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Original Article

Linkage and Association for Bone Mineral Density and Heel Ultrasound Measurements with a Simple Tandem Repeat Polymorphism near the Osteocalcin Gene in Female Dizygotic Twins

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Abstract. In this confirmatory candidate gene study, we investigated possible linkage and association for bone density, heel ultrasound and bone turnover with the osteocalcin gene using the nearby (50–180kb) microsatellite marker D1S3737. Non-identical twin sisters aged 18–75 years at first interview were recruited for the study from the St Thomas' UK Adult Twin Registry with 1366 women being genotyped for marker D1S3737. Linkage, allelic association and joint linkage and association tests were carried out using quantitative transmission disequilibrium tests (QTDT), along with post-hoc multivariate tests of linkage and association. Phenotypes tested were bone mineral density (BMD) at the spine, left forearm and left total hip; quantitative ultrasound measurements of the heel including velocity of ultrasound (VOS) and broadband ultrasound attenuation (BUA); and bone turnover markers, urine deoxypyridinoline (DPD), serum osteocalcin, bone specific and total alkaline phosphatase (ALP). BMD and ultrasound variables showed evidence of pleiotropic linkage ($p = 0.05$) and association ($p = 0.02$) with the marker in postmenopausal women. Bone markers showed little or no evidence of linkage and association for any age group. Evidence for pleiotropic linkage appeared to be strongest for BUA and spine BMD in postmenopausal women. The univariate test statistic for BUA was $\chi^2_1=12.8$ ($p = 0.0003$), equivalent to a LOD score of 2.8. DPD showed borderline evidence of linkage to the marker for women of all ages. Multivariate model-fitting showed allele 10 to be negatively associated with BMD, VOS and BUA via a common pathway, suggesting the putative functional polymorphism affects both bone content and structure through shared underlying metabolic pathways. It is likely that the alleles are in linkage disequilibrium with functional polymorphism(s) in or nearby the osteocalcin gene, which may contribute to the onset of osteoporosis.

Keywords: Bone mineral density; Bone turnover; Calcaneal ultrasound; D1S3737 marker genotype; Menopause; Osteocalcin gene

Introduction

Previous twin and family studies have shown that bone mineral density (BMD), broadband ultrasound attenuation (BUA), velocity of sound (VOS) and bone turnover are under strong independent genetic influence [1–3]. BMD is commonly used for diagnosis and to assess the osteoporotic process and risk of fracture. More recently, quantitative ultrasound measurements (QUS) including BUA and VOS have also been shown to be of potential diagnostic value [1]. BMD and QUS are measures of bone content and possibly architecture, respectively. Genetic factors have been shown to account for up to 80% of the variance of BMD, 60% of QUS and 70% for markers of bone turnover.

Osteocalcin is synthesized by osteoblasts and is the most abundant non-collagenous protein in bone. The human osteocalcin gene, also referred to as BGLAP (bone gamma-carboxyglutamate (gla) protein), has been mapped to the long arm of chromosome 1 (1q21-1q23) [4]. It has four exons and codes for a polypeptide

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consisting of a 23 amino acid signal peptide, a 26 amino acid propeptide and a mature protein of 49 amino acids. The three residues of γ-carboxyglutamic acid at positions 17, 21 and 24 are essential for osteocalcin to bind calcium and thereby interact with hydroxyapatite forming the bone mineralized matrix [5]. It is also subject to multiple regulatory controls. For example, the promoter contains specific elements that are thought to interact with different nuclear transcriptional factors, steroid hormones and vitamins resulting in the regulation of gene expression [6]. We therefore consider the osteocalcin gene to be an excellent candidate to study association with osteoporosis and fractures. The D1S3737 marker used for this study is estimated to be between 50kb (http://genome.ucsc.edu) and 180kb [4] from the BGLAP gene.

Despite knowledge of genetic contribution to osteoporosis and fracture, gene location and allelic variation conferring osteoporotic risk are less well characterized in terms of the number of genes involved, effect size and the way they interact with other risk factors. Here we study the osteocalcin gene, which has not been studied as extensively as other candidates for osteoporosis and test for interaction with menopausal status [7]. To date, only a C/T polymorphism at the promoter of the osteocalcin gene has been identified in healthy Japanese women and the T allele shown to be negatively associated in postmenopausal women with BMD at multiple sites [8]. A recent report in Caucasians failed to show any association with BMD and serum osteocalcin levels with this polymorphism [9]. Conflicting results for association studies are not uncommon, as illustrated by vitamin D and estrogen receptor candidate gene studies for osteoporosis [10]. Factors responsible for the conflicting results include small sample sizes, association (or lack of association) due to population sub-structure and confounding factors such as age, menopausal status and genetic heterogeneity.

To test for the gene's possible role in osteoporosis, we genotyped 1366 non-identical (dizygotic or DZ) female twins for the microsatellite marker D1S3737, previously reported to be linked to the osteocalcin gene and associated with BMD at the left hip femoral neck [4]. DZ twins offer the advantages of being matched for age and that family data can be used to test and control for potential confounding due to population stratification and admixture. The risk of non-paternity in twins is also considerably reduced compared to that of siblings, which in turn reduces the possibility of misleading results due to misspecification of family relationships [11].

Materials and Methods

Study Population

The St Thomas' UK adult twin registry is a volunteer registry consisting of over 4000 same-sex twin pairs ranging from 18 to 76 years of age at first interview. Twins were recruited after national media campaigns as previously described [12]. All subjects for this study were healthy dizygotic twin sisters and did not suffer from diseases affecting bone at first interview. Ethics approval was obtained from the hospital ethics committee and fully informed written consent was obtained from all subjects. The twins completed a nurse-administered questionnaire providing details in their lifestyles, medical, drug, and obstetric and gynecological histories. Postmenopausal status was based on recall of menopause and confirmed by serum sex hormone measurements where possible. DNA samples were extracted from EDTA whole blood, drawn from the twins using a commercial kit (Nucleon BACC3, Nucleon Biosciences, Glasgow, UK). Zygosity was determined by questionnaire and in doubtful cases this was confirmed by multiplex DNA fingerprinting.

Measurements

BMD was measured at the lumbar spine (L1–4), left forearm and left hip using dual-energy X-ray absorptiometry on a Hologic QDR-2000 (Hologic, Waltham, MA, USA). Calcaneal ultrasound measurements, BUA and VOS, were measured at the left heel using a McCue Cuba Clinical scanner (McCue Ultrasonics, Hampshire, UK). Biochemical bone markers including early morning fasting urinary deoxypyridinoline (DPD), serum osteocalcin, total alkaline phosphatase (ALP) and bone specific ALP were measured as previously described [3].

Microsatellite marker-based genotyping was undertaken using standard ABI PrismTM (PE Biosystems, Foster City, CA) fluorescence-based genotyping methodologies. Marker loci were amplified in 5μl single-plex polymerase chain reactions (PCRs) in 384-well microtitre plates. Genotypes were generated as part of a large scale, high throughput genome screen of over 3000 individuals with variable numbers of completed genotypes for each marker (Gemini Genomics, unpublished). For marker D1S3737, this resulted in 509 sib-pairs and 348 individual twins being genotyped, with non-genotyped co-twins missing at random. Amplification products were pooled, precipitated, combined with loading buffer, formamide and an internal size standard (GS400HD, PE Biosystems), then size-separated and detected using ABI PrismTM 377 automated sequencers (PE Biosystems). The D1S3737 alleles were sized, identified and coded using methods previously described [13] and were re-coded to correspond with published alleles [4]. Alleles not previously described were designated new codes. Allele size order and frequency were correlated to recode alleles using the 'Allele Gold-Standardisation' function of PhenobaseTM (Gemini Genomics, Cambridge, UK), a proprietary database of clinical and genetic information.

Genetic and Statistical Analysis

Human genetic studies classically utilize identical (monozygotic or MZ) and DZ twins to make indirect genetic and environmental inferences about well-defined phenotypes. Twins, like other relatives, can also be used for linkage and association studies [11]. A commonly used test for linkage and association in family data is the Transmission Disequilibrium Test (TDT) for qualitative traits and this has recently been extended to quantitative traits [14]. The advantage of more recent TDT type tests is that they are able to directly address confounding due to population stratification and the combined effects of linkage and association [15]. We used DZ twins to carry out tests of non-parametric linkage and association and tests for combined linkage and association using the software package, Quantitative TDT or QTDT [16].

Linkage analysis using QTDT is based on allele-sharing probabilities and variance components analysis. Single point identity by descent (IBD) allele sharing probabilities were inferred for sib-pairs using genehunter 2 [17], with an average IBD probability of 0.79 (SD ± 0.11) and 75% of IBDs with a probability of over 70%. If both linkage and association are detected in separate analyses, Fulker et al. [18] also propose testing for linkage while simultaneously modeling association, to provide a test of whether the locus is a functional candidate or whether it is merely in disequilibrium with a trait locus. If linkage is reduced to zero while modeling association, it can be concluded that the locus could be the functional polymorphism, rather than just a locus in disequilibrium with a disease locus. In the case of microsatellite markers, a reduction in linkage would provide further evidence of linkage disequilibrium.

Violations of non-normality and linkage outliers [19] were identified using standard regression diagnostics applied to the revised Haseman and Elston sib-pair analysis [20].

Population data provide a simple and powerful design to test for association between phenotype and allelic markers. Concerns about 'spurious' associations may arise however, in data with unidentified population strata, where subpopulations exhibit different trait prevalences (or means) and frequencies for the locus under study. One-way to address concerns about population stratification is to test for association using within-family controls [14]. The trade-off for conducting tests robust to population stratification however, is a (potentially large) reduction in power to detect allelic association [21]. In light of such power considerations, we conducted tests in the following order for:

1. population stratification
2. multi-allelic total association and
3. allele-wise total association,

where 'total' refers to a single measure of association, which has not been partitioned into between and within family components [18]. For this test, 1366 individuals were available, further increasing power to detect association compared to robust tests in which complete family data are required (509 sib-pairs). If no evidence of confounding due to population stratification was observed, the more powerful test of total association was utilized. Alternatively, if stratification was detected, an orthogonal model robust to population stratification was used.

Multiallelic association included all alleles in one test as a categorical variable with allele frequencies of $<5\%$ being pooled. For allele-wise tests of association, a standard biometric additive model was implemented, in which the phenotype was repeatedly regressed on each allele type, with genotypes coded as 1, 0 and -1. For bi-allelic loci, these categories would represent AA, Aa and aa individuals, respectively, but for microsatellite markers, the -1 category represents the remaining pooled alleles. To alleviate the problem of multiple testing and to prevent reports of association based on very few individuals, allele-wise association tests were conducted for only seven out of the 17 marker alleles with frequencies of $\geqslant 5\%$.

We also conducted multivariate analyses of linkage using methods described in detail elsewhere [22] and multivariate allelic association [21] using Mx software, to test for pleiotropic effects and whether identified risk-conferring alleles affected phenotypes through the same or different pathways. MZ twins not genotyped for the marker D1S3737 (MZ pairs=590), were also included in the model to increase the power to resolve between QTL and polygenic effects, by further decomposing the shared familial component into additive polygenic and shared environmental components using a classic twin design [7]. Pleiotropic effects were tested for, by comparing the fit with all QTL effects included (full model), to that of a submodel with all QTL effects removed. The difference between model fit-statistics are asymptotically χ^2 with degrees of freedom equal to the difference in the number of parameters fitted. The model assumes a multivariate normal (MVN) distribution between variables. This was tested for using Hadi's method implemented in the statistical package Stata version 7.0. A total of 5 MVN outliers were identified and removed before multivariate analysis.

All tests in the study considered menopausal status either as a covariate incorporated in a regression analysis [16] or by analyzing postmenopausal women alone [7]. We hypothesized that the osteocalcin gene might have a differential effect on pre- and post-menopausal women. Age was controlled for in the matched design of robust association tests and was included as a covariate in total tests of association. Weight was not included as a covariate, since weight has a genetic component to its etiology (with a heritability estimate from these data of 75%; 95% CI 69–81%), which overlaps with the genetic variation of BMD and ultrasound measures. The genetic correlations between weight and total spine BMD and BUA, for example, are 0.28 (95% CI 0.26–0.34) and 0.29 (95% CI 0.24–0.37), respectively (data not shown).

Significance Levels

The study involved tests of linkage and association to a single marker rather than a genome-wide scan. Nevertheless, having demonstrated a multi-allelic (or locus) association between the marker and phenotype, we then attempted to identify specific associated allele(s), which resulted in multiple testing. We therefore calculated the effective number of independent tests performed, by accounting for the correlation between bone measures using a simple regression based method [23]. As a guideline, this established a threshold significance level that accounted for multiple testing to be $p<=0.001$. Multiple testing is a problem that afflicts linkage and association studies and here we follow the practice of making explicit all tests conducted, rather than only present significant results found [24].

Results

Study Population

Basic demographic data, bone measurements and laboratory results for the 1366 study subjects are summarized in Table 1. The numbers of pre- and postmenopausal women were approximately equal. The weight, height, body mass index (BMI) and smoking habit did not differ, but as expected, age, BUA, VOS, BMD at the three sites, DPD, osteocalcin, total and bone ALP differed by menopausal status.

Table 2 shows the allelic frequencies of D1S3737. There are 17 alleles with allele 10 (repeat number equal to 15) being the most common at 26.0%. This is followed by allele 7 (20.6%), 4 (15.8%), 5 (12.0%), 3 (10.0%), 6 (6.2%) and 13 (5.7%). Major allelic frequencies are similar to those previously reported [4].

Table 2. D1S3737 allelic frequencies for the study population of female Caucasian siblings (n=1366)

Allele	Repeat no.	Frequency (%)
1	24	0.2
2	23	0.4
3	22	10.0
4	21	15.8
5	20	12.0
6	19	6.2
7	18	20.6
8	17	0.7
9	16	1.4
10	15	26.0
11	14	0.2
12	13	0.3
13	12	0.1
16	9	0.4
17	8	0.0
18	7	5.7
19	6	0.0
Total		100

Linkage

Table 3 shows the results for tests of linkage, association and combined linkage and association for the whole group with menopausal status included as a covariate. For these univariate analyses, BUA and DPD showed limited evidence of linkage to D1S3737 for the whole group, but bone density variables did not. Linkage is indicated by the χ^2 statistic being significantly greater than zero. For BUA, $\chi^2 = 1.16$ ($p = 0.28$) and for DPD, $\chi^2 = 4.26$ ($p = 0.04$).

In tests using only postmenopausal women, evidence for linkage to the marker did not appear to alter for any of the variables, apart from BUA. When BUA was tested

Table 1. Characteristics of the study group. Values are means with standard deviation in brackets. The sum of subject numbers of the pre- and postmenopausal groups is less than the total number in the whole group, since the menopausal status for some women (3%) was unknown

	Whole group (n=1366)	Premenopausal group (n=691)	Postmenopausal group (n=630)
Age, years	47.3 (11.2)	39.1 (8.4)	56.0 (6.7) ***
Weight, kg	64.3 (15.8)	63.6 (16.2)	65.0 (15.4)
Height, m	1.58 (0.25)	1.59 (0.25)	1.58 (0.25)
BMI	25.1 (4.6)	24.6 (4.7)	25.4 (4.3)
% Ever smoked	47.9	46.3	49.5
Spine BMD, g/cm^2	1.007 (0.147)	1.027 (0.129)	0.961 (0.159) ***
Left forearm BMD, g/cm^2	0.558 (0.057)	0.571 (0.046)	0.542 (0.064) ***
Left hip BMD, g/cm^2	0.926 (0.127)	0.943 (0.130)	0.881 (0.132) ***
BUA, dB/MHz	76.0 (18.4)	79.0 (17.8)	73.5 (18.4) ***
VOS, m/s	1660 (51.1)	1675 (46.2)	1644 (51.8) ***
DPD, nmol/mmol creatinine	5.35 (2.30)	5.54 (2.66)	5.10 (1.67) *
Osteocalcin, ng/ml	9.19 (3.51)	8.64 (2.87)	9.84 (4.05) ***
Bone specific ALP, U/l	19.6 (16.7)	14.3 (7.0)	25.8 (21.8) ***
Total ALP, U/l	68.9 (19.0)	64.1 (16.1)	74.3 (20.6) ***

* $p<0.05$; *** $p<0.001$ comparing between the pre- and postmenopausal groups.

Table 3. QTDT results for whole group: Bone mineral density (BMD) for total lumbar spine, left forearm and left total hip; heel ultrasound measurements including velocity of sound (VOS) and broadband ultrasound attenuation (BUA), and the bone turnover markers deoxypyridinoline (DPD), osteocalcin, bone specific and total alkaline phosphatase (ALP). Tests for linkage, association and joint linkage and association include menopausal status (MPS) as a covariate. Additional tests for linkage in postmenopausal women for BUA and DPD yielded significant results for BUA, but not DPD (see text). Results are for the whole group, except R^2 (and associated p values) for bone density and structure variables, which are for postmenopausal women only. Allele-wise association tests were carried out for alleles with frequencies >5% (i.e. alleles 3, 4, 5, 6, 7, 10 and 18); results are presented if the p value =<0.10. The microsatellite marker D1S3737 was estimated to have a heterozygosity of 83%

Phenotype	Linkage with MPS covariate				Association, robust to PS with MPS covariate						Linkage and association with MPS covariate	
					Multi-allelic			Allele-wise				
	n pairs	V_p	χ^2_1	p	n	R^2	p	Allele	β	p	χ^2_1	p
Bone density and structure												
Spine BMD, g cm^2	509	0.0217	0.00	1	1362	4.1%	0.03	10	−0.01	0.04	0.00	1
Left forearm BMD, g/cm^2	503	0.0032	0.00	1	1357	2.4%	0.36	10	−0.01	0.10	0.00	1
Left hip BMD, g/cm^2	509	0.0161	0.00	1	1360	4.3%	0.02	10	−0.01	0.04	0.00	1
VOS, m s	490	2614	0.00	1	1325	6.4%	0.003	10	−3.79	0.10	0.00	1
BUA, dB/MHz	490	338	1.16	0.28	1324	5.2%	0.004	10	−1.66	0.04	1.19	0.28
Bone marker turnover												
DPD, nmol/l	306	3.02	4.26	0.04	768	2.7%	0.09				3.50	0.06
Osteocalcin, µg/l	280	13.3	0.06	0.81	705	1.9%	0.34				0.12	0.73
Specific ALP, U/l	257	0.98	0.59	0.44	667	2.9%	0.14				0.51	0.48
Total ALP, U l	464	369.7	0.46	0.50	1244	1.5%	0.16				0.38	0.54

Abbreviations: V_p, total phenotypic variance; R^2, percentage of total phenotypic variance explained by the marker; β, regression coefficient effect size measured in specified units; PS, population stratification.

for linkage to D1S3737 in postmenopausal women (DZ pairs = 195), the linkage test statistic increased to χ^2_1 = 6.9 (p = 0.009). Evidence for BUA linkage to the marker increased further when 13 pairs of identified linkage outliers [20] were removed from the analysis (DZ pairs = 182), with χ^2_1 = 12.8 (p = 0.0003) or a lod score of 2.8. Although only suggestive for a genome-wide scan, this result is highly significant for a point-wise test at p<=0.001.

By contrast, evidence of DPD linkage to the marker for women of all ages disappeared when 27 identified outlying sib-pairs were removed from the analysis. With outliers removed (DZ pairs = 482), the test statistic became insignificant with χ^2_1 = 0.59 (p = 0.44).

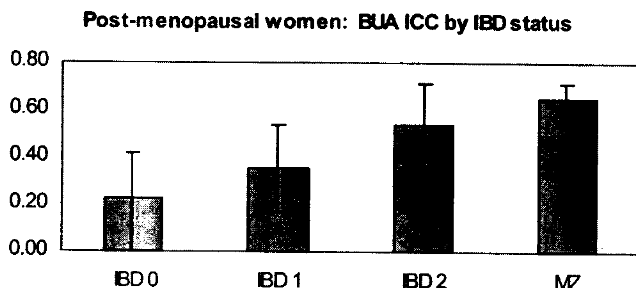

Fig. 1. Intraclass correlation (ICC) of DZ twins by category of identity by descent (IBD) and MZ twins for Broadband ultrasound attenuation (BUA) in postmenopausal women. The small confidence interval for MZ twins reflect the larger MZ sample sizes for BUA (MZ pairs=516) compared to the total number of genotyped DZ twin pairs (490 pairs). MZ data are from the St Thomas' UK adult twin volunteer database [12].

Figure 1 illustrates the linkage detected for BUA in postmenopausal women. Results with MZ twins from our previous studies [12] are included in the histogram for comparative purposes, since these twins share loci that are identical by descent for genotypes across the entire genome (i.e. genome-wide IBD2). The higher intraclass correlation for DPD in MZ twins reflects that genes in addition to osteocalcin are involved in the etiology of the phenotype.

Multivariate Linkage Analysis

Table 4 presents the results for multivariate linkage. Two analyses were conducted, using postmenopausal women for bone content and ultrasound variables and women of all ages for bone marker variables.

In a 5df linkage test for BMD at the spine, left forearm and hip and VOS and BUA in postmenopausal women, there was marginal evidence of pleiotropy at p = 0.13 (including all data) and p = 0.05 with 13 outlying sib-pairs removed from the analysis. The variables showing the strongest pleiotropic relationship with the osteocalcin gene were spine BMD (p = 0.02) and BUA (p = 0.001), followed by BMD at the left forearm and VOS (both p = 0.09).

The bone marker variables DPD, serum osteocalcin and specific and total ALP showed little evidence of pleiotropy. The test statistic using all data declined from χ^2_4 = 6.8 (p = 0.15) to χ^2_4 = 3.2 (p = 0.52) with 27 outliers removed. Neither test was statistically significant.

Table 4. Multivariate tests of linkage for (a) BMD total lumbar spine, forearm and left total hip; heel ultrasound measurements VOS and BUA in postmenopausal women and (b) bone turnover markers DPD, osteocalcin, bone specific and total ALP for women of all ages. Results are presented for all data (super column 1) and data with outliers removed (super column 2). Outliers were identified using standard regression diagnostics applied to the revised Haseman and Elston [20]. The full model used was a Cholesky decomposition with the same number of additive polygenic and unique environmental variance components as there were measured variables [22]. The full model also included a QTL variance component with (a) 5 factor loadings and (b) 4 factor loadings. The test for pleiotropy involved dropping all QTL effects (a 5 df and 4 df test, respectively). Tests for each specific QTL path were only conducted if an overall effect was detected, to confirm that more than one variable was involved in the pleiotropic effect

	All data				Outliers removed			
	χ^2	df	p	n	χ^2	df	p	n
(a) Postmenopausal women, BMD ultrasound								
Full 5 QTL	8.4	5	0.13	195 (516)	11.0	5	0.05	182 (516)
Full – QTL_{Spine}	4.9	1	0.03		5.5	1	0.02	
Full – $QTL_{Forearm}$	3.6	1	0.06		2.9	1	0.09	
Full – QTL_{Hip}	1.6	1	0.20		1.0	1	0.33	
Full – QTL_{Vos}	2.8	1	0.10		3.0	1	0.09	
Full – QTL_{BUA}	7.9	1	0.005		10.1	1	0.001	
(b) All women, bone markers								
Full – 4 QTL	6.8	4	0.15	509 (602)	3.2	4	0.52	482 (602)

Abbreviations: df, degrees of freedom; n, number of DZ (MZ) pairs used in the test.

Association

No evidence for confounding due to population stratification (at $p \leqslant 0.05$) was found for any tests of association and therefore stratification tests are not presented. Two types of total association tests were conducted, multi-allelic and allele-wise (Table 3, super column 2). Multi-allelic tests showed that for bone density and ultrasound variables, a significant proportion of phenotypic variance was attributable to D1S3737 ranging between 1–2% for the whole group and 2–6% in post-menopausal women (Table 3, R^2). All multi-allelic associations were significant for these variables, except for BMD at the left forearm. None of the multi-allelic tests for bone turnover markers were significant, for either the whole group or postmenopausal women.

In allele-wise tests of association, BMD at the spine and hip and BUA showed significant association with allele 10 for the whole group. This reflected a significant underlying negative association in postmenopausal women between allele 10 and all the BMD and QUS variables, with allele 10 accounting for a significant proportion of all BMD and QUS variances for this group of women (1–2%).

Table 5 presents association results between D1S3737 allele 10 and BMD and QUS by menopausal status. No allelic association was observed for premenopausal women for any of the phenotypes. Association was observed in postmenopausal women for all BMD and QUS phenotypes ($p \leqslant 0.05$). Corrected for multiple testing, only BMD at the spine was statistically significant at the nominal threshold of $p \leqslant 0.001$.

For every additional copy of allele 10, the additive genetic effect on the phenotypic means for BMD at the spine, forearm and hip, VOS and BUA in postmenopausal women were respectively (mean ± SD (%)): -0.037 g/cm^2 ± 0.01 (−4%); -0.0085 g/cm^2 ± 0.004 (−1.6%); -0.026 g/cm^2 ± 0.008 (−3%): -7.07 m/s ± 3.39 (−0.5%); -3.02 dB/MHz = 1.2 (−4%). The phenotypic means for each genotype category also suggest that allele 10 might behave in a (partially) recessive manner, with homozygous postmenopausal women having consistently lower BMD and QUS means compared to the other two genotypes (Table 5). However, univariate tests for recessive effects only yielded statistically significant results for BUA ($\beta = 4.04$, $p = 0.025$).

Association has previously been demonstrated between D1S3737 and BMD at the left femoral neck in postmenopausal women [4]. In addition to total BMD at the left hip, we also had BMD measured at the left femoral neck available for this study (data not presented). We found some evidence in support of the reported positive association between allele 6 and BMD at the left femoral neck in postmenopausal women (for our data $\beta = 0.029$, $p = 0.06$), but not for the reported (marginal) result of negative association with allele 7 (for our data $\beta = 0.003$, $p = 0.70$). However, the strongest statistical association observed in our data for BMD at the left femoral neck in postmenopausal women was with allele 10 ($\beta = -0.021$, $p = 0.007$), and this was not observed by Raymond et al. [4].

Table 5. Regression analyses for allele 10 of D1S3737 with bone mineral density (BMD) at three sites and heel ultrasound measurements velocity of sound (VOS) and broadband ultrasound attenuation (BUA) for the whole group, pre- and postmenopausal women. p values were obtained using an additive biometrics model with genotypes x/x, x/10 and 10/10 parametized as −1, 0 and +1, respectively. The percentage comparisons presented in the column headed 10/10 vs x/x are made between individuals homozygous for allele 10 and individuals with no copies of the allele. Hence these are larger than the estimated additive effects presented in the text

Genotype group	x/x			x/10			10/10			10/10 vs x/x Difference	Additive test, p value
	No.	Mean	SD	No.	Mean	SD	No.	Mean	SD		
Whole group											
Spine BMD, g/cm^2	758	1.015 ±	0.15	501	1.002 ±	0.14	103	0.980 ±	0.15	−3.52%	0.02
Left forearm BMD, g/cm^2	755	0.560 ±	0.06	500	0.557 ±	0.05	102	0.551 ±	0.06	−1.66%	0.13
Left hip BMD, g/cm^2	758	0.932 ±	0.13	499	0.916 ±	0.12	103	0.920 ±	0.14	−1.32%	0.07
VOS, m/sec	738	1661 ±	50.8	490	1661 ±	51.9	97	1650 ±	49.5	−0.66%	0.19
BUA, dB/MHz	738	76.4 ±	18.2	489	76.2 ±	18.9	97	72.2 ±	16.6	−5.59%	0.12
Premenopausal group											
Spine BMD, g/cm^2	369	1.049 ±	0.12	254	1.036 ±	0.12	55	1.052 ±	0.12	0.29%	0.63
Left forearm BMD, g/cm^2	366	0.574 ±	0.04	254	0.571 ±	0.04	55	0.572 ±	0.04	−0.24%	0.59
Left hip BMD, g/cm^2	369	0.965 ±	0.12	253	0.950 ±	0.11	55	0.980 ±	0.12	1.59%	0.84
VOS, m/s	357	1674 ±	44.9	250	1676 ±	47.3	51	1666 ±	45.6	−0.42%	0.83
BUA, dB/MHz	357	79.2 ±	18.1	250	78.6 ±	18.3	51	78.9 ±	16.0	−0.41%	0.78
Postmenopausal group											
Spine BMD, g/cm^2	351	0.987 ±	0.17	228	0.956 ±	0.15	46	0.899 ±	0.16	−8.83%	0.0003
Left forearm BMD, g/cm^2	351	0.546 ±	0.07	227	0.540 ±	0.06	45	0.526 ±	0.07	−3.79%	0.049
Left hip BMD, g/cm^2	351	0.903 ±	0.13	227	0.875 ±	0.11	46	0.849 ±	0.13	−5.89%	0.001
VOS, m/s	344	1647 ±	53.9	221	1642 ±	51.7	44	1629 ±	43.9	−1.08%	0.04
BUA, dB/MHz	344	74.5 ±	17.8	220	73.5 ±	19.7	44	64.8 ±	14.1	−13.04%	0.009

Table 6. Correlations (lower diagonal) and significance values (upper diagonal) for bone mineral density (BMD) total lumbar spine, left forearm and left total hip; velocity of sound (VOS) and broadband ultrasound attenuation (BUA), and the bone turnover markers deoxypyridinoline (DPD), serum osteocalcin, bone specific and total alkaline phosphatase (ALP). Shading indicates the higher correlations between BMD/QUS variables and lower between bone turnover markers

	Spine	LF	Left hip	VOS	BUA	DPD	Osteocalcin	ALP_{SP}	ALP_{TOTAL}
Spine	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.000
Left Forearm	0.6	1	0.00	0.00	0.00	0.05	0.00	0.00	0.000
Left hip	0.70	0.64	1	0.00	0.00	0.03	0.00	0.00	0.000
VOS	0.35	0.31	0.33	1	0.00	0.43	0.05	0.00	0.000
BUA	0.47	0.42	0.47	0.52	1	0.66	0.00	0.43	0.000
DPD	−0.14	−0.07	−0.08	0.03	−0.02	1	0.00	0.00	0.002
Osteocalcin	−0.26	−0.18	−0.23	−0.08	−0.15	0.15	1	0.00	0.002
ALP_{SP}	−0.23	−0.22	−0.21	−0.30	0.03	0.13	0.19	1	0.002
ALP_{TOTAL}	−0.17	−0.17	−0.17	−0.17	−0.12	0.22	0.15	0.20	1

Multivariate Analysis of Allelic Association

Table 6 demonstrates the multivariate character of the data. Loosely, the relationships were highest between BMD and QUS variables, followed by those between BMD/QUS and bone turnover and between the bone turnover variables themselves. The negative association between bone turnover and bone density indicates that markers for high bone turnover were (weakly) related to low bone density and structure.

Given the association between bone density and ultrasound variables and allele 10 of the D1S3737 marker, we tested whether the functional polymorphism influenced these measures via the same or different pathways using structural equation modeling including age as a covariate [21].

Figure 2a and b illustrate the two alternate hypotheses. Ignoring genotypic effects (the triangular variables in the diagram), Fig. 2 depicts the general model - a five variable phenotypic factor analysis, with age regressed upon the latent factor. Only one latent factor was retained for the analysis, since additional latent factors explained negligible proportions of the variance. This reflects that BMD and QUS both measure unitary aspects of underlying bone strength. However, as might be anticipated from inspecting Table 6, BUA and VOS did not load as strongly on the underlying factor as compared to the BMD measures (0.60 and 0.49

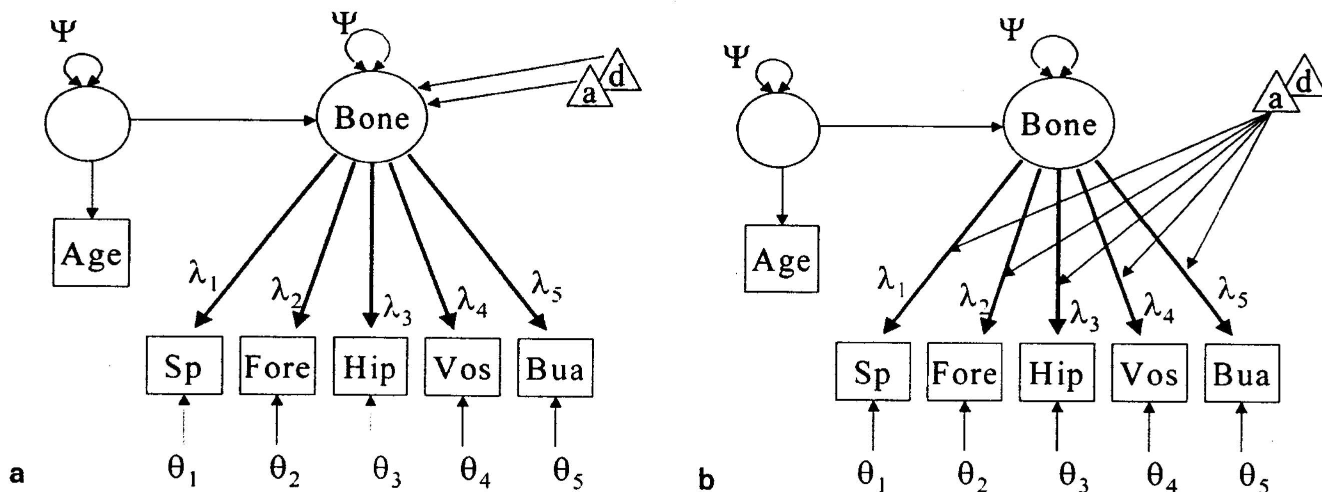

Fig. 2. Multivariate tests of total association for allele 10 with bone density and QUS variables in postmenopausal women. **a** Common path means model; **b** different path means model. Squares represent measured phenotypes; circles: latent variables; triangles: allelic effects. The additive genetic term is a measure of phenotypic mean deviation from the latent variable intercept, while the dominance term is a measure of heterozygote deviation from the mid-point of the additive model. Abbreviations: a, additive genetic allelic affect (coded −1, 0, 1 for genotypes x/x, x/10 and 10/10, respectively); d, dominant genetic allelic effect (coded 0, 1, 0 respectively); λ, factor path loading; θ, measurement error; Ψ, latent variable variance, set to equal 1 in the model; C, covariance between variables for relatives (not shown in diagram). Mean intercepts for the measured and latent variables are also not depicted.

respectively, compared to 0.8 for all three BMD body sites). VOS also showed more measurement error than any of the other variables (0.70 compared to an average of 0.43 for the remaining variables).

The additive and dominant allelic effects in the common path model (Fig. 2a), impact upon the latent construct of bone strength, rather than any of the five individual measures of bone strength. For the different paths model (Fig. 2b), allelic affects were considered directly for each measured variable, with deviation terms for each intercept and factor loading. The general model is a submodel of the common and different path models, so the two types of genetic model can be compared by way of the general model [21].

Results for multivariate association are presented in Table 7. The best fitting model was the common path model including additive genetic effects without dominance ($\chi^2_1 = 5.9$, $p = 0.02$). The different path models showed some evidence for the functional polymorphism acting in a recessive manner for the main effects ($p = 0.08$) and interaction terms ($p = 0.03$), but these models did not fit as well as the equivalent common path models (see Table 7 legend and Different versus Common result, $p = 0.41$). By contrast, the common path model showed little evidence for overall recessive effects (Common model 3 vs Common model 1, $\Delta\chi^2_1 = 1.8$, $p = 0.18$).

Discussion

Evidence for pleiotropic linkage and association with marker D1S3737 in postmenopausal women has been presented for BMD and ultrasound variables. Bone turnover marker variables did not appear to show reliable linkage or association with the genetic marker. Multivariate linkage in postmenopausal women ($p = 0.05$),

Table 7. Multivariate tests of association comparing allelic effects in different and common pathway models. Four allelic submodels were tested: (1) main additive genetic effect(s) (intercept only); (2) main additive effect + additive regression coefficient (i.e. genetic interaction with factor loadings in the different path model); (3) main additive and dominant effects; and (4) main additive and dominant effects + additive and dominant regression coefficients (different path model only). The general model is a submodel of the genetic effects models, so a decision can be made as to which model fits the data better, by taking the difference in fit function between the two models of interest and equating this to an asymptotically χ^2 distribution with degrees of freedom equal to the difference in the number of fitted parameters. The best fitting model is printed in bold with an asterisk

Model	Different			Common			Different vs common		
	χ^2	df	p	χ^2	df	p	$\Delta\chi^2$	Δdf	p
1 Main: (a)	7.5	5	0.18	5.9	1	**0.015***	1.6	4	0.81
2 Main: (a) + b(a)	15.7	10	0.11						
3 Main:(a & d)	17	10	0.08	7.7	2	0.015	8.2	8	0.41
4 Main: (a&d) + b(a & d)	34	20	0.03						

suggested that QTL pleiotropic linkage effects were strongest for BUA (p = 0.001) and spine BMD (p = 0.02). Multivariate association showed the functional polymorphism(s) to have a similar additive genetic effect upon all five measures of BMD and ultrasound via a common pathway (p = 0.02). Two key results, BUA linkage ($p = < 0.001$) and spine BMD allelic association (p = 0.0003). both showed significance at $p <= 0.001$, with all univariate tests of association and pleiotropy for BMD and ultrasound measures significant at $p <= 0.05$. The reason why univariate linkage tests failed to show evidence for all BMD and QUS variables is likely to be due to insufficient power. This is partly supported by evidence in these data for pleiotropic linkage (Table 4). There is also evidence from other studies for linkage to the same region of chromosome one (1q21-23) for normal variation in BMD in young [25] and postmenopausal Caucasian women [4].

Linkage studies for complex traits are notoriously underpowered to detect genes of small effect [26]. Power to detect linkage in small samples is negligible if the recombination fraction between the marker and gene is greater than zero or the gene effect size accounts for less than 10% of phenotypic variation. For this study, power calculations showed that, for example, we only had 5% power to detect linkage to a gene accounting for 10% of phenotypic variation with a recombination fraction of zero, a false positive error rate of $\alpha = 0.001$ and a sample size of 509 sib-pairs. However, things improved somewhat if the gene effect size was larger than this or if evidence for linkage was considered at a higher false positive error rate (say, $0.001 < \alpha < 0.10$). For example, if a QTL effect size for our data was large ($\geqslant 15\%$) and the recombination fraction equaled zero, our power to detect the QTL would range between 60 and 90% (for Type I error rates of 0.001 to 0.10).

Unlike linkage, power to detect association for this study was high at $p <= 0.05$. Assuming a QTL effect size explained 10% of the phenotypic variation, power to detect association remained at 90% or more for the majority of power calculations we considered, although power reduces dramatically if marker:QTL allele frequencies are not closely matched. Although the functional polymorphism is close to D1S3737 in genetic terms, the marker is still estimated to be about 50–180kb from the osteocalcin gene, which is consistent with our observation that linkage estimated in the presence of association did not decline compared to linkage alone [15].

Allele 10 is the most frequent allele of the D1S3737 marker, with a frequency of about 26%, and the homozygous group accounting for almost 7% of all genotypes in women. In the postmenopausal group. 46 out of 630 (7.3%) were homozygous for allele 10 and had significantly lower BMD and QUS phenotypes than women carrying none or one copy of allele 10. Discovery of the functional polymorphism associated with this marker should provide some useful insights into the etiology of osteoporosis.

Study limitations include that only one genetic marker was available for analysis. For the Gemini Genomics genome scan, the nearest neighboring markers flanking the osteocalcin gene were more than 10cM away and unlikely to exhibit significant linkage disequilibrium with the candidate marker. A fine map of the region is required to further locate functional polymorphisms. Osteoporosis is currently defined as a disease of low bone mass and increased fracture risk. Although bone density, structure and turnover used in this study are known to be major determinants of fracture-risk, fracture information on sufficient numbers of subjects was not available at the time of study.

Finally, the generalizability of results from twin studies to singleton populations are sometimes questioned, in part due to the unusual birth history of twins. Twin study design assumptions need to be empirically tested if results are to be considered unbiased and representative of singleton populations. To this end we have previously demonstrated that twins are the same for these data as age-matched singletons for levels and variation of bone density, osteoarthritis, blood pressure, height, menopausal status and other relevant lifestyle factors such as alcohol consumption and overall tobacco consumption [12].

This study confirms previous reports of linkage and association to marker D1S3737 [4] and that the BGLAP gene does not appear to be associated with serum osteocalcin levels [9]. In addition, we show that a polymorphism(s) in or nearby the osteocalcin gene acts pleiotropically upon BMD and QUS variables.

In conclusion, this study, as well as demonstrating the potential importance of the osteocalcin gene, illustrates the importance of menopausal status in detecting linkage and association for bone measurements. In postmenopausal women, bone density and structure variables showed evidence of linkage and association with marker D1S3737, while for bone turnover markers, this was not the case for any age group. In these data, linkage to D1S3737 was reliably observed for BUA in postmenopausal women with evidence of pleiotropic linkage for all bone content and structure variables. The D1S3737 marker was also pleiotropically associated with BMD at the spine, left forearm and left femoral neck, BUA, VOS in postmenopausal women, but again. for bone turnover markers there appeared to be no relationship. Allele 10 is likely to be in linkage disequilibrium with a functional polymorphism in, or nearby, the osteocalcin gene that plays a wide-ranging role in determining bone content, matrix and structure, especially after the menopause. Further studies to screen the osteocalcin gene to identify these functional polymorphisms are warranted.

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