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# Relationship of Lrp5rs121908669 (G171R) Genotypes to Femur T-Score in Pre and Post-Menopausal Women

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## ABSTRACT

*Studies have proven the relationship of LRP5 to BMD, some of its mutations are with increased BMD and some decreased BMD. According to NCBI, LRP5G171R is a proven pathogenic mutation for a disease associated with HBM. There are very few studies regarding this mutation and there is no genotyping study for it. It has also been studied only in groups with increased BMD, so we don't have any idea about its relationship with other cases of BMD. This current study has investigated the distribution of LRP5rs121908669 using a PCR-RFLP and DNA sequencing in Syrian pre and post-menopausal women. As final result, GG is an independent protective factor against abnormal BMD, GA is an independent pathogenic factor, i.e. GA is a prognostic factor for abnormal BMD. Whereas, AA is a non-independent protective factor and a non-independent pathogenic factor for osteopenia. Genotyping of LRP5G171R or the genetic factor in general can be added as a risk factor within the FRAX application.*

**Keywords:** LRP5G171R; LRP5rs121908669; Femur T-score; PCR-RFLP; Pathogenic mutation

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## INTRODUCTION

BMD is a highly heritable trait, with an heritability estimate of 50%-80% depending on skeletal locations and ethnicity [1,2]. Bone mass, a major determinant of the risk of osteoporotic fracture, increases during childhood and adolescence, reaching a peak at about the age of 20 years. Twin and family studies indicate that genetic factors account for approximately 75 percent of the variation in peak bone mass, although the genes that contribute to this variation are largely unknown [3-6].

Some bone disorders has been shown to be due to an inherited problem of function of the gene for low density Lipoprotein Receptor related Protein 5 ( LRP5) [7]. This protein is involved in the Wnt signaling pathway, acting as a co receptor for Wnt, a developmental protein and as a target for the inhibitory effects of Dickkopf (Dkk), another

developmental protein on Wnt signaling [8,9]. The studies suggest a link between Wnt signaling through LRP5 and bone density [10]. LRP5 gene turned out to be an important regulator of peak bone mass in vertebrates. Mutations in LRP5 gene may cause high or low bone mass [11-13]. Both may lead to osteoporosis and fractures. Many of LRP5 mutations cause HBM. They can differ from each other by the location of impact within the skeleton. Some have an increased trabecular bone density (osteosclerosis), whereas others have a cortical bone thickening (hyperostosis). There are many genetic causes for increased bone density.

The mutation LRP5rs121908669 (G171R) that causes the high bone mass phenotype is located in the amino terminal part of the gene. It increases bone density affecting mainly the cortices of the long bones and the skull, thickened cortical bone with no alteration in external shape, dense cranial basis, osteoporosis. It is phenotypically normal individuals with dense bones (High Bone Mass) HBM. The normal inhibition of Wnt signaling was defective in the presence of the G171R mutation. The antagonistic protein Dickkopf could not bind to mutated LRP5, resulting in a constitutive activity of the Wnt signaling pathway. This mutation causes an increase in mineral density in the cortical bone in majority and the installation of the femur bone mostly cortical bone. Thus, it may be useful to correlate the bone mineral density in the femoral neck to the mutation.

## MATERIALS AND METHODS

The study included 150 participants who visited rheumatology clinic at Tishreen university hospital, Lattakia, Syria, between March 2019 and September 2021, interspersed with interruptions due to the Corona pandemic. The work was approved by the ethics committee in Syrian ministry of high education and written informed consent was obtained from all the participants. All of the participants were interviewed with a structured questionnaire before BMD measurement. The contents of the questionnaire included socio demographic characteristics, work habits, physical activity and medication history, as well as starting and onset of menstrual, pregnancy and number of children, history of family orthopedic complaint. Measurements of height and weight were taken and body mass index ($kg/m^2$) was calculated. All participants were women with pre menopause or post menopause. They were from different families. Blood phosphorous and calcium values were collected from patients' files.

**Bone densitometry:** The Bone Mineral Density (BMD; $g/cm^2$) of the lumbar spine (L1-L4) and left femur as measured by Dual energy X-ray Absorptiometry (DXA). All DXA scans were conducted by a specially trained specialist. BMD Results were converted to age and gender specific Z-score matched normal Caucasians. The samples were classified into 3 groups (normal, osteopenia, osteoporosis) according to the world health organization classification of T-score values.

***In silico* Study:** An *in silico* study on NCBI was done in 2021; it was found that there were thousands of mutations for 569 genes associated with osteoporosis. There were 2 pathogenicity proven point mutations for osteoporosis only without any other diseases with a predictive effect on protein of 87% according to the bioinformatics application of SNP predict. Currently, there are greater numbers of genes related to osteoporosis, numbering 855 genes and dozens of point mutations that prove pathogenic to osteoporosis without other diseases (Table 1).

**Table 1: Results of an *in silico* study (2021) to determine the pathogenetic SNPs for osteoporosis.**

| Number of genes | Number of SNPs | Clinical significance | Names of gene |
|---|---|---|---|
| 569 | Thousands | Coding/ non coding protein | - |
| 483 | Thousands | Coding protein | - |
| 7 | Dozens | Pathogenic for osteoporosis and other diseases | BMND7,BMND8, BMND4,CALCR, COL1A1,COL1A2, LRP5 |
| 2 | 3 | Pathogenic only for osteoporosis | COL1A2,LRP5 |
| The chosen SNP is LRP5rs121908669. | | | |

**DNA extraction:** Blood samples were collected by EDTA anticoagulant container tubes (2.5 ml blood from each participating woman) from participants in Tishreen university hospital, Lattakia, Syria. The samples were kept at -20°C. Work has been completed in the biotechnology laboratories of the atomic energy authority, Damascus, Syria, where DNA was isolated from samples using the (QIAamp DNA Blood Mini kit, Qiagen, Germany), according to the manufactures procedure and was stored at -20°C. The total DNA of each sample was measured by using a spectrophotometer followed by quantity ultraviolet light.

**LRP5rs121908669 mutation analysis:** The studied mutation was selected using the software. The prediction ratio for its effect on protein was 87%. In 2021, an *in silico* study was conducted on NCBI with regard to the genes of osteoporosis. It was found that there are only two genes with 3 point mutations proven pathogenic for osteoporosis without other diseases. One of them is LRP5rs121908669.

LRP5rs121908669 polymorphism of exon 3 was amplified using specific forward primer: (5'-TCTGTGTTAGCTGCTTCTCTT-3') and Reverse primer 5′- CCAGGACTGCGTGGGTA -3′.

The primers were manufactured using (Polygen primer designer device, Germany). The stock concentration was 52.51 n.mol/ml for reverse primer and 63.60 n.mol/ml for forward primer. Both were diluted with dual distillation water (ddw) (10X).

The Polymerase Chain Reaction (PCR) was performed in a total volume of 25 µl containing 5 µl of genomic DNA, 5 µl PCR buffer, 1 µl dNTPs, 2 µl of each primer and 1 µl of Taq DNA polymerase. PCR program included initial denaturation at 95°C for three minutes followed by 40 cycles of 95°C for 45 seconds, 52°C for 45 seconds and 72°C for 60 seconds with a final extension at 72°C for 7 minutes. PCR reaction was conducted in a PCR T100 thermo cycler. The amplification PCR products were run on 2% agarose gel stained with DNA Safe Stain Dye and visualized under UV light. The positive result produced bands 259 base pair (bp) (=259 bp) which indicates to presence of the fragment which we were choose for detection the mutation.

The Restriction Fragment Length Polymorphisms (RFLP) of the *LRP5* gene was carried out by PCR product digestion for 16 h at 37°C with 0.8 µl Bfi1. Then, 15 µl of the digested PCR products were added to 3 µl (6X) loading dye and loaded on 3.5% agarose gel and run at 80V for 60 minutes. PCR products for LRP5rs121908669 were then visualized using gel documentation system BIO-RAD (Gel-DocSy1-L8-M5). The lengths of the digest product were 192pb × 67bp; 259pb; 259bp × 192 × 67bp for normal sample, mutant sample, hetero mutant sample, respectively. There was no positive or negative control sample.

All were confirmed by direct sequencing using SeqStudio genetic analyzer (Applied bio systems, USA). The cycle sequencing reaction was performed in a 10 µl volume containing 1 µl of the terminator ready reaction, 5 p. mol of either the forward or reverse primer and 10 mg of purified PCR product. The thermal cycle protocol was 95°C for 4 minutes followed by 30 cycles at 96°C for 10 seconds, 50°C for 5 seconds and 60°C for 4 minutes. Centri-Sep columns (Princeton separations, Adelphia, NJ, USA) were used for the effective and reliable removal of excess dye terminators from completed DNA sequencing reactions. Data were compared and aligned with different sequences using the NCBI BLAST assembled genomes tool.

**Statistical study:** We examined cross sectional data of 150 participants. Related samples McNemar change test was used to exams the variant significant under 95% confidence level ($\alpha \leq .050$) and studying a null hypothesis interested in LRP5 G171R genotypes of distribution across femur T-score values.

We also used automated cross tab to identify the odd risk for femur T-score values observing when LRP5 G171R genotypes excess comparing with the possibility to observe femur T-score values when LRP5 G171R genotypes absence.

Finally, the *Chi square tests* were used to estimate the correlation between LRP5 G171R genotypes and each of the femur T-score values under 95% confidence ($\alpha \leq .050$).

## RESULTS AND DISCUSSION

It is the first study of its kind in the world to link the genotypes of LRP5rs121908669 (G171R) with femur T-score. There are only two studies in the world on LRP5 G171R. The study that discovered the mutation by chance. Also the study that confirms that the mutation is linked with a HBM and is a cause of ado1 disease, which has non syndromic high bone mass, since they do not suffer from an enlarged mandible and a torus palatinus. One of the weaknesses of that study is that the studied group is only those with high bone density and their families. While our study included (high, low and normal) BMD to find out the credibility of its association with HBM, to study the effect of race on expression of LRP5G171R. There is also no study that studies the genotyping of this mutation.

We note that the significant chance to occurrence GG genotype in normal, osteopenia, osteoporosis femur T-score values are 2.5%, 0.00%, 0.00% respectively. Hence we reject the null hypothesis an retain the alternative hypothesis which indicate that the distributions of different values across normal, osteopenia, osteoporosis femur T-score values, respectively and GG genotype are significantly variant with confidence level of 95% or more ($p \leq .05$).

There are significant correlation between normal, osteopenia femur T-score values $^{*}$GG genotype (*Chi square*=13.750, p=.000<.05), (*Chi square*=10.765, p=.001<.05), respectively. There is no significant correlation between osteoporosis femur T-score values $^{*}$GG genotype (*Chi square*=.689, p=.407>.05).

The odds for the present of normal, osteopenia, osteoporosis femur T-score values were $^{*}$.1.830 times greater, .366 times les, .732 times less, respectively when GG genotype exists compare with their odd without GG genotype exists.

A carrier of GG genotype is a non-independent predisposing factor for normal T-score and osteopenia T-score. The GG genotype may be associated with cases of normal T-score or osteopenia T-score depending on environmental, clinical or genetic factors that support or cancel the effect of the genotype. The GG genotype is not a predisposing factor for osteoporosis T-score, nor is it associated with any osteoporotic case. In general, it can be said that the GG genotype is an independent protective factor against osteoporosis.

We note that the significant chance to occurrence AA genotype in normal, osteopenia, osteoporosis femur T-score values are 0.00%, 0.01%, 28% respectively. Hence we reject the null hypothesis and retain the alternative hypothesis which indicates that the distributions of different values across normal, osteopenia, respectively and AA genotype are significantly variant with confidence level of 95% or more ($p \leq .05$). We retain the null hypothesis which indicates that the distributions of different values across AA genotype and osteoporosis femur T-score values are equally likely with confidence level of 95% or more ($p \leq .05$).

There are significant correlation between normal, osteopenia femur T-score values $^{*}$AA genotype (*Chi square*=8.683, p=.003<.05), (*Chi square*=7.731, p=.005<.05), respectively. There is no significant correlation between osteoporosis femur T-score values $^{*}$AA genotype (*Chi square*=.204, p=.651>.05).

The odds for the present of normal, osteopenia, osteoporosis femur T-score values were $^{*}$.558 times less, 7.231 times greater, 1.282 times greater, respectively when AA genotype exists compare with their odd without AA genotype exists.

AA genotype is a non-independent predisposing factor for normal T-score and osteopenia T-score. The AA genotype can be associated with cases of normal T-score or osteopenia depending on environmental, clinical or genetic factors that support or cancel the effect of the genotype. The AA genotype is not a predisposing factor for osteoporosis T score, but it can be associated with cases of osteoporosis, explaining that these people have an interaction between genes that makes this genotype cause osteoporosis or presence of other genes or mutations that cause osteoporosis, or presence of environmental or clinical factors predisposing to the disease. In general, it can be said that AA genotype is a non-independent influencing factor with its positive and negative influence.

We note that the significant chance to occurrence GA genotype in normal, osteopenia, osteoporosis femur T-score values are 0.00%, 8.7%, 57.5% respectively. Hence we reject the null hypothesis and retain the alternative hypothesis which indicates that the distributions of different values across normal and GA genotype are significantly variant with confidence level of 95% or more ($p \leq .05$). We retain the null hypothesis which indicates that the distributions of

different values across GA genotype and osteopenia, osteoporosis femur T-score values, respectively are equally likely with confidence level of 95% or more ($p \leq .05$).

There are no significant correlation between normal, osteopenia, osteoporosis femur T-score values, respectively [*]GA genotype (*Chi square*=3.463, p=.063>.05), (*Chi square*=2.263, p=.133>.05), (*Chi square*=.344, p=.557>.05), respectively.

The odds for the present of normal, osteopenia, osteoporosis femur T-score values were [*].712 times less, 1.652 times greater, 1.297 times greater, respectively when GA genotype exists compare with their odd without GA genotype exists.

GA genotype is not a predisposing factor for normal T-score, nor is it associated with any normal T-score. In general, it can be said that the GA genotype is an independent protective factor for a normal T-score, meaning it is associated with pathological cases. GA genotype is not a predisposing factor for T-score (osteopenia, osteoporosis), but it can be associated with cases of osteopenia or osteoporosis, explaining that these people have an interaction between genes that makes this genotype cause osteoporosis or osteopenia, the presence of genes or other mutations that cause osteopenia or osteoporosis or the presence of environmental or clinical factors predisposing to the disease.

## CONCLUSION

As final result, GG is an independent protective factor against abnormal BMD, *i.e.* GG is a prognostic factor for the absence of abnormal BMD. GA is an independent pathogenic factor, *i.e.* GA is a prognostic factor for abnormal BMD. Whereas, AA is a protective factor and a pathogenic factor for osteopenia but not independently and may be associated with all bone conditions (normal, osteopenia, osteoporosis) and cannot be considered as a prognostic factor for any bone condition. Genotyping of LRP5G171R or the genetic factor in general can be added as a risk factor within the FRAX application.

## DECLARATION

I confirm that this work is a part of an approved PhD thesis which was approved by university board's decision No.1698 of 05/02/2019 and this work is an original and has not been published elsewhere, nor is it currently under consideration for publication elsewhere.

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