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**Linkage disequilibrium between polymorphisms in the human
TNFRSF1B gene and their association with bone mass in
perimenopausal women**

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Abstract

Osteoporosis is a multifactorial disease with a strong genetic component characterised by reduced bone density and increased fracture risk. A candidate locus for regulation of hip bone mineral density (BMD) has been identified on chromosome 1p36 by linkage analysis. One of the positional and functional candidate genes located within this region is the tumour necrosis factor receptor superfamily member 1B (*TNFRSF1B*). In order to investigate whether allelic variation in *TNFRSF1B* contributes to regulation of bone mass, we studied several polymorphisms of this gene in a population based cohort study of 1240 perimenopausal women from the UK. We studied a T676G change in exon 6 (196:Met-Arg) and three SNPs (G593A, T598G, and T620C) in the 3'UTR of the gene. The 3'UTR SNPs were in strong linkage disequilibrium (LD) with each other ($p < 0.00001$), and the exon 6 SNP was in LD with G593A and T598G ($p < 0.00001$). We found no association between T676G alleles and BMD at the spine or hip. However, haplotype analysis showed that subjects homozygous for the A593-T598-C620 haplotype ($n=85$) had femoral neck BMD values 5.7% lower than those who did not carry the haplotype ($n=1155$; $p < 0.00008$) and this remained significant after correcting for confounding factors and multiple testing ($p < 0.0009$). Regression analysis showed that the ATC haplotype accounted for 1.2% of the population variance in hip BMD and was the second strongest predictor after body weight. In summary, our work supports the view that allelic variation in the 3'UTR of *TNFRSF1B* gene contributes to the genetic regulation of bone mass, with effects that are specific for femoral neck BMD.

Introduction.

Osteoporosis is a common disease with a strong genetic component characterised by low bone mineral density (BMD), microarchitectural deterioration of bone tissue and an increased risk of fracture after minimal trauma (1). Twin and family studies have shown that peak bone mass is under strong genetic control with estimates of heritability ranging from 50% for wrist BMD to 85% for spine and hip BMD in various studies (2-4). Segregation analysis has shown that BMD is a complex phenotype that is regulated by interplay between environmental factors and several genes each with modest effects on bone mass and bone turnover (2). Several candidate genes have been studied in relation to BMD, include the vitamin D receptor (*VDR*) (5), the oestrogen receptor (6,7), the *COL1A1* gene (8), and the *TGF- β 1* gene (9), however, current evidence suggests that allelic variants of these genes accounts for only a small portion of the population variance in BMD (10). Linkage studies in man have identified quantitative trait loci on chromosomes 1p36, 2p23-p24, 4q32-34, 5q33-35, 6p11-12 and 11q12-13 that show probable or definite linkage to BMD in families (11-14).

One of the candidate loci for regulation of BMD in man is on chromosome 1p36. This was initially identified by linkage analysis in sib-pairs (11) and it's candidacy was subsequently confirmed by variance components linkage analysis in a separate set of families (15). The tumour necrosis factor receptor superfamily member 1 B (*TNFRSF1B*) is a strong positional and functional candidate gene within this region (16). The *TNFRSF1B* gene encodes the 75Kd tumour necrosis factor receptor, which is highly expressed in osteoclast precursors and plays an important role in mediating the effects of TNF on osteoclastogenesis (17). In a previous study, evidence of an allelic association between a specific haplotype of the 3' untranslated region (UTR) of *TNFRSF1B* and spine BMD was observed but the study population was small and the finding has not yet been confirmed in other populations (18). In order to further investigate the candidacy of *TNFRSF1B* as a genetic determinant of BMD we have now examined the relationship between four common polymorphisms of *TNFRSF1B* gene and BMD in a population based sample of 1240 healthy perimenopausal women from the UK. The polymorphisms studied have previously been described in the *TNFRSF1B* gene and were chosen because of their potential effects on receptor function. One is a T to G substitution within exon 6 (T676G) which results in an

amino acid substitution (Met→Arg) at codon 196, whereas the other three polymorphisms are situated close to each other in the 3'UTR of the mRNA. Previous studies have shown evidence of linkage and association between the T676G coding polymorphism and phenotypes relevant to cardiovascular disease such as blood pressure and HDL levels (19). This polymorphism has also been associated with rheumatoid arthritis (20) and systemic lupus erythematosus (21). The 3'UTR polymorphisms have been reported to be associated with obesity (22) and Crohn's disease (23).

Results

The average age of the study subjects was (mean \pm SEM) 47.60 ± 0.04 years. Approximately 76% of the women were premenopausal and the average time elapsed since menopause was 5.01 ± 0.29 years in the postmenopausal group. 159 (12.8%) women were taking hormone replacement therapy (HRT) and 102 (8.2%) have had hysterectomy.

Genotype frequencies for the *TNFRSF1B* polymorphisms were in Hardy-Weinberg equilibrium and were as follows: T676G (TT = 50.9%, TG = 42.9%, GG = 6.7%); G593A (AG = 48.6%, GG = 28.1%, AA = 23.3%); T598G (TT = 90.3%, GT = 9.7%, GG = 0%); T620C (TC = 47.9%, TT = 38.8%, CC = 13.3%). Allele frequencies for these polymorphisms were similar to those previously reported in Caucasians (19,24,25). Linkage disequilibrium (D') values between the polymorphisms at the *TNFRSF1B* locus are shown in Table 1. Strong and highly significant linkage disequilibrium (LD) was observed between the three polymorphisms located in the 3'UTR. The polymorphism T676G located in exon 6 showed LD only with G593A and T598G.

The relationship between genotype and BMD values at the lumbar spine and femoral neck adjusted for age, height, weight, menopausal status, and HRT use are shown in Table 2. There was a significant association ($p = 0.02$) between G593A genotypes and femoral neck BMD although this was primarily driven by lower BMD values in A/A homozygotes. Trends for association were also observed with T598G ($p = 0.09$) and T620C ($p = 0.05$). There was no significant association between any of the polymorphisms and lumbar spine BMD.

Since strong linkage disequilibrium was observed between the 3'UTR polymorphisms, we investigated the relationship between BMD and genotype groups defined by the combination of the three 3'UTR polymorphisms. The genotypes for each polymorphism were coded as 1, 2 or 3 corresponding to homozygote wild type, heterozygote or homozygote variant, respectively (i.e. the genotype 121 corresponds to 593G/G-598T/G-620T/T). Thirteen different 3'UTR genotype combination were observed in our population and their frequencies are shown in Figure 1. The relationship between 3'UTR genotype combination and femoral neck BMD values, adjusted for age, height, weight, menopausal status, and duration of HRT use, are shown in Figure 1. Similar analysis results for lumbar spine were not statistically significant (data not shown). Individuals bearing the genotype 313 (corresponds to 593A/A-598T/T-620C/C) had significantly lower adjusted femoral neck BMD values (least square mean \pm SD; 0.843 ± 0.012 ; $n = 85$) than those without this genotype (0.894 ± 0.003 ; $n = 1155$; $p = 0.00008$). The results remained statistically significant after correction for multiple testing ($p = 0.0009$). A similar but non-significant trend was observed for lumbar spine BMD (1.044 ± 0.016 vs. 1.065 ± 0.004 ; $p = 0.20$).

The genotype 313 contains two copies of the ATC haplotype. In view of this, the relationship between this haplotype and BMD was investigated. Individuals with the genotypes 322, 222, 221 and 212 were excluded from the analysis because the haplotype phase could not be inferred from the DNA sequencing results. In the resulting cohort of 821, subjects were coded according to whether they had 2 copies [1,1], one copy [1,0] or no copies [0,0] of the ATC haplotype. These haplotype groups were then investigated in relation to BMD and other variables. We analysed the data using a multiple linear regression model in which the haplotype copy number was entered in the regression model, as were other predictors of BMD such as age, weight, height, menopausal status, and HRT use. Results for LS and FN BMD are shown in Table 3. The model shown in Table 3 explained 13.3% and 17.3% of the total observed variance in LS and FN BMD, respectively. Body weight, height and years since menopause (YSM) were the most significant independent predictors of LS BMD. The ATC haplotype copy number was not a significant determinant of LS BMD but it was the second most significant independent predictor for FN BMD ($p = 0.001$) after body weight ($p < 0.001$). Height and YSM were also significant independent predictors for FN BMD. However, Body weight accounted for the

majority of the total observed variance in LS (~12%) and FN (~15%) BMD. The ATC haplotype accounted for 1.2% of the total observed variance in FN BMD and was the second strongest predictor. Analysis of the relationship between ATC haplotype and BMD by general linear model ANOVA showed that adjusted FN BMD values were significantly lower in those with two copies of the ATC haplotype (0.843 ± 0.012 ; $n = 85$) than those with one copy (0.891 ± 0.009 ; $n = 160$) or no copies of the ATC haplotype (0.895 ± 0.005 ; $n = 576$; $p < 0.001$) see Figure 2. Similar results were obtained when only premenopausal women were included in the analysis (adjusted FN BMD for ATC [1,1] = 0.853 ± 0.014 ($n = 66$); ATC [1,0] = 0.900 ± 0.010 ($n = 124$); ATC [0,0] = 0.896 ± 0.005 ($n = 443$); $p = 0.001$).

We then extended the haplotype analysis to include the entire population of samples ($n=1240$) by using the programme haplotyper (26) which predicts haplotypes for individuals with unknown haplotype phase. Results were similar to those described above (i.e. women with two copies of the ATC haplotype had the lowest adjusted FN BMD (0.843 ± 0.012 ; $n = 85$) compared to those with one copy (0.892 ± 0.005 ; $n = 514$) or no copies of the ATC haplotype (0.896 ± 0.004 ; $n = 641$; $p < 0.001$)) and no other haplotype was significantly associated with BMD. The T676G polymorphism did not give additional information when it was included in the haplotype analysis along with the 3'UTR polymorphisms. The ATC haplotype remained the only haplotype significantly associated with FN BMD whether it was associated with the T (TATC) or the G allele (GATC) of the T676G polymorphism (data not shown).

Discussion

The cytokine TNF- α has important effects on bone turnover, by modulating differentiation and function of cells of the osteoblast and osteoclast lineage (27). Studies *in vitro* have shown that TNF- α can promote osteoclast differentiation independent of RANK/RANKL (28) and evidence has been presented to suggest that oestrogen deficiency causes bone loss *in vivo*, by upregulating production of TNF- α (29), which, acts through the p55 TNF receptor, encoded by the *TNFRSF1A* gene, to augment osteoclastogenesis (30). Conversely, TNF induced activation of the p75 TNF- α receptor, encoded by the *TNFRSF1B* gene, has been found to suppress osteoclastogenesis *in vitro* (17). These data indicate that the skeletal response to TNF depends on a balance between expression and activation of the *TNFRSF1A* and *TNFRSF1B* gene products.

In this study, we investigated the relationship between four polymorphisms in the *TNFRSF1B* gene and BMD in women from Northeast of Scotland. The T676G is a coding polymorphism (196:Met \rightarrow Arg) located in exon 6 of the gene whereas G593A, T598G and T620C are non-coding polymorphisms located close to each other in the 3'UTR region of the gene.

No association with the T676G polymorphism and BMD was found but there was a strong association between BMD and haplotypes defined by the 3'UTR polymorphisms. This association was highly significant even after correction for multiple testing and remained so when confounding factors such as age, weight, height, menopausal status, and HRT use were taken into account. Whilst individual polymorphisms in the 3'UTR were only weakly associated with BMD, there was a strong association between carriage of the A593 –T598 –C620 (ATC) haplotype and BMD. Thus, in both multiple regression and ANOVA analysis, individuals homozygous for the ATC haplotype had the lowest FN BMD values compared to heterozygotes or those who did not carry the ATC haplotype. Our previous studies (7) along with the data presented here illustrate the importance of haplotype analysis in association studies such as these. Whilst the effect of the ATC haplotype on FN BMD was modest (~1.2%), it is of a similar magnitude to other candidate genes which have been implicated in the regulation of BMD such as VDR (10,31) and oestrogen receptor (7,32). Furthermore, our study was sufficiently powered to detect such an effect. Based on the 3'UTR genotype frequencies observed in our population,

computer simulation showed that our study had 82% and 99% power to detect changes in BMD Z-scores (standard deviation unit) of 0.05 and 0.10, respectively.

These findings are in broad agreement with the work of Spotila *et al* who also reported a positive association between polymorphisms in the 3'UTR of the *TNFRSF1B* gene and BMD in women from the USA (18). In the Spotila's study however, the association was observed with LS BMD rather than FN BMD and the low BMD haplotype was AGT rather than ATC. Possible explanations for the differences between studies include the fact that patients in the Spotila's study were drawn from three different ethnic backgrounds and the fact that the results were based on a sample size much smaller than the population studied here. In this regard, it is of interest that the original family based studies by Devoto *et al* showed evidence of linkage to hip BMD rather than spine BMD (11,15), which is in keeping with the association with FN BMD which we report here. The fact that specific candidate genes and loci affect BMD differently at different skeletal sites is entirely in keeping with evidence from human and animal studies which shows that the effects of genetic factors on BMD regulation are site specific (14,33).

Further work will be required to investigate the mechanisms by which the *TNFRSF1B* 3'UTR regulate BMD. Variations in the 3'UTR of genes are known to alter the stem-loop structure of mRNA and to affect mRNA processing and stability (34-37). In order to assess the possible impact of the 3'UTR polymorphisms on RNA structure, we performed computer simulation studies using RNAdraw (38) to study the predicted effects of the ATC haplotype on RNA secondary structure. This showed that the presence of the ATC haplotype creates changes in the stem-loop structure (compared to the most common haplotype) which potentially could affect mRNA processing, translation, or stability (data not shown). Although these data suggest that the ATC haplotype may influence gene function by an effect on mRNA structure, functional studies will be required to confirm this. Another possibility is that the ATC haplotype may simply be in linkage disequilibrium with a functional polymorphism elsewhere in the *TNFRSF1B* gene or in a gene nearby. Neither of these possibilities seems likely; the only other known functional polymorphism in *TNFRSF1B* is the T676G coding change and we found no association between this polymorphism and BMD. The nearest functional candidate gene nearby is *TNFRSF8*, which ends approximately 60Kb upstream of *TNFRSF1B*. Although this is also a candidate for

regulation of BMD and is within the range of expected LD, we have found no association between polymorphisms of the *TNFRSF8* gene and BMD in the same population (Tasker, Albagha & Ralston; unpublished data).

We conclude that, in this relatively large population, allelic variations at the *TNFRSF1B* locus accounts for part of the heritable component of FN BMD, raising the possibility that polymorphisms of the *TNFRSF1B* gene could be useful genetic markers for bone mass and susceptibility to osteoporotic fracture. Further studies in other populations will be required to confirm this finding and to determine the molecular mechanisms responsible.

Subjects and Methods

Subjects

The study group comprised 1240 women aged 45-54 who were randomly selected from a large population based BMD screening programme for osteoporotic fracture risk (39). The screening program involved 7000 women who were selected randomly from the Community Health Index records, a database of all patients registered with a general practitioner from a 25-mile radius of Aberdeen, a city with a population of 500,000 in the North East of Scotland. Women were invited by letter to undergo BMD measurements between 1990-1994 and 5119 of the 7000 invited (73.1%) attended for evaluation. Participants were weighed on a set of balance scales calibrated to 0.05 kg (Seca, Hamburg, Germany) and height was measured using a stadiometer (Holtain Ltd, Crymych, United Kingdom). All participants completed a questionnaire on menopausal status, and use of Hormone Replacement Therapy (HRT). Information from this questionnaire was combined to identify 5 categories as follows: 1= premenopausal, no HRT; 2 = perimenopausal, no HRT; 3 = postmenopausal, no HRT; 4 = postmenopausal, previous HRT users; 5 = postmenopausal, current HRT user. Women were classified as premenopausal if they were menstruating regularly, as perimenopausal if menstruation was irregular and/or up to 6 months had elapsed since their last period and postmenopausal if menstruation had ceased for 6 months or more. All participants gave written informed consent to being included in the study, which was approved by the Grampian Research Ethics Committee.

Bone mineral densitometry measurements

The bone mineral density measurements (BMD) of the left proximal femur (the femoral neck, FN) and lumbar spine, LS (L2-4) were performed by dual energy X-ray absorptiometry using a Norland XR26 densitometer (Norland Corp, Wisconsin, USA). Calibration of the machine was performed daily, and quality assurance checked by measuring the manufacturer's lumbar spine phantom at daily intervals and a Hologic spine phantom at weekly intervals. The *in vivo* precision for the XR26 was 1.95% for the lumbar spine (LS), and 2.31% for the femoral neck (FN).

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes. Genotypes for the *TNFRSF1B* exon 6 polymorphism (T676G; GenBank accession no. M32315) were determined by PCR-RFLP analysis as described previously (25). The G593A, T598G, and T620C polymorphisms located in the 3'UTR of the *TNFRSF1B* gene (GenBank accession no. U52165) were detected by direct DNA sequencing. A 192 bp PCR product containing the three polymorphisms was amplified as described previously (18). PCR products were then cleaned using ExoSAP IT™ kit (USB Corporation, Cleveland, Ohio, USA) and sequenced using the DYEnamic ET dye terminator cycle sequencing kit (Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, UK). DNA sequencing was performed in 96-well plates which were run on a MEGABACE-1000 automated DNA sequencer (Amersham Pharmacia Biotech UK Ltd, Buckinghamshire, UK). To check for genotyping errors, 8 DNA samples were randomly selected from each 96-well plate and re-sequenced (n= 104). All genotypes were identical to those obtained from the first round of sequencing.

Statistical methods

Statistical analysis was carried out using Minitab version 12 (Minitab, Inc., Coventry, UK). Differences in BMD between the genotypes were tested using a General Linear Model analysis of variance (ANOVA) adjusting for age, weight, height, menopausal status, and duration of HRT use. Stepwise multiple linear regression was used to test for independent predictors of BMD by entering genotypes, age, weight, height, menopausal status, and duration of HRT use into the model. The Bonferroni correction method was used to correct for multiple testing. Linkage disequilibrium (LD) between the polymorphisms, expressed as D', was analysed using the EH and 2BY2 algorithms (40,41). Haplotype inference for individuals with ambiguous haplotype phase was performed using the Haplotyper programme (26). Calculation of the study power was performed using the "Power and sample size" utility of the Minitab® software

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	T676G	G593A	T598G	T620C
T676G	-			
G593A	0.286 [*]	-		
T598G	0.513 [*]	1.000 [*]	-	
T620C	0.077	0.404 [*]	1.000 [*]	-

Table 1. Linkage disequilibrium at the *TNFRSF1B* locus

Values are shown for D'. * Significant at $p < 0.00001$.

Albagha et al, Table 1

	Lumbar Spine BMD			p-value	Femoral Neck BMD			p-value
	TT (n=611)	TG (n=509)	GG (n=80)		TT (n=611)	TG (n=509)	GG (n=80)	
T676G*	1.064 ± 0.006	1.059 ± 0.006	1.078 ± 0.016	0.54	0.892 ± 0.005	0.886 ± 0.005	0.897 ± 0.013	0.53
G593A	GG (n=348) 1.062 ± 0.008	GA (n=603) 1.068 ± 0.006	AA (n=289) 1.056 ± 0.009	0.51	GG (n=348) 0.894 ± 0.006	GA (n=603) 0.896 ± 0.005	AA (n=289) 0.874 ± 0.007	0.02
T598G	TT (n=1120) 1.062 ± 0.004	TG (n=120) 1.073 ± 0.013	GG (n=0) NA	0.42	TT (n=1120) 0.889 ± 0.003	TG (n=120) 0.908 ± 0.010	GG (n=0) NA	0.09
T620C	TT (n=481) 1.064 ± 0.006	TC (n=594) 1.066 ± 0.006	CC (n=165) 1.052 ± 0.011	0.53	TT (n=481) 0.897 ± 0.005	TC (n=594) 0.891 ± 0.005	CC (n=165) 0.871 ± 0.009	0.05

Table 2. Lumbar spine and femoral neck BMD values in relation to *TNFRSF1B* genotypes

Values are least square mean ± SD BMD values in g/cm², adjusted for age, weight, height, menopausal status, and HRT use.

*Data for T676G were available for 1200 subjects.

Albagha et al, Table 2

<i>Lumbar Spine BMD</i>				<i>Femoral Neck BMD</i>			
Predictor	Coefficient	R^2	p value	Predictor	Coefficient	R^2	p value
Weight (kg)	0.0040	0.116	<0.001	Weight (kg)	0.0038	0.153	<0.001
Height (cm)	0.0036	0.130	<0.001	ATC Haplotype	-0.0195	0.165	0.001
YSM**	-0.0036	0.137	0.025	Height (cm)*	0.0019	0.171	0.009
ATC Haplotype	-0.0074	0.138	0.332	YSM**	-0.0030	0.178	0.014
Age (y)	-0.0024	0.139	0.496	Age (y)	0.0026	0.179	0.343
HRT use (yrs)	-0.0023	0.139	0.627	HRT use (yrs)	-0.0021	0.179	0.563
Constant	0.3485	--	0.118	Constant	0.2212	--	0.202
<i>Final Adjusted $R^2 = 0.133$</i>				<i>Final Adjusted $R^2 = 0.173$</i>			

Table 3. Multiple linear regression analysis of BMD.

*Study subjects were classified into 3 genotype groups: [1,1], [1,0], and [0,0] corresponding to individuals with 2 copies, one copy, and subjects without the ATC haplotype, respectively. ** YSM: years since menopause.

Albagha et al, Table 3

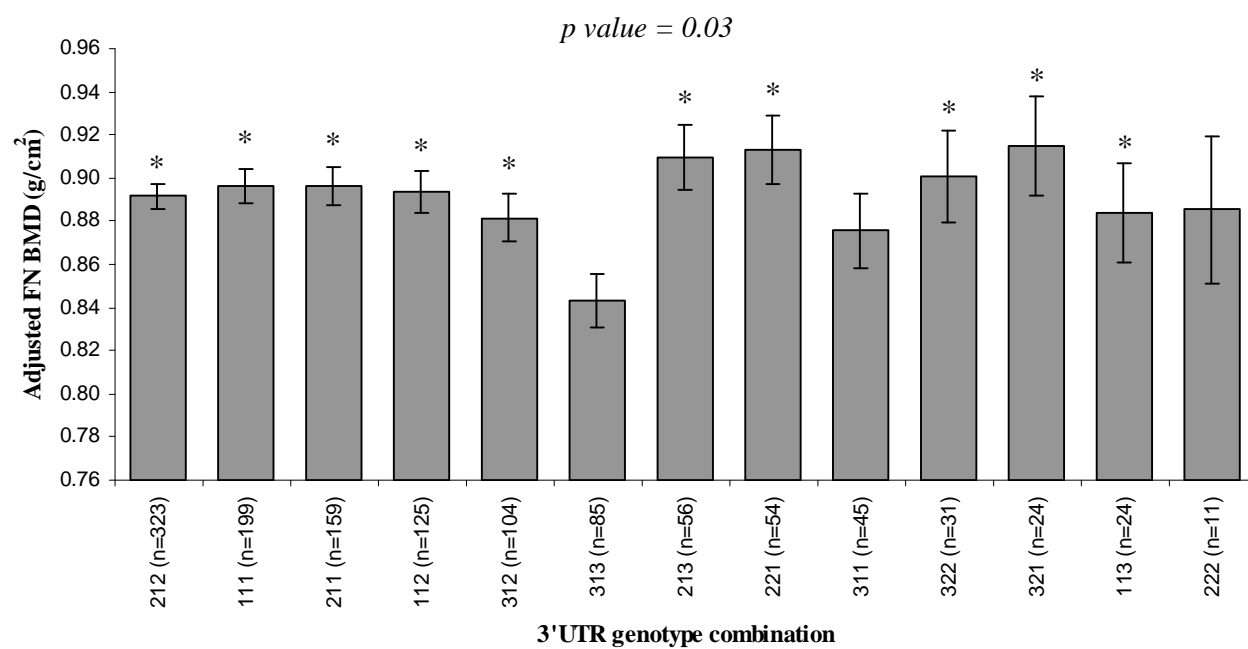


Figure 1. *TNFRSF1B* 3'UTR genotype combinations and FN BMD. * Significantly different from 313 genotype ($p < 0.001$).

Albagha et al, Figure 1

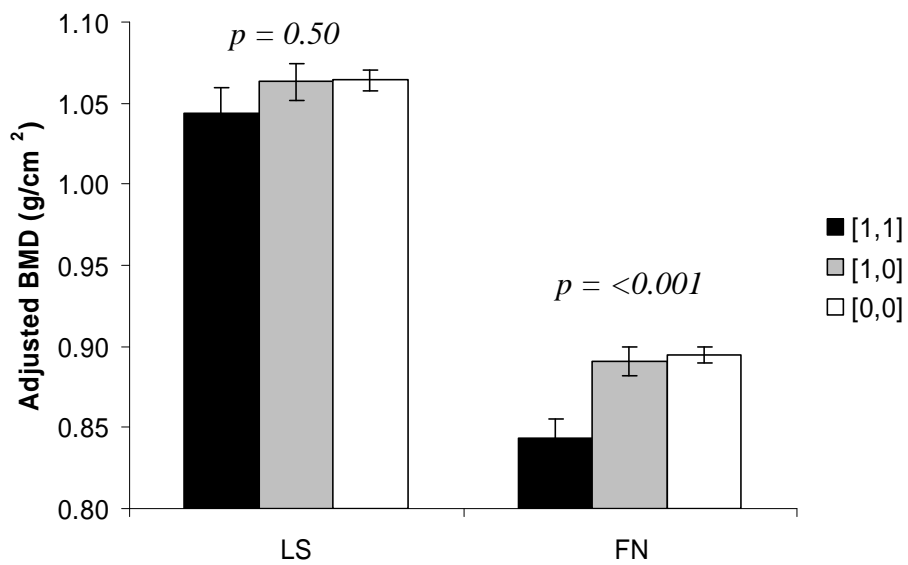


Figure 2. Lumbar Spine (LS) and femoral neck (FN) BMD values in relation to the *TNFRSF1B* 3'UTR genotypes defined by the ATC haplotype status. Subjects were coded according to whether they had 2 copies [1,1], one copy [1,0] or no copies of the ATC haplotype.

Albagha et al, Figure 2