

Relationship of LRP5rs121908669(G171R) genotypes to femur T-score values

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Abstract

Background and aims: Among some studied cases, there is a decrease in bone mineral density in the femoral region without decrease in bone mineral density in the lumbar region in postmenopausal women. The femoral bone is mostly cortical bone. The *lrp5* gene is closely related to the cortical bone. LRP5G171R(LRP5rs121908669) is a proven pathogenic single nucleotide polymorphism SNP for a disease associated with high bone mass HBM. There is no genotyping study for it until now. There is no idea about its relationship with other cases of BMD. **Methods:** LRP5rs121908669 was diagnosed using PCR-RFLP and DNA sequencing in Syrian pre & post-menopausal women. Related-Samples McNemar Change Test was used under 95% confidence level ($\alpha \leq .050$) to study distribution LRP5G171R genotypes across femur T-score values. Odds Ratio test was used to identify the odd risk for femur T-score values when LRP5G171R genotype is absence or existed. Chi-Square Tests(χ^2) were used to estimate the correlation between genotypes and each of femur T-score under 95% confidence ($\alpha \leq .050$). **Results:** Significant chance to occurrence GG genotype, CC genotype, GC genotype in normal, osteopenia, osteoporosis femur T-score values are (GG 2.5%, 0.00%, 0.00%, CC 0.00%, 0.01%, 28%, GC 0.00%, 8.7%, 57.5%), respectively. There are significant correlation between GG genotype and normal, osteopenia femur T-score values, but no significant correlation with osteoporosis femur T-score values $\chi^2=13.750$, $p = .000 < .05$, $\chi^2=10.765$, $p = .001 < .05$, $\chi^2 = .689$, $p = .407 > .05$, respectively. There are significant correlation between CC genotype and normal, osteopenia femur T-score values, but no significant correlation with osteoporosis femur T-score values with $\chi^2 = .683$, $p = .003 < .05$, $\chi^2 = 7.731$, $p = .005 < .05$, $\chi^2 = .204$, $p = .651 > .05$, respectively. There are no significant correlation between GC genotype and normal, osteopenia, osteoporosis femur T-score values with $\chi^2 = 3.463$, $p = .063 > .05$, $\chi^2 = 2.263$, $p = .133 > .05$, $\chi^2 = .344$, $p = .557 > .05$, respectively. **Discussion:** It is the first genotyping of LRP5G171R in the world. Normal genotype GG increase the likelihood of normal femur BMD. homozygous genotype CC and heterozygous GC genotype increase the likelihood of osteoporosis or osteopenia in femur bone. There are often associations in the distribution between carriers of GC or CC and carriers with osteoporosis or osteopenia in femur bone. **Conclusion:** In general, LRP5G171R is not only related to HBM, it is related to normal, osteopenia, osteoporosis. Thus, NCBI should be informed that LRP5G171R should be classified clinically as conflicting information, not pathogenic to ADO1 as it is classified until now. Also, LRP5G171R can be added as a risk factor within the FRAX application. LRP5G171R can be both a diagnostic and a therapeutic goal for osteoporosis.

Keywords: CC genotype, femur T-score, GG genotype, GC genotype, LRP5G171R, LRP5rs121908669.

INTRODUCTION

Bone mass is a major determinant of the risk of osteoporotic fracture [1,2]. Twin and family studies indicate that genetic factors account for approximately 75% of the variation in peak bone mass [1,3,4]. Therefore, studying the genetic factor will be important for early intervention and prevention of osteoporosis. especially with the challenges facing dual energy X-ray absorptiometry DEXA

Bone mineral density BMD is a highly heritable trait, with a heritability estimate of 50% to 80% [5,6]. Therefore, there is a high risk to be inherited it to generations.

The LRP5 gene turned out to be an important regulator of peak bone mass in vertebrates [7]. Single nucleotide polymorphisms SNPs in the LRP5 gene may cause high or low bone mass[8-10]. Both may lead to osteoporosis and fractures[8].

LRP5rs121908669 (G171R) that causes the high bonemass phenotype is located in the aminoterminal part of the gene. Some genetic information about this SNP is explained in the table below table.1[11]

Table.1: Some genetic information about LRP5 G171R(LRP5rs121908669) from NCBI

Name	Gene ID: 4041 /LRP5
Description	LDL receptor-related protein 5
Location	11:68312591-68449275
Cytogenetic region	11q13.2
SNV	G>C 511
EXON	3/23
RefSeqGene	143636 bp
Protein change	G171R
OMIM:	603506
Condition	ADO1

It increases bone density affecting mainly the cortices of the long bones and the skull, with no alteration in external shape. It is phenotypically normal. It is a cause of autosomal dominant osteopetrosis type1 (ADO1) disease, which has non- syndromic high bone mass[1,7], since ADO1 carriers do not suffer from an enlarged mandible and a torus palatinus[10]. The normal inhibition of Wnt signaling was defective in the presence of LRP5G171R. The antagonistic protein Dickkopf could not bind to mutated LRP5, resulting in constitutive activation of the Wnt signaling pathway[1].

Linkage analyses in people at high-risk for rare metabolic bone diseases should also yield important clues to the pathogenesis of osteoporosis[12]. There is no study related to this SNP in different BMD groups. This SNP causes an increase in BMD in the cortical bone in majority[9]. The installation of the femur bone is mostly cortical bone[13]. Thus, it may be useful to correlate the BMD in femur region with this SNP. The aim of this study is to answer this question" Can LRP5G171R be an early predictor of osteopenia or osteoporosis?

MATERIALS AND METHODS

The study included 150 participants who visited rheumatology clinic at Tishreen University Hospital, Lattakia, Syria, throughout the period between March 2019 and September 2021, which was interspersed with interruptions due to the Corona pandemic. The work was approved by the Ethics Committee in Syrian Ministry of High Education, and prior written consents were obtained from all the participants.

All of the participants were interviewed using a structured questionnaire. The questionnaire included socio-demographic characteristics, work habits, physical activity, medication history, age, age of beginning and end of menstrual, pregnancy and number of children, history of family orthopedic complaint, clinical history of bone pain, measurements of height and weight, body mass index BMI (kg/m²), data of fractures, lumbar and femur Z-score, lumbar and femur T-score. 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. The controls had high or normal T-score for both femur and lumbar T-score. All patients with hypertension, diabetes, osteomalacia, surgical menopause and cancer were excluded.

Bone densitometry

The bone mineral density (BMD; g/cm²) of the lumbar spine (L1-L4) and left femur as measured by dual energy X-ray absorptiometry (DXA) (Medix DR, France). All DXA scans were conducted by a specially trained specialist. BMD Results were converted to age- and gender-specific Z-score that matched normal Caucasians. The samples were classified into 3 groups (normal, osteopenia, and osteoporosis) according to the World Health Organization classification of T-score values.

Insilico Study:

An Insilico study on NCBI was done in 2021. It was found that there were thousands of mutations for 569 genes associated with osteoporosis. There were two proven pathogenic SNPs for osteoporosis only without any other diseases with a predictive effect on protein of 87% according to the bioinformatics application of SNP PREDICT[14,15]. Currently, there are greater numbers of genes related to osteoporosis, numbering 855 genes, and dozens of SNPs that are pathogenic proven for osteoporosis without other diseases as shown in table.2[14]

Table.2: results of an Insilico study(2021) to determine the pathogenetic SNPs for osteoporosis

Number of genes	Number of SNPs	Clinical significance	Names of genes
569	Thousands	Coding/noncoding 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

DNA Extraction:

Blood samples were collected using EDTA anticoagulant container tubes (2.5 ml blood from each participant) in Tishreen University Hospital, Lattakia, Syria. The samples were kept at -20 °C. Work had 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 manufacturer's procedures and was stored at -20°C. The total DNA of each sample was measured by using a spectrophotometer followed by a quantity Ultraviolet light.

LRP5rs121908669 SNP analysis:

The studied SNP was selected using the software <https://loschmidt.chemi.muni.cz/predictsnp/>. The Prediction ratio for its effect on the protein was 87%. In 2021, an Insilico study was conducted on NCBI concerning the genes of osteoporosis. It was found that there are only two genes with 3 SNPs proven pathogenic for osteoporosis without other diseases. One of them is of LRP5rs121908669.

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

Primers were designed using <https://www.ncbi.nlm.nih.gov/tools/primer-blast>, and <https://bioinfo.ut.ee/primer3-0.4.0/>. The primers were manufactured using (a polygon primer designer device, in 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 thermocycler (Mastercycler, Eppendorf, Germany). 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 the presence of the fragment which was chosen to detect this SNP.

Restriction enzymes for RFLP were chosen from <https://nc2.neb.com/NEBcutter2/>. The Restriction Fragment Length Polymorphisms (RFLP) of the LRP5 gene was carried out by PCR product digestion for 16h at 37 °C with 0.8 µl BfiI (MBI Fermentas, Vilnius, Lithuania). Then, 15 µl of the digested PCR products were added to 3 (6X) loading dye and loaded on 3.5% agarose gel, and run at 80V for 60 minutes. PCR products for L 5rs121908669 were then visualized using the gel documentation system BIO-RAD (Gel-DocSy1-L8-M5). The lengths of the digested product were 192bp*67bp; 259bp; 259bp*192*67bp for the normal genotype GG, Hhomozygous genotype CC, and heterozygous genotype GC, respectively. The ladder is 20pb. There was no positive or negative control sample.

All were confirmed by direct sequencing using SeqStudio Genetic Analyzer (Applied Biosystems, USA). The cycle-sequencing reaction was performed in a 10 µl volume containing 1 µl of the ready reaction of the terminator, 5 p.mol of either the forward or reverse primer, and 10 ng of purified PCR product (ExoSAP-IT kit; Amersham BioSciences, Piscataway, NJ, USA). 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 (ABI GeneAmp PCR System 9700, Applied Biosystems). Centri-Sep columns (Princeton Separations, Adelphia, NJ, USA) were used for the effective and reliable removal of excess dye terminators (DyeEx 2.0, Qiagen, Germany) from completed DNA sequencing reactions. Data were compared and aligned with different sequences using the NCBI BLAST Assembled Genomes tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Statistical study:

Statistical analysis was performed using SPSS computer software version 20 (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp; 2011). Related-Samples McNemar Change Test was used to examine the correlation in the distribution between the presence of LRP5G171R genotypes and BMD of femur region under 95% confidence level ($\alpha \leq .050$), and to study a null hypothesis concerning LRP5G171R genotypes of distribution across femur T-score values. Chi-Square Test was used to estimate the correlation between LRP5G171R genotypes and BMD of femur region which are of under 95% confidence ($\alpha \leq .050$). Odd Ratio test was used to identify the odd risk for femur T-score values when LRP5G171R genotype is absence or existed.

RESULTS:

All data about age, age of beginning and end of menstrual, pregnancy and number of children, history of family orthopedic complaint, bone complaint, measurements of height and weight, body mass index BMI (kg/m²), data of fractures, classification of cases according to WHO*, are contained in the table below table.3. All participants had normal blood concentrations of calcium and phosphorous.

Table.3: Clinical, laboratory, demographic and radiological information for participants

Variable	Case
Total number	150
Age	60(40, 80)
Age of beginning of menstrual	14(11, 17)
Age of end of menstrual	50.5(46, 55)
Weight	69.5(40,99)
Height	165(150,180)
BMI	29.69(17.99, 41.4)
Data on fractures(YES/NO)	85/65
History of family orthopedic complaint(YES/NO)	56/94
Clinical history of bone complaint(YES/NO)	139/11
L2-L4(lumbar) Z-score	(-4.1, 3.1)
L2-L4 (lumbar)T-score	(-5.6, 1.2)
Femur Z-score	(-1.9, 1.1)
Femur T-score	(-2.2, 1.1)
Normal(T-score \geq 1) *	74
Osteopenia (-2.5) < T-score <(-1)	48
Osteoporosis T-score \leq (-2.5)	28
Total	150

* World Health Organization Definition of Osteoporosis by T-score values

LRP5rs121908669 detection and genotyping were determined using PCR-RFLP figure.1 and DNA sequencing figure.2. It was found that there were 97(64.66%) GG genotype, 20 (13.3%) homozygous genotype CC, 33 (22%) heterozygous genotype GC.

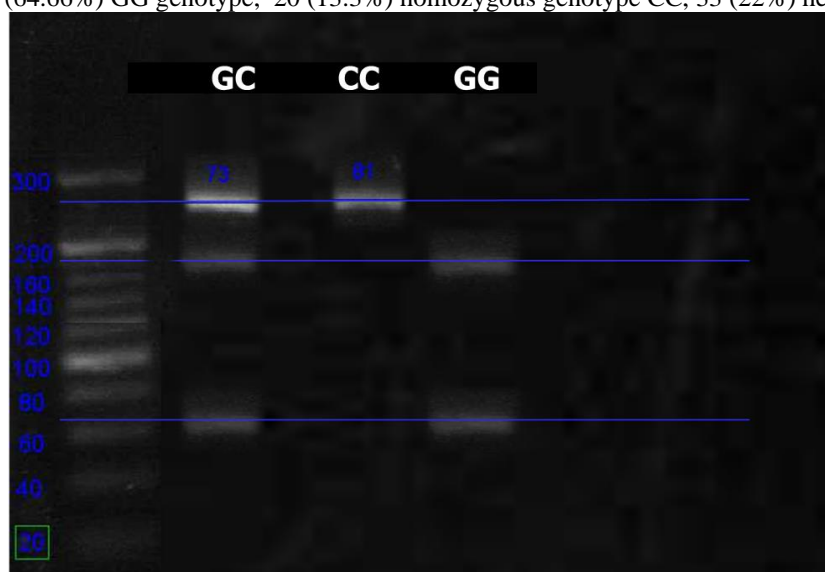


Figure.1: Agarose gel electrophoresis of PCR products for the BfiI polymorphism: the far left lane, 20bp DNA ladder, The rest of lanes,GG genotype, CC genotype, GC genotype.

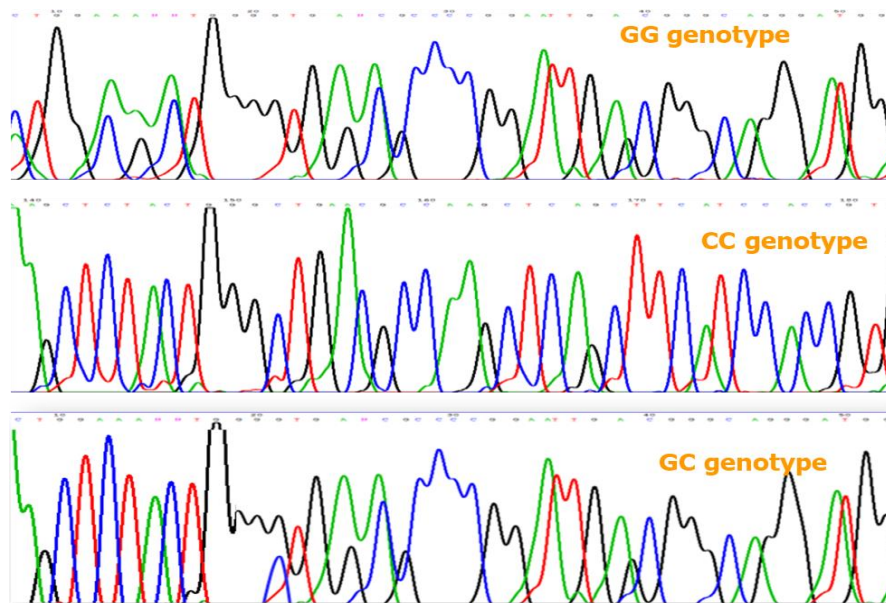


Figure.2: GG(GGGGT),CC(GGGCT),GC(GGG(G/C)T) genotypes of LRP5G171R by DNA sequencing analysis

As for the results of the applied statistical studies, they are shown in the table below

Table 4: Results of Related-Samples McNemar Change Test, Risk estimate tests, Chi-Square Tests to evaluate the relationship of genotypes to femur T-score values.

Genotype	Groups	McNemar %	OR(Odds Ratio)	CI(95%Confidence Interval)	Chi-square	P
GG	normal	2.5	1.830	1.344- 2.493	13.750	0.000
	osteopenia	0	0.366	0.185- 0.723	10.765	0.001
	osteoporosis	0	0.732	0.346- 1.547	0.689	0.407
CC	normal	0	0.558	0.417- 0.746	8.683	0.003
	osteopenia	1	7.231	1.056- 49.516	7.731	0.005
	osteoporosis	28	1.282	0.426- 3.856	0.204	0.651
GC	normal	0	0.712	0.514- 0.986	3.463	0.063
	osteopenia	8.7	1.652	0.818- 3.334	2.263	0.133
	osteoporosis	57.5	1.297	0.535- 3.149	0.344	0.557

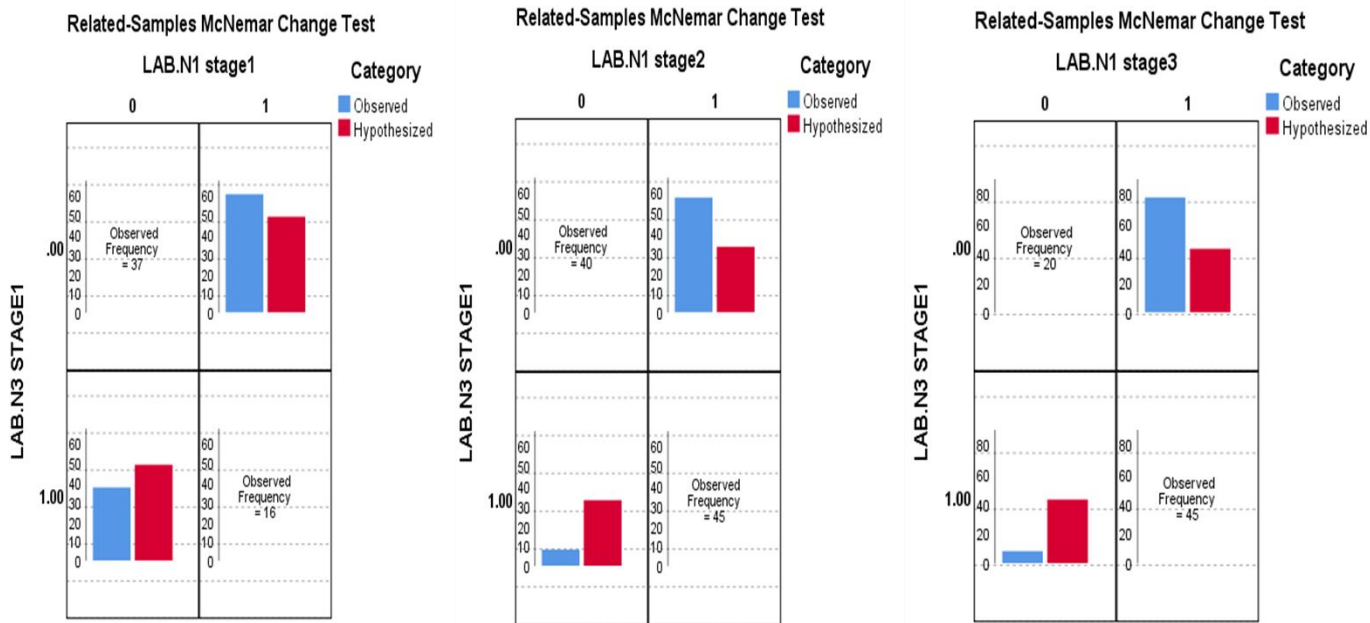


Figure 3: Related-Samples McNemar Change Test between GG genotype and normal femur T-score(LAB.N1 stage1), osteopenia femur T-score(LAB.N1 stage2), osteoporosis femur T-score(LAB.N1 stage3).

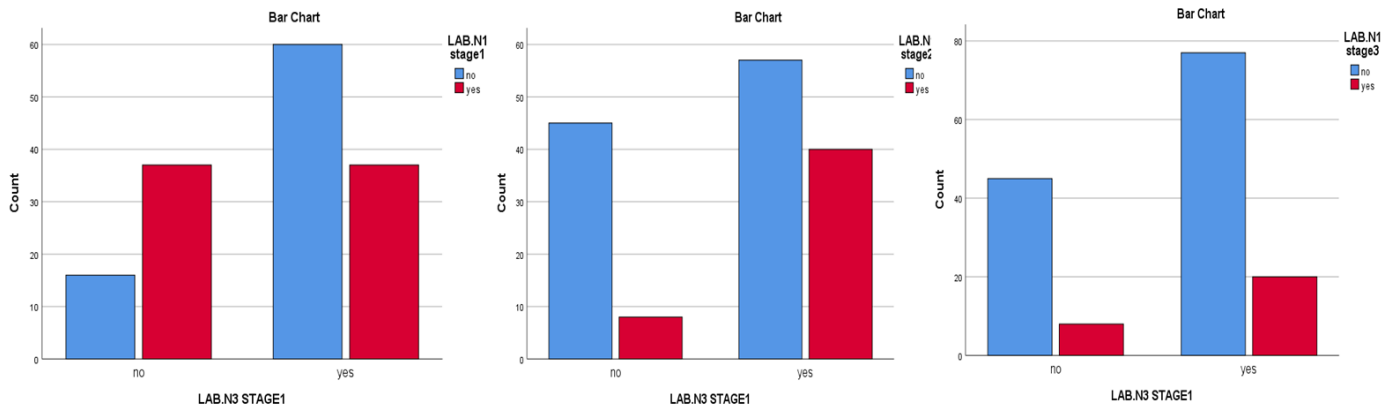


Figure 4: Bar chart to the frequency between normal femur T-score(LAB.N1 stage1) osteopenia femur T-score(LAB.N1 stage2), osteoporosis femur T-score(LAB.N1 stage3) and GG genotype.

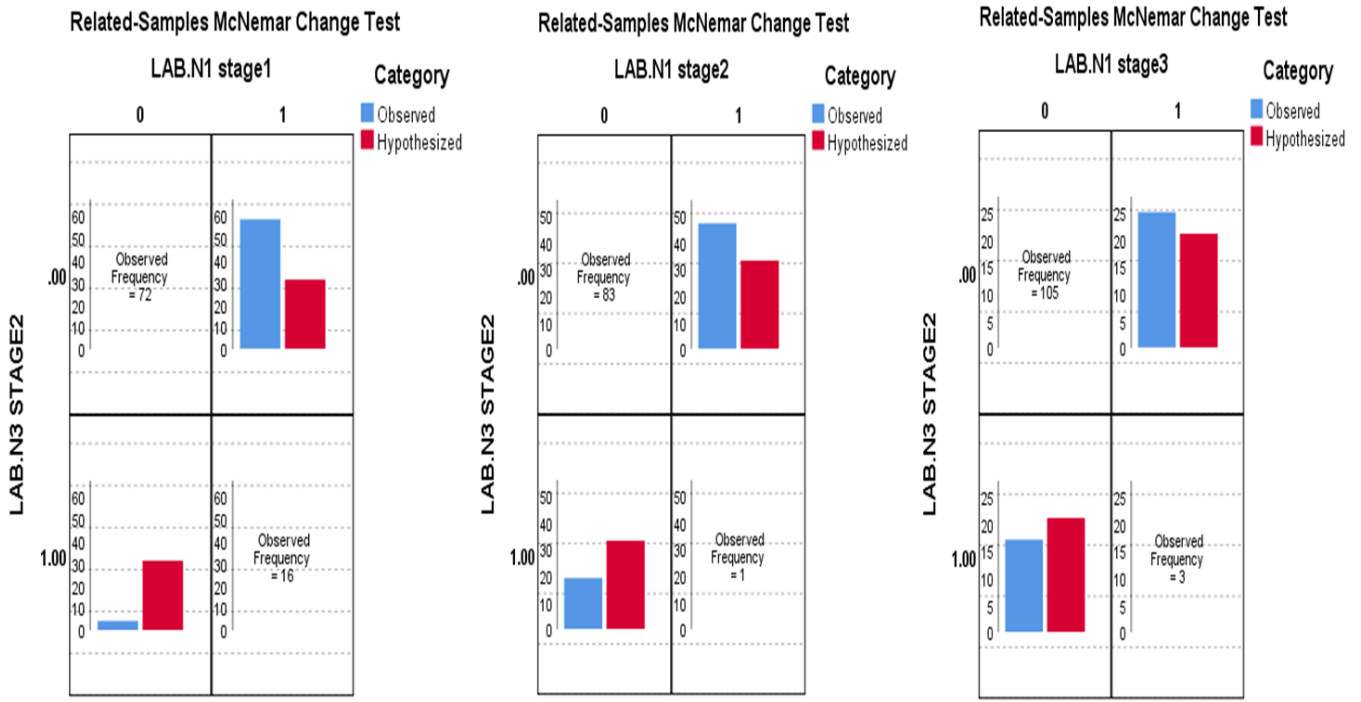


Figure 5: Related-Samples McNemar Change Test between CC genotype and normal femur T-score(LAB.N1 stage1), osteopenia femur T-score(LAB.N1 stage2), osteoporosis femur T-score(LAB.N1 stage3).

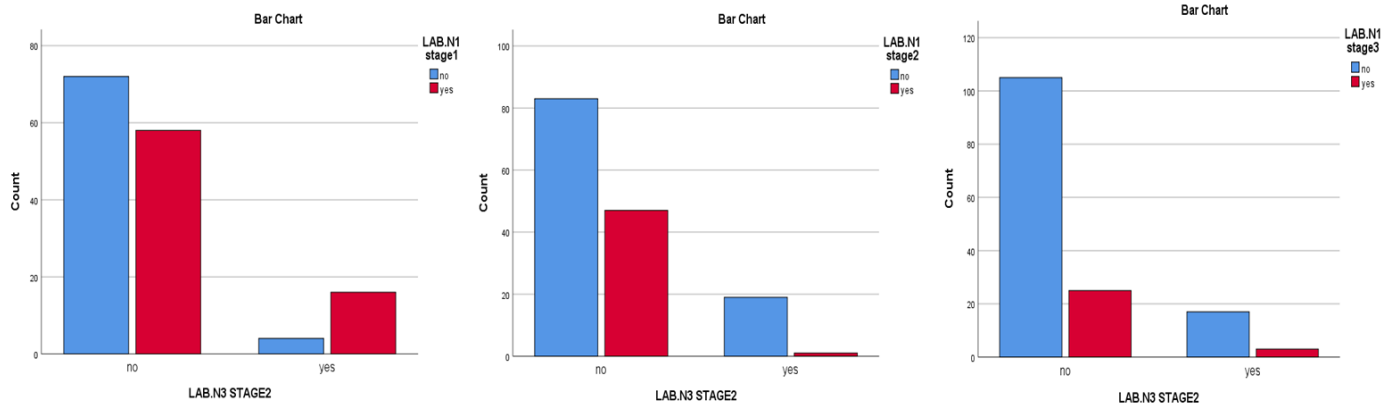
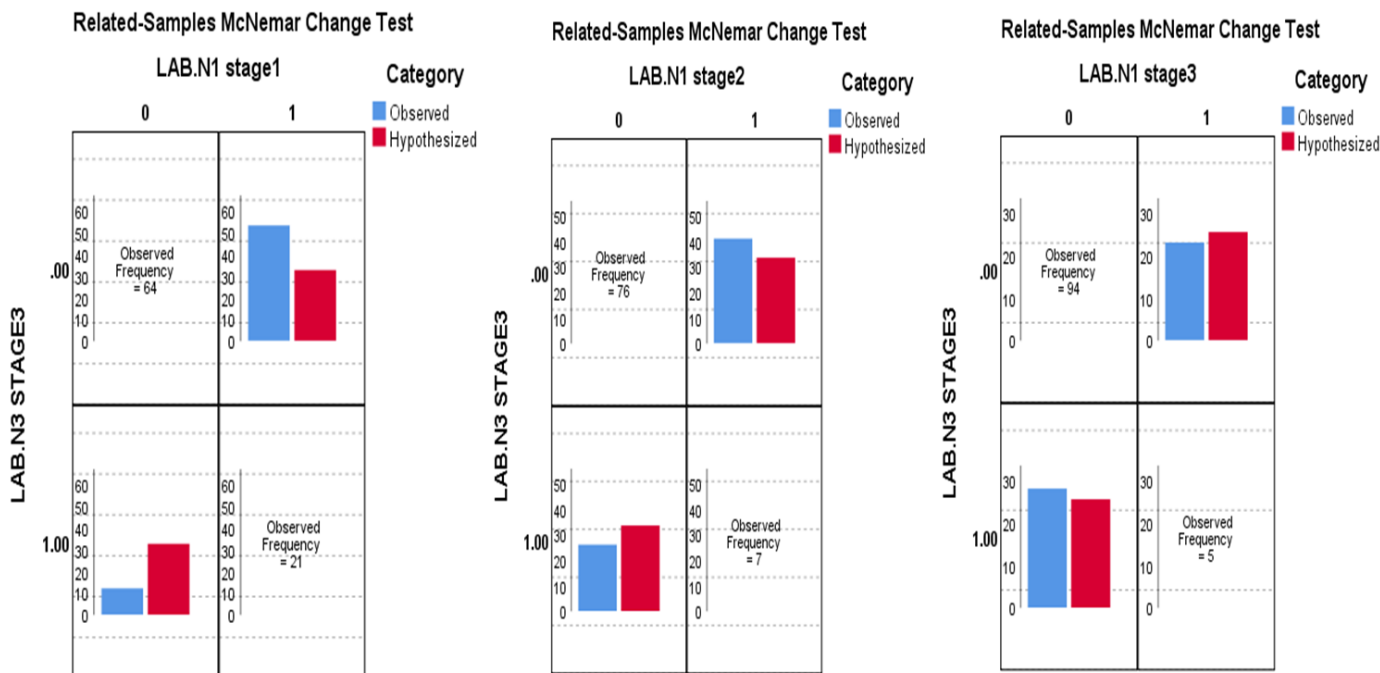


Figure 6: Bar chart to the frequency between normal femur T-score(LAB.N1 stage1), osteopenia femur T-score(LAB.N1 stage2), osteoporosis femur T-score(LAB.N1 stage3) and CC genotype.



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Figure 7: Related-Samples McNemar Change Test between GC genotype and normal femur T-score(LAB.N1 stage1), osteopenia femur T-score(LAB.N1 stage2), osteoporosis femur T-score(LAB.N1 stage3).

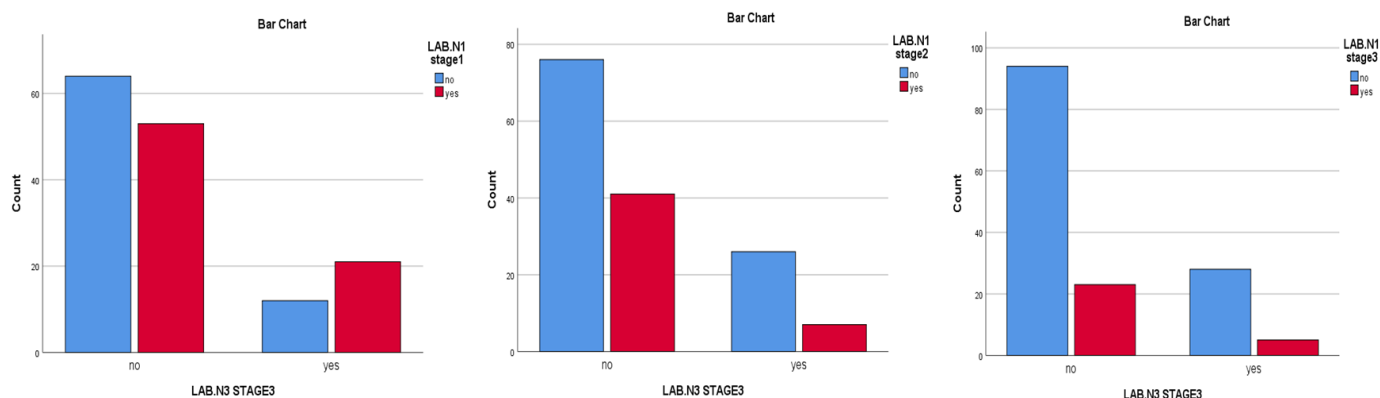


Figure 8: Bar chart to the frequency between normal femur T-score(LAB.N1 stage1) osteopenia femur T-score(LAB.N1 stage2), osteoporosis femur T-score(LAB.N1 stage3) and GC genotype

DISCUSSION:

It is the first study of its kind in the world to link the genotypes of LRP5rs121908669 (G171R) with femur T-score values. There is only one study related to this SNP.

The significant chance of occurrence of GG genotype in normal, osteopenia, osteoporosis femur T –score values are 2.5%, 0.00%, 0.00%, respectively. So, the null hypothesis is rejected and the alternative hypothesis is retained which indicates that the distributions of different values (normal, osteopenia, osteoporosis) femur T –score across GG genotype are significantly variant with a confidence level of 95% or more ($p \leq .05$), table.4, figure.3.

There are significant correlations between normal, osteopenia femur T –score values and GG genotype with $\chi^2 = 13.750$, $p = .000 < .05$, $\chi^2 = 10.765$, $p = .001 < .05$, respectively. There is no significant correlation between osteoporosis femur T –score values and GG genotype with $\chi^2 = .689$, $p = .407 > .05$, table.4.

The odds for the presence of normal, osteopenia, and osteoporosis femur T –score values were *.1830 times greater, * .366 times less, and *.732 times less, respectively when GG genotype exists compare with their odd without GG genotype exists, table.4, figure.4.

GG genotype is a factor that reduces the risk of osteopenia and osteoporosis and increases the likelihood of obtaining a normal

BMD. This corresponds to Van Wesenbeeck E et al. 's study2003 [7].

The significant chance of occurrence of CC genotype in normal, osteopenia, and osteoporosis femur T –score values are 0.00%, 0.01%, 28%, respectively. So, the null hypothesis is rejected and the alternative hypothesis is retained which indicates that the distributions of CC genotype across normal and osteopenia, respectively, are significantly variant with a confidence level of 95% or more ($p \leq .05$). the null hypothesis is retained which indicates that the distributions of CC genotype across osteoporosis femur T –score values are equally likely with a confidence level of 95% or more ($p \leq .05$), table.4, figure.5.

There are significant correlation between normal and osteopenia femur T –score values and CC genotype $\chi^2= 8.683$, $p = .003 < .05$, $\chi^2= 7.731$, $p = .005 < .05$, respectively. There is no significant correlation between osteoporosis femur T –score values and CC genotype $\chi^2=.204$, $p = .651 > .05$, table.4.

The odds presence of normal, osteopenia and osteoporosis femur T –score values were *.558 times less, *.7231 times greater, and *1.282 times greater, respectively when CC genotype exists compared with their odd without CC genotype exists, table.4, figure.6.

CC genotype is a factor that increases the risk of osteopenia and osteoporosis and reduces the likelihood of obtaining a normal BMD. This contradicts Van Wesenbeeck E et al. 's study2003 [7].

The significant chance of occurrence of GC genotype in normal, osteopenia, osteoporosis femur T –score values are 0.00%, 8.7%, and 57.5%, respectively. So, the null hypothesis is rejected and the alternative hypothesis is retained which indicates that the distributions of GC genotype across normal femur T- scores are significantly variant with a confidence level of 95% or more ($p \leq .05$). The null hypothesis is retained which indicates that the distributions of GC genotype across osteopenia, and osteoporosis femur T –score values, respectively, are equally likely with a confidence level of 95% or more ($p \leq .05$), table.4, figure.7.

There are no significant correlation between normal, osteopenia, or osteoporosis femur T –score values respectively, and GC genotype $\chi^2=.3.463$, $p = .063 > .05$, $\chi^2=.2.263$, $p = .133 > .05$, $\chi^2=.344$, $p = .557 > .05$, respectively, table.4.

The odds for the presence of normal, osteopenia, and osteoporosis femur T –score values were *.712 times less, *1.652 times greater, and *1.297 times greater, respectively when GC genotype exists compare with their odd without GC genotype exists, table.4, figure.8.

GC genotype is a factor that increases the risk of osteopenia and osteoporosis and reduces the likelihood of obtaining a normal BMD. This contradicts Van Wesenbeeck E et al. 's study2003 [7].

The limitations of this study are the small size of the sample, no positive control for RFLP, and the necessity to sequence all hot spots for the LRP5 gene.

CONCLUSION:

GG normal genotype increase the likelihood of normal femur BMD. Homozygous genotype CC and heterozygous genotype GC increase the likelihood of osteoporosis or osteopenia in femur bone. There are often associations in the distribution between carriers of GC or CC and carriers with osteoporosis or osteopenia in femur bone. In general, LRP5G171R is not only related to HBM, it is related to normal, osteopenia, osteoporosis. Thus, NCBI should be informed that LRP5G171R should be classified clinically as conflicting information, not pathogenic as it is classified until now. Also, Genotypes of LRP5G171R can be added as a risk factor within the FRAX application. . LRP5G171R can be both a diagnostic and a therapeutic goal for osteoporosis.

Author Contributions: Conceptualization, E.SH and H.Y. and A.H. ; methodology, E.SH., H.Y., A.H., W.A, B.H., and A.D. ; software, E.SH; validation, H.Y , A.H. , W.A, A.D., and B.H.; formal analysis, E.SH; investigation, E.SH, A.D., and B.H.; resources, E.SH; data curation, E.SH.; writing—original draft preparation, E.SH.; writing—review and editing, E.SH, H.Y., A.H., W.A ; visualization, H.Y.; A.H. supervision H.Y, A.H and A.KH.; project administration, W.A, A.H and H.Y. ; funding acquisition, E.SH. All authors have read and agreed to the published version of the manuscript.”

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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.

Ethical approval statement: The work was approved by the Ethics Committee in Syrian Ministry of Higher Education and written informed consent was obtained from all the participants according to the Declaration of Helsinki.

Informed Consent Statement: “Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patient(s) to publish this paper.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author

(Eiman M. Shahrour), upon reasonable request. All relevant material is included in this publication

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Conflict of Interests

Eiman shahrour , Bassel AL-Halabi , Amir N Dabboul, Walid Al-achkar, Abd Alrazak Hassan, Atieh Khamis, and Haissam Yazigi declare that they have no conflict of interest.

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