses for the false-discovery rate.

We used multiple-variable logistic-regression models to evaluate the impact of variables on lifetime fractures and fractures \( \geq 50 \) years of age. In these models, age and duration of care increased the odds of both fracture outcomes (Table 5). Additionally, the CC genotype reduced the odds of lifetime fracture (OR 0.33, 95% CI 0.12, 0.87) and fracture \( \geq 50 \) years of age (OR 0.31, 95% CI 0.11, 0.87). The relationship between the CC genotype and fracture was similar when including all women regardless of race and when including two subjects with very high T-scores (Table 5). The CC genotype was no longer a significant predictor of lifetime fracture or fractures \( \geq 50 \) years of age when controlling for the false-positive discovery rate. Only in models that included all subjects (n = 3564) did the GT genotype become significant, reducing the odds of fracture over age 50 (OR 0.76, 95% CI 0.60, 0.97).

In multiple linear-regression models predicting the lowest hip T-score (Table 6), significant covariates included age, duration of care, weight, and tobacco use. Neither the

| Table 3. Spearman Correlation Coefficients Between T-Scores and Predictors |
|-----------------------------|-----------------|-----------------|-----------------|
| Predictor                   | Sample Size     | Correlation Coefficient | P Value         |
| Spine T-score               |                 |                 |                 |
| Age, y                      | 3454            | −0.174           | <0.001          |
| Care duration, d            | 3454            | 0.108            | <0.001          |
| Weight, kg                  | 3419            | 0.318            | <0.001          |
| Smoking\( ^a \)             | 3359            | 0.004            | 0.812           |
| Lowest hip T-score          |                 |                 |                 |
| Age, years                  | 3328            | −0.371           | <0.001          |
| Care duration, d            | 3328            | 0.195            | <0.001          |
| Weight, kg                  | 3323            | 0.389            | <0.001          |
| Smoking\( ^a \)             | 3271            | 0.009            | 0.603           |

\(^a\)Smoking was analyzed using a scale, where 1 = current, 2 = prior, and 3 = never use. Statistically significant findings are highlighted in bold text.

| Table 4. Single-Variable Logistic-Regression Models Predicting Odds of Osteoporosis and Fractures |
|-----------------------------|-----------------|-----------------|-----------------|
| Predictor                   | \( n \)         | Osteoporosis    | Fracture \( \geq 50 \) Years of Age | Lifetime Fracture |
| Age, per y                  | 3446            | 1.08 (1.07, 1.09) | 1.05 (0.64, 1.72) | 1.03 (1.03, 1.04) |
| Duration of care, per y     | 3424            | 0.90 (0.89, 0.92) | 0.01 (0.71) | 4.29 (0.01, 4128) |
| Weight, per kilogram        | 3480            | 0.95 (0.94, 0.95) | 1.00 (0.89, 1.00) | 1.00 (1.00, 1.00) |
| Never, relative to ever, smoker | 3345          | 1.05 (0.88, 1.26) | 1.07 (0.92, 1.23) | 0.98 (0.85, 1.13) |
| AC, relative to AA, genotype| 3382            | 1.21 (0.97, 1.52) | 1.03 (0.86, 1.24) | 1.01 (0.88, 1.27) |
| CC, relative to AA, genotype| 3382            | 0.66 (0.20, 2.25) | 0.31 (0.11, 0.83) | 0.32 (0.13, 0.82) |
| GT, relative to GG, genotype| 3480            | 1.04 (0.87, 1.24) | 1.01 (0.88, 1.16) | 0.95 (0.83, 1.10) |
| TT, relative to GG, genotype| 3480            | 0.98 (0.70, 1.36) | 0.88 (0.67, 1.14) | 0.91 (0.70, 1.18) |

Numbers in the table are ORs, followed by their 95% CI in parentheses. Smoking was recorded as current, prior, or never. We also analyzed smoking as a three-level factor and again as a dichotomous variable (ever vs never); results were nearly identical to analyses where smoking was categorized as ever vs never. Because smoking affects fracture risk for years after smoking cessation, and the date of cessation was not recorded for prior smokers, we chose to report the odds of each outcome for never (vs ever) smokers. Here, we present the single-variable models when excluding non-Caucasian subjects and two outliers with very high hip T-scores (\( n = 3478 \)). Statistically significant findings are highlighted in bold text.
The table represents models, excluding non-Caucasian subjects and two individuals with unusually high BMD (n = 3478). Numbers in the table are ORs with 95% CIs. In multivariate models—including all subjects regardless of race, minus two outliers with high T-scores (n = 3562)—the AC genotype increased the odds of osteoporosis (OR 1.34, 95% CI 1.02, 1.78), and the CC genotype reduced the odds of fracture over age 50 (OR 0.30, 95% CI 0.10, 0.84, P = 0.020). In models, including subjects (n = 3564), the AC genotype increased the odds of osteoporosis (OR 1.34, 95% CI 1.02, 1.77, P = 0.038), and the CC genotype reduced the odds of fracture over age 50 (OR 0.32, 95% CI 0.12, 0.84, P = 0.020). In multiple-variable analyses, where the ECE1 −338(G/T) and −839(A/C) SNPs were analyzed using an interaction term, neither allele was a significant predictor of any study outcomes. The Benjamini-Hochberg method of controlling the false-positive discovery rate showed that age and care duration remained significant predictors of osteoporosis. The Benjamini-Hochberg method of controlling the false-positive discovery rate showed that age and care duration remained significant predictors of fracture ≥50 y of age and lifetime fracture. Statistically significant findings are highlighted in bold text.

**ECE1** b −839(A/C) nor **ECE1** b −338(G/T) was significant in models predicting the spine and lowest hip T-score. Findings were similar when we included all subjects regardless of race, with or without the two outliers with high T-scores. In multiple-variable models entering the A/C and G/T alleles with an interaction term, the alleles were not significant predictors of any study outcomes.

| Table 5. Multiple-Variable Logistic-Regression Models Predicting Odds of Osteoporosis and Fracture Using the A/C Allele as a Three-Factor Variable |
|---------------------------------|-----------------|-----------------|
|                                | Osteoporosis    | Fracture ≥50 Years |
| Age, per y                     | 1.05 (1.04, 1.06) P < 0.001 | 1.05 (1.05, 1.07) P < 0.001 |
| Care duration, per d           | 0.98 (0.95, 1.00) P = 0.049 | 1.01 (1.02, 1.06) P < 0.001 |
| Weight, per kg                 | 0.95 (0.94, 0.95) P < 0.001 | 1.00 (0.99, 1.00) P = 0.373 |
| Never, relative to ever, smoker | 0.95 (0.78, 1.16) P = 0.569 | 0.96 (0.83, 1.12) P = 0.575 |

| AC, relative to AA, allele      | 1.34 (1.02, 1.78) P = 0.038 | 1.10 (0.89, 1.38) P = 0.324 |
| CC, relative to AA, allele      | 0.64 (0.17, 2.47) P = 0.500 | 0.31 (0.11, 0.87) P = 0.026 |
| GT, relative to GG, allele      | 0.98 (0.79, 1.21) P = 0.858 | 0.97 (0.83, 1.13) P = 0.683 |
| TT, relative to GG, allele      | 0.88 (0.58, 1.33) P = 0.475 | 0.90 (0.67, 1.22) P = 0.539 |

The table represents models, excluding non-Caucasian subjects and two individuals with unusually high BMD (n = 3478). Findings were similar when including all subjects regardless of race and excluding two individuals with high T-scores (n = 3562), except that the AC genotype was borderline significant in models predicting the lowest hip T-score (β = 0.0845, P = 0.071). In multiple-variable analyses, where the ECE1 −338(G/T) and −839(A/C) SNPs were analyzed using an interaction term, neither allele was a significant predictor of spine or hip T-score. Statistically significant findings are highlighted in bold text.

| Table 6. Multiple Linear-Regression Models Predicting Spine and Hip T-Scores, Using the A/C Allele as a Three-Factor Variable |
|---------------------------------|-----------------|-----------------|
|                                | Spine T-Score   | Lowest Hip T-Score |
|                                | B   | SE   | T Value | P Value   | B   | SE   | T Value | P Value   |
| Intercept                      | −1.903 | 0.329 | −5.781 | <0.001 | −1.097 | 0.187 | −5.861 | <0.001 |
| Age, per y                     | −0.0189 | 0.003 | −6.197 | <0.001 | −0.034 | 0.002 | −19.350 | <0.001 |
| Care duration, per d           | 0.011 | 0.008 | 1.307 | 0.191 | 0.017 | 0.005 | 3.669 | <0.001 |
| Weight, per kg                 | 0.030 | 0.002 | 17.966 | <0.001 | 0.021 | 0.001 | 22.529 | <0.001 |
| Never, relative to ever, smoker | 0.040 | 0.057 | 0.697 | 0.486 | 0.074 | 0.032 | 2.291 | 0.022 |
| AC, relative to AA, genotype   | −0.064 | 0.083 | −0.772 | 0.440 | −0.072 | 0.047 | −1.525 | 0.127 |
| CC, relative to AA, genotype   | −0.411 | 0.346 | −1.186 | 0.235 | −0.090 | 0.194 | −0.463 | 0.643 |
| GT, relative to GG, genotype   | 0.031 | 0.061 | 0.504 | 0.614 | −0.017 | 0.034 | −0.508 | 0.612 |
| TT, relative to GG, genotype   | 0.024 | 0.115 | 0.205 | 0.838 | −0.044 | 0.066 | −0.668 | 0.504 |

R² = 0.11  R² = 0.26
We further explored the relationship between genotypes and spine and femoral neck BMD. In these analyses, subjects with the AC genotype had lower femoral neck BMD compared with subjects with the AA genotype (0.870 ± 0.147 vs 0.885 ± 0.142 g/cm², P = 0.05). Subjects with the AC genotype had similar spine BMD to subjects with the AA genotype (1.099 ± 0.195 vs 1.108 ± 0.199 g/cm², P = 0.321). We found no difference in spine or femoral neck BMD by the GG, GT, or TT genotype (data not shown).

3. Discussion

In the current study, we tested the hypothesis that ECE1 isoform b promoter polymorphisms at ECE1 b −338(G/T) and ECE1 b −839(A/C) on chromosome 1 were associated with BMD and clinical fractures in postmenopausal women. We used banked DNA and medical records’ data from postmenopausal women participating in the Marshfield Clinic PMRP to test our hypothesis. In multivariate models, the AC genotype increased the odds of osteoporosis, whereas the CC genotype reduced the odds of lifetime fracture and fracture ≥50 years of age. However, findings were no longer significant when controlling the false-discovery rate. We found no consistent association between the ECE1 b −338(G/T) allele and BMD, osteoporosis, or fracture.

Whereas our results might seem discrepant, with the AC allele associated with increased osteoporosis risk and CC associated with decreased fracture, such findings do not negate the potential importance of our study. Most GWAS analyses are conducted using simple additive models of genotype effects. However, it is well known that for individual loci, additive, dominance, and sometimes epistatic effects are potentially relevant to the biology [16]. A simple example is ABO blood types, where A and B alleles are dominant to O and co-dominant with each other. The situation of a heterozygous genotype having a more extreme phenotype than either homozygous genotype has also been well described in the past, and this condition is called overdominance or underdominance. Overdominance of global fitness in malarial regions is thought by some to be the mechanism underlying the persistence of the sickle hemoglobin allele in the human population. The example of sickle cell also points out the importance of potential gene–environment interactions, as the increased fitness of hemoglobin allele/sickle hemoglobin individuals is limited to regions where malaria is endemic. Other potential explanations for overdominance also exist, including the tested locus being in linkage disequilibrium with the biologically relevant allele and false-positive association arising as a result of a small sample size. Our data cannot distinguish among these alternatives, and any questions about our study results are best be addressed by replication in an independent study population.

In mice, we previously found that variation in the genomic region harboring the Ece1 allele affected bone size and accounted for 40% of the variance in bone biomechanics and BMD in an intercross of recombinant congenic strains HcB-8 and HcB-23 (5-7). We confirmed the effect in fully congenic strains [8]. We demonstrated that ECE1-dependent ET1 signaling affects bone physiology in in vitro, ex vivo, and in vivo studies. Addition of exogenous big ET1 to immortalized mouse osteoblast cultures increased mineralization and decreased sclerostin production by upregulating microRNA 1263-p [5, 6]. Addition of ET1 simulated mechanical loading, when added to human bone core cultures ex vivo [7]. Congenic mice, identical at all loci except for Ece1, demonstrated variation in bone size and strength [8]. Ablation of the endothelin receptor A in osteoblasts decreased BMD in 12-week-old mice [9]. In human GWASs, SNP rs7521902, located close to ECE1 on chromosome 1p36.12, was significantly associated with lumbar spine and femoral neck BMD and fracture in women [12]. Additionally, the University of Michigan PheWeb (http://pheweb.sph.umich.edu/) reported significant associations between the A/C allele and risk of rib (P = 0.022), femur (P = 0.023), and vertebral (P = 0.032) fractures. Whereas this database does not adjust for covariates known to increase fracture risk, its results support the findings of the current study.

Limited human phenotyping is a weakness of the current study. In our earlier mouse experiments, we were able to evaluate multiple phenotypes and perform destructive biomechanical testing [5–7]. Those experiments revealed that bone cross-sectional geometry was the feature most strongly associated with genotype and correlated strongly with mechanical...
performance. DXA-determined BMD is a composite measure that incorporates both true volumetric density and bone size. Another possible explanation for the BMD vs fracture discrepancy is the small number (n = 23, <1%) of individuals harboring the CC genotype.

Our study has multiple strengths. First, we identified the ET1 signaling axis as potentially affecting skeletal health from in vitro [5, 6], ex vivo [7], and in vivo studies [18] and then tested the hypothesis that ET1 signaling affects skeletal health in postmenopausal women. The study was population based and controlled for several factors known to affect bone health. Weaknesses include a focus on Caucasian women, analysis of clinical rather than all fractures or fragility fractures, extraction of data from medical records rather than prospectively, and limited power to detect weak associations between the genotypes and study outcomes. Indeed, a small sample size is potentially our greatest weakness, as GWASs typically recruit much larger groups of subjects.

Our data and previous studies demonstrate that the ET1 signaling axis, and specifically ECE1, affects BMD. ET1 signaling is responsive to mechanical load [7], and allelic differences in murine Ece1 lead to differences in bone size and strength [18, 19] at skeletal maturity. Thus, we hypothesize that determination of ECE1 b status at an early age might permit loading intervention to increase peak bone mass and decrease lifetime risk of osteoporosis and fracture. Confirmation of findings and extension to other groups are needed in other, larger cohorts.

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