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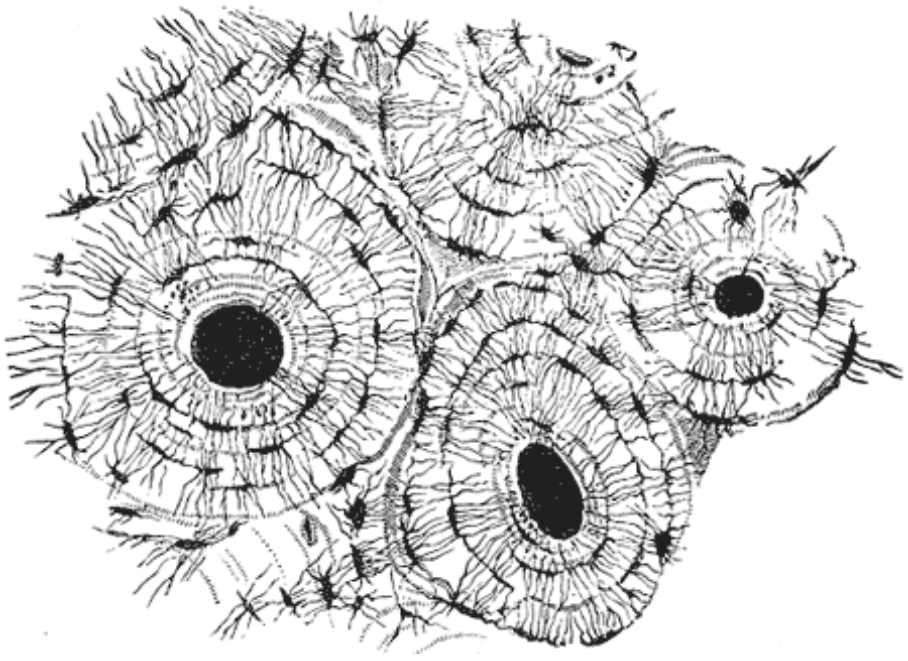
Tesis Doctoral, **2017**



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ANÁLISIS DE LA INFLUENCIA DE FACTORES GENÉTICOS Y AMBIENTALES EN EL NIVEL DE MASA ÓSEA EN ADULTOS JÓVENES

María Correa Rodríguez

Tesis Doctoral Internacional – Internacional PhD Thesis

Programa de Doctorado en Medicina Clínica y Salud Pública

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ANÁLISIS DE LA INFLUENCIA DE FACTORES GENÉTICOS Y AMBIENTALES EN EL NIVEL DE MASA ÓSEA EN ADULTOS JÓVENES

ANALYSIS OF THE INFLUENCE OF GENETIC AND ENVIRONMENTAL FACTORS ON THE LEVEL OF BONE MASS IN YOUNG ADULTS

Esta Tesis Doctoral ha sido realizada bajo la dirección de:

Dra. Blanca María Rueda Medina

Dra. Jacqueline Schmidt Rio-Valle

Universidad de Granada

Las directoras de la Tesis Doctoral, Dra. Blanca María Rueda Medina y Dra. Jacqueline Schmidt Rio-Valle, informan que los trabajos de investigación que se exponen en la memoria de la Tesis Doctoral titulada "*ANÁLISIS DE LA INFLUENCIA DE FACTORES GENÉTICOS Y AMBIENTALES EN EL NIVEL DE MASA ÓSEA EN ADULTOS JÓVENES*" han sido realizados bajo nuestra dirección por la doctoranda Dª. María Correa Rodríguez, en el Departamento de Enfermería de la Universidad de Granada, encontrándola conforme para ser presentada y aspirar al Grado de Doctor por el tribunal que en su día se designe.

Garantizamos, al firmar esta Tesis Doctoral, que el trabajo ha sido realizado por la doctoranda bajo la dirección de las directoras de la Tesis y hasta donde nuestro conocimiento alcanza, en la realización del trabajo, se han respetado los derechos de otros autores a ser citados, cuando se han utilizado sus resultados o publicaciones.

Dra. Blanca María Rueda Medina

Dra. Jacqueline Schmidt Rio-Valle

La doctoranda

María Correa Rodríguez

Memoria presentada por Dª María Correa Rodríguez para optar al grado de Doctor por la Universidad de Granada.

El presente trabajo de investigación ha sido realizado bajo la supervisión de la Dra. Blanca María Rueda Medina y la Dra. Jacqueline Schmidt Rio-Valle.

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A mis padres

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ABREVIATURAS

ADN	Ácido desoxirribonucleico
AD-SOS	Velocidad del sonido de amplitud dependiente (*Amplitude dependent speed of sound*)
ANCOVA	Análisis de la covarianza
ARN	Ácido ribonucleico
BH	Benjaminin y Hochberg
BMC	Contenido mineral óseo (*Bone mineral content*)
BMU	Unidades básicas multicelulares (*Basic multicelular units*)
BUA	Atenuación ultrasónica de banda ancha (*Broadband ultrasound attenuation*)
CI	Intervalo de confianza
CNV	Variación del número de copias (*Copy number variations*)
DAQS	Índice de calidad antioxidante de la dieta (*Dietary antioxidant quality score*)
DE	Desviación estándar
DMO	Densidad mineral ósea
DPA	Absorciometría fotónica dual (*DPA-dual photon absorciometry*)
DXA	Absorciometría de rayos X de doble energía (*Dual energy X ray absorciometry*)
eQTL	Locus regulador de la expresión (*Expression quantitative trait loci*)
GEFOS	Factores genéticos de la osteoporosis (*Genetic factors for osteoporosis*)
GENYO	Centro Pfizer-Universidad de Granada-Junta de Andalucía de genómica e investigación oncológica
GH	Hormona de crecimiento
GSK3β	Glucógeno-sintetaso-kinasa 3β
GWAS	Estudio de asociación del genoma completo (*Genome-wide association study*)
IL	Interleucina
IMC	Índice de masa corporal

IPAQ Cuestionario internacional de actividad física (*International physical activity questionnaire*)

ISCD Sociedad internacional de densitometría clínica (*International society for bone densitometry*)

LD Desequilibrio de ligamiento

LDL Lipoproteína de baja densidad

MAF Frecuencia del alelo menor

MROS Fracturas osteoporóticas en hombres (*Osteoporotic fractures in men*)

OMS Organización mundial de la salud

PCP Polaridad celular planar

PCR Reacción en cadena de la polimerasa

pDXA Absorciometría de rayos X de doble energía periférica (*Pheripheral dual energy X ray absorciometry*)

PMO Pico de masa óseo

pQCT Tomografía computarizada periférica (*Pheripheral quantitative computed tomography*)

PTH Hormona paratiroidea o paratohormona

PTHrP Proteína relacionada con la hormona paratiroidea

QCT Tomografía computarizada (*Quantitative computed tomography*)

QMR Resonancia magnética cuantitativa (*Quantitative magnetic resonance*)

QUI Índice cuantitativo ultrasónico (*Quantitative ultrasound index*)

QUS Ultrasonido óseo cuantitativo (*Quantitative ultrasound*)

RA Absorciometría radiográfica (*Radiographic absorciometry*)

SEIOMM Sociedad española de investigación en osteoporosis y metabolismo mineral

SI Índice de consistencia (*Stiffness index*)

SNP Polimorfismo de un solo nucleótido (*Single nucleotide polymorphism*)

SOS Velocidad del sonido (*Speed of sound*)

SPA Absorciometría fotónica simple (*Single photon absorptiometry*)

Tag-SNPs Marca de polimorfismo de un solo nucleótido (SNPs-etiqueta)

TGF-beta	Factor de crecimiento transformante beta
TNF	Factor de necrosis tumoral
US	Ultrasonidos
UVB	Irradiación solar ultravioleta tipo B
VNTR	Variable de repeticiones en tándem (*Variable number tandem repeats*)
µMR	Microrresonancia magnética (*Magnetic resonance microscopy*)

ABREVIATURAS GENES

CCDC170	Dominio enrollado en bucle que contiene 170 (*Coiled-coil domain containing 170*)
DKK1	Proteína relacionada con Dickkopf 1 (*Dickkopf-related protein 1*)
ESR1	Receptor estrógeno 1 (*Estrogen receptor 1*)
ESR2	Receptor estrógeno 2 (*Estrogen receptor 2*)
GPATCH1	Dominio parche-G que contiene 1 (*G-patch domain containing 1*)
LRP5	Receptor de la lipoproteína de baja densidad 5
LRP6	Receptor de la lipoproteína de baja densidad 6
OPG	Osteoprotegerina
RANK	Receptor activador del factor nuclear κ B
RANKL	Ligando de receptor activador para el factor nuclear κ B
RSPO3	R-espondina 3 (*R-spondin 3*)
SOST	Esclerostina
SPTBN1	Espectrina beta, no eritrocítica 1 (*Spectrin beta, non-erythrocytic 1*)
TMEM135	Proteína transmembrana 135 (*Transmembrane protein 135*)
VDR	Receptor de la vitamina D
WNT16	Miembro de la familia Wnt 16 (*Wnt family member 16*)
WNT5	Miembro de la familia Wnt 5 (*Wnt family member 5*)

RESUMEN

La osteoporosis es una enfermedad ósea caracterizada por la disminución de la densidad mineral ósea (DMO), el deterioro de la microarquitectura del hueso y el incremento del riesgo de fracturas. Está bien establecido que la osteoporosis es una enfermedad compleja determinada por múltiples factores genéticos y ambientales como la nutrición, la actividad física o la composición corporal entre otros. La adquisición del pico de masa óseo (PMO), definido como la cantidad de masa ósea alcanzada al final de la maduración esquelética, se ha identificado como un factor predictor del riesgo de osteoporosis.

En la valoración de la masa ósea, el ultrasonido óseo cuantitativo (QUS) se ha propuesto como una técnica apropiada para la estimación de la salud ósea. La técnica QUS proporciona información de la arquitectura ósea reflejando así su microarquitectura, elasticidad y conectividad. Además, es un método no invasivo, conveniente, portátil y de bajo coste económico. Los parámetros de ultrasonido cuantitativo están determinados por factores genéticos, con una heredabilidad estimada del 74%. No obstante, la mayoría de los estudios que han investigado previamente la influencia de los factores genéticos en los parámetros óseos, se han centrado principalmente en la densidad mineral ósea (DMO) evaluada mediante absorciometría de rayos X de doble energía (DXA). Por otro lado, numerosos factores modificables como la actividad física, la ingesta nutricional o la composición corporal se han postulado como determinantes en la mineralización ósea. Sin embargo, el papel de los factores genéticos y ambientales en las propiedades óseas determinadas mediante QUS no se ha investigado en profundidad. Además, un número muy limitado de estudios previos han analizado los marcadores genéticos y ambientales que influyen en el estatus óseo en la adultez temprana, un periodo crucial para la adquisición del PMO. Teniendo en cuenta todas estas evidencias, el objetivo de esta Tesis Doctoral fue avanzar en el conocimiento sobre los factores genéticos y ambientales que

podrían estar influyendo en la adquisición de la masa ósea en las etapas tempranas de la vida.

Mediante una estrategia de selección de genes candidatos se investigó el posible papel de los genes *VDR, ESR1, RANKL, RANK, OPG, LRP5, SOST, WNT16, SPTBN1, RSPO3, CCDC170, DKK1, GPATCH1 y TMEM135* como nuevos marcadores genéticos en la adquisición de masa ósea en adultos jóvenes. Los resultados obtenidos sugieren que variantes genéticas de los genes *WNT16*, *LRP5* y *RSPO3* podrían jugar un papel relevante en la adquisición del PMO en edades tempranas. En cambio, nuestros resultados no evidenciaron ninguna asociación significativa entre variantes genéticos del *VDR, SOST, ESR1, RANKL, RANK, OPG, SPTBN1, CCDC170, MBL2/DKK1, TMEM135 y GPATCH1* y el parámetro de ultrasonografía, sugiriendo que estos marcadores genéticos no serían determinantes en los niveles de mineralización en etapas tempranas. Además, tras realizar un estudio de interacción génica se pudo observar una interacción entre el SNP rs9340799 del gen *ESR1* y el SNP rs3736228 del gen *LRP5* que alcanzó un alto grado de significación estadística.

En relación a los factores ambientales, los hallazgos del presente trabajo sugieren que la masa magra y la actividad física intensa son importantes determinantes de la masa ósea en adultos jóvenes. Además, nuestros resultados apuntan que la ingesta de antioxidantes de alta calidad puede influir en los niveles de masa ósea en mujeres jóvenes.

En conclusión, los datos obtenidos en la presente Tesis Doctoral muestran que tanto los factores genéticos (genes *WNT16*, *LRP5* y *RSPO3)* como ambientales (nivel de actividad física, masa magra e ingesta de antioxidantes) estarían jugando un papel clave en la adquisición de la masa ósea en la adultez temprana. Así, en base al conocimiento actual se puede comenzar a esbozar un modelo de factores implicados en los niveles de masa ósea en adultos jóvenes. La identificación y caracterización de los marcadores genéticos y

factores ambientales involucrados facilitará el desarrollo de nuevas estrategias terapéuticas y actuaciones preventivas frente a la osteoporosis en esta etapa.

SUMMARY

Osteoporosis is a skeletal disease characterised by diminished bone mineral density (BMD), deterioration in bone microarchitecture, and an increased risk of fracture. It is well established that osteoporosis is a complex disease determined by multiple genetic and environmental factors such as nutrition intake, physical activity and body composition among others, modulating individual susceptibility. The acquisition of peak bone mass (PBM), defined as the amount of bone tissue present at the end of the skeletal maturation, has been identified as a predictor of the risk of osteoporosis.

In assessing bone mass, quantitative ultrasound (QUS) has been proposed as an appropriate technique for estimating bone health. The QUS technique provides information on bone architecture reflecting its microarchitecture, elasticity and connectivity. In addition, it is a non-invasive, convenient, portable and inexpensive method. Quantitative ultrasound parameters are determined by genetic factors, with an estimated heritability of 74%. However, most studies that have previously investigated the influence of genetic factors on bone parameters have mainly focused on bone mineral density (BMD) assessed by dual energy X-ray absorptiometry (DXA). On the other hand, numerous modifiable factors such as physical activity, nutritional intake or body composition have been postulated as determinants in bone mineralization. However, the role of genetic and environmental factors in the bone properties determined by QUS has not been investigated in depth. In addition, a limited number of previous studies have analysed genetic and environmental markers that influence bone status in early adulthood, a crucial period for PMO acquisition. Taking into account all this evidence, the objective of this Doctoral Thesis was to advance knowledge about the genetic and environmental factors that could be influencing the acquisition of the bone mass in the early stages of life.

The possible role of the *VDR*, *ESR1, RANKL, RANK, OPG, LRP5, SOST, WNT16, SPTBN1, RSPO3, CCDC170, DKK1, GPATCH1* and *TMEM135* genes as novel genetic markers in the acquisition of bone mass were investigated using a candidate gene approach. The results suggest that genetic variants of the *WNT16, LRP5* and *RSPO3* genes could play a relevant role in the acquisition of PBM at early ages. In contrast, our results did not show any significant association between genetic variants of *VDR, SOST, ESR1, RANKL, RANK, OPG, SPTBN1, CCDC170, MBL2/DKK1, TMEM135* and *GPATCH1* and ultrasonography parameter, suggesting that these genetic markers are not determinants of bone mineralization at an early life stage. In addition, in the interaction analysis, we identified an interaction between rs9340799 SNP of the *ESR1* gene and rs3736228 SNP of the *LRP5* gene, which reached a high level of statistical significance.

Regarding environmental factors, the findings of this study suggest that lean mass and intense physical activity are important determinants of bone mass in young adults. In addition, our results indicate that the intake of high-quality antioxidants may influence bone mass in young women.

In conclusion, the data obtained in this Doctoral Thesis show that genetic factors (*WNT16, LRP5* and *RSPO3* genes) and environmental factors (physical activity, lean mass and antioxidant intake) may play a key role in the acquisition of bone mass in early adulthood. Thus, based on current knowledge, it is possible to outline a model of factors involved in bone mass in young adults. The identification and characterisation of the genetic markers and environmental factors involved will promote the development of new therapeutic strategies and preventive actions against osteoporosis at this stage.

I. INTRODUCCIÓN

1. EL TEJIDO ÓSEO

1.1. Componentes del tejido óseo

El hueso es un tejido conjuntivo especializado que ejerce fundamentalmente funciones de protección, soporte y metabolismo al ser un reservorio iónico (Robling, Castillo, & Turner, 2006). En función de su estructura macroscópica se distinguen dos tipos de hueso: el denominado cortical o compacto y el hueso trabecular o esponjoso (Clarke, 2008). El hueso cortical o compacto está constituido por una masa densa y sólida que constituye aproximadamente el 80% del esqueleto. Por el contrario, el hueso trabecular o esponjoso, que conforma el 20% restante del esqueleto, está formado por una red tridimensional de estructuras ramificadas denominadas trabéculas. El hueso trabecular es metabólicamente más activo que el compacto, con una tasa de renovación anual del 25%, frente al 2-3% del hueso compacto. Así, el hueso trabecular es más sensible a los cambios bioquímicos, hormonales y nutricionales por lo que es más susceptible de sufrir mayores pérdidas de tejido.

El tejido óseo está constituido por una matriz extracelular y por elementos celulares (Buckwalter, Glimcher, Cooper, & Recker, 1996). La matriz ósea está formada por un componente orgánico formado fundamentalmente por fibras colágenas tipo I, junto con proteínas reguladoras (osteocalcina, osteopontina u osteonectina) y un componente inorgánico constituido por cristales de calcio y fósforo denominados hidroxiapatita (Aszódi, Bateman, Gustafsson, Boot-Handford, & Fässler, 2000). El componente celular óseo lo constituyen las células osteoprogenitoras, los osteoblastos, los osteocitos y los osteoclastos (Figura 1). Las células osteoprogenitoras o células madre óseas son células indiferenciadas de origen mesenquimal con capacidad diferenciadora a osteoblastos. Los osteoblastos se originan a partir de las células osteoprogenitoras y son responsables de la producción de componentes de la matriz ósea (Capulli, Paone, & Rucci, 2014). Los osteocitos conforman el 90% del total de las células en el hueso y se originan a partir de

los osteoblastos, que han perdido la capacidad de síntesis y quedan embebidos en la matriz ósea calcificada (Franz-Odendaal, Hall, & Witten, 2006). Por último, los osteoclastos son células multinucleadas que derivan de células hematopoyéticas de la línea monocito-macrófago, cuya principal función es la resorción ósea (Boyle, Simonet, & Lacey, 2003).

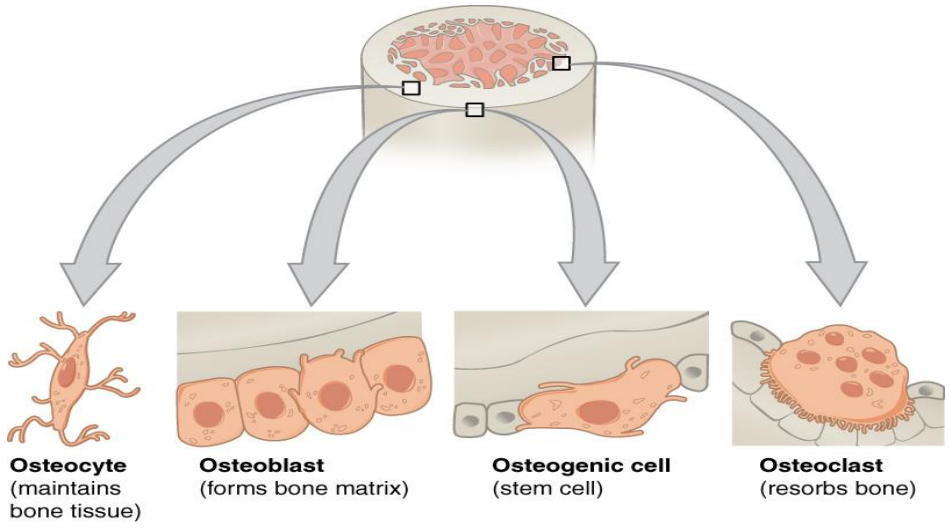


Figura 1. Células óseas.

Representación de los cuatro tipos de células que se encuentran en el tejido óseo. Las células osteogénicas son indiferenciadas y se convierten en osteoblastos. Cuando los osteoblastos quedan atrapados dentro de la matriz calcificada, su estructura y función cambian, y se convierten en osteocitos. Los osteoclastos se desarrollan a partir de monocitos y macrófagos y difieren en apariencia de otras células óseas. Ilustración de Anatomy & Physiology. Fuente: http://cnx.org/content/col11496/1.6/

1.2. Remodelado óseo

El hueso es un tejido dinámico que se encuentra en un proceso continuo de destrucción y formación denominado remodelado óseo. Anualmente se renueva aproximadamente el 10% del esqueleto. El proceso de remodelado óseo es imprescindible para mantener las funciones del esqueleto, así como para reparar microfracturas. A nivel microscópico el remodelado óseo se produce en las denominadas unidades básicas multicelulares *(BMU - Basic multicelular units),* que integran grupos de osteoblastos y osteoclastos (Andersen et al., 2009). La acción de las BMUs constituye un

proceso cíclico de remodelado óseo que consta de las siguientes fases sucesivas (Sims & Gooi, 2008) (Figura 2):

1. **Fase de activación.** Proceso determinado por la presencia de microfracturas o algún tipo de estrés mecánico o químico por el cual factores locales y sistémicos activan el ciclo de remodelado, a través de la estimulación y activación de los osteoblastos. Así se produce la activación, migración y diferenciación de células hematopoyéticas precursoras de la estirpe osteoclástica. Así surgen los osteoclastos maduros multinucleados.

2. **Fase de resorción.** Se inicia por los osteoclastos y tiene una duración aproximada de 1-3 semanas. Como resultado final de la resorción osteoclástica, se generan unas cavidades en el hueso que reciben el nombre de lagunas Howship en el hueso trabecular o canal Haversiano en el hueso cortical. Cuando finaliza el periodo resortivo, el osteoclasto entra en apoptosis.

3. **Fase de inversión.** Entre el período resortivo y formativo tiene lugar una fase intermedia de aparente inactividad, que tiene una duración de 1-2 semanas. En esta fase, se deposita una capa de material rico en glicoproteínas sobre la superficie resorbida que se denomina "línea de cementación", sobre la cual los osteoblastos formarán la nueva matriz.

4. **Fase de formación.** Los precursores osteoblásticos se diferencian en osteoblastos maduros y ocupan la zona excavada por los osteoclastos depositando osteoide, una sustancia orgánica que posteriormente se mineralizará. Esta fase dura 1-3 meses. Una vez que la nueva unidad estructural ósea está completamente formada, finaliza esta fase. La superficie ósea se cubre de células delimitantes aplanadas. Posteriormente,

se inicia un tiempo de reposo o **fase quiescente** hasta que comienza un nuevo ciclo de remodelado.

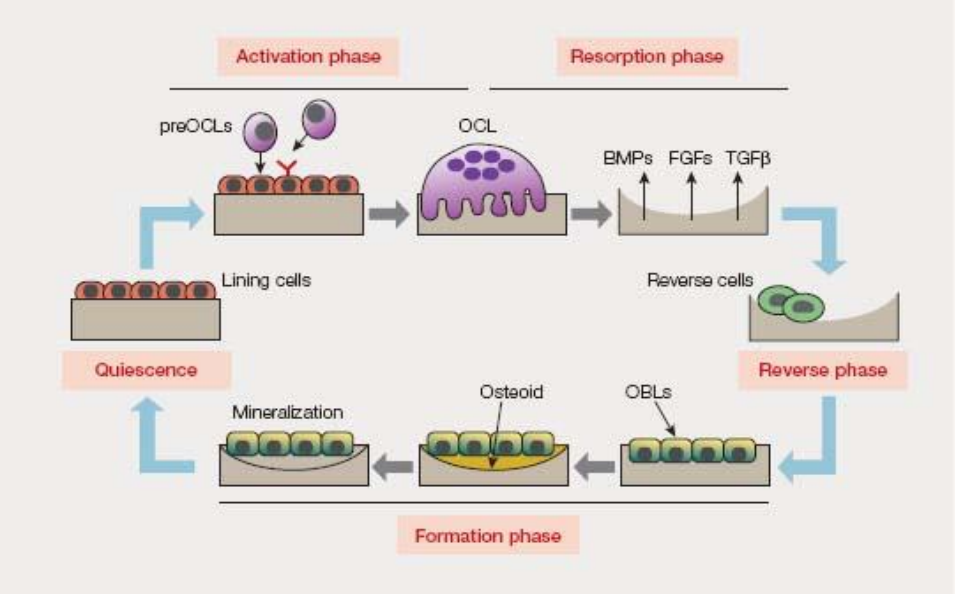


Figura 2. El proceso de remodelado óseo.

La remodelación ósea comienza con la activación de las células de revestimiento, que aumentan la expresión de RANKL. RANKL interactúa con su receptor RANK, desencadenando así la diferenciación osteoclástica (fase de activación). Los osteoclastos reabsorben el hueso (fase de resorción), permitiendo así la liberación de factores habitualmente almacenados en la matriz ósea (BMPs, TGFβ, FGFs) que reclutan osteoblastos en el área resorbida. Una vez reclutados, los osteoblastos producen la nueva matriz ósea y promueven su mineralización (fase de formación), completando así el proceso de remodelación ósea. Abreviaturas: BMPs, proteínas morfogenéticas óseas; FGF, factores de crecimiento de fibroblastos; Pre-OCLs, preosteoclastos; OCL, osteoclastos; OBLs, osteoblastos; TGFβ, factor de crecimiento transformante.
Fuente:http://www.medicographia.com/wp-content/pdf/Medicographia105.pdf
(Albergaria, 2010).

1.2.1. Regulación del proceso de remodelado óseo

El remodelado óseo es un proceso complejo, controlado por una red de factores conectados entre sí que se produce a lo largo de toda la vida. El proceso se encuentra regulado por factores locales (citoquinas inflamatorias: IL-1,TNFI, IL-6, proteínas morfogenéticas del hueso, el factor de crecimiento transformador beta (TGF beta) o la proteína relacionada con la hormona paratiroidea - PTHrP) así como por factores

sistémicos que se agrupan en: factores hormonales (hormona paratiroidea-PTH, calcitonina, insulina, leptina, hormona del crecimiento-GH, vitamina D, glucocorticoides, estrógenos, andrógenos y hormas tiroideas, como las más representativas) y factores mecánicos (Phan, Xu, & Zheng, 2004) (Figura 3).

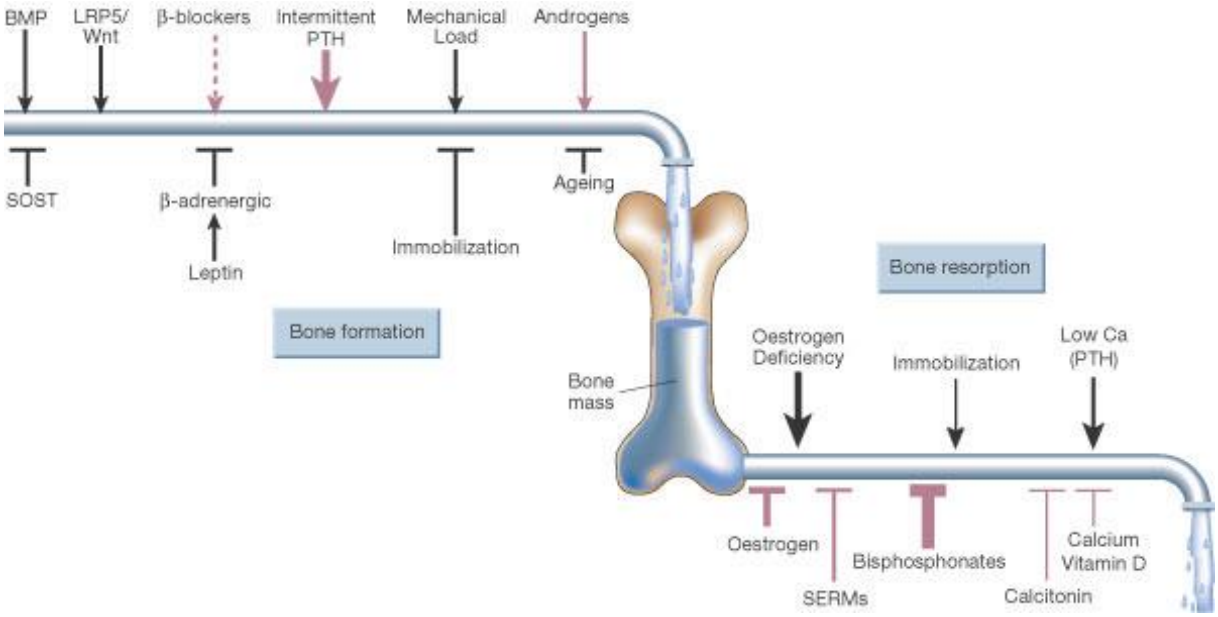


Figura 3. Factores reguladores de la masa ósea.

Representación esquemática del sistema que mantiene los niveles de masa ósea. Se enumeran los estimuladores fisiológicos (azul) y farmacológicos (naranja) y los inhibidores de la formación y la resorción ósea. El impacto relativo, si se conoce, está representado por el grosor de las flechas. Las líneas continuas son las terapias actuales y las líneas de puntos son las posibles terapias. Abreviaturas: BMP, proteína morfogenética ósea; SOST, esclerostina; LRP5, lipoproteína de baja densidad (LDL) relacionados con la proteína del receptor 5-; PTH, hormona paratiroidea; SERM, modulador selectivo del receptor de estrógeno (Harada & Rodan, 2003).

A continuación, se describen las principales vías moleculares que regulan el complejo proceso de remodelado óseo.

- **Sistema RANKL/RANK/OPG**

El sistema RANKL/RANK/OPG juega un papel fundamental en la vía de la osteoclastogénesis involucrada en el proceso de remodelación ósea (Boyce & Xing, 2008) (Figura 4). Cuando el RANKL *(receptor activator of nuclear factor KB ligand)*, secretado por

los osteoblastos, interacciona con su receptor RANK *(receptor activator of nuclear factor KB)*, presente en la membrana de los progenitores de los osteoclastos, se activan diversas vías de señalización en el precursor de osteoclastos que inducen a la expresión de genes, inhibiendo la apoptosis de los osteoclastos y promoviendo su diferenciación y activación, induciendo así la resorción ósea.

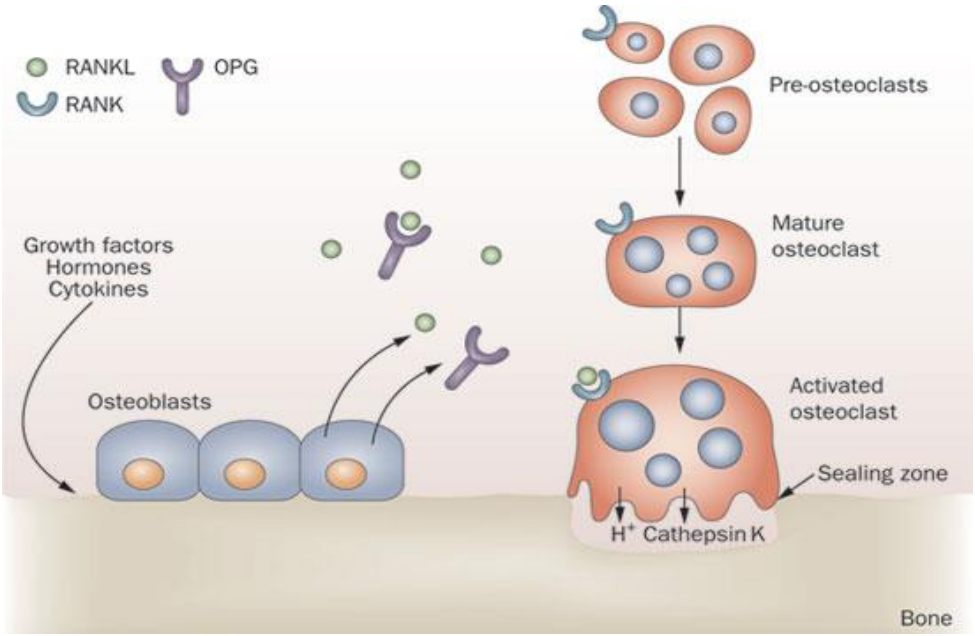


Figura 4. El sistema RANKL-RANK-OPG en el remodelado óseo.

RANKL expresado por células de linaje de osteoblasto se une a RANK en la superficie de pre-osteoclastos y osteoclastos maduros, lo que resulta en aumento de la resorción ósea a través de un aumento en la diferenciación de los osteoclastos, la actividad y la supervivencia. OPG es un 'receptor de señuelo', también producido por osteoblastos, que se une a RANKL, impidiendo la unión de RANKL a RANK e inhibiendo así la resorción ósea. El balance de RANKL y OPG determina la tasa final de reabsorción ósea. (Lewiecki, 2011)

Por otro lado, los osteoblastos también producen osteoprotegerina (OPG), una proteína soluble que forma parte de la superfamilia de los receptores del factor de necrosis tumoral (TNF). La OPG es un inhibidor que se fija al RANKL e impide la interacción de este con su receptor RANK. Este bloqueo conlleva la inhibición de la formación de los osteoclastos y, en consecuencia, la resorción ósea.

- **Vía de señalización Wnt**

La vía de señalización Wnt se ha establecido como una de las más relevantes en la modulación de la actividad osteoblástica (Krishnan, Bryant, & Macdougald, 2006). En la vía Wnt participan una serie de proteínas, que regulan distintos aspectos del metabolismo óseo, activando dos grandes vías intracelulares: la vía beta-catenina, vía clásica o canónica y la vía no canónica. La vía canónica Wnt, es la más conocida y la que regula principalmente la formación ósea (Baron & Rawadi, 2007). La vía canónica de señalización depende fundamentalmente de la estabilidad de la beta-catenina en el citoplasma del osteoblasto y su posterior traslocación al núcleo, donde modula la transcripción de varios genes. En condiciones basales, cuando la vía Wnt no se encuentra activada, la beta-catenina es fosforilada por diferentes quinasas, fundamentalmente la glucógeno-sintetaso-kinasa 3β (GSK3β), y degradada en los proteosomas. Así, los niveles intracelulares de beta-catenina se mantienen bajos. No obstante, cuando se activa la vía Wnt, por la unión de los ligandos Wnt a su receptor, se inhibe la actividad fosforilativa de la GSK3β impidiendo la degradación de la beta-catenina. Así, la beta-catenina se acumula en el citoplasma del osteoblasto y se trasloca al núcleo, donde regula la expresión de genes de la vía canónica Wnt (Figura 5). Los ligandos Wnt actúan a través de la fijación a un complejo co-receptor de la membrana celular, consistente en un receptor frizzled (Fz) y en una proteína relacionada con el receptor LDL (low density lipoprotein receptor-related protein 5, 6: LRP5/6). La esclerotina (SOST) es una molécula inhibidora que se fija a LRP5/6, impidiendo la formación del complejo LRP5/6-*frizzled*-Wnt (Moester, Papapoulos, Löwik, & Van Bezooijen, 2010). Otras moléculas antagonistas son las proteínas de la familia *dikkopf,* destacando el tipo 1 (DKK1) (Morvan et al., 2006). La unión Dkk a LRP5/6 inhibe la vía canónica de señalización Wnt mediante la reducción del número de receptores LRP.

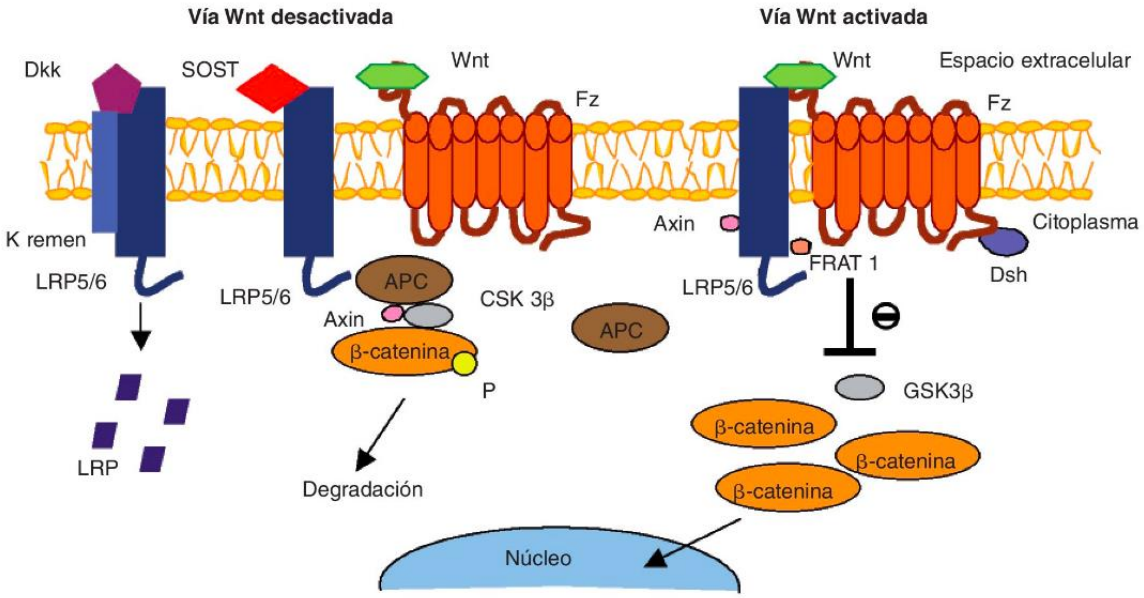


Figura 5. Vía de señalización beta-catenina.

Vía de señalización Wnt en el osteoblasto. Cuando la vía está activada, Wnt forma un complejo con el receptor frizzled (Fz) y LRP5/6; beta-catenina se disocia de una serie de cofactores (APC, Axin y GSK3β), impidiendo así su fosforilación. Los cofactores Axin, Frat1 y Dsh provocan una inhibición en la enzima glucógeno-sintetaso-quinasa (GSK3β), lo que permite alcanzar un nivel estable de beta-catenina en el citosol, que es traslocada al núcleo, donde activa genes que promueven la diferenciación del osteoblasto. En la forma opuesta a la anterior, la proteína Fz no está unida al ligando Wnt y no se produce señal de activación. Los factores inhibidores Dkk y SOST pueden acoplarse a LRP5/6 y bloquear la señal Wnt; en esta situación, no se produce la inhibición de GSK3β y la beta-catenina es fosforilada (P) y transportada al proteosoma para su degradación. APC: factor supresor tumoral (adenomatous polyposiscoli tumor) (Escobar-Gómez, Jódar, & Hawkins, 2009).

Por otro lado, existen otras vías alternativas denominadas no canónicas, que incluyen la vía dependiente del calcio (Wnt/Ca^2) y la vía de polaridad celular planar (PCP). Diferentes proteínas WNT, como WNT5a o WNT16, parece que son capaces de activar la osteoclastogénesis a través de estas vías no-canónicas (Baron & Kneissel, 2013). No obstante, el papel en el metabolismo óseo de estos mecanismos de señalización es menos conocido.

- **Estrógenos**

Los estrógenos desempeñan un papel determinante en la regulación ósea (Khosla, Oursler, & Monroe, 2012) (Figura 6). A nivel molecular, los estrógenos llevan a cabo sus funciones a través de las dos isoformas del receptor estrogénico, alfa y beta. El mecanismo de acción de los estrógenos se produce por la interrelación ligando-receptor y la presencia de diferentes co-activadores o represores, que determinan la respuesta específica de la célula (Imai et al., 2009). En los osteoclastos, los estrógenos disminuyen la resorción ósea mediante la regulación de factores pro-resortivos que son inhibidos, (RANKL, IL1, IL6, TNF) y factores anti-resortivos que son estimulados (TGF, OPG). Además, los estrógenos inducen la apoptosis, lo que lleva a la disminución del número de los osteoclastos (Kameda et al., 1997). Sobre los osteoblastos, se ha observado que los estrógenos producen un aumento de su formación y proliferación y una disminución de su apoptosis. Los estrógenos también disminuirían la apoptosis en el caso de los osteocitos. El déficit de estrógenos se asocia con un desequilibrio entre los procesos de resorción y formación ósea. Aunque pueden existir múltiples mecanismos, los efectos de los estrógenos sobre la disminución de la apoptosis osteoblástica, del estrés oxidativo y la actividad NF-kB (RANKL) parecen ser mediadores clave para la formación ósea (Khosla et al., 2012).

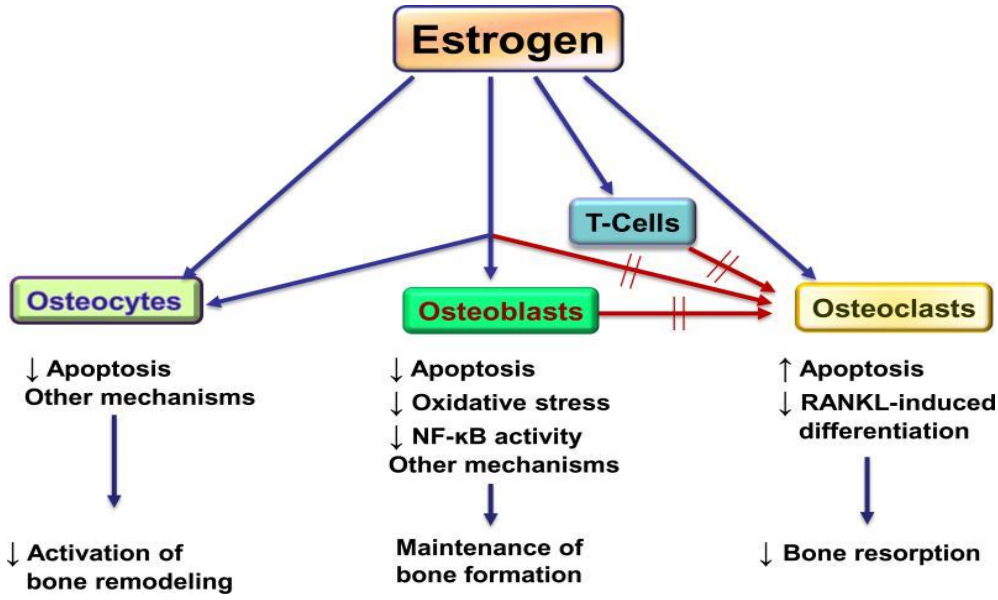


Figura 6. Modelo de trabajo de los estrógenos en la regulación del recambio óseo a través de los efectos sobre osteocitos, osteoblastos, y osteoclastos (Khosla et al., 2012).

- **Vitamina D**

La forma activa de la vitamina D (1,25 (OH) 2 D_3) ejerce su función biológica a través de la unión a su receptor (VDR) que se encuentran en los osteoblastos. A nivel celular, el complejo 1,25 (OH) 2 D_3-receptor de la vitamina D actúa como factor de transcripción promoviendo la diferenciación de los osteoblastos así como la regulación de la producción de proteínas tales como el colágeno, la fosfatasa alcalina y la osteocalcina, fundamentales en el proceso de remodelación ósea (Bikle, 2012). Además, la 1,25 (OH) 2 D_3 induce la expresión del RANKL en la membrana celular de los osteoblastos e inhibe la secreción de OPG, regulando tanto la formación como la resorción ósea (Yamamoto et al., 2013).

1.3. Ganancia de hueso: pico de masa óseo

El remodelado óseo es un proceso que tiene lugar durante la etapa del crecimiento y la vida adulta. La diferencia entre la cantidad de hueso que se destruye y la que se forma

se conoce como balance óseo, siendo su valor positivo durante el crecimiento hasta el alcanzar el denominado pico de masa óseo (PMO). El PMO se define como la cantidad máxima de hueso adquirida al final de la maduración esquelética (Heaney et al., 2001; Hendrickx, Boudin, & Van Hul, 2015) (Figura 7). El PMO se adquiere entre los 20 y 30 años de edad, produciéndose esta adquisición más prematuramente en las mujeres. No obstante, en los hombres el PMO es aproximadamente un 20-30% superior (Berger et al., 2010; Matkovic et al., 1994).

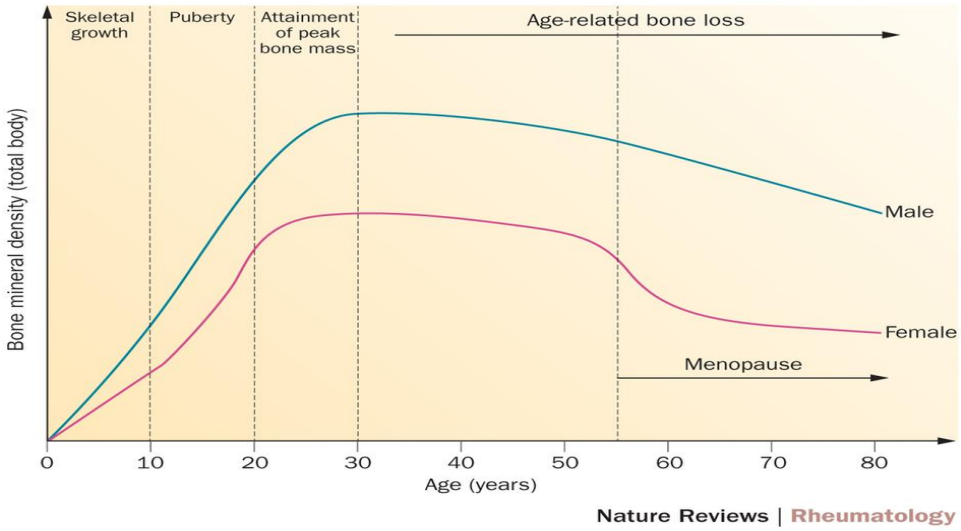


Figura 7. Descripción general de los valores de masa ósea durante la vida, destacando la importancia del PMO y posterior tasa de disminución de la masa ósea.

Cuanto menor sea el valor de PMO, mayor será el riesgo de fragilidad ósea en etapas posteriores de la vida. Factores relacionados con la edad como la menopausia en las mujeres, el estilo de vida y los antecedentes genéticos del individuo, serán también determinantes del riesgo de fractura osteoporótica (Hendrickx et al., 2015).

El PMO está determinado fundamentalmente por factores genéticos, endocrinos y ambientales. Estudios realizados en gemelos y familias han estimado que la heredabilidad para el PMO es de hasta un 75% (Kelly et al., 1993; Peacock, Turner, Econs, & Foroud, 2002).

Un óptimo PMO se asocia con una mayor masa ósea y, consecuentemente, actúa como un factor determinante para prevenir y frenar la pérdida de masa ósea en la etapa

adulta (Heaney et al., 2001). Por lo tanto, maximizar la adquisición del PMO en edades tempranas es una de las estrategias fundamentales para minimizar la pérdida de hueso relacionada con la edad.

1.4. Pérdida de hueso

El predominio de los mecanismos de destrucción de hueso sobre los de formación, como consecuencia del desequilibrio en las unidades celulares básicas, da lugar a un balance óseo negativo que normalmente comienza en la quinta-sexta década de la vida y de forma especial en la mayoría de mujeres tras la menopausia, produciéndose una pérdida de masa ósea anual (Boot et al., 2010). La disminución patológica de la masa ósea que tiene lugar como consecuencia de una disfunción en el remodelado óseo, es decir, un desequilibrio entre los procesos de formación y destrucción del hueso a favor de estos últimos, da lugar a la aparición de osteoporosis (Clarke & Khosla, 2010).

2. OSTEOPOROSIS

2.1. Definición de osteoporosis

La osteoporosis es una enfermedad metabólica crónica que afecta de forma generalizada al sistema esquelético. Se caracteriza por la pérdida de masa ósea y por el deterioro de la microarquitectura del hueso, con el consecuente incremento de la fragilidad ósea y una mayor susceptibilidad a las fracturas (Consensus development conference: diagnosis, prophylaxis, and treatment of osteoporosis, 1993). Con posterioridad a esta definición se ha incluido el concepto de resistencia ósea, que refleja la integración de la densidad y la calidad ósea (NIH Consensus Development Panel on Osteoporosis Prevention, Diagnosis, and Therapy, 2001). La densidad ósea es un aspecto cuantitativo determinado por el PMO y por la pérdida ósea. Por el contrario, la calidad ósea es un aspecto cualitativo que hace referencia a la microarquitectura del hueso, el grado de recambio, las microfracturas y el grado de mineralización (Seeman, 2008).

La osteoporosis es considerada un proceso prevenible y tratable. Sin embargo, la ausencia de sintomatología hasta la aparición de las primeras consecuencias clínicas, las fracturas por fragilidad, hace difícil el diagnóstico en fases tempranas. Estudios previos han evidenciado que se trata de una patología infradiagnosticada, donde el 95% de los pacientes que presentaban una fractura por fragilidad no tenían un diagnóstico previo de osteoporosis (Chesnut et al., 2001; Delmas et al., 2004).

La Organización Mundial de la Salud (OMS) estableció en 1994 una definición de osteoporosis basada en la valoración ósea mediante absorciometría de rayos X de doble energía (DXA) (Tabla 1) (Kanis, 1994). Esta clasificación, aunque ha permitido homogeneizar universalmente el criterio diagnóstico de la osteoporosis, tiene limitaciones ya que los criterios fueron establecidos para mujeres postmenopáusicas de raza blanca y para mediciones realizadas con DXA en el fémur proximal o columna lumbar (Kanis et

al., 2008). El empleo del índice T-score, que se define como el número de desviaciones estándar por las que la densidad mineral ósea (DMO) difiere respecto al valor medio esperado de un adulto joven y sano del mismo sexo, debe limitarse a mujeres postmenopáusicas y hombres con edades ≥ 50 años. En mujeres premenopáusicas y hombres con edades < 50 años se recomienda la utilización del Z-score, que refleja el número de desviaciones estándar por las que la DMO de un individuo difiere del valor medio de una población de similar sexo y edad definido. En edades infantiles no está indicada la utilización del T-score ni el Z-score, ya que no existen valores de referencia estandarizados en la actualidad. Otra posible limitación de los criterios diagnósticos establecidos por la OMS, es que deja abierta la definición de osteoporosis para diferentes técnicas densitometrías y áreas de medición.

Tabla 1. Criterios diagnósticos de osteoporosis según la OMS

Normalidad	DMO >-1 T-score
Osteopenia	DMO entre -1 y -2,5 DE T- score
Osteoporosis	DMO ≤ -2,5 DE T-score
Osteoporosis establecida	DMO ≤ -2,5 DE T-score más fractura por fragilidad

Abreviaturas: T-score: desviaciones estándar por las que la DMO difiere respecto al valor medio esperado de un adulto joven y sano del mismo sexo. DE: Desviación estándar de la media.

Además de la clasificación diagnóstica de la osteoporosis, desde el punto de vista causal, se pueden diferenciar dos tipos de osteoporosis (Dobbs, Buckwalter, & Saltzman, 1999):

A. Osteoporosis primaria. Es el grupo más frecuente e incluye tres tipos:

a. Osteoporosis idiopática juvenil. Característica de mujeres premenopáusicas y hombres jóvenes. La causa es desconocida. Afecta por igual a ambos sexos y se caracteriza por la aparición brusca de dolor óseo y fracturas ante mínimos traumatismos.

b. **Osteoporosis postmenopáusica.** Los síntomas suelen aparecer en mujeres de 51 a 75 años de edad, aunque pueden empezar antes o después de esas edades.
c. **Osteoporosis senil.** Está relacionada con el envejecimiento y con el desequilibrio entre la velocidad de destrucción y de formación ósea. En general, afecta a mayores de 70 años y es más frecuente en las mujeres que en los varones.

B. **Osteoporosis secundaria.** Este tipo de osteoporosis puede ser consecuencia de ciertas enfermedades (anorexia nerviosa, insuficiencia renal crónica, enfermedad inflamatoria intestinal, etc.), trastornos hormonales (hipertiroidismo, hiperparatiroidismo, hipogonadismo, etc.) o ciertos fármacos (corticosteroides, barbitúricos, anticonvulsivantes o cantidades excesivas de hormona tiroidea).

2.2. Epidemiología y relevancia clínica de la osteoporosis

La osteoporosis es considerada un grave problema de salud pública que afecta a más de 200 millones de personas en todo el mundo. La OMS estima que más de 75 millones de personas en Europa, Japón y los Estados Unidos padece osteoporosis, siendo la enfermedad metabólica ósea más común después de los 50 años (Kanis & Glüer, 2000). En la Unión Europea, se estimó que, en el año 2010, 27.5 millones de personas padecían osteoporosis (22 millones de mujeres y 5.5 millones de hombres) (Hernlund et al., 2013). En España se calcula que aproximadamente 2 millones de mujeres y 800.000 varones padecen osteoporosis, estimándose una prevalencia del 26,07% en mujeres mayores de 50 años (Díaz Curiel et al., 2001) y del 8,1% en hombres mayores de 50 años (Naves et al., 2005).

Las fracturas osteoporóticas más comunes son las de las vértebras (columna vertebral), el fémur proximal (cadera) y el antebrazo distal (muñeca). Las fracturas provocan un aumento significativo de la morbimortalidad y generan incapacidad funcional y pérdida de la calidad de vida (Adachi et al., 2010). En el año 2000, se calcula que se produjeron en todo el mundo un total de 9 millones de fracturas osteoporóticas (Cooper et al., 2011; Kanis et al., 2012). Debido al progresivo envejecimiento de la población, en 2050 se prevé que la incidencia mundial de las fracturas de cadera se incremente en un 240% en las mujeres y un 310% en los hombres (Gullberg, Johnell, & Kanis, 1997). El coste económico asociado exclusivamente a esta patología supuso aproximadamente 37 billones de euros en el año 2010. En el 2025 se espera un aumento del 25% en el coste, debido al envejecimiento progresivo de la población (Hernlund et al., 2013).

A pesar de ser la osteoporosis un problema socio-sanitario de gran magnitud debido a su elevada prevalencia, morbimortalidad asociada, tasas de hospitalización, gasto sanitario y deterioro de la calidad de vida, menos del 30% de los pacientes están diagnosticados y menos del 10% reciben tratamiento.

3. MEDICIÓN DE LA MASA ÓSEA

En la actualidad existen multitud de técnicas para la valoración de la masa ósea: absorciometría radiográfica (RA-Radiographic absorciometry), absorciometría fotónica dual (DPA-Dual photon absorciometry), tomografía computarizada (QCT-Quantitative computed tomography), tomografía computarizada periférica (pQCT-Pheripheral quantitative computed tomography), absorciometría de rayos X de doble energía (DXA-Dual energy X ray absorciometry), absorciometría de rayos X de doble energía periférica (pDXA-Pheripheral dual energy X ray absorciometry), absorciometría fotónica simple (SPA-single photon absorptiometry), ultrasonido óseo cuantitativo (QUS-Quantitative

ultrasound), resonancia magnética cuantitativa (QMR-Quantitative magnetic resonance) y microrresonancia magnética (μMR-Magnetic resonance microscopy).

A continuación, se describe la técnica DXA al ser la referente en el diagnóstico de la osteoporosis y la técnica QUS, que ha sido la técnica de elección para la valoración de la masa ósea en el presente trabajo.

3.1. Absorción de rayos X de doble energía (DXA)

La DXA es la técnica de referencia para la evaluación de la masa ósea. De forma general, se realiza a nivel lumbar (L1-L4 o L2-L4) y/o femoral (cuello, trocánter o triángulo de Ward). La técnica se basa en la medición de la transmisión de un haz de fotones de rayos X con dos picos de energía (de alta y baja energía) a través de un determinado sector anatómico. Es un método ampliamente extendido en la actualidad por su baja radiación, su precisión y su fiabilidad (coeficiente de variación: 0,5–3%; error de exactitud: 3–5%) (Blake & Fogelman, 2007). Por el contrario, tiene la desventaja del alto coste, el gran tamaño y la necesidad de personal especializado para su manejo.

Los equipos DXA calculan la densidad mineral ósea en g/cm^2 (BMD - *Bone mineral density*), el contenido mineral óseo en g (BMC - *Bone mineral content*), el área (cm^2), la altura (cm) y el grosor (cm) del área anatómica evaluada. Además, proporcionan el índice T-score y el índice Z-score descritos anteriormente.

En los últimos años, también se han desarrollado equipos DXA periféricos, para medir la masa ósea en diversas localizaciones como falanges, radio distal o calcáneo. A diferencia de las mediciones con DXA central, para el DXA periférico no existe consenso en los criterios diagnósticos de osteoporosis (Eis & Lewiecki, 2006).

3.2. Ultrasonido óseo cuantitativo (QUS)

El ultrasonido óseo cuantitativo (QUS) es una técnica que utiliza ultrasonidos (US) para la valoración de la masa ósea. La onda de US se propaga a través del hueso y permite cuantificar propiedades mecánicas del hueso como la atenuación y la velocidad de transmisión, reflejando así su densidad, arquitectura, conectividad y elasticidad (Chin & Ima-Nirwana, 2013). En la actualidad, existen dispositivos de US para distintas localizaciones anatómicas que incluyen el calcáneo, las falanges, la tibia, la rótula o el radio. En base a lo establecido por la Sociedad Internacional de Densitometría Clínica (ISCD), la única medida de QUS reconocida como determinante del estado óseo es el calcáneo (Krieg et al., 2008). El QUS en el calcáneo presenta ventajas en relación a otras áreas anatómicas. El calcáneo es un hueso constituido en el 95% por hueso trabecular, donde se produce un recambio óseo más acelerado respecto al hueso cortical, reflejando mejor las alteraciones metabólicas (Töyräs, Nieminen, Kröger, & Jurvelin, 2002). Además, está formado por dos superficies laterales paralelas que se rodean de una capa mínima de tejido conectivo y presenta fácil accesibilidad.

El QUS refleja las características del hueso a través de los siguientes parámetros: la velocidad del sonido (SOS - *Speed of sound*) y la atenuación ultrasónica de banda ancha (BUA - *Broadband ultrasound attenuation*). La variable SOS refleja la velocidad del ultrasonido dentro del hueso, expresado como m/s, mientras que la variable BUA expresa la atenuación de ultrasonidos a través del hueso en dB/MHz. Dispositivos QUS más sofisticados proporcionan parámetros de ultrasonido adicionales como la velocidad del sonido de amplitud dependiente (AD-SoS - *Amplitude dependent speed of sound, m/s),* el índice cuantitativo ultrasónico (QUI - *Quantitative ultrasound index*) o el índice de consistencia (SI - *Stiffness index*). Tanto el índice de ultrasonido cuantitativo [0,41 × (BUA + SoS) - 571] como el índice de consistencia [(0,67 × BUA) + (0,28 × SoS) - 420] derivan de la combinación matemática de los parámetros SOS y BUA.

El QUS es una técnica no invasiva que recientemente se ha propuesto como alternativa al DXA para la valoración ósea. En comparación con DXA, las ventajas del QUS incluyen la ausencia de radiaciones ionizantes, el bajo coste económico, la portabilidad de los equipos, la rapidez en la exploración y la facilidad de uso al no requerir de personal especializado (Glüer, 1999).

La correlación entre los parámetros del QUS (BUA y SOS) y el DXA, aunque estadísticamente significativa, es baja-moderada con valores variables dependiendo del equipo de US utilizado y el área anatómica evaluada (He et al., 2000). Estos datos, en línea con estudios *in vitro,* confirman que el QUS, a diferencia del DXA, proporciona información relacionada con la calidad ósea como la elasticidad, la resistencia y la microarquitectura del hueso (Bauer et al., 2007; Moayyeri et al., 2012; Stewart, Kumar, & Reid, 2006). Una desventaja de los equipos de US para la valoración ósea, a diferencia de los DXA, es la falta de consenso sobre el punto de corte para establecer el diagnóstico de osteoporosis.

La evidencia actual refuerza la pertinencia del uso de los equipos de US en la estimación del riesgo de fractura en poblaciones con distintas características y grupos de edad (Hernández et al., 2004; Maggi et al., 2006; Sosa et al., 2005). Un reciente meta-análisis con 21 estudios independientes, concluye que los parámetros SOS, BUA y QUI predicen el riesgo de fractura independientemente de la DMO (Moayyeri et al., 2012). Así, la técnica QUS se ha propuesto como un sustituto potencial al DXA, permitiendo una mayor accesibilidad a la población general. Actualmente, los equipos de US han adquirido popularidad como un método de cribado útil para identificar individuos en riesgo de padecer osteoporosis.

4. FACTORES DETERMINANTES DE OSTEOPOROSIS

Los factores de riesgo de la osteoporosis se definen como aquellas variables que se asocian con un mayor riesgo de padecer la enfermedad y, consecuentemente, con el aumento de las fracturas por fragilidad. La osteoporosis es una enfermedad compleja en la que, tanto en su inicio como en su progresión, intervienen un número indeterminado de variables que se puede clasificar en factores no modificables y factores modificables (Figura 8).

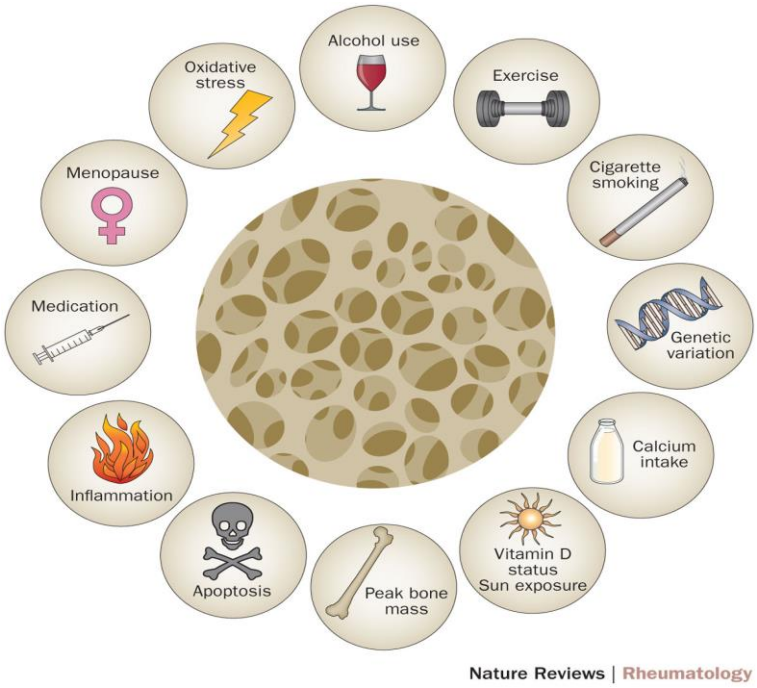


Figura 8. Factores que influyen en el desarrollo de la osteoporosis.

Tanto los factores hereditarios como los no hereditarios contribuyen al desarrollo de la osteoporosis primaria, considerándose así un trastorno multifactorial. La combinación y el impacto de los diferentes factores de riesgo presentes determina el riesgo de fragilidad ósea y fracturas. (Hendrickx et al., 2015)

4.1. Factores de riesgo no modificables

4.1.1. Edad

El aumento de la edad es un factor de riesgo independiente para la osteoporosis. A partir de la tercera y cuarta década de la vida la masa ósea disminuye (Demontiero, Vidal, & Duque, 2012). En las mujeres, la pérdida de masa ósea es más pronunciada en la primera década tras la menopausia siendo la incidencia de la enfermedad mayor que en los hombres (Steiger, Cummings, Black, Spencer, & Genant, 1992). Sin embargo, en edades superiores a los 65 años, tiende a igualarse la disminución de la masa ósea en ambos sexos (Lang et al., 2012).

Con la edad se incrementa la incidencia de esta patología al modificarse las condiciones fisiológicas, produciéndose un balance óseo negativo al disminuir la actividad osteoblástica (Boskey & Coleman, 2010). Además, con el envejecimiento aparece una disminución de la absorción intestinal de calcio y otros nutrientes, una reducción de los niveles estrogénicos en las mujeres y, de forma genérica, una disminución de la actividad física entre otros.

4.1.2. Sexo

La osteoporosis es más frecuente en el sexo femenino (Cawthon, 2011). Las mujeres son más susceptibles a padecer la enfermedad al alcanzar un PMO menor que el alcanzado por los hombres.

Las hormonas sexuales juegan un papel fundamental en la disminución de la resorción ósea. Como ya se ha comentado, los estrógenos inhiben la apoptosis de los osteoblastos y favorecen la de los osteoclastos. Por este motivo, el déficit de estrógenos que se produce en las mujeres tras el cese de la actividad ovárica en la menopausia, acelera la tasa de

pérdida de masa ósea. Así pues, el tiempo transcurrido desde el inicio de la menopausia es un factor de riesgo determinante para promover una disminución de la masa ósea. La menopausia precoz o la menarquia tardía son factores asociados a un nivel de masa ósea bajo (Qiu et al., 2013; Sioka et al., 2010).

4.1.3. Factores étnicos

Los datos de estudios epidemiológicos han mostrado que la osteoporosis es más frecuente en individuos de raza caucásica y asiática frente a individuos de raza negra. Se ha evidenciado que en las mujeres de raza blanca el riesgo de fractura es mayor que en las mujeres de raza negra (Cauley, 2011; Cauley et al., 2005).

4.1.4. Factores genéticos

La osteoporosis es una enfermedad poligénica determinada por numerosas variantes genéticas, que contribuyen cada una de ellas con un efecto moderado al riesgo de padecer la enfermedad (Stewart & Ralston, 2000). Estudios familiares han estimado una heredabilidad comprendida entre el 50 y el 80% para la variabilidad de la masa ósea en diferentes fenotipos: 84% para la DMO central (Arden, Baker, Hogg, Baan, & Spector, 1996) o 74 % para QUS del calcáneo (Howard, Nguyen, Harris, Kelly, & Eisman, 1998; Karasik et al., 2002).

4.2. Factores de riesgo modificables

4.2.1. Variables antropométricas

Un peso igual o inferior a 57 kg y un IMC menor de 19 kg/m^2 son considerados factores predictores de un nivel bajo de masa ósea. Una talla baja también se ha descrito

como un factor de riesgo de fractura (Asomaning, Bertone-Johnson, Nasca, Hooven, & Pekow, 2006; Compston et al., 2014).

El peso es un factor predictor de la masa ósea, de tal manera que la delgadez constituye un factor de riesgo de osteoporosis, mientras que la obesidad ejerce un papel protector (Albala et al., 1996). El efecto protector de la masa grasa sobre la masa ósea puede explicarse por factores de carga. En este sentido, en individuos con un mayor peso corporal se produce un mayor efecto osteoblástico, debido a que someten su esqueleto a estímulos con mayor carga mecánica (Zhao et al., 2007). Por otro lado, la correlación entre la masa grasa y la masa ósea podría deberse a factores hormonales, debido al aumento de los niveles de estrógenos producidos por los adipocitos o a la resistencia a la insulina asociada a la obesidad, que influye en la secreción de hormonas con actividad ósea como la leptina (Legiran & Brandi, 2012).

No existe consenso acerca de la relación independiente entre la masa magra, la masa grasa y la masa ósea. En estudios previos se observó que, aunque la masa grasa y la masa magra correlacionan positivamente con la masa ósea, la correlación entre la masa grasa y la masa ósea es menor (Ho-Pham, Nguyen, & Nguyen, 2014; Lorentzon, Landin, Mellström, & Ohlsson, 2006). En esta línea, la mayoría de los autores han sugerido que dicha asociación posiblemente sea resultado de la mayor carga mecánica que una masa corporal elevada supone, a la que el esqueleto responde con una estimulación de la actividad osteoblástica (Baptista et al., 2012; Luis Gracia-Marco et al., 2011). Por lo tanto, los sujetos con mayor adiposidad presentan mayores niveles de masa ósea como consecuencia del incremento de la masa magra, que se produce para satisfacer las demandas durante la locomoción en estos individuos (Jeddi et al., 2015).

4.2.2. Factores nutricionales

La ingesta dietética es un factor crucial en la prevención de la osteoporosis (Cashman, 2007; New et al., 2000). Las dietas hipocalóricas durante la infancia y la adolescencia se han asociado a un déficit en el crecimiento y la mineralización ósea (Compston, Laskey, Croucher, Coxon, & Kreitzman, 1992; Devlin et al., 2010). Por el contrario, trabajos previos han descrito una asociación entre las dietas hiperproteicas y mayores niveles de masa ósea. No obstante, los resultados obtenidos hasta la fecha no son concluyentes (Alexy, Remer, Manz, Neu, & Schoenau, 2005; Bonjour, 2011; Chevalley, Bonjour, Ferrari, & Rizzoli, 2008).

En relación a la ingesta cálcica, un aporte inferior a las recomendaciones favorece una mayor resorción ósea al representar el hueso el 99% de la reserva cálcica total del organismo. La ingesta recomendada de calcio varía según la edad y circunstancias concretas como el embarazo y la lactancia, oscilando entre los 1000-1500 mg/día (Ross et al., 2011). En trabajos previos, se ha descrito una correlación positiva entre la ingesta de calcio y la adquisición del PMO en edades tempranas (Bailey, Martin, McKay, Whiting, & Mirwald, 2000; Nieves et al., 2010; Peters, Verly Jr, Marchioni, Fisberg, & Martini, 2012). Sin embargo, en otros estudios en los que la masa ósea se determinaba mediante DXA y/o ultrasonido cuantitativo los hallazgos eran negativos, evidenciando así la controversia existente (Babaroutsi, Magkos, Manios, & Sidossis, 2005; De Smet et al., 2015; Tai, Leung, Grey, Reid, & Bolland, 2015).

Por otro lado, el hueso es el mayor depósito de fósforo en el organismo acumulándose el 85% del fósforo total. Las necesidades de fósforo varían entre 800-1000 mg/día (Institute of Medicine Standing Committee on the Scientific Evaluation of Dietary Reference Intakes, 1997). No es habitual detectar deficiencias en la ingesta de fósforo ya que se encuentra disponible en un gran número de alimentos y su biodisponibilidad es alta. Sin embargo, una ingesta excesiva puede desequilibrar la relación calcio/fósforo,

provocando una menor absorción del calcio que puede causar una disminución de la mineralización ósea (Basabe Tuero et al., 2004; Loughrill, Wray, Christides, & Zand, 2016).

La vitamina D también juega un papel fundamental en la mineralización ósea, facilitando una absorción óptima de calcio y fósforo a nivel intestinal y renal (Holick, 1996; Thomas & Briot, 2016). Las necesidades diarias recomendadas varían alrededor de 400-1000 UI (Ross et al., 2011). La dieta no aporta las cantidades necesarias, siendo la síntesis que se produce en la piel, a partir de la exposición solar, la mayor fuente de vitamina D. Trabajos previos han investigado la asociación entre suplementos de vitamina D y la mineralización ósea, evidenciando una falta de consenso en los resultados (Abrahamsen et al. 2010; Avenell et al. 2005; Reid, Bolland, & Grey 2014).

Recientemente, se ha analizado la posible influencia de la ingesta de nutrientes antioxidantes incluyendo vitamina C, E, A, el zinc o el selenio sobre la masa ósea. Los antioxidantes actúan neutralizando los radicales libres e inhibiendo del daño oxidativo a las células del hueso y, por lo tanto, podrían influir en la patogénesis de la osteoporosis (Basu, Michaëlsson, Olofsson, Johansson, & Melhus, 2001; Yalin et al., 2005). Aunque varias líneas de investigación sugieren que existe una estrecha relación entre la ingesta de antioxidantes y la masa ósea, los resultados son inconsistentes (Chuin et al., 2009; De França, Camargo, Lazaretti-Castro, & Martini, 2013; Rivas et al., 2012; Sugiura et al., 2011; Wolf et al., 2005).

4.2.3. Actividad física

El ejercicio físico es uno de los factores más directamente relacionados con la mineralización ósea debido a su efecto osteogénico (Todd & Robinson, 2003; Vicente-Rodríguez, 2006). Por el contrario, el sedentarismo o las situaciones de inmovilización conllevan a una disminución de la masa ósea (Chastin, Mandrichenko, Helbostadt, &

Skelton, 2014). En base a la evidencia disponible, está ampliamente aceptado que los deportes que implican actividades de carga y alto impacto son los más eficaces para aumentar la mineralización ósea y disminuir el riesgo de fracturas (Behringer, Gruetzner, McCourt, & Mester, 2014; Etherington et al., 1996; French, Fulkerson, & Story, 2000).

La actividad física juega un papel determinante en la formación y el remodelado óseo que se produce en edades tempranas (Karlsson, Nordqvist, & Karlsson, 2008; Nilsson, Ohlsson, Odén, Mellström, & Lorentzon, 2012). En la madurez, el ejercicio físico también actúa como un factor preventivo de la osteoporosis, disminuyendo las pérdidas de masa ósea asociadas a la edad. Aunque numerosos estudios han identificado una relación positiva entre la actividad física y los niveles de masa ósea (Daly, 2007; Guadalupe-Grau, Fuentes, Guerra, & Calbet, 2009; Karlsson et al., 2008), otros han descrito mínimas o nulas ganancias en la masa ósea (Chubak et al., 2006; Matos, Lopes da Silva, Martinez de Oliveira, & Castelo-Branco, 2009). Probablemente, la disparidad de resultados obtenidos sea consecuencia de la amplia variedad de métodos disponibles para cuantificar el ejercicio físico.

4.2.4. Hábitos tóxicos

El tabaquismo es un factor de riesgo para el desarrollo de osteoporosis (Wong et al., 2007). A través de mecanismos de acción directos se provoca un daño sobre el hueso, inhibiendo la actividad osteoblástica. Además, el consumo de tabaco disminuye la absorción intestinal de calcio. Varios estudios han identificado una asociación positiva entre el hábito tabáquico y menores niveles de mineralización ósea y, en consecuencia, un mayor riesgo de fracturas (Høidrup et al., 2000; Olofsson et al., 2005; Ortego-Centeno et al., 1997; Ward & Klesges, 2001).

Por otro lado, la ingesta de grandes cantidades de alcohol se ha asociado con un mayor riesgo de osteoporosis al provocar un efecto inhibitorio sobre los osteoblastos,

disminuir la absorción intestinal del calcio y alterar su metabolización así como la de la vitamina D (Berg et al., 2008; Mikosch, 2014). La ingesta de alcohol también se ha relacionado con una mayor frecuencia de caídas, que conlleva a un incremento del riesgo de fracturas (Kool, Ameratunga, & Jackson, 2009). No obstante, un consumo moderado de alcohol en mujeres se ha asociado con mayores niveles de masa ósea (Ganry, Baudoin, & Fardellone, 2000; Hagberg et al., 2001).

4.2.5. Fármacos

Los glucocorticoides inhiben el crecimiento óseo actuando directamente sobre los osteoblastos (Canalis & Delany, 2002). El amplio uso de los glucocorticoides en la práctica clínica ha provocado que la osteoporosis esteroidea sea la causa más frecuente de osteoporosis secundaria (Briot & Roux, 2015). Además de los glucocorticoides, los fármacos antiepilépticos, la tiroxina, los fármacos inhibidores de la aromatasa y la heparina, entre otros, se han asociado con una disminución de la masa ósea y el aumento del riesgo de fracturas (Mazziotti, Canalis, & Giustina, 2010; Panday, Gona, & Humphrey, 2014).

4.2.6. Exposición solar

La vitamina D que se aporta al organismo proviene en más de un 90% de la exposición solar, mientras que un 10% aproximadamente es aportado por la dieta. Así, la exposición solar es fundamental para mantener unos niveles óptimos de vitamina D (Johnson, 2010). En la epidermis, a partir de la irradiación solar ultravioleta tipo B (UVB), se transforma el 7-dehidrocolesterol en pre-vitamina D3, que se convierte en vitamina D3 o colecalciferol (Nair & Maseeh, 2012). En trabajos previos se ha descrito cómo una deficiencia de vitamina D, que se traduce en una disminución de la masa ósea y la adquisición de un menor PMO, puede ser consecuencia de la falta de exposición solar (Kruavit et al., 2012; Weaver, Passmore, Collins, & Fung, 2010).

5. ESTUDIO DE LAS BASES GENÉTICAS DE LOS FENOTIPOS RELACIONADOS CON LA OSTEOPOROSIS

5.1. Estrategias en la determinación de bases genéticas de fenotipos complejos

En las últimas décadas, la secuenciación del genoma humano, junto con la identificación de un elevado abanico de marcadores genéticos y el desarrollo de diferentes estrategias de genotipado, ha provocado un importante avance en la investigación genómica de enfermedades y/o fenotipos complejos. La identificación del componente genético de enfermedades complejas es de gran relevancia, ya que permitirá aportar nuevos datos sobre los mecanismos fisiopatológicos subyacentes en estas enfermedades, así como contribuirá a la identificación de nuevas dianas terapéuticas.

5.1.1. Marcadores genéticos

Un marcador genético se define como una secuencia de ADN (ácido desoxirribonucleico) con un componente variable, que permite identificar diferencias entre individuos y que está localizado en una posición concreta del genoma. Al componente variable de los marcadores genéticos se le denomina polimorfismo y a cada una de las variantes de un marcador genético alelo. Cuando un individuo tiene dos alelos iguales se dice que es homocigoto para el marcador genético y cuando tiene dos alelos diferentes se denomina heterocigoto.

En la actualidad existe una gran variedad de marcadores genéticos que se utilizan para el estudio de la genética de las enfermedades complejas. Entre ellos, se encuentran el número variable de repeticiones en tándem (VNTR - *Variable number tandem repeats),* la

variación del número de copias (CNV - *Copy number variations*) y los polimorfismos de un único nucleótido (SNP - *Single nucleotide polymorphism*).

Los VNTRs son repeticiones seriadas de nucleótidos que generalmente son de pequeño tamaño, denominándose microsatélites. Los CNV se definen como segmentos de ADN con una longitud mayor o igual a un 1kb que presentan un número de copias variable en relación a un genoma de referencia. Por otro lado, el estudio de SNPs es la estrategia utilizada por excelencia para estudiar las bases genéticas de patologías complejas (Risch, 2000). Un SNP es una variación de un único nucleótido en una posición concreta del genoma que afecta a una sola base (A-adenina, T-tiamina, C-citosina o G-guanina), siendo la frecuencia del alelo minoritario (MAF) al menos del 1% en la población (Figura 9). Los SNPs se encuentran tanto en las regiones codificantes (exones) como no codificantes (intrones y región promotora) de los genes. Los SNPs que se localizan en regiones codificantes se pueden clasificar como SNP sinónimos, cuando el cambio de la base de ADN no produce un cambio en la secuencia aminoacídica de la proteína, y SNP no sinónimos, cuando el cambio de la base de ADN produce un cambio en la secuencia aminoacídica de la proteína que puede originar un cambio en la funcionalidad de la proteína. Por otro lado, los SNPs pueden localizarse en regiones no codificantes, ya sea en la región promotora del gen, en regiones intrónicas o en regiones intergénicas. Estas variantes génicas pueden alterar secuencias reguladoras o alterar el procesamiento del ARNm (ácido ribonucleico mensajero).

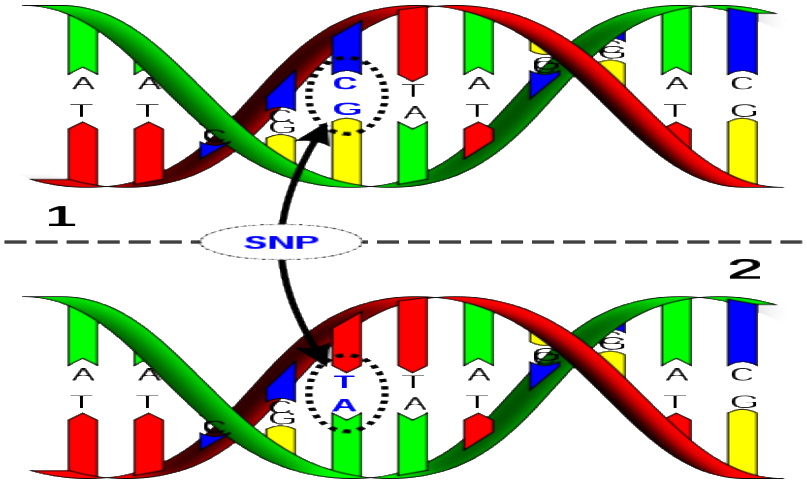


Figura 9. Polimorfismos de un único nucleótido (SNP).

Los SNPs que contribuyen a la variación de los niveles de expresión de ARNm o proteínas se denominan eQTL (*Expression quantitative trait loci*). Los eQTLs se pueden clasificar como cis-eQTLs, aquellos que mapean cerca de la posición del gen cuya expresión ha sido analizada, y trans-eQTLs, aquellos identificados en otras regiones del genoma.

Por otro lado, se ha demostrado que entre determinados SNPs puede existir algún grado de desequilibrio de ligamiento (LD). Esto quiere decir que dichas variantes tienden a heredarse juntas con una mayor frecuencia a la que cabría esperar por el azar, originándose así bloques de ligamiento. La combinación de los alelos que componen dichos bloques es lo que se denomina haplotipos (Figura 10). El LD se estima mediante dos parámetros: D′ mide el nivel de heredabilidad conjunta de los dos polimorfismos y r^2 indica el coeficiente de correlación entre los dos SNPs.

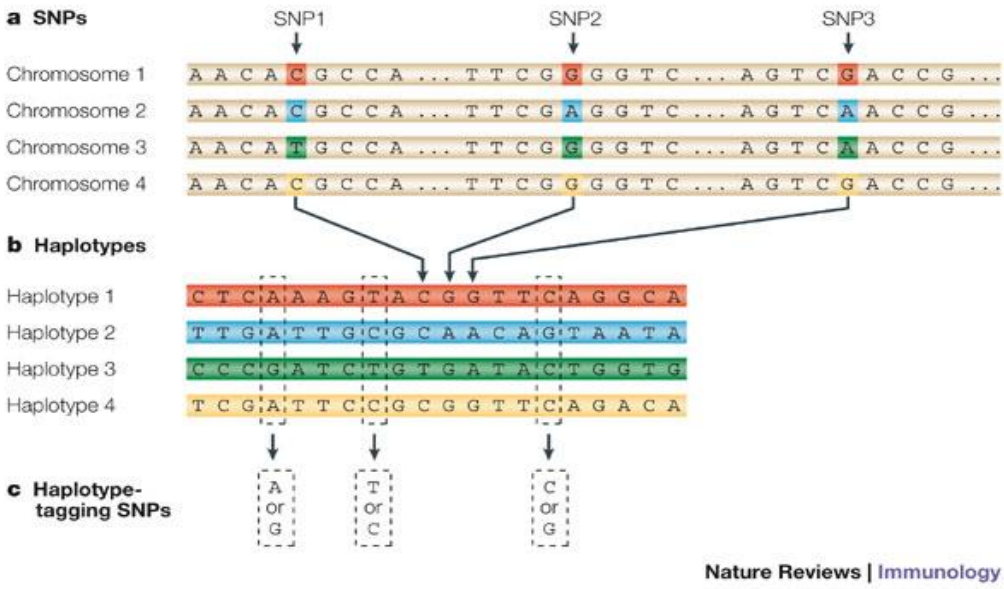


Figura 10. SNPs, haplotipos y tag-SNPs.

A) Se muestran cuatro versiones de la misma región cromosómica tomada de diferentes individuos. Toda la secuencia de ADN es idéntica en estos cromosomas, salvo en tres posiciones en las que se localizan polimorfismos de un solo nucleótido (SNPs). Cada SNP tiene dos posibles alelos. El primer SNP (SNP1) tiene los alelos C y T.

B) Muchos SNP se heredan juntos en bloques de desequilibrio de ligamiento. La combinación concreta de alelos de los distintos SNP que están en un mismo bloque constituye un haplotipo característico de ese bloque. Así, un haplotipo consiste en una combinación de alelos de diferentes SNPs que se heredan en bloque. Se muestran aquí 4 haplotipos diferentes con los genotipos observados para 20 SNPs. Cada haplotipo se diferencia del resto sólo en los alelos de los tres SNPs que se muestran en el panel a.

C) Un grupo de SNPs de cada haplotipo pueden ser representativos de todo ese haplotipo (por lo que reciben el nombre de tag-SNPs o SNP-etiqueta). De este modo, no es necesario genotipar todos los SNPs del genoma, sino sólo los tag-SNP. Así, en el panel b el genotipado de los SNP1, SNP2 y SNP3 de los 20 SNPs representados sería suficiente para identificar de manera única estos 4 haplotipos y caracterizar la variabilidad genética de esta región. (Hafler & Jager, 2005).

5.1.2. Estudios de asociación genética

Para determinar si una variante genética se asocia a una enfermedad y/o fenotipo complejo se realizan estudios de asociación genética, utilizando diseños de casos-controles o diseños de cohortes. En los estudios de casos-controles, se compara la distribución de frecuencias de marcadores genéticos en los casos (individuos afectados) y los controles (individuos sanos) de una misma población. Por otro lado, en los estudios de casos se

estudian los sujetos de una muestra poblacional con unas características determinadas y se analiza la posible asociación entre un fenotipo y el factor genético de interés. El tipo de fenotipo y/o enfermedad a estudiar va a determinar el diseño utilizado.

Los estudios de asociación pueden clasificarse en dos clases en función de la existencia de una hipótesis previa para la selección de los genes o no. La estrategia de hipótesis a priori se utiliza en los estudios de genes candidatos, en los que se investigan una o varias variantes genéticas frente a una enfermedad o fenotipo determinado. Los genes se seleccionan en base a su posición genómica en un cromosoma (candidato posicional) o a sus productos proteicos (candidato funcional). La estrategia de asociación de genes candidatos ha sido ampliamente utilizada y ha permitido la identificación de numerosos marcadores genéticos implicados en enfermedades y/o fenotipos complejos.

Por otro lado, la estrategia de estudios sin hipótesis a priori es la utilizada por los estudios de asociación del genoma completo (GWAS - *Genome wide association study*) en los que se analizan un gran número de SNPs (entre 500.000 y 1.000.000) distribuidos a lo largo de todo el genoma, con el fin de identificar asociaciones entre ciertos fenotipos o enfermedades con variaciones genéticas específicas. A lo largo de los últimos años, el desarrollo de los GWAS ha dado lugar a un avance muy significativo en el conocimiento de las bases genéticas de enfermedades y fenotipos complejos como la obesidad (Locke et al., 2015), la hipertensión (Levy et al., 2009), el cáncer (Michailidou et al., 2015) o la diabetes (Sladek et al., 2007).

Por último, los estudios de interacciones génicas investigan las posibles interacciones genéticas, profundizando así en la etiopatogenia de las enfermedades complejas (Lin, Chu, Lin, Yang, & Su, 2015). La multitud de estudios de genes candidatos y, más recientemente, los estudios GWAS que se han desarrollado han contribuido enormemente a la mejora del conocimiento de las bases genéticas de fenotipos complejos.

Sin embargo, una fracción de heredabilidad continúa siendo desconocida y podría explicarse por las interacciones que se producen.

5.2. Genética de la osteoporosis y fenotipos relacionados: estudios previos

Numerosos estudios de asociación génica se han centrado en la identificación de determinantes genéticos asociados con fenotipos indicadores de los niveles de mineralización ósea y, por lo tanto, relacionados con el desarrollo de la osteoporosis.

5.2.1. Estudios de genes candidatos

En relación a los estudios de genes candidatos, los genes más ampliamente estudiados han sido aquellos que regulan vías del metabolismo óseo y, por consiguiente, que intervienen en la variabilidad de la masa ósea, permitiendo así la identificación de numerosos marcadores genéticos asociados a fenotipos relacionados con la osteoporosis. La mayor parte de los trabajos que han investigado los determinantes genéticos de la osteoporosis, se han centrado mayoritariamente en la identificación de SNPs asociados con la DMO o el riesgo de fracturas (Figura 11). En relación a los factores genéticos asociados con las mediciones óseas obtenidas mediante ultrasonografía, los estudios publicados son más limitados.

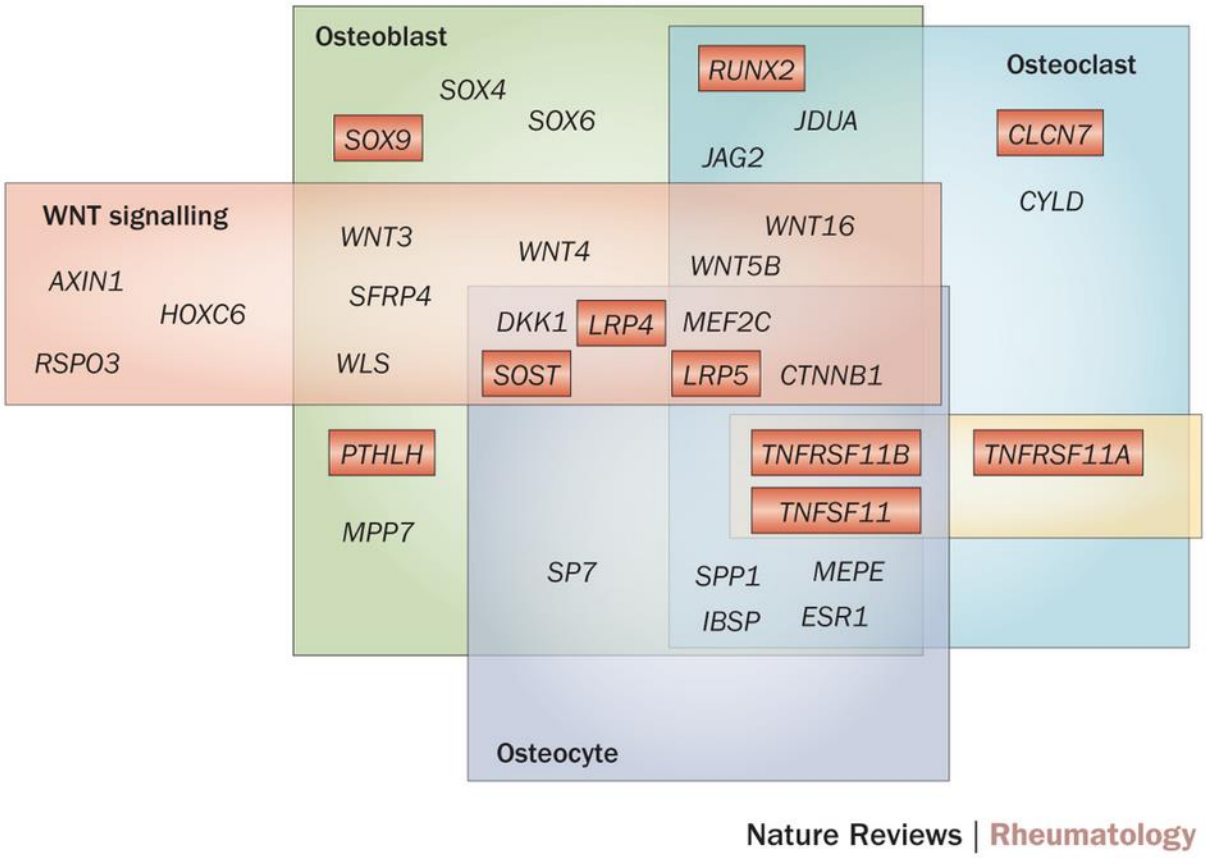


Figura 11. Genes asociados con la DMO o el riesgo de fracturas.

Los genes representados tienen papeles directos o indirectos en los tres principales tipos de células óseas (osteocitos, osteoblastos y osteoclastos) y están implicados en vías que regulan la osteoblastogénesis y la osteoclastogénesis. (Hendrickx et al., 2015).

- ***Polimorfismos del gen VDR.*** Uno de los genes más ampliamente estudiados en la genética de la osteoporosis, por su papel clave en la homeostasis del calcio, regulación del crecimiento de los huesos, la diferenciación celular, la absorción de calcio intestinal y la secreción de hormona paratiroidea, ha sido el gen *VDR* (Goltzman, Miao, Panda, & Hendy, 2004). En 1994, Morrison et al. fueron los primeros en correlacionar variantes genéticas del *VDR* con los niveles de mineralización ósea (Morrison et al., 1994). A partir de ese momento, se han realizado numerosos estudios de asociación entre polimorfismos del *VDR* incluyendo *TaqI* (rs731236), *BsmI* (rs1544410), *FokI* (rs2228570) y *Apa1* (rs7975232), y fenotipos relacionados con la osteoporosis, fundamentalmente la DMO y el riesgo de fracturas, mostrando resultados contradictorios (Canto-Cetina et al., 2015; González-Mercado et al., 2013; Ji, Yao, Sun, Li, & Han, 2010; Macdonald et al., 2006;

Qin, Dong, Zeng, Liu, & Liao, 2013; Uitterlinden et al., 2006; Wang et al., 2013; Zintzaras, Rodopoulou, & Koukoulis, 2006). No obstante, la mayoría de estos trabajos se han llevado a cabo en poblaciones mixtas constituidas en gran medida por mujeres postmenopáusicas. Así, el rol del gen *VDR* en la adquisición de la masa ósea en la adultez temprana, periodo en el que se produce la adquisición del PMO, ha sido escasamente investigado (Abrams et al., 2005; Gunnes, Berg, Halse, & Lehmann, 1997; McGuigan et al., 2002). Además, los limitados estudios que analizaron la asociación entre polimorfismos del *VDR* y parámetros QUS, han evidenciado una falta de consistencia en los hallazgos (Babaroutsi et al., 2005; Kim, Kim, Kim, Ho Kim, & Lee, 2007; Koh et al., 2004; Laaksonen, Kärkkäinen, Outila, Rita, & Lamberg-Allardt, 2004; Omasu et al., 2004; Zajickova, Zofkova, & Hill, 2005).

- ***Polimorfismos del gen ESR.*** Los estrógenos desempeñan un papel relevante en la adquisición y mantenimiento de la masa ósea (Manolagas, O'Brien, & Almeida, 2013). Tanto los receptores de estrógenos α o tipo 1 (ESR1) como ß o tipo 2 (ESR2) se expresan en las células óseas. El *ESR1,* principal mediador de la acción estrogénica en el hueso, tiene una importancia fundamental en la regulación de su remodelado óseo (Khalid & Krum, 2016). En 1996, Kobayashi et al. describieron por primera vez la asociación entre SNPs del gen *ESR1* y la mineralización ósea (Kobayashi et al., 1996). A partir de ese momento, un gran número de trabajos han investigado la influencia de variantes genéticas del gen *ESR1*, incluyendo *PvuII* (rs2234693) y *XbaI (rs9340799)*, en fenotipos óseos determinados por DXA (Ioannidis et al., 2004; Koller et al., 2013; Massart et al., 2009; Rivadeneira et al., 2009) o QUS (Albagha et al., 2005; Elfassihi et al., 2010; Giguère, Dodin, Blanchet, Morgan, & Rousseau, 2000; Moayyeri et al., 2014), evidenciando resultados contradictorios. No obstante, a pesar de los avances que se han producido en el conocimiento de los marcadores genéticos del *ESR1* asociados con parámetros óseos, el posible papel del gen *ESR1* en la adquisición de la masa ósea en edades tempranas no ha sido ampliamente investigado.

- ***Polimorfismos del sistema RANKL-RANK-OPG.*** El sistema formado por *RANKL/RANK/OPG* juega un papel crucial en la regulación de la resorción ósea y del remodelado óseo (Boyle et al., 2003). Por lo tanto, los genes de esta vía han sido seleccionados como candidatos funcionales en numerosos estudios de asociación. Trabajos previos han identificado asociaciones significativas entre SNPs del sistema *RANK/RANKL/OPG* y la DMO (Choi et al., 2005; Hsu et al., 2006; Kim et al., 2007; Paternoster et al., 2010; Roshandel et al., 2010; Shang, Lin, & Cui, 2013; Yamada, 2003). No obstante, la posible influencia del sistema *RANK/RANKL/OPG,* como determinante genético de parámetros óseos evaluados mediante ultrasonografía ósea, no ha sido previamente investigada.

- ***Polimorfismos del gen LRP5 y el gen SOST.*** Los genes de la vía de señalización Wnt (wingless) han sido objeto de numerosos estudios de asociación en los últimos años debido a su implicación en el metabolismo óseo, modulando tanto la diferenciación como la actividad de las células óseas (Anastasilakis, Polyzos, & Toulis, 2011). Entre los componentes más estudiados de esta vía se encuentran los genes *LRP5/6*, que median en la vía Wnt de señalización canónica (Gong et al., 2001), y el gen *SOST*, que es un antagonista que interactúa con *LRP5* y *LRP6* inhibiendo la función de los osteoblastos (Li et al., 2005; Moester et al., 2010). Trabajos previos han evidenciado una relación entre la DMO y el gen co-receptor de la vía de señalización Wnt (*LRP5*) o el gen antagonista de Wnt (*SOST*) (Baroncelli et al., 2006; Bollerslev et al., 2005; Ferrari et al., 2004; He et al., 2015; Kumar et al., 2011; Markatseli et al., 2011; Piters et al., 2012; Saarinen et al., 2007; Velázquez-Cruz et al., 2014; Xiong et al., 2007). En relación a la posible asociación con parámetros óseos determinados mediante ultrasonido cuantitativo (QUS), hasta la fecha sólo dos estudios han analizado marcadores genéticos del gen *LRP5* (Kumar et al., 2011; Saarinen et al., 2007) mientras que el gen *SOST* no ha sido previamente investigado.

- ***Polimorfismos del gen WNT16.*** Existen otras vías alternativas no-canónicas que emplean diferentes mediadores, cuya función en el metabolismo óseo resulta menos conocida. Recientemente, se ha evidenciado la importancia de la proteína WNT16, que interviene en la regulación de la masa ósea cortical y la resistencia ósea mediante la inhibición de la osteoclastogénesis (Kobayashi, Uehara, Koide, & Takahashi, 2015; Logan & Nusse, 2004). WNT16 puede activar una cascada de señalizaciones en la vía canónica y no-canónica en los osteoblastos, mientras que en los osteoclastos sólo activa la vía no-canónica. Estudios previos han descrito una asociación entre marcadores genéticos del gen *WNT16* y diversos fenotipos óseos incluyendo la DMO, parámetros QUS o el riesgo de fracturas (García-Ibarbia et al., 2013; Hendrickx et al., 2014; Zheng et al., 2012). No obstante, el posible papel del gen *WNT16* en la adquisición del PMO a edades tempranas no ha sido previamente investigado.

5.2.2. Estudios de asociación del genoma completo (GWAS)

La gran mayoría de estudios GWAS publicados hasta la fecha se han centrado en la búsqueda de variantes en genes asociados a los niveles de DMO y el riesgo de fractura (Estrada, Styrkarsdottir, & Evangelou, 2012; Koller et al., 2013; Medina-Gomez et al., 2012; Paternoster et al., 2010; Richards et al., 2008; Richards et al., 2009; Rivadeneira et al., 2009; Styrkarsdottir et al., 2009, 2010). Estos estudios, han permitido la identificación de hasta 61 regiones del genoma, asociadas significativamente con los niveles de DMO y/o riesgo de fracturas en adultos (Richards, Zheng, & Spector, 2012) (Tabla 2).

Tabla 2. Regiones del genoma asociadas con DMO a nivel de significación estadística de GWAS*

Locus	Gen	*P*	Locus	Gen	*P*
1p31.3	*WLS* (*GPR177*)	$2.6x10^{-13}$	10q24.2	*CPN1*	$9.0x10^{-10}$
1p36	*ZBTB40*	$7.4x10^{-57}$	11p12	*LRP4*, *ARHGAP1* y *F2*	$5.1x10^{-18}$
1p36.12	*WNT4*	$9.6x10^{-11}$	11p14.1	*DCDC5*	$2.2x10^{-11}$
1q24.3	*DNM3*	$8.5x10^{-15}$	11p14.1	*LIN7C* y *DCDC5*	$4.9x10^{-8}$
2p16	*SPTBN1*	$2.3x10^{-18}$	11p15	*SOX6*	$1.1x10^{-32}$
2p21	*PKDCC*	$1.3x10^{-9}$	11q13.2	*LRP5*	$2.1x10^{-26}$
2q13	*ANAPC1*	$1.5x10^{-9}$	12p11.22	*KLHDC5* y *PTHLH*	$1.9x10^{-12}$
2q14.1	*INSIG2*	$1.2x10^{-10}$	12p13.33	*ERC1* y *WNT5B*	$5.6x10^{-12}$
2q24	*GALNT3*	$3.9x10^{-30}$	2q13	*SP7*	$3.0x10^{-20}$
3p22	*CTNNB1*	$4.4x10^{-25}$	12q13.12	*DHH*	$1.2x10^{-15}$
3q13.2	*KIAA2018*	$4.1x10^{-10}$	12q23.3	*C12ORF23*	$9.6x10^{-10}$
3q5.31	*LEKR1*	$4.5x10^{-12}$	13q14	*RANKL*	$2.0x10^{-21}$
4p16.3	*IDUA*	$5.2x10^{-15}$	14q32	*MARK3*	$5.2x10^{-16}$
4q21.1	*MEPE*, *SPP1 y IBSP*	$1.2x10^{-27}$	14q32.12	*RPS6KA5*	$2.0x10^{-15}$
5q14	*MEF2C*	$4.5x10^{-61}$	16p13.11	*NTAN1*	$1.7x10^{-10}$
5q31	*ALDH7A1*	$6.4x10^{-6}$	1p13.3	*AXIN1*	$1.0x10^{-16}$
6p1.1	*SUPT3H* y *RUNX2*	$5.6x10^{-11}$	16p13.3	*C16ORF38* y *CLCN7*	$1.5x10^{-16}$
6p22.3	*CDKAL1* y *SOX4*	$2.7x10^{-13}$	16q12.1	*CYLD*	$1.9x10^{-22}$
6q22	*RSPO3*	$8.1x10^{-12}$	16q23	*ADAMTS18*	$2.1x10^{-8}$
6q25.1	*C6ORF97* y *ESR1*	$4.0x10^{-35}$	16q24	*FOXL1* y *FOXC2*	$1.0x10^{-14}$
7p14.1	*STARD3NL*	$3.8x10^{-38}$	17p.3	*SMG6*	$9.8x10^{-19}$
7q21.3	*FLJ42280* y *SHFM1*	$9.4x10^{-12}$	17q12	*CRHR1*	$1.4x10^{-8}$
7q21.3	*SLC25A13*	$8.1x10^{-48}$	17q21	*SOST*	$2.0x10^{-11}$
7q31.31	*WNT16* y *FAM3C*	$3.2x10^{-51}$	17q2	*HDAC5*	$1.7x10^{-8}$
7q36.1	*ABCF2*	$7.3x10^{-9}$	17q24.3	*SOX9*	$1.9x10^{-11}$
8q13.3	*XKR9* y *LACTB2*	$1.9x10^{-8}$	18p11.2	*FAM210A*	$4.9x10^{-8}$
8q24	*OPG*	$3.2x10^{-39}$	18q21.33	*RANK*	$1.6x10^{-17}$
9q34.11	*FUBP3*	$3.4x10^{-22}$	19q13.11	*GPATCH1*	$6.6x10^{-11}$
1p11.23	*MPP7*	$2.4x10^{-16}$	20p12	*JAG1*	$3.1x10^{-19}$
10q21.1	*MBL2* y *DKK1*	$1.6x10^{-12}$	Xp2231	*FAM9B* y *KAL1*	$1.2x10^{-8}$
10q22.3	*KCNMA1*	$5.0x10^{-19}$			

**El nivel de significación estadística GWAS se define como $P < 5x10^{-8}$* (Richards et al., 2012).

En relación a la valoración ósea mediante QUS, por el momento sólo se ha publicado un estudio GWAS realizado en el contexto del consorcio GEFOS (*Genetic factors for osteoporosis*), que ha revelado nuevas asociaciones entre numerosos marcadores genéticos y parámetros QUS (BUA y SOS) (Tabla 3). Cabe destacar que, en este estudio, se han mostrado asociaciones significativas entre parámetros QUS y variantes genéticas que previamente ya se habían descrito como polimorfismos asociados con la DMO y el riesgo de fracturas (Moayyeri et al., 2014).

Tabla 3. Regiones de genoma asociadas con los niveles de BUA del calcáneo a nivel de significación estadística GWAS*

Locus	Gen candidato	SNP	Valor P para BUA
2p16.2	*SPTBN1*	rs11898505	$4.24x10^{-13}$
6q22.33	*RSPO3*	rs7741021	$9.26x10^{-21}$
6q25.1	*CCDC170*	rs4869739	$1.93x10^{-9}$
6q25.1	*ESR1*	rs3020331	$2.91x10^{-9}$
		rs2982552	$1.70x10^{-10}$
7q31.31	*WNT16*	rs2908007	$4.32x10^{-35}$
10q21.1	*MBL2/DKK1*	rs7902708	$1.30x10^{-8}$
11q14.2	*TMEM135*	rs597319	$8.23x10^{-14}$
19q13.11	*GPATCH1*	rs10416265	$2.37x10^{-13}$

**El nivel de significación estadística GWAS se define como* $P < 5x10^{-8}$ (Moayyeri et al., 2014)

5.2.3. Estudios de interacciones génicas

El análisis de las posibles interacciones genéticas es clave para profundizar en la etiopatogenia de las enfermedades complejas como la osteoporosis (Lin et al., 2015). No obstante, a pesar de que se ha sugerido que dichas interacciones podrían jugar un papel importante en la osteoporosis acentuando los fenotipos del hueso (DMO, QUS, etc.) y, en consecuencia, aumentando el riesgo de padecer la enfermedad, hasta la fecha se han publicado escasos estudios (Giguère et al., 2000; Koh et al., 2004; Patel et al., 2000; Rivadeneira et al., 2006; Yang et al., 2013; Zupan et al., 2009). Además, la gran mayoría

de los trabajos se han centrado en la medición de la masa ósea mediante DXA. El conocimiento de la posible influencia de interacciones genéticas en los parámetros QUS es muy limitado (Giguère et al., 2000; Koh et al., 2004; Patel et al., 2000).

II. JUSTIFICACIÓN, OBJETIVOS Y DISEÑO EXPERIMENTAL

1. JUSTIFICACIÓN

La osteoporosis es la enfermedad metabólica ósea más frecuente en los países occidentales. La adquisición de un óptimo nivel de masa ósea a edades tempranas es esencial para prevenir la aparición de osteoporosis en la edad adulta. A pesar de ello, son escasos los estudios realizados con objeto de determinar los factores genéticos y ambientales asociados con la adquisición de un adecuado nivel de mineralización ósea en adultos jóvenes. La mayoría de los estudios publicados hasta la fecha se han focalizado en la caracterización de factores genéticos y ambientales asociados con fenotipos relacionados con la osteoporosis en poblaciones mixtas o de adultos mayores (generalmente mujeres postmenopáusicas).

Además, los trabajos previos se han centrado mayoritariamente en la identificación de marcadores genéticos y factores ambientales asociados con la DMO en diferentes áreas anatómicas determinadas con DXA. En relación a los factores genéticos asociados con las mediciones óseas obtenidas mediante ultrasonografía, los estudios publicados son limitados. En ese sentido, al ser el parámetro QUS un fenotipo poligénico en el que numerosos genes intervienen con un efecto moderado, aún quedarían numerosos marcadores genéticos por identificar.

La identificación de los marcadores genéticos que predisponen a una baja masa ósea en la adultez temprana permitiría identificar precozmente individuos con alto riesgo de desarrollar osteoporosis en la edad adulta. De esta forma, se podrían implementar estrategias tempranas de prevención con el objetivo de optimizar la adquisición de un adecuado nivel de masa ósea. Por otro lado, es de gran interés la determinación de los factores ambientales que contribuyen en mayor medida en la adquisición del máximo potencial de mineralización para detectar conductas susceptibles de modificación y mejora que garanticen un adecuado nivel de masa ósea en esta etapa del ciclo vital.

2. OBJETIVOS

2.1. Objetivo general

Analizar la influencia de factores genéticos y ambientales en la adquisición de los niveles de masa ósea en adultos jóvenes.

2.2. Objetivos específicos

1. Identificar nuevos marcadores genéticos asociados con el nivel de masa ósea mediante estrategia de genes candidatos en adultos jóvenes (publicaciones 1, 2, 3, 4, 5 y 6).
2. Determinar la influencia de factores modificables (antropometría, dieta y actividad física) en el nivel de masa ósea en adultos jóvenes (publicaciones 7 y 8).
3. Investigar la posible existencia de interacciones genéticas que estén actuando en la adquisición de la masa ósea en la adultez temprana (publicación 9).

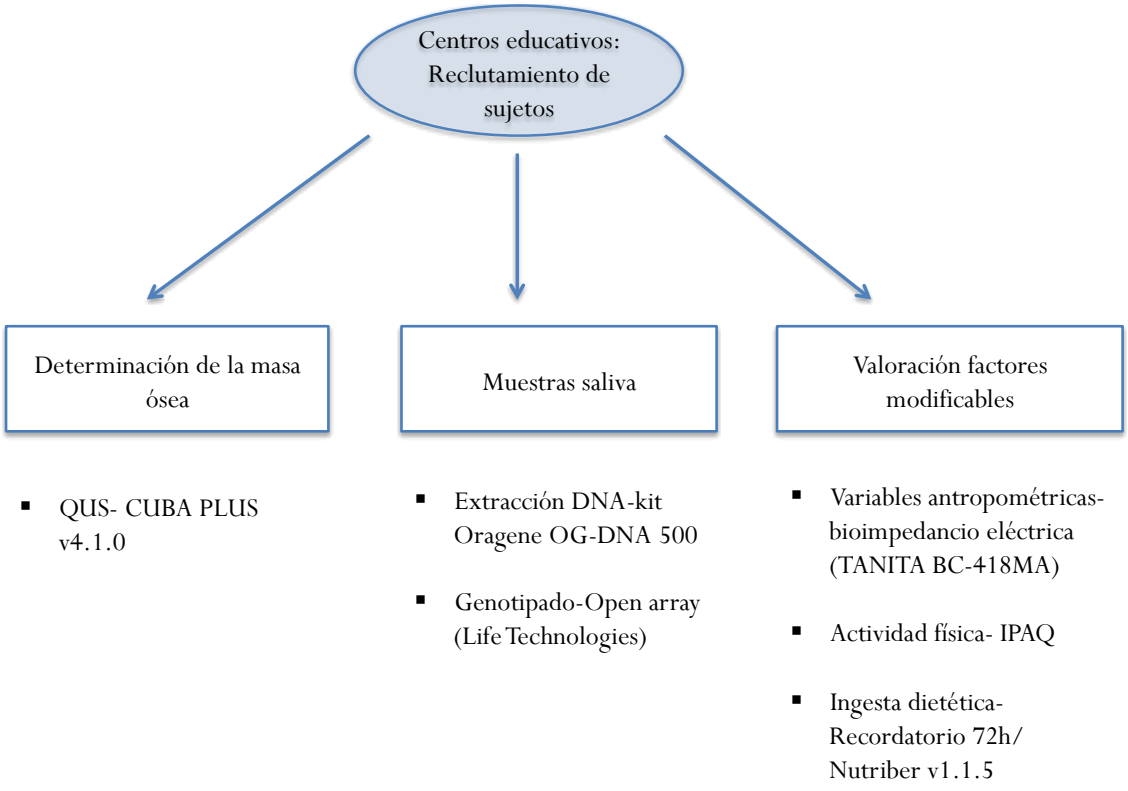
Los títulos de las publicaciones relacionadas con estos objetivos son los siguientes:

1. *A cross-sectional study of the association of VDR gene, calcium intake and heel ultrasound measures in early adulthood.*
2. *Polymorphisms in WNT16 gene are associated with calcaneus ultrasound parameter in Spanish young adults.*
3. *The rs3736228 polymorphism in the LRP5 gene is associated with calcaneal ultrasound parameter but not with body composition in a cohort of Caucasians young adults.*
4. *Association study of estrogen receptor alpha gene polymorphisms with bone mass assessed by quantitative ultrasound in young adults.*

5. *Association study of RANKL/RANK/OPG polymorphisms with heel quantitative ultrasound in young adults.*
6. *RSPO3 gene polymorphism is associated with ultrasound bone measurement in young adults.*
7. *The effects of body composition, dietary intake and physical activity on calcaneus quantitative ultrasound in Spanish young adults.*
8. *Dietary antioxidant quality score (DAQs) is associated with calcaneal quantitative ultrasound in young women.*
9. *Identifying SNP-SNP interactions associated with bone quantitative ultrasound parameter in early adulthood.*

3. DISEÑO EXPERIMENTAL

Para desarrollar el trabajo de la presente Tesis Doctoral, se ha llevado a cabo un estudio transversal en el que participaron cinco centros educativos de la provincia de Granada (España). La recogida de la muestra se realizó entre enero del año 2014 y octubre del año 2015. El protocolo experimental de reclutamiento de individuos, recogida de datos y de muestras biológicas fue aprobado por el Comité de Ética de Investigación Humana de la Universidad de Granada (Anexo 3). Se planteó el diseño experimental que se representa en la Figura 12.



3.1. Población de estudio

La población estuvo constituida por adultos jóvenes sanos de origen caucásico. Debido a que el reclutamiento de los sujetos se produjo de forma progresiva, el número de individuos incluidos varía en los diferentes trabajos presentados. La franja de edad de los participantes estuvo comprendida entre los 18 y 25 años (edad media: 20.41 ± 2.69 años). La presencia de enfermedades óseas, endocrinas o metabólicas, la toma de suplementos farmacológicos de calcio y vitamina D, así como el tratamiento previo o actual con fármacos que pudieran afectar a la mineralización ósea, se consideraron como criterios de exclusión. Todos los voluntarios se reclutaron tras mostrar su consentimiento por escrito (ANEXO 4). A todos los participantes se les realizó una valoración del nivel de masa ósea en el calcáneo mediante ultrasonografía, así como la determinación de sus hábitos de vida (ingesta dietética y nivel de actividad física) y parámetros antropométricos. Además, se les solicitó la donación de una muestra biológica de saliva.

3.2. Determinación de la masa ósea

La valoración de la masa ósea se realizó mediante ultrasonografía en la zona media del calcáneo derecho. El equipo de ultrasonidos utilizado fue el CUBA PLUS v4.1.0. (McCue Ultrasonics Limited, Compton, Winchester, UK). El parámetro de valoración ósea obtenido fue la atenuación ultrasónica de banda ancha (BUA - *Broadband ultrasound attenuation*, dB/MHz). Debido a las características técnicas del equipo de ultrasonidos utilizado, otros parámetros como SOS, SI o índice QUS no fueron evaluados. El equipo se calibró diariamente siguiendo las instrucciones del fabricante utilizando su correspondiente fantoma normalizado.

3.3. Recogida de datos sobre factores ambientales

3.3.1. Selección de factores ambientales

Inicialmente se realizó una revisión bibliográfica en la que se seleccionaron los factores ambientales que previamente se habían asociado significativamente con fenotipos relacionados con la osteoporosis (DMO, QUS, BMC, etc.) en distintas cohortes poblacionales. En base a la evidencia disponible, en esta Tesis Doctoral se seleccionaron variables antropométricas, nutricionales y de actividad física como factores no modificables.

3.3.2. Parámetros antropométricos

La altura corporal se midió con un estadiómetro de pared en posición vertical a 0,1cm y se registró en centímetros. La composición corporal (peso, masa magra, masa grasa y porcentaje de masa grasa) se midió con una balanza electrónica (TANITA BC-418MA), con una precisión de $\pm$ 100gr y se registró en kg. El IMC fue calculado como el peso corporal dividido por la altura al cuadrado (kg/m^2).

3.3.3. Hábitos dietéticos

La ingesta calórica total, así como la ingesta de macronutrientes y micronutrientes, se estimó de forma indirecta mediante un recordatorio de 72h (dos días laborales y un festivo). Posteriormente, las ingestas dietéticas fueron analizadas con el programa informático Nutriber versión 1.1.5 (ANEXO 5).

3.3.4. Actividad física

El nivel de actividad física se estimó mediante el cuestionario internacional de actividad física IPAQ *(International physical activity questionnaire*) (ANEXO 6).

3.4. Obtención de ADN, selección de marcadores genéticos y genotipado

3.4.1. Obtención de ADN

Se recogió a los participantes una muestra de saliva mediante un método no invasivo a través el kit Oragene OG-DNA 500 (DNA genotek, Ontario, Canada). La extracción del ADN se llevó a cabo de forma manual siguiendo las instrucciones del fabricante mediante procedimientos estándar. Una vez completada la extracción, se determinó la concentración de ADN de cada muestra y se procedió a la normalización de la concentración para su posterior genotipado.

3.4.2. Selección de genes y marcadores genéticos

En la presente Tesis Doctoral se analizaron 14 genes candidatos en base a su implicación en los mecanismos reguladores del proceso de remodelado óseo y su

asociación previa con osteoporosis y/o fenotipos relacionados en estudios previos (Tabla 4). Los marcadores genéticos seleccionados para cada uno de ellos fueron:

- *VDR:* como marcadores genéticos se seleccionaron tres SNPs: rs2228570 o FokI, rs731236 o TaqI y rs9729 en base a su asociación previa con la DMO y su relevancia funcional sobre los niveles de expresión del gen *VDR* (Grundberg et al., 2007; Whitfield et al., 2001).

- *WNT16:* se seleccionaron seis SNPs rs3801387, rs3801385, rs2908007, rs2908004, rs2707466 y rs2536184 que cubren la mayor parte de la variabilidad genética dentro y alrededor del este gen en poblaciones caucásicas, tal y como se describió previamente (Hendrickx et al., 2014).

- *RANKL, RANK, OPG, LRP5, SOST, ESR1:* se eligieron como marcadores genéticos varios SNPs para cada gen que habían mostrado una asociación con los niveles de DMO y/o en estudios de genes candidatos y/o GWAS (Estrada et al., 2012; Koller et al., 2013; Moayyeri et al., 2014; Paternoster et al., 2010; Richards et al., 2008; Roshandel et al., 2010).

- *SPTBN1, RSPO3, CCDC170, DKK1, GPATCH1 y TMEM135:* para estos genes se seleccionaron como marcadores genéticos los SNPs que habían mostrado asociación significativa ($P < 5.3x10^{8}$) con los niveles de QUS en el único en estudios GWAS previo (Moayyeri et al., 2014).

Tabla 4. Relación de genes y polimorfismos analizados en la presente Tesis Doctoral.

Cromosoma	Gen	SNP	Allel0	MAF en este estudio	HWE (p)
2	*SPTBN1*	rs11898505	G>A	0.37	0.75
6	*RSPO3*	rs7741021	A>C	0.39	0.84
6	*CCDC170*	rs4869739	A>T	0.35	0.22
6	*ESR1*	rs3020331	C>T	0.43	0.10
6	*ESR1*	rs2982552	C>T	0.49	0.19
6	*ESR1*	rs2982575	C>T	0.49	0.17
6	*ESR1*	rs2504063	G>A	0.47	0.51
6	*ESR1*	rs2234693	T>C	0.44	0.19
6	*ESR1*	rs9340799	A>G	0.34	0.75
7	*WNT16*	rs2908007	T>C	0.18	0.33
7	*WNT16*	rs2908004	T>C	0.22	0.25
7	*WNT16*	rs3801387	T>C	0.10	0.78
7	*WNT16*	rs3801385	A>G	0.09	0.33
7	*WNT16*	rs2707466	G>A	0.22	0.39
7	*WNT16*	rs2536184	G>A	0.03	0.09
8	*OPG*	rs4355801	A>G	0.39	0.17
8	*OPG*	rs3102735	T>C	0.12	0.35
8	*OPG*	rs2073618	G>C	0.46	0.63
10	*MBL2/DKK1*	rs7902708	G>C	0.11	0.77
11	*TMEM135*	rs597319	A>G	0.31	0.19
11	*LRP5*	rs2306862	C>T	0.19	0.32
11	*LRP5*	rs599083	T>G	0.39	0.06
11	*LRP5*	rs556442	A>G	0.42	0.88
11	*LRP5*	rs3736228	C>T	0.17	0.46
12	*VDR*	rs2228570	G>A	0.36	0.49
12	*VDR*	rs9729	C>A	0.44	0.64
12	*VDR*	ss731236	C>T	0.40	0.82
13	*RANKL*	rs9594759	T>C	0.48	0.40
13	*RANKL*	rs12585014	G>A	0.18	0.11
13	*RANKL*	rs7988338	G>A	0.19	0.40
13	*RANKL*	rs2148073	C>G	0.18	0.60
17	*SOST*	rs4792909	G>T	0.42	0.31
17	*SOST*	rs851054	A>G	0.38	0.06
17	*SOST*	rs2023794	T>C	0.05	0.18
18	*RANK*	rs1805034	C>T	0.41	0.26
18	*RANK*	rs12458117	G>A	0.19	0.35
18	*RANK*	rs3018362	A>G	0.32	0.18
19	*GPATCH1*	rs10416265	A>G	0.32	0.06

SNP Polimorfismos de un solo nucleótido; MAF Frecuencia del alelo menor; HWE Equilibrio de Hardy-Weinberg.

3.4.3. Genotipado

Las muestras de ADN una vez normalizadas se enviaron para su genotipado al Centro Pfizer-Universidad de Granada-Junta de Andalucía de genómica e investigación oncológica (GENYO). Este centro cuenta, dentro de su plataforma de apoyo a la investigación, con la Unidad de Genómica y Genotipado, que ofrece el genotipado de SNPs. Las plataformas de elección fueron la tecnología "Genotyping Open array" (Life Technologies) y la tecnología "Taqman" (AppliedBiosystems).

3.5. Análisis estadístico

El análisis de los datos referentes a la valoración del nivel de masa ósea y su relación con los distintos factores genéticos y ambientales incluidos en la presente Tesis Doctoral se realizó utilizando el programa SPSS versión 21.0. (SPSS, Chicago, IL, USA). La asociación de los SNPs seleccionados con los valores óseos se investigó mediante un modelo de regresión lineal tras ajustar por variables de confusión. Además, se realizó un análisis de la covarianza (ANCOVA) para analizar la influencia de los genotipos de cada SNP en los niveles de masa ósea considerando un modelo genético dominante y recesivo. Para explorar las correlaciones entre los factores modificables (variables antropométricas, nivel de actividad física e ingesta dietética) y los valores de BUA en el calcáneo se utilizó el coeficiente de correlación de Pearson y de Spearman (r). La asociación entre los valores BUA (variable dependiente) y las variables ambientales se analizó mediante un modelo de regresión lineal ajustando por variables de confusión. En relación a las interacciones genéticas o genético-ambientales asociadas con la masa ósea, el análisis estadístico se llevó a cabo mediante el programa STATA versión 11.0 (STATA Corporation, College Station, TX). El nivel de significación se estableció para p valor < 0.05.

Por otro lado, se evaluó que todos los SNPs incluidos en esta Tesis Doctoral se encontraran en equilibrio de Hardy-Weinberg con un nivel de significancia al 1%. Además, el umbral de éxito de genotipado para cada variante genética analizada en el estudio se estableció en el 95%. El desequilibrio de ligamiento (LD) entre las variantes genéticas de cada gen y la estimación de haplotipos se calcularon con el software Haploview V4.1 (Barrett, Fry, Maller, & Daly, 2005). El poder estadístico de los diferentes análisis fue calculado por medio del software Quanto versión 1.2 (Gauderman & Morrison, 2006). Se utilizó la corrección de Bonferroni para comparaciones múltiples en los análisis independientes de cada gen.

III. PUBLICACIONES

1. A cross-sectional study of the association of VDR gene, calcium intake and heel ultrasound measures in early adulthood

[Correa-Rodríguez, M., Schmidt Rio-Valle, J., González-Jiménez, E., & Rueda-Medina, B. (2016). A cross-sectional study of the association of VDR gene, calcium intake, and heel ultrasound measures in early adulthood. Calcified Tissue International, 98(3), 226–34].

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ORIGINAL RESEARCH

A Cross-Sectional Study of the Association of *VDR* Gene, Calcium Intake, and Heel Ultrasound Measures in Early Adulthood

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Abstract The acquisition of a high adult peak bone mass (PBM) is considered an important determinant of osteoporotic risk later in life. Genetic and environmental factors determine optimal PBM acquisition in early adulthood. The aim of this study was to test the association of vitamin D receptor (*VDR*) gene polymorphisms and dietary calcium intake with the bone mass of young adults. The study population comprised a total of 305 individuals (mean age 20.41; SD 2.36) whose bone mass was assessed through heel ultrasound [quantitative ultrasound measurements (QUS)] measurements (BUA, dB/MHz). The *FokI* G/A, rs9729 G/T, and *TaqI* G/A polymorphisms were selected as genetic markers of *VDR*. A significant difference in BUA values was observed according to gender (females 82.96; SD 15.89 vs. males 97.72; SD 16.50; $p < 0.00001$). The mean dietary calcium intake of the study group (827.84 mg/day; SD 347.04) was lower than the dietary reference intake for young adults (1000 mg/day) and had no association with BUA. None of the three *VDR* polymorphisms tested showed an association with BUA. Similarly, the analysis of *VDR* 3′ haplotypes, estimated using rs9729 and *Taq1* as tag SNPs, did not reveal any significant association with QUS traits. Our results confirm the existence of different heel QUS for women and men, as well as a tendency towards low calcium consumption by young adults, and they also suggest that the *VDR* gene does not play a major role in the genetic determination of QUS parameter in early adulthood.

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Keywords Broadband ultrasound attenuation · Vitamin D receptor gene · Young adults · Peak bone mass · Calcium intake

Introduction

Osteoporosis is a multifactorial disease influenced by both genetic and environmental determinants, characterized by diminished bone mineral density (BMD), deterioration in bone microarchitecture, and an increased risk of fracture [1]. Bone mass in adulthood depends on the peak bone mass (PBM: amount of bone tissue present at the end of skeletal maturation) and rate of bone loss in late adulthood. The acquisition of a high adult PBM is considered an important determinant of osteoporotic risk later in life [2]. PBM is usually reached between 25 and 35 years of age and occurs earlier in women than in men [2, 3]. The interaction of genetic and environmental factors (body mass, nutrient intake, and physical activity) determines the acquisition of optimal PBM.

Among the environmental factors, calcium intake plays an important role in skeletal calcium retention during growth. It is considered that variations in calcium intake early in life may account for as much as a 5–10 % difference in PBM. Several studies support the fact that adequate calcium intake in adolescence/early adulthood improves PBM [4–6].

It is well established that genetic factors are major contributors to bone mass and density, accounting for up to 80 % of the variability in different bone traits (central BMD, hip fracture, heel quantitative ultrasound, bone loss, and PBM) [3, 7, 8]. Many of the studies aiming to characterize the genetic factors influencing bone mass have focused extensively on the vitamin D receptor (*VDR*) gene.

VDR has been considered an interesting candidate gene for bone traits due to its relevant role in regulating calcium absorption, bone remodeling, and the mineralization rate [9]. VDR is a steroid receptor that acts as a transcription factor and mediates the action of vitamin D in skeletal metabolism, including calcium absorption and the regulation of osteoblast differentiation [9]. *VDR* genetic studies generally use populations of adult-elderly subjects and dual-energy X-ray absorptiometry (DEXA) BMD measurements [10–13]. In contrast, the role of the *VDR* gene in bone gain during early adulthood, a period that corresponds to the most crucial years of PBM attainment, has not been thoroughly investigated. Only a few studies have been conducted, and these show conflicting results [14–17]. Similarly, studies that have sought to investigate the association of different *VDR* polymorphisms with bone properties other than BMD, such as those determined by quantitative ultrasound measurements (QUS) have led to contradictory results [18–21].

QUS, including broadband ultrasound attenuation (BUA), is a non-invasive technique useful in assessing bone structure and bone mass and provides determinants of bone structure beyond those associated with DXA [22]. Its portability, non-invasiveness, radiation-free, and low cost make QUS as an excellent alternative technique for assessing bone mass in healthy populations. QUS measurements reflect not only BMD but also additional information on microstructure, bone elasticity, and connectivity, which are related to bone mass [23–25]. A recent meta-analysis including elderly people showed that heel QUS predicts the risk of fractures independently of BMD [22]. Furthermore, recent studies have demonstrated that heel QUS measurements are useful for assessing PBM during early adulthood [26, 27].

In this study, we took advantage of heel QUS to assess the bone properties of a healthy young adult population with the aim of testing the association of *VDR* gene polymorphisms with QUS measurements. Thus, an association study involving three *VDR* polymorphisms (*FokI* G/A, rs9729 G/T, and *TaqI* G/A) was conducted. In addition, we wanted to evaluate whether variations in dietary calcium intake are associated with heel QUS measurements in our population.

Methods

Subjects

Three hundred and five healthy individuals of Caucasian ancestry (223 female and 82 male; mean age 20.41; SD 2.36; min. 18 max. 25; age range 7) were recruited from five public academic centers in Granada (Spain), located in the main districts of the city. A member of the research team visited the subjects at their academic centers and explained the objectives and characteristics of the study. Body weight measurements were taken (after removing shoes and heavy outer clothing) using a TANITA BC-418MA. Height was measured without shoes using a Harpenden stadiometer. Height and weight were used to calculate body mass index (BMI, kg/m^2). The subjects completed standardized questionnaires and were interviewed about the following aspects: medication, lifestyle factors, and alcohol consumption. Dietary calcium intake (DCI) was assessed using the 72-h recall method considering intakes on Thursday, Friday, and Saturday. To improve the accuracy of the food descriptions, standard household measures, pictorial food models (Dairy Food Council, USA), and ad hoc food photographs were employed during the interviews to define amounts when requested. The interviews lasted half an hour and the food-intake data were checked by a nutrition and diet expert. Food records were converted to nutrient intake using a computerized nutrient analysis program (Nutriber 1.1.5). Written informed consent was obtained from all individual participants and the study was approved by local ethics committees and conducted in accordance with the Declaration of Helsinki. Subjects with a history of bone disease, metabolic or endocrine diseases, and hormone-replacement therapy that could affect bone mass were excluded.

QUS Measurement

Bone quality was assessed by ultrasound measurements at the right calcaneus, which provides information on bone quality via different parameters including BUA, SOS (speed of sound), stiffness index, and the QUS index. The calcaneus bone is used for QUS assessment because it contains a large percentage of trabecular bone, which has high metabolic turnover rate [22]. The CUBA clinical ultrasound bone densitometer (McCue Ultrasonics Limited, Compton, Winchester, UK), which provides information on only BUA (attenuation of sound waves as they pass from the transmitting transducer to the receiving transducer, dB/MHz), was used to perform the QUS measurements.

Daily calibrations were made with a physical phantom to control the long-term stability of the apparatus.

VDR Genetic Marker Selection and Genotyping

Saliva samples for DNA extraction were collected from the study participants using the OG-500 Collection Kit (DNA GenotekInc, Ontario, Canada). The DNA was isolated from the saliva samples according to the manufacturer's protocol.

Three single-nucleotide polymorphisms (SNPs) (rs2228570 or *FokI*, rs731236 or *TaqI*, and rs9729) of the *VDR* gene were selected as genetic markers on the basis of their previous association with BMD levels and functional relevance in *VDR* gene expression levels [28, 29]. The rs2228570 C>T substitution leads to a new start codon that generates a longer *VDR* protein with lower transcriptional activity [28]. The rs9729 and rs731236 SNPs are tag SNPs for a haplotype in the 3′-UTR region, certain alleles of which have been shown to lead to *VDR* over expression [29].

The *FokI* and *TaqI* VDR polymorphisms were genotyped using a real-time allelic discrimination assay. Polymerase chain reaction (PCR) was performed using commercially available TaqMan probes in an ABI 7900 real-time PCR instrument (Life Technologies). A High-Resolution Melting (HRM) protocol was used for rs9729 SNP genotyping using forward primer "CTAAACGAGT CAATCCCCTCAT" and reverse primer "GCCCTCCTC TGTCAGTTTTCC" diluted in the Melt Doctor HRM Master Mix (Applied Biosystems). A real-time PCR was performed using the integrated cycler/fluorometer7900-upgraded equipment (Applied Biosystems) and monitored using fluorescent DNA intercalating dyes present in the Melt Doctor HRM Master Mix. The PCR protocol was as follows: denaturation of 10 min at 95 °C, 45 cycles of amplification (15 s at 95 °C and 30 s at 60 °C), and elongation during 5 min at 72 °C. The HRM curve was obtained through denaturation at 95 °C for 10 s, cooling to 60 °C for 1 min, and a temperature increase from 95 °C to 60 °C for 15 s with a 1 % ramp rate. The results were analyzed using the High Resolution Melt Software v3.0.1.

To guarantee the accuracy of the genotyping, duplicate samples and negative controls were included in all PCR reactions. In addition, 10 % of the samples were sequenced using Sanger technology, showing 100 % identical genotypes.

Statistical Analysis

All analyses were carried out using SPSS software, version 10.0 (SPSS, Chicago, IL, USA). Analyses were performed separately for males and females. The student's *t* test between means was used to assess the differences between males and females in population variables. Pearson correlations were performed to determine the relationships between BUA and weight, height, BMI, and DCI.

The relationship of the *VDR* genotypes and haplotypes with weight, height, BMI, and calcium intake variables, within each gender, were tested through variance analysis (ANOVA). Covariance analysis (ANCOVA) was employed to determine differences in QUS measurements according to genotype or haplotype, after adjusting for the following confounders: age, weight, and height.

The selected SNPs of the *VDR* gene were tested for Hardy–Weinberg equilibrium using an χ^2 test. Haploview software (Broad Institute of MIT and Harvard) was used to obtain pair-wise linkage disequilibrium (D') and correlation coefficients (r^2). p values <0.05 were considered to be statistically significant. The statistical power of the study was estimated using Quanto version 1.2 software (Department of Preventive Medicine, University of Southern California, CA) considering a BUA mean of 86.93, SD 22.32, 5 % type I error, MAFs of 0.36–0.44, 305 individuals, and an additive genetic model.

Results

The anthropometric characteristics, calcium intake, and mineralization levels of the 305 study subjects (combined and by gender) are shown in Table 1. As expected, significant differences in body weight ($p < 0.001$), height ($p < 0.001$) and BMI ($p < 0.005$) were observed between males and females. The mean BUA for the total population was 86.93 (SD 22.32) (dB/Mhz), similar to that observed for young adults in previous studies [26, 30].

A significant difference in calcaneus BUA levels was observed when gender was considered. Males had a significantly higher BUA than females (females 82.96; SD 15.89 vs. males 97.72; SD 16.50; $p < 0.00001$) (Table 1). This difference between males and females still remained significant after adjustment for BMI ($p < 0.00001$). When the correlation of BUA with anthropometric characteristics was analyzed, significant correlations with weight, height, and BMI were observed. Considering gender, only weight and BMI evidenced significant positive correlations with BUA in both males and females (Table 2).

The mean DCI of the study population was 827.84 (SD 347.04) mg/day, which is lower than the dietary reference intake (DRI) for the Spanish population [31] and the DRI reported by the Institute of Medicine of The National Academies [32]. Seventy-two percent of individuals consumed <1000 g of calcium per day. No significant differences in DCI were observed between males and females (Table 1). The correlation analysis of DCI with BUA showed no significant interaction (height, weight, and BMI were considered as confounding factors) (Table 2).

The three SNPs selected as genetic markers (*Fok1* G/A; *TaqI* G/A; rs9729 G/T) for the *VDR* gene were observed to be in Hardy–Weinberg equilibrium (HWE) (Table 3). The genotype frequencies were as follows: *Fok1* (AA, 13.6 %; AG, 44.0 %; GG, 42.4 %); rs9729 (TT, 18.9 %; TG 50.7 %; GG 30.5 %); and *TaqI* (AA, 35.6 %; AG, 48.7 %; GG, 15.8 %). The observed minor allele frequency (MAF)

Table 1 Characteristics of study subjects

Characteristic	Females ($n = 223$)		Males ($n = 82$)		All ($n = 305$)	
	Mean	SD	Mean	SD	Mean	SD
Age (years)	20.27	2.33	20.79	2.39	20.41	2.36
Height (m)	1.63 **	0.06	1.76	0.06	1.66	0.08
Weight (kg)	59.65**	10.33	73.67	12.34	63.42	12.54
BMI (kg/m^2)	22.37 *	3.84	23.73	3.55	22.73	3.80
Calcium intake IDC (mg/day)	814.6	343.9	862.1	356.9	827.6	347.6
BUA (dB/Mhz)	82.96**	15.89	97.72	16.50	86.93	17.32

* $p < 0.005$ between females and males

** $p < 0.0001$ between females and males

Table 2 Analysis of the correlations of BUA and independent variables

Characteristic	Females ($n = 223$)		Males ($n = 82$)		All ($n = 305$)	
	Correlation with BUA	p	Correlation with BUA	p	Correlation with BUA	p
Height (m)	0.063	0.174	0.141	0.103	0.312	0.000**
Weight (kg)	0.321	0.000**	0.298	0.003**	0.439	0.000**
BMI (kg/m^2)	0.307	0.000**	0.282	0.005**	0.334	0.000**
Calcium intake IDC (mg/day)	0.009	0.446	0.163	0.072	0.072	0.106

** $p < 0.0001$

Table 3 Minor allele frequency (MAF) and Hardy–Weinberg equilibrium (HWE) p value for the FoK1, rs9729, and Taq1 *VDR* polymorphisms

Polymorphism	Allele			HWE
	Nucleotide	Frequency	Frequency HapMap-CEPH	p
Fok1 rs2228570	A	0.36	0.41	0.49
rs9729	A	0.44	0.47	0.64
Taq1 rs731236	G	0.40	0.36	0.82

for the three SNPs was very similar to that reported for the European-CEPH population panel (Table 3).

The analysis of subject characteristics and QUS measurement according to genotype is described in Table 4 for the sexes combined, and in Tables 5 and 6 for females and males, respectively. None of the SNPs showed association with weight, height, and BMI. In addition, DCI did not differ between the *VDR* genotypes of the three tested SNPs.

The association between individual polymorphisms and QUS measurements was tested using ANCOVA, adjusted for age, weight, and height. No significant association of the *Fok1*, rs9729, and *Taq1* polymorphism genotypes with QUS measurements was found (Table 4). Subjects with the TT homozygote genotype for rs9729 showed the lowest BUA values but no statistically significant differences were observed.

VDR 3′ haplotypes were estimated using rs9729 and Taq1 as tag SNPs, as described previously [29]. We found that the two genetic markers were in strong LD ($D' = 0.97$; $r^2 = 0.86$) in our population. Among the six possible haplotypic combinations, the three most common were Hap1/Hap1 present in 30.0 % of the individuals, Hap1/Hap2 that accounted for 46.1 % of the population, and Hap2/Hap2 accounting for 15.8 % (Table 7). These three mayor haplotypes showed frequencies similar to those recorded in previous studies [29]. In a similar way to that observed in the individual SNP analysis, none of the haplotypes revealed statistically significant associations with any of the subject characteristics, including QUS traits (Table 7).

Discussion

In spite of the importance attributed to PBM acquisition for preventing osteoporosis later in life, there is little data on bone mass in healthy young adults. For that reason, this study involved a population of well-characterized healthy

Table 4 Subject characteristics and QUS measurement according to genotype ($n = 305$)

%	FokI							rs9729							TaqI						
	AA		AG		GG		*p*	TT		TG		GG		*p*	AA		AG		GG		*p*
	Mean 13.6	SD	Mean 44.0	SD	Mean 42.4	SD		Mean 18.9	SD	Mean 50.7	SD	Mean 30.5	SD		Mean 35.6	SD	Mean 48.7	SD	Mean 15.8	SD	
Age (years)	20.37	2.34	20.54	2.28	20.23	2.43	0.558	20.28	2.24	20.30	2.35	20..72	2.45	0.361	20..42	2.37	20.44	2.39	20.13	2.18	0.714
Weight (kg)	62.37	12.29	63.12	11.73	64.16	13.56	0.675	64.10	12.88	63.18	12.69	63.78	12.15	0.854	63.47	12.17	63.70	12.83	63.21	13.05	0.971
Height (m)	1.66	0.08	1.66	0.07	1.67	0.09	0.389	1.67	0.07	1.66	0.08	1.66	0.08	0.494	1.66	0.08	1.67	0.08	1.67	0.07	0.620
BMI (kg/m^2)	22.50	3.57	22.74	3.70	22.80	4.04	0.912	22.90	4.09	22.72	3.58	22.77	4.03	0.958	22.98	4.15	22.72	3.54	22..37	4.00	0.655
DCI (mg/day)	830.6	387.4	794.0	325.8	864.0	357.7	0.275	851.7	358.6	832.4	335.1	801.0	363.9	0.666	830.4	368.1	825.3	337.9	836.8	345.9	0.980
BUA adjusted (dB/Mhz)	87.46	17.77	87.05	18.32	86.95	17.48	0.682	83.79	16.68	87.15	18.02	85.01	16.47	0.145	85.24	16.29	89.14	18.41	87.03	17.07	0.110

p values were determined by analysis of variance except for BUA which were analyzed after adjusting for age, weight, and height

Table 5 Subject characteristics and QUS measurement according to genotype in females ($n = 223$)

%	FokI							rs9729							TaqI						
	AA		AG		GG		*p*	TT		TG		GG		*p*	AA		AG		GG		*p*
	Mean 14.1	SD	Mean 47.3	SD	Mean 38.6	SD		Mean 17.2	SD	Mean 52.3	SD	Mean 30.5	SD		Mean 36.4	SD	Mean 48.4	SD	Mean 15.2	SD	
Age (years)	20.10	2.16	20.52	2.29	19.93	2.40	0.209	20.16	2.33	20.10	2.24	20.69	2.50	0.244	20.35	2.39	20.21	2.31	20.03	2.20	0.788
Weight (kg)	57.74	8.11	60.79	10.54	58.96	10.87	0.265	58.97	8.35	59.45	10.02	60.81	11.84	0.602	60.78	11.87	59.30	9.67	58.81	8.88	0.540
Height (m)	1.63	0.04	1.63	0.06	1.63	0.06	0.575	1.64	0.05	1.63	0.06	1.63	0.05	0.361	1.62	0.05	1.63	0.06	1.65	0.05	0.097
BMI (kg/m^2)	21.72	2.88	22.61	3.81	22.30	4.23	0.523	22.00	3.55	22.32	3.49	22.80	4.55	0.557	23.09	4.62	22.16	3.38	21.50	3.13	0.096
DCI (mg/day)	827.2	417.6	787.2	331.8	847.0	333.9	0.497	807.5	331.6	834.7	348.8	778.8	346.0	0.577	819.6	356.6	813.4	343.4	807.0	336.9	0.983
BUA adjusted (dB/Mhz)	83.42	15.20	83.29	16.86	82.86	16.07	0.816	81.84	12.77	84.42	17.35	82.97	16.03	0.196	81.24	15.79	84.85	17.29	80.58	12.35	0.123

p values were determined by analysis of variance except for BUA which were analyzed after adjusting for age, weight, and height

Table 6 Subject characteristics and QUS measurement according to genotype in males ($n = 82$)

%	FokI							rs9729							TaqI						
	AA		AG		GG		*p*	TT		TG		GG		*p*	AA		AG		GG		*p*
	Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD	Mean	SD		Mean	SD	Mean	SD	Mean	SD	
	12.2		35.4		52.4			23.2		46.3		30.5			33.3		49.4		17.3		
Age (years)	21.20	2.78	20.62	2.30	20.81	2.40	0.805	20.53	2.09	20.92	2.58	20.80	2.38	0.845	20.63	2.35	21.05	2.53	20.36	2.20	0.600
Weight (kg)	76.75	12.18	71.50	12.14	74.42	12.55	0.438	74.64	14.15	74.47	13.32	71.73	9.19	0.645	71.33	9.49	75.28	12.99	73.57	15.61	0.449
Height (m)	1.75	0.08	1.75	0.06	1.76	0.07	0.636	1.73	0.06	1.76	0.066	1.77	0.07	0.202	1.77	0.07	1.76	0.06	1.73	0.06	0.267
BMI (kg/m^2)	24.94	4.50	23.23	3.29	23.79	3.49	0.423	24.68	4.59	23.94	3.60	22.68	2.18	0.158	22.67	2.34	24.19	3.56	24.42	5.11	0.169
DCI (mg/day)	841.2	292.1	817.8	308.0	896.9	401.7	0.647	938.3	402.6	825.7	294.8	859.5	409.3	0.538	861.5	404.7	855.8	325.3	907.0	369.1	0.897
BUA adjusted (dB/Mhz)	100.00	19.82	100.28	17.32	95.47	15.16	0.186	93.74	20.91	100.34	16.70	96.76	11.68	0.226	96.78	11.68	100.18	16.68	94.07	22.75	0.514

p values were determined by analysis of variance except for BUA which were analyzed after adjusting for age, weight, and height

young Caucasian individuals and it provides novel data about bone mass as indicated by QUS measurements at the time of PBM acquisition.

Interestingly, we observed that the BUA values were significantly higher in males compared to females. This agrees with a previous study that reported gender-dependent differences in bone mass for both children and adults [33–35]. It has been postulated that sex-dependent differences in anthropometric features, mainly weight and BMI, may account for the variations in bone mass between males and females [36, 37]. Our data support this hypothesis since we observed a positive correlation between weight and BMI with BUA levels in males and females.

Another interesting finding was that calcium intake was below the DRI (1000 mg/day) in 72 % of the study subjects, reinforcing previous literature showing that DCI in young adults is lower than the current RDA [38, 39]. Thus, we could confirm a tendency in young Caucasian adults in the Spanish population towards lower levels of dietary calcium. Although calcium has been reported as an important nutrient for improving PBM, previous studies using QUS have shown a lack of association between DCI and ultrasound measurements [30, 40]. Furthermore, a recent meta-analysis examining the effectiveness of calcium supplements in increasing BMD in healthy children found that calcium supplements had no effect on BMD [41]. Again, our results support this as we failed to detect a significant correlation between calcium intake and BUA values.

After adjusting for confounding factors, an analysis of the three *VDR* polymorphisms tested did not reveal any evidence of association with BUA. In addition, we performed, for the first time, an analysis of *VDR* 3′ haplotypes that revealed no evidence of association with QUS traits. The lack of association seen in our study is unlikely to be due to low statistical power since the total sample size analyzed represents a power of 80 % to detect fourfold increments between minor allele homozygotes and carriers of the major allele, assuming MAFs of 0.36–0.44 at the 5 % significance level. However, for associations with less-fold increments (twofold to threefold), the study power decreases to 50 %.

As in other recent studies, we observed no association of the *VDR* gene with bone properties at the time of PBM acquisition. A longitudinal study of Norwegian children and adolescents found no association of Bsm1 polymorphism with BMD [17]. Similarly, a lack of association of *Fok1* with BMD was reported in healthy adolescent girls [30].

In relation to the quantitative ultrasound properties of bone, few previous studies have analyzed the various *VDR* polymorphisms. Our results are consistent with many which found no relationship between the *Bsm1, Taq1*, and

Table 7 Subject characteristics and QUS measurement according to haplotype

	BUA Adjusted (dB/Mh)											
	Males				Females				All			
	%	Mean	SD	*p*	%	Mean	SD	*p*	%	Mean	SD	*p*
Hap1/Hap1	29.6	97.63	11.09	0.417	30.0	81.23	15.45	0.388	30.0	85.67	16.01	0.313
Hap2/Hap2	17.3	94.07	22.75		15.8	80.58	12.35		15.8	84.60	17.07	
Hap3/Hap3	–	–	–		0.7	85.00	12.72		0.7	85.00	12.72	
Hap1/Hap2	43.2	101.23	16.63		46.1	84.81	17.27		46.1	89.14	18.40	
Hap1/Hap3	3.7	90.00	16.82		5.1	80.67	18.99		5.1	82.53	18.40	
Hap2/Hap3	6.2	92.80	16.87		2.4	95.50	21.92		2.4	93.57	16.48	

p value was determined after adjusting for age, weight, and height

FokI polymorphisms with QUS parameters [19, 20]. A recent study showed that polymorphisms at the 3′ end of the *VDR* gene (*BsmI, ApaI, TaqI*) are not related to heel ultrasound values in post-menopausal women of Caucasian origin [19]. In addition, another study reported a lack of association between Bsm1 polymorphisms in a group of young Japanese women [20]. Similarly, in a recent GWAS meta-analysis focused on characterizing the genetic determinants of heel bone properties measured with QUS, no association with *VDR* genetic variants was detected [42].

In contrast to both these findings and our results, two studies reported an association of the *FokI* and *BsmI VDR* polymorphisms and calcaneal QUS [21, 43]. The *FokI* Ff genotype was found to be associated with QUS in Finnish adolescent boys but not in girls, in a study involving 124 individuals. The discordance in the results might be related to insufficient statistical power owing to the small sample size. The reported association between *BsmI* polymorphisms and calcaneal QUS was found in a population of post-menopausal Korean women [43]. In this case, differences in ethnicity, age, and environmental factors could account for the difference in association results. Therefore, the findings from this and previous studies point to the fact that *VDR* polymorphisms do not play a major role in QUS in young Caucasian populations.

On the other hand, due to the important role of *VDR* gene as a regulator of bone properties, several studies have been conducted to investigate its role in osteoporosis and BMD. Although *VDR* was one of the first genes reported to have links and association with osteoporosis and BMD [44], its implication is now questioned. Several independent studies have demonstrated no associations of *VDR* polymorphisms with BMD, which agrees with our findings [10, 12]. A large-scale analysis conducted by the GENOMOS consortium involving more than 25,000 European subjects found no association between five *VDR* SNPs, including *FokI* and *TaqI,* and BMD [45]. Similarly, a large-scale population-based study showed no evidence of association between common polymorphisms of the *VDR* gene and BMD in a population of over 3000 British women [12].

The results of this cross-sectional study are subject to the following limitations. The use of a 72-h recall method could result in underreporting of calcium dietary intake. However, as the 72-h recall was interviewer-driven using standard household measures and pictorial food models, and food-intake data was checked by a nutrition and diet expert, the margin of error is expected to be low. Thus, it is unlikely that the non-significant association of DCI and BUA observed could be attributable to measurement errors. Secondly, although DXA is considered the gold standard method in the assessment of bone mass, BUA measured at the heel has shown moderate correlation with heel bone mineral density (BMD) [22]. In addition, heel ultrasounds are also safer, cheaper, and more portable than DXA. These characteristics of heel ultrasounds support the use of the QUS methodology as an alternative to DXA. As we mentioned previously, another limitation of this study is the lack of statistical power to detect interactions of low-fold increments (twofold to threefold) of genetic markers. Moreover, statistical power to detect the significant differences in heel BUA would be much lower in sex-specific analyses than the calculated statistical power for overall sample analysis. Future studies including larger population sizes are necessary to address this issue. On other hand, only three common *VDR* SNPs were analyzed, and it would be possible that the role of non-tested *VDR* polymorphisms remains undetected.

As well as analyzing the association between calcium and the *VDR* gene in QUS traits, this study provides new information on bone mass in young adults, determined using the ultrasound technique. There are very few ultrasound studies on adults. In conclusion, our results confirm the existence of different heel QUS for women and men, as well as a tendency towards low calcium consumption in young adults. Additionally, the results do not support the implication of the *VDR* gene in bone mass as assessed by heel ultrasound measurements in early adulthood.

Further replication studies and meta-analysis are necessary to fully discount the implication of the *VDR* gene as a genetic marker of QUS traits and PBM.

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Authors Contribution Correa-Rodríguez M monitored data collection, wrote the statistical analysis plan, cleaned and analyzed the data, and drafted and revised the paper. Rueda-Medina B analyzed the data, and drafted and revised the paper. Schmidt Rio-Valle Jacqueline and González-Jiménez Emilio analyzed the data and revised the draft paper.

Compliance with Ethical Standards

Conflict of interest María Correa-Rodríguez, Jacqueline Schmidt Rio-Valle, Emilio González-Jiménez, and Blanca Rueda-Medina declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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2. Polymorphisms in *WNT16* gene are associated with calcaneus ultrasound parameter in Spanish young adults

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ORIGINAL ARTICLE

Polymorphisms of the *WNT16* gene are associated with the heel ultrasound parameter in young adults

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Abstract
Summary Bone mineral content is influenced by genetic factors. We investigated the role of *WNT16* in bone properties determined using quantitative ultrasound (QUS) on young adults. Three *WNT16* genetic markers (rs2908007, rs2908004, and rs2707466) were found to have a significant association with the broadband ultrasound attenuation (BUA) measurement, suggesting that *WNT16* influences bone mass in young adults.
Introduction The aim of this study was to investigate whether genetic markers on the *WNT16* gene are associated with bone mass, as assessed using QUS in a population of healthy young Spanish adults.
Methods A cross-sectional study was conducted on 575 individuals (mean age 20.41±2.69). Bone quality was assessed using BUA measurements (dB/MHz) on the right calcaneus. Six single nucleotide polymorphisms (SNPs) (rs2908007, rs2908004, rs3801387, rs3801385, rs2707466, and rs2536184) covering the *WNT16* gene were selected as genetic markers and genotyped to test their association with BUA variations.
Results The rs2908007, rs2908004, and rs2707466 SNPs were found to have a significant association with BUA (p=0.004, p=0.001, and p=0.004, respectively).
Conclusion We demonstrate for the first time that *WNT16* genetic polymorphisms influence QUS traits in a population of young adults. This finding suggests that *WNT16* might be an important genetic factor in determining peak bone mass acquisition.

Keywords Calcaneal ultrasound · Peak bone mass · Polymorphisms · *WNT16* · Young adults

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Introduction

Osteoporosis is a complex disease characterized by diminished bone mineral density (BMD), deterioration in bone microarchitecture, and an increased risk of fracture [1]. Susceptibility to this condition depends on both genetic and environmental determinants. Twin and family studies have shown the genetic contribution to different traits related to osteoporosis with heritability estimates, reaching 84 % for central BMD [2], 74 % for heel quantitative ultrasound (QUS) measurements [3, 4], and 50–85 % for peak bone mass (PBM) [5, 6].

In order to identify the genetic factors involved in different bone mineral traits throughout life, genetic linkage analyses, candidate gene studies, genome-wide association studies (GWAS), and meta-analyses have been conducted. Among others, the *WNT16* gene has been strongly associated with BMD, hip geometry parameters, fracture risk, and bone acquisition [7–15].

The *WNT16* gene, located on chromosome 7q31.31, codes for a protein belonging to the Wnt family, which is known to regulate bone homeostasis [16]. Wnt proteins activate β-catenin-dependent canonical and β-catenin-independent non-canonical signaling pathways. Wnt16 is a non-canonical WNT ligand that regulates bone resorption inhibiting osteoclastogenesis [17]. The importance of Wnt16 in the regulation of bone metabolism has been confirmed in Wnt16 ($Wnt16^{-/-}$) knockout (KO) mouse models [7, 11].

WNT16 was identified as one of the loci significantly associated with QUS parameters in a population of elderly Caucasian adults [10]. Recently, the Genetics Factors for Osteoporosis (GEFOS) consortium conducted the largest GWAS to date, focused on characterizing genetic factors associated with bone parameters assessed by ultrasonography, reporting the association of the single nucleotide polymorphism (SNP) rs2908007 with calcaneal measurements including broadband ultrasound attenuation (BUA) [9].

PBM attainment during early adulthood is an important determinant in osteoporosis risk later in life [18]. Identifying genetic factors that influence bone mineral accretion during childhood and adolescence is important as it contributes to the early identification of individuals at high risk of developing osteoporosis when they get older. To this end, determining bone properties using ultrasonography to further analyze genetic factors is of interest since it avoids young people being exposed to radiation [19, 20]. Interestingly, in previous studies, PBM acquisition was assessed using heel QUS during early adulthood [21, 22].

Although recent GWAS involving populations of children and adolescents have identified specific loci associated with BMD early in life [7–23], little is known about the genetic determinants of bone accretion assessed by ultrasound measurements.

Taking into consideration all this evidence, we aimed to investigate whether genetic polymorphisms in the *WNT16* gene are associated with bone mass, as assessed by quantitative ultrasound in a population of healthy young Caucasian adults.

Methods

Subjects

Five hundred and seventy-five unrelated healthy individuals of Caucasian ancestry (400 females and 175 males, median age 20.41±2.69) were recruited from different academic centers in Granada (Spain). Physical activity (PA) and its frequency were determined using the self-administered International Physical Activity Questionnaire (IPAQ) [24] that calculates the respective total minutes for vigorous PA, moderate PA, and walking. A metabolic equivalent (MET)-min was derived by multiplying the respective total minutes with the MET value of vigorous PA (MET=8.0), moderate PA (MET=4.0), and walking (MET=3.3), and then adding all three together (www.ipaq.ki.se). A wide range of PA activity level was found in study population from 0 MET (none PA) to 56,640 MET-min. Most of the individuals showed a MET-min value corresponding with moderate PA activity (median 1770 MET-min). Dietary calcium intake (DCI) was assessed using the 72-h recall method, considering intakes on Thursday, Friday, and Saturday. Food records were converted to nutrient intake using a computerized nutrient analysis program (Nutriber 1.1.5). For all subjects, a detailed medical history was obtained. In particular, subjects with a history of bone disease, metabolic or endocrine diseases, hormonal contraceptive therapy, or medication that could affect bone mass were excluded from the study. Informed consent was obtained from all individual participants, and the study was approved by the local ethics committees and conducted in accordance with the Declaration of Helsinki.

Anthropometry

Weight to the nearest 0.1 kg was recorded using a body composition analyzer (TANITA BC-418MA), and height was measured to the nearest 0.1 cm using a Harpenden stadiometer. Both measurements were taken without shoes. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). The same trained research assistant performed all the measurements.

QUS measurement

Bone quality was assessed through ultrasound measurements on the right calcaneus (BUA, dB/MHz) using the CUBA clinical ultrasound bone densitometer (McCue Ultrasonics Limited, Compton, Winchester, UK). The calcaneus is used for QUS assessment because it contains a large percentage of trabecular bone, which has high metabolic turnover [25]. Daily calibrations were made with physical phantoms to control the long-term stability of the apparatus.

WNT16 genetic marker selection and genotyping

Saliva samples for DNA extraction were collected from the study participants using the OG-500 Collection Kit (DNA Genotek Inc, Ontario, Canada). The DNA was isolated from the saliva samples according to the manufacturer's protocol.

Six SNPs (rs3801387, rs3801385, rs2908007, rs2908004, rs2707466, and rs2536184) were selected as genetic markers to cover most of the common genetic variation in and around the *WNT16* gene for Caucasian populations as previously described [15].

Genotyping was performed at the Genomic and Genotyping Unit in the GENyO Center (Pfizer-University of Granada-Junta de Andalucía Centre for Genomics and Oncological Research) using OpenArray technology (Life Technologies, Carlsbad, CA, USA). A TaqMan OpenArray genotyping plate was custom-designed including six predesigned TaqMan genotyping assays for each of the selected SNPs. Standard cycling conditions were used, as recommended by the manufacturer. Thermal cycling and fluorescence detection were performed using the QuantStudio 12 K Flex Real-Time PCR System (Applied Biosystems). The

genotyping call rate for the six Taqman assays included in the array was higher than 95 %. To guarantee the accuracy of genotyping, duplicate samples and negative controls were included in all genotyping arrays, presenting 100 % identical genotypes.

Statistical analysis

The chi-squared test was used to determine whether the observed genotype frequencies were compatible with the Hardy-Weinberg equilibrium (HWE). Between-loci linkage disequilibrium was analyzed with Haploview software (Broad Institute of MIT and Harvard) [26]. Linear regression analysis was used to analyze the relationships between the SNPs and calcaneus ultrasounds adjusted for age, sex, weight, height, physical activity, and calcium intake. The results are reported as a percentage change (β) in the standard deviation (SD) with 95 % confidence intervals (95 % CI). Values of less than 0.05 were considered statistically significant. Correction for multiple testing was performed using the Bonferroni method for the six SNPs tested. All statistical analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL, USA).

Results

The characteristics of the 575 study subjects are shown in Table 1. The mean BUA for the total population was 82.03, SD 26.72 (dB/Mhz), similar to that previously observed for young adults [21, 27].

The minor allele frequency (MAF), location, and function of the six SNPs selected as *WNT16* genetic markers are listed in Table 2. The observed MAF of all the SNPs examined in this study was comparable to those reported by the European-CEPH population panel. None of the SNPs failed the missingness test (genotyping >0.05) or frequency test (MAF <0.01) and were observed to be in HWE.

The linkage disequilibrium pattern between the tested SNPs in our population is shown in Fig. 1. As can be seen, there was a tight correlation between alleles at the rs2908004 and rs2707466 loci (r^2=0.98) as previously described [10, 15]. The r^2 values estimated between the other SNPs were very similar to those reported previously for Caucasian populations [10, 15].

Linear regression analysis revealed that the rs2908007, rs2908004, and rs2707466 *WNT16* SNPs were significantly associated with the BUA parameter after adjustments for age, sex, weight, height, PA, and calcium intake (p=0.004, p=0.003, and p=0.004, respectively). After the Bonferroni correction for multiple testing, the association of these three genetic markers with the calcaneus ultrasound was still significant (p=0.020, p=0.015, and p=0.020, respectively). For the remainder of the SNPs tested (rs3801387, rs3801385, and rs2536184), no significant association with QUS measurements was found (Table 3).

Table 1 Characteristics of the study population

Characteristic	Mean±SD
N	575
Age (years)	20.41±2.69
Height (m)	1.67 ±,08
Weight (kg)	63.72±12.82
BMI (kg/m^2)	22.63±3.72
Calcium intake (mg/day)	804.42±356.49
Physical activity (MET/min)[a]	1779.00 (0–56640.0)[b]
Heel ultrasound BUA (dB/Mhz)	82.03±26.72

Data are shown as mean±SD

BMI bone mineral index, *BUA* broadband ultrasound attenuation

[a] The calculation of MET-min is described in the "Methods" section

[b] MET-min are expressed as mean and range (minimum PA level: 0 MET/min; maximum 56,640.0 MET/min)

Discussion

This study involved a population of well-characterized young healthy Caucasian individuals in which we examined the association between six *WNT16* polymorphisms and bone strength as indicated by heel bone QUS measurements. For the first time, we confirmed the association of the rs2908007, rs2908004, and rs2707466 *WNT16* polymorphisms with BUA in young adults. In spite of the relevance attributed to PBM acquisition to prevent osteoporosis later in life, there are a few studies about bone mass as indicated by the QUS measurements in young adults [21, 22]. Most studies generally use populations of adult elderly subjects and BMD measurements to assess bone mass. In this line, this study provides new information (BUA parameter) about the status of bone mass assessed by ultrasonography at the time of PBM acquisition.

These findings agree with earlier studies and provide further confirmation of *WNT16* being a genetic factor influencing heel bone QUS parameters at various ages. A recent meta-analysis of GWAS studies performed by the GEFOS consortium reported that SNP rs2908007 was associated with calcaneal measurements including BUA [9]. Similarly, another study, which analyzed six SNPs of the *WNT16* gene in a cohort of 494 Spanish Caucasian individuals over 49 years of age, showed that rs2908004 and rs2707466 were associated with calcaneal QUS parameters [10].

In addition, these three genetic polymorphisms of the *WNT16* gene have been demonstrated to be associated with BMD at different bone sites [7, 8, 10–15]. The association between rs2908004 and rs2707466 SNPs and wrist fractures was reported

Table 2 Overview of the selected SNPs for WNT16, minor allele frequency (MAF), and Hardy-Weinberg equilibrium (HWE)

SNP	Allele[a]	MAF in this study	MAFHapMapCEU	Function	Location	HWE (*p*)
rs2908007	T/c	0.18	0.38	5′ UTR	121322110	0.33
rs2908004	T/c	0.22	0.36	Missense	121329715	0.25
rs3801387	T/c	0.10	0.26	Intron	121334711	0.78
rs3801385	A/g	0.09	0.09	Intron	121337489	0.33
rs2707466	G/a	0.22	0.41	Missense	121339035	0.39
rs2536184	G/a	0.03	0.17	3′ UTR	121348703	0.09

[a] Capital letters indicate the major allele and small letters indicate the minor allele

in a GWAS meta-analysis [11]. Furthermore, a candidate gene association study in a population of healthy men, which analyzed *WNT16* tagSNPs, confirmed the association of rs2908007 and rs2707466 with BMD [15]. Similarly, another GWAS meta-analysis found associations between rs2908007 and rs2908004 and total body BMD in 2660 children [7].

In contrast, and in agreement with our findings, no significant association between rs3801385, rs2536184, and rs3801387 SNPs with QUS measurements has been reported [10]. Intriguingly, in several studies, the rs3801387 SNP was found to be associated with BMD at different skeletal sites in populations of elderly individuals [12, 14, 15]. Therefore, it could be suggested that the genetic component underlying variability in different bone phenotypes (BMD, BUA, and PBM) might have shared genetic markers, while it is different for others, even in the same gene. Further functional studies focusing on these genetic markers are necessary to elucidate the molecular basis behind these differences.

On other hand, due to the contribution of genetic factors to PBM variability, recent studies have focused on identifying the genes underlying bone accrual. In line with our results, a GWAS study including a population of children found an association of *WNT16* SNPs with BMD [14]. Furthermore, in a GWAS meta-analysis involving a population of 2660 children, the reported association of WNT16 loci was strongest in the younger populations [7]. These results, together with our findings, suggest that the *WNT16* gene might be an important genetic factor contributing to bone mass accrual early in life.

One limitation of our study is that we did not perform functional experiments to determine the mechanisms by which the alleles of the three SNPs (rs2908007, rs2908004, and rs2707466) associated with BUA affect bone strength in the calcaneus. Further functional studies are needed to identify the causal variants and determine their effects on bone acquisition in young adults. On the other hand, calcaneus ultrasonography provides information on bone microstructure, elasticity, and connectivity through various parameters including BUA, SOS, the stiffness index, and QUS index. Due to the technical characteristics of the ultrasound bone densitometer used in this study, we only obtained information on BUA. For this reason, the influence of *WNT16* on other ultrasound parameters has not been evaluated.

In summary, we investigated, for the first time, the influence of the *WNT16* gene on bone strength in young adults. This was assessed using ultrasonography and provided evidence that the rs2908007, rs2908004, and rs2707466 SNPs have a significant association with BUA measurements in young Spanish adults. Further studies on independent

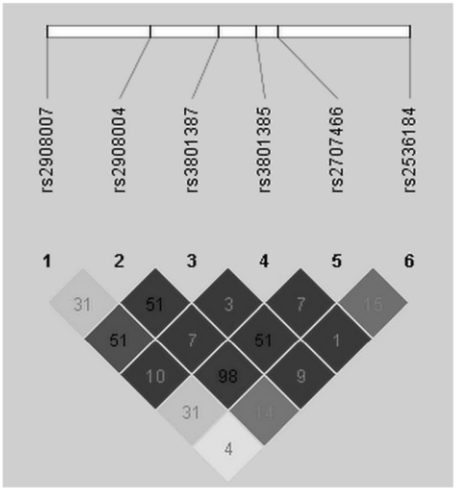

Fig. 1 Linkage disequilibrium pattern between the six tested *WNT16* SNPs (r^2)

Table 3 Linear regression analysis results for the association of SNPs in the *WNT16* gene with BUA

SNP	Heel BUA (Db/MHz)	
	β (95 % CI)	*p*
rs2908007	0.116 (0.211, 1.120)	0.004[a]
rs2908004	−0.133 (−1.247, −0.316)	0.001[a]
rs3801387	0.077 (−0.025, 1.084)	0.057
rs3801385	−0.011 (−2.170, 1.803)	0.781
rs2707466	−0.118 (−1.159, −0.229)	0.004[a]
rs2536184	0.019 (−0.605, 1.063)	0.632

Beta represents the regression coefficient

[a] Associations withstanding Bonferroni correction

populations are necessary to confirm the implication of *WNT16* as a genetic determinant of PBM acquisition as assessed by ultrasonography.

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Compliance with ethical standards All the procedures performed in the study involving human participants were undertaken in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Conflicts of interest None.

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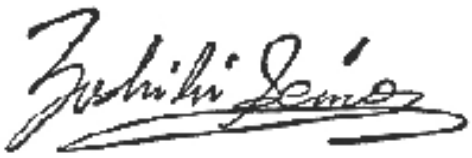

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The rs3736228 polymorphism in the *LRP5* gene is associated with calcaneal ultrasound parameter but no with body composition in a cohort of Caucasians young adults

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Abstract

The aim of the present study was to investigate the possible influence of low-density lipoprotein receptor-related protein 5 (*LRP5*) and sclerostin (*SOST*) genes as genetic factors contributing to calcaneal Quantitative ultrasound (QUS) and body composition variables in a population of Caucasian young adults. The study population comprised a total of 575 individuals (mean age 20.41; SD 2.36) whose bone mass was assessed through QUS to determine Broadband Ultrasound Attenuation (BUA, dB/MHz). Body composition measurements were performed using a Body Composition Analyser. Seven single nucleotide polymorphisms (SNPs) of *LRP5* (rs2306862, rs599083, rs556442 and rs3736228) and *SOST* (rs4792909, rs851054 and rs2023794) were selected as genetic markers and genotyped using a TaqMan OpenArray technology. Linear regression analysis was used to test the possible association of the tested SNPs with QUS and body composition parameters. Linear regression analysis revealed that the rs3736228 SNP of *LPR5* was significantly associated with BUA after adjustments for age, sex, weight, height, physical activity and calcium intake (p=0,028, β (95% CI) = 0,089 (0,099-1,691). For the rest of SNPs no significant associations with the QUS measurement were observed. Regarding body composition, no significant associations were found between *LRP5* and *SOST* polymorphisms and BMI, total fat mass and total lean mass after adjusted by age and sex as covariates. We reported that the rs3736228 *LRP5* genetic polymorphism influences calcaneal QUS parameter in a population of Caucasian young adults. This finding suggests that *LRP5* might be an important genetic marker contributing to bone mass accrual early in life.

Key words: Quantitative Ultrasound; calcaneus; *LRP5*; *SOST*; fat mass; lean mass.

Introduction

Quantitative ultrasound (QUS) is a relatively recent and non-invasive method of assessing bone mass. In addition to bone mineral density (BMD), QUS methods provide structural information on microstructure, bone elasticity and connectivity which may be important in determining the fracture risk [1, 2]. Besides this, QUS is a safe, radiation-free, cost-effective and convenient method compared to dual-energy x-ray absorptiometry (DXA). Heritability (h^2) studies involving twins and families have shown that calcaneal QUS parameters, including broadband ultrasound attenuation (BUA), are under genetic control (h^2 74%) [3, 4]. However, the majority of studies examining genetic influence on bone mass have focused on BMD measured by DXA, overall few studies about the genetic factors contributing to ultrasonography bone parameters have been conducted.

The canonical Wnt signaling pathway plays a main role in the regulation of bone homeostasis, influencing bone formation and resorption [5]. Low-density lipoprotein receptor-related protein 5 (*LRP5*), which encodes a cell surface receptor in the Wnt canonical signal, acts as a regulator of osteoblasts growth and as inhibitor of osteoblast and osteocyte apoptosis [6]. On the other hand, sclerostin (SOST) is a negative regulator of bone formation by inactivating signaling from LRP5/6 receptors in both osteocytes and osteoblast [7, 8]. Single nucleotide polymorphisms (SNPs) of *LRP5* and *SOST* have been analysed for their association with BMD and osteoporotic fractures in different populations yielding inconsistent conclusions [9–18]. Regarding ultrasonography bone parameters, there is limited information about the role of *LRP5* and *SOST* on bone mass status assessed by calcaneal QUS [17, 19, 20].

On the other hand, Wnt/β-catenin signaling is additionally involved in the negative regulation of adipogenesis [21, 22]. Evidence suggests the existence of common genetic factors contributing to both obesity and osteoporosis [23, 24]. In this line, recent studies have evaluated the relationship between *LRP5* and *SOST* polymorphisms and body compositions variables [25–28]. However, a clear relationship has not been elucidated.

On this bases, we aimed to investigate the possible influence of *LRP5* (rs2306862, rs599083, rs556442, rs3736228) and *SOST* (rs4792909, rs851054, rs2023794) polymorphisms as genetic markers contributing to bone mass (calcaneal QUS) and body composition variables (body mass index (BMI), total fat mass and lean mass) in a population of Caucasian young adults.

Material and methods

Study subjects

The population study comprised five hundred and seventy-five healthy individuals of Caucasian ancestry (400 females and 175 males, median age 20,41±2,69), who were agreed to participate in this study and were recruited from five different academic centres of Granada (Spain). All participants were evaluated by means of a detailed medical history. Subjects with any of the following criteria were excluded from the study: history of bone disease, metabolic or endocrine diseases, hormone-replacement therapy or current treatments that could affect bone mass. The study was approved by local ethics committees and conducted in accordance with the Declaration of Helsinki and informed consent was obtained for each participants.

Body composition and lifestyle variables

Body weight, body fat mass and lean mass measurements were performed using Body Composition Analyzer (TANITA BC-418MA®). Height was measured using a Harpenden stadiometer to the nearest 0,1 cm (Holtain 602VR®). Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). Physical activity (PA) were determined using the self-administered International Physical Activity Questionnaire (IPAQ) that calculates the respective total minutes for vigorous PA, moderate PA, and walking [29]. A metabolic equivalent (MET)-min was derived by multiplying the respective total minutes with the MET value of vigorous PA (MET = 8.0), moderate PA (MET = 4.0), and walking (MET = 3.3), and then adding all three together. Dietary calcium intake (DCI) was assessed using 72h recall method. Food records were converted to nutrient intake using a computerized nutrient analysis program (Nutriber 1.1.5).

Heel ultrasound

Bone mass status was assessed by ultrasound measurements at the right calcaneus (BUA, dB/MHz) using the CUBA clinical ultrasound bone densitometer (McCue Ultrasonics Limited, Compton, Winchester, UK). The calcaneus is used for QUS assessment because it contains a high percentage of trabecular bone and it is easy accessibility [30]. A well-trained investigator performed measurements using the same device. Daily calibrations were made with physical phantom to control the long-term stability of the apparatus.

Selection criteria for SNPs and genotyping

Saliva samples for DNA extraction were collected from study participants using the OG-500 Collection Kit (DNA Genotek Inc, Ontario, Canada). DNA was isolated from saliva samples according to manufacturer´s protocol.

Two genes from the Wnt signaling pathway (*LRP5* and *SOST*) identified as important in candidate gene and/or genome wide association studies (GWAS) for osteoporosis related phenotypes were included in this study [9, 11, 31]. A total of four SNPs of *LRP5* (rs2306862, rs599083, rs556442 and rs3736228) and three SNPs of *SOST* (rs4792909, rs851054 and rs2023794) were selected as genetic markers on the basis of their previous association with QUS and/or BMD parameters.

Genotyping was performed at the Genomic and Genotyping unit of GENYO centre (Pfizer-University of Granada-Junta de Andalucía Centre for Genomics and Oncological Research) using the Open Array technology (Life Technologies, Carlsbad, CA, USA). A TaqMan OpenArray genotyping plate was custom designed including seven predesigned TaqMan genotyping assays for each of the selected SNPs. Standard cycling conditions were used as recommended by the manufacturer. Thermal cycling and fluorescence detection were performed using QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). Genotyping call rate for the seven Taqman assays included in the array was higher than 95%. To guarantee accuracy of genotyping duplicate samples and negative controls were included in all genotyping arrays, showing 100% identical genotypes.

Statistical analysis

The chi-squared test was used to determine whether the observed genotype frequencies were compatible with the Hardy-Weinberg equilibrium (HWE). Linear regression analysis was used to analyze the relationships between the SNPs and calcaneus QUS

adjusted for age, sex, weight, height, physical activity and calcium intake. Similarly, the association between the SNPs and BMI, total fat mass and lean mass values were tested using linear regression with adjustments made for age and sex. Each SNP was tested separately. Results are reported as a percentage change (β) in a standard deviation (SD) with 95% confidence intervals (95% CI). P values less than 0.05 were considered to be statistically significant. All statistical analyses were performed using SPSS version 20.0 (SPSS,Chicago,IL,USA).

Results

The characteristics of the 575 study subjects are summarized in Table 1. Minor allele frequency (MAF), location and function of *LRP5* and *SOST* SNPs are showed in Table 2. The observed MAF of all the SNPs analyzed in this study was comparable to those reported for the European-CEU population panel. None of the SNPs failed the missingness test (genotyping >0.05) or the frequency test (MAF<0.01) and were observed to be in HWE.

Association analyses of calcaneal QUS and SNPs in the *LRP5* and *SOST* genes are shown in Table 3 for the combined population as well as stratifying individuals according to gender (Table 3). Interestingly, the linear regression analysis revealed that the rs3736228 *LRP5* SNP was significantly associated with BUA parameter after adjustments for age, sex, weight, height, PA and calcium intake in the whole sample (p=0.028, β (95% CI) = 0.089 (0.099 – 1.691) (Table 3). The analysis of the rs3736228 *LRP5* SNP considering females and males as separate groups only revealed a trend toward significance in females (p=0.092, β (95% CI) = 0.084 (-0.127, 1.671). For the rest of the tested SNPs in *LRP5* (rs2306862, rs599083 and rs556442) and *SOST*

(rs4792909, rs851054 and rs2023794) no significant associations with QUS measurements were found both in the combined and stratified analysis (Table 3).

In addition, the possible relationship between SNPs in the *LRP5* and *SOST* genes with body composition variables was investigated in the combined population (Table 4) and considering the gender (females Table 5, males Table 6). As can be observed no significant association between the *LRP5* and *SOST* polymorphisms and BMI, total fat mass and total lean mass after adjusted by age and sex as covariates was observed.

Discussion

Osteoporosis and obesity are two common complex diseases in which the contribution of genetic factors is well established [23, 24]. In this context, recent lines of evidence suggest the relationship between *LRP5* and *SOST* polymorphisms and bone mass variables and body compositions [25–28].

Taking advantage of the extended characterization of our study cohort for bone status assessed by QUS and body composition variables related to obesity, in this study we investigated the implication of genetic markers of *LRP5* and *SOST* in both mineral accrual and adiposity processes in a population of Caucasian young adults. Our findings provide evidence for the implication of the rs3736228 polymorphism in *LRP5* as a genetic factor influencing calcaneal QUS parameters but not for BMI and body composition in youth. After the stratification of the study population considering gender, the statistically significant association of the rs376228 with BUA could not be detected due to the reduction in sample size (women n=400 and men n=175) resulting in a decrease of statistical power to detect association. In addition, we investigated the possible association of genetic variants within *SOST* gene with calcaneal QUS

parameter however no association of any of the tested genetic markers with BUA parameter was identified.

To our knowledge, only two previous studies have investigated the association of the rs3736228 polymorphism in *LRP5* gene with calcaneal QUS measures in young adults [19, 20]. In the study of Saarinen et al. only 235 healthy young Finnish men were included and genotyped for different SNPs in *LPR5* gene. They observed that the rs3736228 SNP of *LRP5* was significantly associated with BMD and bone mineral content (BMC), but no with calcaneal BUA [20]. Similarly, Kumar et al. conducted an association study testing several SNPs in *LRP5* gene in two women cohorts (elderly women aged 75 years and young women aged 25 years). They did not find association of any of the tested *LRP5* SNPs with BUA parameter in young women [19]. Unfortunately, this study only shows partial results and the p values reached and magnitude effects cannot be observed. There could be different reasons accounting for the lack of concordance between our findings and these studies. Firstly, in the case of Saarinen et al. study considering the rs3736228 SNP MAF and the population size included, it would be possible that the association of this genetic marker remained undetected due to low statistical power. Secondly, another possibility is that differences in gender distribution in the three study populations may cause the lack of concordance in association results. Considering all these arguments, further replication studies are needed to fully clarify the implication of the rs3736228 SNP in QUS parameter in young adults.

On the other hand and in accordance with our findings, the rs3736228 in *LRP5* has been recognized as one of the key genes in bone mass accrual during growth [6]. Early studies evidenced the association of this genetic marker and BMC in adolescents [25, 32]. In addition, it have been reported that the rs3736228 SNP is associated with

different bone phenotypes status in early adulthood assessed by DXA [20, 33] . In the same line, a number of candidate gene based association studies [34, 35], GWAS [10] and meta-analysis of GWAS [9, 11] have found associations between rs3736228 polymorphism of *LRP5* and BMD or/and fracture risk. Thus, together all, these evidences suggest that *LRP5* could be not only a genetic factor for bone phenotypes in elderly but also an important gene influencing peak bone mass (PBM) accrual in adulthood.

For the rest of the tested SNPs in *LRP5* no significant associations with QUS measurements were found. These findings are in line with a previous study that found no association of rs2306862 and rs556442 SNPs with calcaneal BUA [20]. Similarly, our results revealed no significant association of any of the polymorphisms tested in *SOST*, pointing to the fact that *SOST* genes plays no significant role in the genetics of heel QUS in young Caucasian adults. To our knowledge this is the first study in which the possible implication of SNPs in *SOST* as genetic factors of the heel quantitative ultrasound parameter (BUA) has been investigated.

Regarding body composition phenotypes, the analysis of *LRP5* and *SOST* polymorphisms did not reveal any statistical significant association in our population. To our knowledge, only a few studies have sought to investigate the implication of genes of the Wnt pathway in young adults showing contradictory findings. In line with our findings, a previous study reported a lack of association of *LRP5* polymorphisms with obesity phenotypes in 1244 subjects from 411 Chinese nuclear families [27]. By contrary, in other studies it have been suggested that that *LRP5* and *SOST* are genetic factors contributing to obesity [25–28]. It is important to note that these studies are very heterogeneous with respect to population ancestry (Chinese, Iranian, European), study design (population sizes, gender or age/ methodology: nuclear family, case-control,

longitudinal, cross-sectional) and genetic markers selection.

A limitation of the present study is its cross-sectional design that makes difficult to draw conclusions about the bone mass status over time according to genotype. Due to the technical characteristics of the ultrasound bone densitometer used in this study we only obtained information about BUA. Thus, the influence of *LRP5* and *SOST* in other ultrasound parameters such as speed of sound (SOS), stiffness index and QUS index) has not been evaluated. On the other hand, the strengths include that the cohort studied in this study is well-characterized, participants were of similar age and all of them from Caucasian origin, thus negating the effect of population stratification. In addition, we measured total fat mass and lean mass as indices of body composition and investigated the relationship between *LRP5* and *SOST* polymorphisms, calcaneal QUS and body composition phenotypes in a large sample size of young adults.

In conclusion, we reported a significant association of the rs3736228 *LRP5* polymorphism with calcaneal QUS measurement in young adults supporting the role of these gene as a genetic factor contributing to bone mass during early adulthood but not to the adiposity process. Further replication studies are needed to confirm the role of the *LRP5* gene in bone mass accrual during youth.

Conflict of Interest

María Correa-Rodríguez, Jacqueline Schmidt Rio-Valle, and Blanca Rueda-Medina declare that they have no conflict of interest.

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All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Table 1. Characteristics of study population.

Characteristic	Females (n=400)	Males (n=175)	Overall (n=575)
Age (years)	20.37±2.71	20.50±2.69	20.41±2.69
Height (m)	1.63±0.06	1.75±0.07	1.67±0.08
Weight (kg)	59.54±10.19	73.29±13.13	63.72±12.82
BMI (kg/m^2)	22.16±3.60	23.69±3.77	22.63±3.72
Fat mass (kg)	15.48±8.11	11.80±7.33	15.12±9.27
Lean mass (kg)	44.30±3.51	61.35±7.54	48.63±9.67
Calcium intake (mg/day)	789.22±351.28	839.14±366.79	804.42±356.49
Physical activity (MET/min) [a]	2383.57 (0-56640)	3768.56 (0-23184)	2805.08 (0-56640)
Heel ultrasound BUA (dB/Mhz)	78.26±25.45	90.65±27.62	82.03±26.72

Data are shown as mean±SD.
BMI bone mineral index. BUA broadband ultrasound attenuation
[a] The calculation of MET-min is described in the Methods section. MET-min are expressed as mean and range.

Table 2. Overview of SNPs selected as genetic markers in *LRP5* and *SOST* genes.

Chromosome	Gene	SNP	Allele	MAF in this study	MAF HapMapCEU	Position	HWE (p)
11	*LRP5*	rs2306862	C>T	0.189	0.159	68410042	0.32
11	*LRP5*	rs599083	T>G	0.394	0.274	68424878	0.06
11	*LRP5*	rs556442	A>G	0.422	0.279	68425222	0.88
11	*LRP5*	rs3736228	C>T	0.176	0.138	68433827	0.46
17	*SOST*	rs4792909	G>T	0.426	0.385	43721456	0.31
17	*SOST*	rs851054	A>G	0.381	0.389	43759255	0.06
17	*SOST*	rs2023794	T>C	0.053	0.033	43760292	0.18

MAF the minor allele frequency. HWE p value for Hardy-Weinberg equilibrium

Table 3. Regression analysis between SNPs in *LRP5* and *SOST* genes and BUA parameter.

Gene	SNP	Heel BUA (dB/MHz)					
		Overall		Females		Males	
		β (95% CI)	P value	β (95% CI)	P value	β (95% CI)	P value
LRP5	rs2306862	0.052 (-0.277, 1.348)	0.196	0.024 (-0.777, 1.264)	0.639	0.126 (-0.216, 2.658)	0.095
LRP5	rs599083	-0.046 (-0.692, 0.249)	0.356	0.016 (-0.467, 0.615)	0.788	-0.095 (-1.334, 0.403)	0.291
LRP5	rs556442	0.005 (-0.443, 0.504)	0.900	0.013 (-0.492, 0.639)	0.799	0.015 (-0.837, 1.018)	0.847
LRP5	rs3736228	0.089 (0.099, 1.691)	0.028	0.084 (-0.127, 1.671)	0.092	0.113 (-0.425, 3.069)	0.137
SOST	rs4792909	0.048 (-0.168, 0.658)	0.244	0.018 (-0.385, 0.554)	0.723	0.023 (-0.741, 1.003)	0.767
SOST	rs851054	0.005 (-0.365, 0.417)	0.896	-0.05 (-0.696, 0.223)	0.312	0.090 (-0.315, 1.260)	0.238
SOST	rs2023794	-0.037 (-0.900, 0.330)	0.364	-0.044 (-1.071, 0.405)	0.376	0.031(-0.855, 1.305)	0.682

Beta represents the regression coefficient.
P values are shown adjusted for the covariates age. weight. height. physical activity and calcium intake.

Table 4. Regression analysis between SNPs in *LRP5* and *SOST* genes and obesity phenotypes.

Gene	SNP	BMI		Fat mass		Lean mass	
		β (95% CI)	P value	β (95% CI)	P value	β (95% CI)	P value
LRP5	rs2306862	0.069 (-0.046, 0.184)	0.239	0.163 (-0.085, 0.411)	0.197	0.042 (-0.133, 0.190)	0.730
LRP5	rs599083	-0.002 (-0.071, 0.068)	0.964	0.064 (-0.050, 0.248)	0.192	0.014 (-0.072, 0.126)	0.592
LRP5	rs556442	0.027 (-0.040, 0.094)	0.429	0.043 (-0.102, 0.187)	0.563	-0.036 (-0.130, 0.058)	0.451
LRP5	rs3736228	0.079 (-0.033, 0.191)	0.169	0.147 (-0.094, 0.388)	0.231	-0.053 (-0.210, 0.105)	0.510
SOST	rs4792909	-0.044 (-0.103, 0.014)	0.139	-0.033 (-0.159, 0.093)	0.604	-0.029 (-0.110, 0.051)	0.476
SOST	rs851054	-0.002 (-0.057, 0.054)	0.955	-0.021 (-0.141, 0.098)	0.725	-0.017 (-0.095, 0.061)	0.672
SOST	rs2023794	0.033 (-0.054, 0.121)	0.453	0.123 (-0.064, 0.310)	0.198	0.098 (-0.024, 0.220)	0.116

Beta represents the regression coefficient.
P values are shown unadjusted for the covariates age and sex.

Table 5. Regression analysis between SNPs in *LRP5* and *SOST* genes and obesity phenotypes in females.

Gene	SNP	BMI		Fat mass		Lean mass	
		β (95% CI)	P value	β (95% CI)	P value	β (95% CI)	P value
LRP5	rs2306862	0.058 (-0.066, 0.243)	0.263	0.042 (-0.208, 0.491)	0.426	0.036 (-0.098, 0.205)	0.488
LRP5	rs599083	0.011 (-0.075, 0.090)	0.852	0.075 (-0.067, 0.309)	0.206	0.078 (-0.027, 0.136)	0.192
LRP5	rs556442	0.011 (-0.076, 0.095)	0.833	0.005 (-0.179, -0.198)	0.923	-0.072 (-0.143, 0.025)	0.166
LRP5	rs3736228	0.056 (-0.062, 0.210)	0.283	0.027 (-0.225, 0.389)	0.600	-0.009 (-0.145, 0.121)	0.861
SOST	rs4792909	-0.070 (-0.118, 0.022)	0.181	-0.017 (-0.185, 0.132)	0.741	-0.025 (-0.086, 0.052)	0.634
SOST	rs851054	-0.053 (-0.104, 0.033)	0.308	-0.070 (-0.261, 0.048)	0.177	-0.048 (-0.099, 0.036)	0.355
SOST	rs2023794	0.017 (-0.097, 0.135)	0.745	0.041 (-0.158, 0.366)	0.434	0.024 (-0.088, 0.140)	0.652

Beta represents the regression coefficient.
P values are shown unadjusted for the covariates age and sex.

Table 6. Regression analysis between SNPs in *LRP5* and *SOST* genes and obesity phenotypes in males.

Gene	SNP	BMI		Fat mass		Lean mass	
		β (95% CI)	P value	β (95% CI)	P value	β (95% CI)	P value
LRP5	rs2306862	0.048 (-0.150, 0.286)	0.539	0.106 (-0.133, 0.720)	0.177	-0.020 (-0.480, 0.372)	0.803
LRP5	rs599083	-0.039 (-0.159, 0.102)	0.664	0.027 (-0.201, 0.273)	0.762	-0.027 (-0.318, 0.234)	0.766
LRP5	rs556442	0.072 (-0.072, 0.196)	0.359	0.102 (-0.085, 0.430)	0.187	0.007 (-0.250, 0.274)	0.926
LRP5	rs3736228	0.071 (-0.150, 0.406)	0.365	0.148 (-0.021, 1.064)	0.059	-0.084 (-0.837, 0.247)	0.285
SOST	rs4792909	-0.128 (-0.233, 0.024)	0.110	-0.110 (-0.431, 0.077)	0.170	-0.142 (-0.465, 0.023)	0.076
SOST	rs851054	0.059 (-0.071, 0.157)	0.454	0.069 (-0.125, 0.323)	0.384	-0.020 (-0.252, 0.194)	0.798
SOST	rs2023794	0.008 (-0.159, 0.178)	0.915	0.068 (-0.187, 0.475)	0.391	0.022 (-0.282, 0.376)	0.779

Beta represents the regression coefficient.
P values are shown unadjusted for the covariates age and sex.

4. Association study of estrogen receptor alpha gene polymorphisms with bone mass assessed by quantitative ultrasound in young adults

[Manuscrito en proceso de revisión]

Association study of estrogen receptor alpha gene polymorphisms with bone mass assessed by quantitative ultrasound in young adults

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Running Head: Estrogen receptor alpha gene and bone mass

Abstract

Background: Different genetic variants in estrogen receptor alpha (*ESR1*) have been shown to influence bone phenotypes including quantitative bone ultrasound in elderly.

Aim: We aimed to investigate the role of *ESR1* polymorphisms in bone mass assessed by calcaneal quantitative ultrasound (QUS) in a population of young adults.

Subjects and methods: The study sample consisted of 466 healthy individuals of Caucasian ancestry (315 females and 152 males) with a median age 20.39±2.70. Six *ESR1* polymorphisms (rs302033, rs2982552, rs2982575, rs2504063, rs2234693-*PvuII* and rs9340799-*XbaI*) were selected as genetic markers and genotyped. Bone mass in the right calcaneus was estimated with QUS.

Results: In the unadjusted analysis, rs2982575 polymorphism was significantly associated with quantitative ultrasound parameter in the whole sample (p=0.014, β (95% CI) = -0.114 (-1.023, -0.115). However, after adjusting for multiple confounding factors this association did not remain significant. For the rest of the selected polymorphisms in *ESR1,* no significant association was observed with calcaneal parameter. Linkage disequilibrium analysis identified a single LD block for the *ESR1* gene including *PvuII* and *XbaI* SNPs (pair-wise r^2 =0.66).

Conclusion: Our results revealed a lack of significant association between *ESR1* polymorphisms and calcaneal quantitative ultrasound in a cohort of young adults suggesting that *ESR1* gene do not play a major role in the acquisition of bone mass during early adulthood.

Key words: *ESR1*; quantitative ultrasound; polymorphisms; young adults.

Introduction

Osteoporosis is a common skeletal disorder characterized by low bone mass and structural deterioration of bone tissue predisposing to an increased risk of fractures (Cosman et al., 2014). Currently, osteoporosis is a mayor public health concern affecting over 200 million people worldwide (Kanis, 2007). Genetic factors are considered as determinant in several phenotypes relevant to the pathogenesis of osteoporosis including bone microarchitecture assessed by quantitative ultrasound (QUS) (Ralston & de Crombrugghe, 2006). QUS has been proposed as a non-invasive and alternative method to dual-energy X-ray absorptiometry (DXA) for assessment of bone status (Krieg et al., 2008). Twin and family studies have estimated that the heritability (h^2) of QUS ranges from 53 and 74% at calcaneus (Arden, Baker, Hogg, Baan, & Spector, 1996; Lee et al., 2004). However, due to the fact that most previous studies investigating the genetic factors contributing to bone mass have been focused on bone mineral density (BMD) measured by DXA, there is limited evidence on the influence of genetic factors in QUS parameters.

Optimizing peak bone mass (PBM), defined as the amount of bone gained at the end of the skeletal maturation, is a crucial factor for the prevention of osteoporosis later in life (Bonjour, Chevalley, Ferrari, & Rizzoli, 2009). PBM is usually acquired around the age of 30 (Berger et al., 2010; Bonjour et al., 2009) and is known to be genetically determined with heritability estimates reaching 50–85 % (Guéguen et al., 1995; Pocock et al., 1987). Therefore, identifying genetic factors that influence bone accrual during growth is of relevance since could contribute to the early identification of individuals at risk of developing osteoporosis in the elderly.

Estrogens are known to exert beneficial effects on the regulation of skeletal growth and maintenance of bone mass through the estrogen receptor α (*ERα*, *ESR1*) located at 6q25 (Manolagas, O'Brien, & Almeida, 2013). *ESR1*, the major mediator of estrogen action in bone, has been widely studied as a candidate gene in association studies of osteoporosis related phenotypes. In particular, rs2234693-*PvuII* and rs9340799-*XbaI* polymorphisms have shown association with several osteoporosis outcomes but with inconclusive results (Albagha et al., 2005; Binh et al., 2006; Gennari et al., 1998; Ioannidis et al., 2004; Massart et al., 2009; Rojano-Mejía et al., 2014; Sowers, Jannausch, Liang, & Willing, 2004; Tang et al., 2013; Valero et al., 2005; Wang et al., 2012). In recent years, genome-wide association studies (GWAS) and meta-analysis of GWAS have been performed leading to the identification of several genetic variants, including different *ESR1* polymorphisms, involved in bone phenotypes including BMD and QUS measurements (Koller et al., 2013; Moayyeri et al., 2014; Rivadeneira et al., 2009). Most of these studies have been conducted in mixed populations with samples of premenopausal, postmenopausal women and men considering wide age ranges. To the best of our knowledge, there have been limited prior studies carried out to investigate genetic markers that influence bone status in early adulthood, a period that corresponds to the most crucial years of PBM attainment.

In order to contribute to the identification of genetic markers involved in bone mass acquisition early in lifespan, in the present study we aimed to investigate the possible role of *ESR1* as a genetic marker of bone phenotypes assessed by calcaneal QUS in a population of young adults.

Methods

Study subjects

The population study comprised four hundred and sixty-six healthy individuals of Caucasian ancestry (315 females and 151 males, median age 20.38±2.70) from different academic centres of Granada (Spain). Exclusion criteria were history of bone disease, metabolic or endocrine diseases, hormone contraception therapy or current treatments that could affect bone mass. The study was approved by local Ethical Committee and conducted in accordance with the Declaration of Helsinki. A signed and informed consent was obtained for each participant.

Body composition and lifestyle variables

Body weight measurement was performed using Body Composition Analyzer (TANITA BC-418MA®). Height was measured using a Harpenden stadiometer to the nearest 0,1 cm (Holtain 602VR®). Body mass index (BMI) was calculated as weight divided by height squared (kg/m2). Physical activity (PA) were determined using the self-administered International Physical Activity Questionnaire (IPAQ) that calculates the respective total minutes for vigorous PA, moderate PA, and walking (Craig et al., 2003). Dietary calcium intake (DCI) was assessed using the 72-hr recall method that covers intake on a Thursday, Friday and Saturday (Yang et al., 2010). To improve the accuracy of the food descriptions, standard household measures and pictorial food models were employed during the interviews to define amounts when requested. Food records were converted to nutrient intake with Nutriber® software (Nutriber 1.1.5).

Calcaneal ultrasound

QUS of the right calcaneus was determined by measuring Broadband ultrasound attenuation (BUA) (dB/MHz) using the CUBA clinical ultrasound bone densitometer (McCue Ultrasonics Limited, Compton, Winchester, UK). QUS method has been postulated as a non-invasive, portable, inexpensive and useful tool for assessment bone mass alternative to DXA (Krieg et al., 2008). Heritability estimates for QUS of the heel appears to have comparable with BMD measured by DXA (Arden et al., 1996). Daily calibrations were made with physical phantom to control the long-term stability of the apparatus.

ESR1 genetic markers selection and genotyping

Saliva samples for DNA extraction were collected from study participants using the OG-500 Collection Kit (DNA Genotek Inc, Ontario, Canada). DNA was isolated from saliva samples according to manufacturer´s protocol. Six single nucleotide polymorphisms (SNPs) of *ESR1* (rs302033, rs2982552, rs2982575, rs2504063, rs2234693 and rs9340799) previously associated with osteoporosis related phenotypes (BMD and QUS parameters) in candidate gene and/or GWAS were selected as genetic markers in this study (Albagha et al., 2005; Koller et al., 2013; Massart et al., 2009; Moayyeri et al., 2014; Rivadeneira et al., 2009).

Genotyping was performed at the Genomic and Genotyping unit of GENYO centre (Pfizer-University of Granada-Junta de Andalucía Centre for Genomics and

Oncological Research) using the Open Array technology (Life Technologies, Carlsbad, CA, USA). A TaqMan OpenArray genotyping plate was custom designed including six predesigned TaqMan genotyping assays for each of the selected SNPs. Standard cycling conditions were used as recommended by the manufacturer. Thermal cycling and fluorescence detection were performed using QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). Genotyping call rate for the seven Taqman assays included in the array was higher than 95%. To guarantee accuracy of genotyping duplicate samples and negative controls were included in all genotyping arrays, showing 100% identical genotypes.

Statistical analysis

The chi-squared test was used to determine whether the observed genotype frequencies were compatible with the Hardy-Weinberg equilibrium (HWE). Linear regression analysis was used to analyse the relationships between each SNP in *ESR1* and calcaneus QUS unadjusted and adjusted for confounding factors (age, sex, weight, height, physical activity and calcium intake). Results are reported as a percentage change (β) in a standard deviation (SD) with 95% confidence intervals (95% CI) for each copy of the minor allele. Haploview program (Broad Institute of MIT and Harvard) was used to calculate the linkage disequilibrium (LD) coefficient and determine SNPs haplotypes (Barrett, Fry, Maller, & Daly, 2005). P values less than 0.05 were considered to be statistically significant. All statistical analyses were performed using SPSS version 20.0 (SPSS,Chicago,IL,USA).

The statistical power of the study was estimated using Quanto version 1.2 software (Department of Preventive Medicine, University of Southern California, CA)

considering a BUA mean of 81.96 standard deviation (SD) 27.65, 5% type I error, MAFs of 0.34-0.49, 466 individuals and an additive genetic model.

Results

Table 1 summarize the descriptive characteristics of the 466 study subjects by gender and as a whole. The mean calcaneal ultrasound measurement for the total population was 81.96, SD 27.65 (dB/Mhz), similar to that previously observed for young adults (Babaroutsi et al., 2005; Scheffler et al., 2014).

Position, function and minor allele frequency (MAF) of the six *ESR1* SNPs selected as genetic markers are showed in Table 2. None of the SNPs failed the missingness test (genotyping >0.05) or the frequency test (MAF<0.01) and all SNPs were observed to be in HWE.

Association analyses of SNPs in the *ESR1* gene and calcaneal ultrasound parameter without adjustment for confounding factors in the combined population as well as stratifying individuals according to gender are shown in Table 3. Linear regression analysis revealed that the rs2982575 polymorphism was significantly associated with calcaneal QUS in the whole sample (p= -0.014, β (95% CI) = -0.569 (-1.023, -0.115). However, this association did not remain statistically significant after adjusting for multiple covariates such as age, sex, weight, height, PA and calcium intake (Table 4). In the un-adjusted and adjusted analyses, none of the rest of SNPs in *ESR1* (rs3020331, rs2982552, rs2982575, rs2504063, *XbaI* and *PvuII*) were statistically significantly associated with quantitative ultrasound (Tables 3 and 4).

Linkage disequilibrium analysis pattern between the tested SNPs in our population is shown in Figure 1. A single LD block for the *ESR1* gene including *XbaI* and *PvuII* SNPs (pair-wise r^2 =0.66) was identified. The observed *XbaI-PvuII* haplotypes frequencies were: TA 55.0%, CG 34.3% and CA 9.8%. No significant association was observed between none of the possible haplotypic combinations and calcaneal ultrasound after un-adjustesd and adjusted regression analyses (data no shown).

Discussion

Due to the growing occurrence of osteoporosis worldwide, community-based genetic screening programs may become particularly relevant to identify individuals at risk of developing the disease. The use of genetic tests, as a novel preventive approach, might be of great significance in the implementation of early strategies to reduce osteoporosis risk (Andermann & Blancquaert, 2010). Otherwise, given the affordability of the technology and the potential to provide information on bone properties, quantitative ultrasound has been postulate as a valuable technique for assessing bone mass status in primary care services. Thus, the identification of genetic markers associated with calcaneal ultrasound parameter has become an issue of particular interest.

Our results revealed no significant associations of six tested SNPs in *ESR1* gene with calcaneal QUS in a population of young adults. Thus, it could be suggested that QUS as a complex phenotype, may be modulated by other genetic markers beyond *ESR1* in early life stage. In accordance with these findings, in a previous work it was demonstrated the lack of relationship between other phenotype related to osteoporosis and genetic variants in *ESR1*. *XbaI* and *PvuII* polymorphisms were not associated with

BMD in adjusted or unadjusted analyses in a large meta-analysis conducted by the GENOMOS consortium including 18917 individuals from eight European populations (Ioannidis et al., 2004). In this line, in a longitudinal study carried out in a population of Caucasian women, Sawers et al. reported a minimal impact of *XbaI* and *PvuII* genotypes on BMD measurements with respect to other covariates such as BMI (Sowers et al., 2004). Furthermore, no significant associations between BMD at different sites and *XbaI* and *PvuII* SNPs were reported in previous studies conducted in Caucasian women (Gennari et al., 1998; Valero et al., 2005).

In relation to previous studies analysing the role of *ESR1* in bone mass by assessed QUS, Albagha et al. found a significant association between *XbaI-PvuII* haplotypes and calcaneal ultrasound parameter (Albagha et al., 2005). Similarly, Binh et al. identified that *XbaI-PvuII* polymorphisms were associated with speed of sound (SOS) parameter (Binh et al., 2006). First, it is important to consider that although BUA and SOS are both parameters determined by QUS, BUA is influenced by connectivity and trabecular separation and SOS is directly related to the elasticity and density of the bone (Krieg et al., 2008). Thus, on the basis of our findings together with those from Binh et al., it could be postulated that these genetic variants in *ESR1* might influence SOS but not BUA. In addition, it is important to note that these two previous studies have been conducted in populations of postmenopausal women. Therefore, the possibility that *XbaI* and *PvuII ESR1* genetic variants could influence bone phenotypes only later in life when the level of oestrogens decreases should be considered. To confirm these hypotheses, further replication studies in independent populations of young adults would be of interest.

Regarding rs3020331, rs2982552, rs2982575, rs2504063 SNPs in *ESR1* gene, our findings revealed no significant associations with calcaneal QUS suggesting that these polymorphisms might not play a relevant role in bone gain during early adulthood. In contrast, rs3020331 and rs2982552 polymorphisms were identified as genetic determinants of heel bone properties in European subjects in a meta-analysis of GWAS conducted by GEFOS/GENOMOS (Genetic Markers of Osteoporosis) consortium (Moayyeri et al., 2014). Moreover, BMD was reported to be associated with rs2982575 and rs2504063 genetic variants in previous meta-analysis of GWAS conducted in Caucasian women (Koller et al., 2013; Rivadeneira et al., 2009). It would be relevant to consider that most meta-analysis, in order to maximize sample size and statistical power, have been conduced in combined samples of different ages and do not perform stratified analysis by age ranges. As we analysed a cohort including only young adults (18-25 years), again a possible reason for discrepancies may be caused by differences in population age range between the studies of Koller et al. (20-45 years), Rivadeneira et al. (18-96 years) and Moayyeri et al. (25-80 years) and our study cohort. Therefore, similarly to that observed for *XbaI* and *PvuII*, our findings raise the possibility that these other *ESR1* polymorphisms could be genetic markers for osteoporosis-related phenotypes later in life but not for bone mass accrual in early stages. However, given the current limited evidence concerning the potential role of *ESR1* gene polymorphisms in bone gain during early adulthood, it is difficult to completely exclude the possibility that these genetic variants are likely to be causal variants involved in PBM acquisition. Thus, further studies in young adults and functional studies are needed to confirm the preliminary findings of the present study.

There were potential limitations to this study. Due to its cross-sectional design, no causal conclusions can be drawn. In addition, we cannot discard that the limited

statistical power could contribute to lack of reported significant associations since the sample size analysed represents a power of 50 % to detect four fold increments of QUS traits in our cohort assuming MAFs of 0.34–0.49 at the 5 % significance level.

In summary, we investigated the possible influence of *ESR1* gene polymorphisms on bone mass status assessed by calcaneus QUS in a cohort of young adults. Our results suggest that *ESR1* polymorphisms do not contribute to heel ultrasound measurement in early adulthood.

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Conflict of Interest

The authors declare that they have no conflict of interest.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Table 1. Descriptive characteristic of study participants.

Characteristic	**Females** (n=315)		**Males** (n=152)		**Overall** (n=466)	
	mean	SD	mean	SD	mean	SD
Age (years)	20.31	2.67	20.54	2.76	20.38	2.70
Height (m)	1.63	0.06	1.75	0.06	1.67	0.08
Weight (kg)	60.01	10.71	73.55	13.24	64.40	13.20
BMI (kg/m^2)	22.31	3.81	23.70	3.82	22.79	3.87
Calcium intake (mg/day)	787.98	335.17	827.583	365.11	800.81	345.26
Physical activity (MET/min)[a]	2256.17	(0-15624.00)	3889.25	(0-23184.00)	2785.34	(0-23184.00)
Calcaneal ultrasound (dB/Mhz)	77.62	28.80	91.01	27.31	81.96	27.65

BMI bone mineral index; BUA broadband ultrasound attenuation.
[a] MET-min are expressed as mean and range.

Table 2. General information for the selected single-nucleotide polymorphisms of *ESR1* (6q25).

Marker ID	Chr position	Function	Alleles[a]	MAF	HWE[b]
rs3020331	151687645	Intron	C/T	0.43	0.10
rs2982552	151738428	Intron	C/T	0.49	0.19
rs2982575	151748656	Intron	C/T	0.49	0.17
rs2504063	151769572	Intron	G/A	0.47	0.51
rs2234693	151842200	Intron	T/C	0.44	0.19
rs9340799	151842246	Intron	A/G	0.34	0.75

HWE Hardy–Weinberg; MAF minor allele frequency.

[a] The second allele is the minor allele.

[b] p values of HWE equilibrium test.

Table 3. Unadjusted analysis of association between *ESR1* gene and quantitative bone ultrasound.

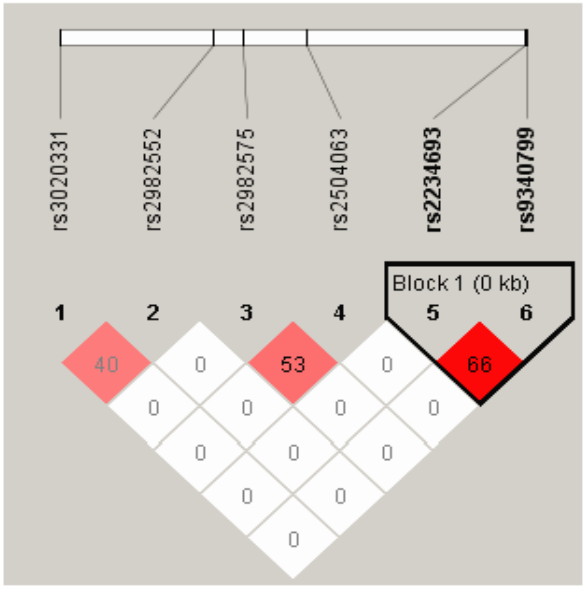
SNP	Calcaneal ultrasound (dB/MHz)								
	Overall			Females			Males		
	β	(95% CI)	P value	β	(95% CI)	P value	β	(95% CI)	P value
rs3020331	0.015	(-0.481, 0.678)	0.739	0.019	(-0.610, 0.855)	0.742	0.021	(-0.785, 1.019)	0.798
rs2982552	-0.024	(-0.664, 0.383)	0.599	0.030	(-0.451, 0.781)	0.599	-0.112	(-1.555, 0.280)	0.172
rs2982575	-0.114	(-1.023, -0.115)	0.014	-0.067	(-0.966, 0.241)	0.238	-0.083	(-1.054, 0.338)	0.311
rs2504063	-0.027	(-0.616, 0.334)	0.561	-0.076	(-0.923, 0.170)	0.176	0.108	(-0.290, 1.460)	0.188
rs2234693	0.039	(-0.289, 0.721)	0.401	0.025	(-0.465, 0.740)	0.654	0.004	(-0.852, 0.901)	0.956
rs9340799	-0.010	(-0.717, 0.579)	0.835	0.025	(-0.644, 1.022)	0.655	-0.037	(-1.208, 0.762)	0.655

Table 4. Adjusted analysis of association between *ESR1* gene and quantitative bone ultrasound*.

SNP	Calcaneal ultrasound (dB/MHz)								
	Overall			Females			Males		
	β	(95% CI)	P value	β	(95% CI)	P value	β	(95% CI)	P value
rs3020331	0.005	(-0.526, 0.585)	0.917	-0.005	(-0.752, 0.692)	0.934	0.004	(-0.876, 0.918)	0.963
rs2982552	-0.028	(-0.662, 0.340)	0.529	0.016	(-0.516, 0.693)	0.773	-0.107	(-1.516, 0.301)	0.188
rs2982575	-0.070	(-0.797, 0.092)	0.120	-0.070	(-0.972, 0.212)	0.207	-0.078	(-1.025, 0.349)	0.333
rs2504063	-0.023	(-0.577, 0.335)	0.602	-0.085	(-0.952, 0.116)	0.124	0.101	(-0.347, 1.446)	0.227
rs2234693	0.005	(-0.461, 0.516)	0.911	0.015	(-0.512, 0.679)	0.782	-0.009	(-0.921, 0.828)	0.917
rs9340799	0.011	(-0.543, 0.704)	0.800	0.037	(-0.538, 1.092)	0.504	-0.029	(-1.165, 0.807)	0.720

*Adjusted for sex, age, weight, height, physical activity and calcium intake.

Figure 1. Location and pair-wise linkage disequilibrium values of *ESR1* polymorphisms in Caucasian young adults.



Darker color indicates higher LD and lighter color indicates less LD (r^2).

5. Association study of *RANKL/RANK/OPG* polymorphisms with heel quantitative ultrasound in young adults

[Correa-Rodríguez, M., Schmidt Rio-Valle, J., & Rueda-Medina, B. (2016). Association study of *RANKL/RANK/OPG* polymorphisms with heel quantitative ultrasound in young adults. *Nursing Research*, In-press]

Factor de impacto en JCR 2015: 1.856; ranking 14/116 Categoría Nursing; Q1.

Nursing Research

Nov 02, 2016

RE: NRES-D-16-00006R1, titled "Association study of RANKL/RANK/OPG polymorphisms with heel quantitative ultrasound in young adults"

Dear Dr. Correa-Rodríguez,

Congratulations! I'm pleased to inform you that the paper titled "Association study of RANKL/RANK/OPG polymorphisms with heel quantitative ultrasound in young adults" whose authors are Correa-Rodriguez M, Schmidt-RioValle J and Rueda-Medina B has been accepted for publication in *Nursing Research*.

Congratulations again, and thank you for submitting your interesting and important work to the journal.

Sincerely,

Kathleen T. Hickey, EdD, RN, FNP, ANP, APNG, FAHA, FAAN
Action Editor, Special Issue on Omics in Nursing Science
Nursing Research

Susan J. Henly, PhD, RN, FAAN
Editor
Nursing Research

Association study of *RANKL/RANK/OPG* polymorphisms with heel quantitative ultrasound in young adults

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Short title: *RANKL/RANK/OPG* genes and quantitative ultrasound

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Conflict of interest

The authors declare that they have no conflict of interest.

Abstract

Background: The receptor activator of the nuclear factor-kappa B ligand (*RANKL*), the receptor activator of nuclear factor-kappa B (*RANK*), and the osteoprotegerin (*OPG*) signalling pathway play an important role in the regulation of bone remodelling and osteoclast differentiation. Quantitative ultrasound (QUS) is a relatively recent and non-invasive method providing structural information on microstructure, bone elasticity and connectivity. However, in contrast to bone mineral density (BMD) measurements, the possible association of the *RANKL/RANK/OPG* pathway with heel QUS has not previously been analysed.

Objectives: The aim of this study was to assess, for the first time, the contribution of the *RANKL/RANK/OPG* pathway genes in the genetic background of heel QUS parameters.

Methods: Ten single nucleotide polymorphisms (SNPs) of *RANKL (*rs9594759, rs12585014, rs7988338, rs2148073), *RANK* (rs1805034, rs12458117, rs3018362), and *OPG* (rs4355801, rs3102735, rs2073618) were selected as genetic markers and genotyped using Open Array technology in 575 self-reported Caucasian individuals aged 18 to 25. Bone mass in the right calcaneus was estimated with QUS to obtain the broadband ultrasound attenuation (BUA) measurement (dB/MHz). Linear regression analyses were performed to test the possible association between the SNPs and BUA.

Results: Linear regression analysis of all the tested SNPs revealed no significant association with the BUA parameter after adjusting for age, sex, weight, height, physical activity, and calcium intake. The lowest p value was observed for the rs9594759 *RANKL* polymorphism and heel QUS (p=0.064, β (95% CI) = -0.075 (-0.960, 0.028)).

Conclusion: Our results suggest that polymorphism of the *RANKL, RANK* and *OPG* genes does not make a significant genetic contribution to heel ultrasound measurements in a population of young Caucasian adults. Further studies replicating the results in independent populations are needed to support these initial findings.

Key words: Quantitative Ultrasound; calcaneus; *RANKL/RANK/OPG*; polymorphisms.

Osteoporosis is a skeletal disorder characterised by compromised bone strength that increases fracture risk (Cosman et al., 2014). Although osteoporosis is a multi-factorial disease involving both environmental and genetic factors, family and twins studies suggest that genetic factors explain approximately 84% of the variation in central bone mineral density (BMD) (Ralston, 2010). Due to the progressive ageing of the population, osteoporosis is considered a growing public health issue worldwide and significant economic costs are associated with osteoporotic fractures (Harvey, Dennison, & Cooper, 2010). Therefore, population-wide prevention strategies are required to reduce osteoporosis risk. For this reason, novel diagnosis tools based on the detection of molecular markers for the disease represent an excellent preventive strategy that could lead to the early identification of individuals at risk of osteoporosis and advance preventive programmes to improve the health and nursing care of these patients and reduce the impact of this disease. To this end, identifying genetic factors that contribute to an increased risk of osteoporosis is of great interest.

In order to identify the genes involved in bone remodelling, candidate gene association studies (Luo et al., 2014; Park et al., 2014), genome-wide association studies (GWASs) (Bae et al., 2016; Koller et al., 2010), and meta-analyses (Estrada, Styrkarsdottir & Evangelou, 2012; Rivadeneira et al., 2009) have been conducted leading to the identification of several loci associated with BMD.

Among the numerous genetic markers that have been shown to influence BMD, rs4355801, rs3102735, rs2073618, rs9594759, rs12585014, rs7988338, rs2148073, rs1805034, rs12458117 and rs3018362 single-nucleotide polymorphisms (SNPs), mapping to *TNFSF11*, *TNFRSF11A* and *TNFRSF11B* genes, have been reported to be associated with bone-related phenotypes in both candidate gene studies (Roshandel et al., 2011; Yamada, 2003) and GWAS (Paternoster, Lorentzon et al., 2010; Rivadeneira et al., 2009; Styrkarsdottir et al., 2008; Styrkarsdottir et al., 2009). It is important to note that most of these genetic studies, in order to maximize sample size and statistical power, do not include analyses stratified according to different age ranges (Estrada et al., 2012; Rivadeneira et al., 2009). Thus, to date there has been no evidence presented of the possible influence of the *RANKL, RANK* and *OPG* genes on bone mass in early adulthood, when peak bone mass is acquired.

TNFSF11, which encodes the RANK ligand (*RANKL*), *TNFRSF11A,* which encodes receptor activator of nuclear factor-kappa B (*RANK*), and *TNFRSF11B,* which encodes osteoprotegerin (*OPG*), define the RANKL/RANK/OPG signalling pathway that plays an important role in the regulation of bone remodelling and

osteoclast differentiation (Boyce & Xing, 2008). RANKL interaction with RANK stimulates osteoclast differentiation and inhibits osteoclast apoptosis while OPG antagonises the interaction between RANKL and RANK and regulates bone resorption by inhibiting osteoclast formation (Kohli & Kohli, 2011).

When assessing the bone mass of osteoporosis patients, BMD is usually obtained through dual-energy x-ray absorptiometry (DXA). In addition to this, calcaneal quantitative ultrasound measurement (QUS) has recently been postulated as a useful non-invasive technique for assessing bone structure. According to the International Society of Clinical Densitometry (ISCD), calcaneus QUS is the only recognised measure of QUS as a determinant of bone health status (Krieg et al., 2008) .The calcaneus is used for QUS assessment because it contains a high percentage of trabecular bone and is easily accessible (Töyräs, Nieminen, Kröger, & Jurvelin, 2002). Compared to DXA, QUS is gaining popularity for bone assessment in healthy populations because of its non-invasiveness, convenience, portability, and low cost. In addition to this, QUS measurements are correlated with BMD and are clinically important in providing determinants of bone structure beyond those associated with DXA (Moayyeri et al., 2012). Evidence suggests that QUS measurements reflect not only BMD but also provide additional information on microstructure, bone elasticity and connectivity (Krieg et al., 2008). In addition, previous studies have shown that QUS of the calcaneus predicts osteoporosis fracturing independently of BMD (Moayyeri et al., 2012). The affordability of the technology and the potential to provide information on bone properties make QUS an appropriate technique for assessing bone mass in primary care.

Similarly to BMD, heritability (h^2) studies have shown that calcaneal QUS parameters, including broadband ultrasound attenuation (BUA), are under significant genetic control. Heritability estimates (h^2) (percentage of variance of a trait explained by genetic factors) for calcaneal QUS measurements are as high as 74% (Karasik et al., 2010). However, most studies examining genetic influence on skeletal traits have focused on BMD measured by DXA in postmenopausal women and older men (Roshandel et al., 2011; Velázquez-Cruz et al., 2014; Wang et al., 2013).

By contrast, little is known about the genetic factors contributing to ultrasonography bone parameters. A few GWAS and candidate gene studies, have demonstrated the association of genetic markers in the *WTN16* gene with heel QUS (Correa-Rodríguez, Schmidt-RioValle, & Rueda-Medina, 2015a; García-Ibarbia et al., 2013; Moayyeri et al., 2014), while for other genes, such as VDR, no evidence of association has been observed (Correa-

Rodríguez, Schmidt-RioValle, González-Jiménez, & Rueda-Medina, 2015b; Moayyeri et al., 2014; Omasu et al., 2004; Zajickova, Zofkova, & Hill, 2005).

In spite of being interesting candidate genes for their implication in bone metabolism, the possible associations of *RANKL, RANK* and *OPG* genes with heel QUS have not been analysed previously. Taking into consideration this evidence, we aimed to determine, for the first time, whether genetic polymorphisms in the *RANKL, RANK* and *OPG* system were associated with calcaneal bone properties assessed by QUS in a population of young adults.

METHODS

Subjects

Five hundred and seventy-five self-reported unrelated healthy individuals of Caucasian ancestry (400 (69.6%) females and 175 (30.4%) males, mean age 20. 41±2. 69) were recruited from various academic centres in Granada (Spain). To assess physical activity (PA), we used the self-administered short version of the International Physical Activity Questionnaire (IPAQ). The questionnaire is a valid and reliable instrument for measuring PA in European adult populations (Craig et al., 2003). It calculates total hours spent performing vigorous and moderate PA and walking. A metabolic equivalent (MET)-min was derived by multiplying the respective total minutes with the MET value of vigorous PA (MET = 8.0), moderate PA (MET = 4.0), and walking (MET = 3.3), and then adding all three together. Dietary calcium intake (DCI) was assessed using the 72-hr recall method that covers intake on a Thursday, Friday and Saturday (Yang et al., 2010). To improve the accuracy of the food descriptions, standard household measures and pictorial food models were employed during the interviews to define amounts when requested. Food records were converted to nutrient intake with Nutriber® software, a computerised nutrient analysis program designed for collecting and analysing dietary recalls and then calculating dietary calcium intake (mg) (Mataix & Collado, 2006).

For each participant, height was measured to the nearest 0.1 cm using a Harpenden stadiometer, and weight to the nearest 0.1 kg using a Body Composition Analyser (TANITA BC-418MA). Weight and height measurements were made without shoes. The same trained research assistant performed all the measurements. Subjects with a history of bone disease, metabolic or endocrine diseases, hormonal contraceptive therapy or medications that

could affect bone mass were excluded. Written informed consent was obtained from each participant and the study was approved by the relevant ethics committees and conducted in accordance with the Declaration of Helsinki.

QUS measurement

QUS measurements were taken of the right calcaneus using the CUBA clinical ultrasound bone densitometer (McCue Ultrasonics Limited, Compton, Winchester, UK) to determine the broadband ultrasound attenuation (BUA). BUA refers to the slope between attenuation of sound signals and its frequency, and it was measured as dB/MHz. The same QUS device was used in all the academic centres and was calibrated each day using the physical phantom provided by the manufacturer. QUS is a non-invasive technique useful in assessing bone structure and bone mass in healthy populations. Since our study population comprised young healthy individuals, we considered QUS the ideal technique for assessing bone mass.

SNP selection and Genotyping

Saliva samples for DNA extraction were collected from study participants using the OG-500 Collection Kit (DNA Genotek Inc, Ontario, Canada). DNA was isolated from the saliva samples according to the manufacturer´s protocol. We selected ten SNPs (rs4355801, rs3102735, rs2073618, rs9594759, rs12585014, rs7988338, rs2148073, rs1805034, rs12458117 and rs3018362) in *RANKL/RANK/OPG* as genetic markers based on their previous association with BMD in GWAS and/or candidate gene studies (Paternoster, Lorentzon et al., 2010; Richards et al., 2008; Rivadeneira et al., 2009; Styrkarsdottir et al., 2009). Genotyping was performed at the Genomic and Genotyping unit of the GENYO centre (Pfizer-University of Granada-Junta de Andalucía Centre for Genomics and Oncological Research) using the Open Array technology (Life Technologies, Carlsbad, CA, USA). A TaqMan OpenArray genotyping plate was custom designed, including predesigned TaqMan genotyping assays for each of the selected SNPs. Standard cycling conditions were employed as recommended by the manufacturer. Thermal cycling and fluorescence detection were performed using the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). The genotyping call rate for the ten SNPs included in the array was higher than 95%. To guarantee the accuracy of the genotyping, duplicate samples and negative controls were included in all genotyping arrays, giving 100% identical genotypes.

Statistical analysis

The Hardy-Weinberg equilibrium (HWE) was tested for each SNP using the chi-squared test. For all analyses the genotype of each SNP was encoded as 0, 1, 2, where 0 and 2 denotes homozygotes with major allele and minor allele respectively and 1 codes heterozygotes. First, each SNP was tested separately for association with BUA values and then the Haploview program (Broad Institute of MIT and Harvard) was used to calculate the linkage disequilibrium (LD) coefficient and determine SNPs haplotypes (Barrett, Fry, Maller, & Daly, 2005). The association between each SNP as well as the haplotypes and BUA values was tested using linear regression with adjustments made for age, sex, weight, height, physical activity and calcium intake. The results were reported as the change of the outcome variable (β coefficient) with 95% confidence intervals (CIs). Values less than 0.05 were considered to be statistically significant. All analysis were performed using SPSS version 20.0 (SPSS,Chicago,IL,USA).

The statistical power of the study was estimated using Quanto version 1.2 software (Department of Preventive Medicine, University of Southern California, CA). Considering a BUA mean of 86.93 standard deviation (SD) 22.32, 5% type I error, MAFs of 0.13-0.48 and 575 individuals, 80% power was estimated to detect differences of 0.4 SD and 50% power to detect differences of 0.3 SD under an additive genetic model (Gauderman & Morrison, 2006).

RESULTS

The characteristics of the 575 study subjects (400 females and 175 males) have been published previously (Correa-Rodríguez et al., 2015a). The mean and SD or range for 575 subjects´ descriptive characteristics are as follows: age 20.41 ± 2.69 years; weight 63.72 ± 12.82 kg; height 1.67 ± 0.08 m; calcium intake 804.42 ± 356.49 mg/day; PA 1779.00 (0-56640) MET/min; and BUA 82.03 ± 26.72 (dB/Mhz).

Minor allele frequency (MAF), location and function of the ten SNPs selected as *RANKL, RANK* and *OPG* genetic markers are listed in Table 1. The observed MAFs of all the SNPs examined in this study were comparable to those reported for the 1000 Genomes project. None of the SNPs failed the missingness test (genotyping >0.05) or the frequency test (MAF<0.01) and were observed to be in HWE.

Linear regression analysis revealed no significant association of the 10 tested SNPs (rs4355801, rs3102735, rs2073618, rs9594759, rs12585014, rs7988338, rs2148073, rs1805034, rs12458117 and rs3018362) with the

BUA parameter after adjustments for age, sex, weight, height, PA and calcium intake (Table 2). A trend of association with heel QUS was only observed for the rs9594759 *RANK* polymorphism (p=0.064, β (95% CI) = -0.075 (-0.960, 0.028)) (Table 2).

The linkage disequilibrium pattern between the tested SNPs in our population is shown in Figure 1. The Haploview software analysis identified one haplotype block for the *RANKL* gene including the rs12585014, rs7988338 and rs2148073 polymorphisms (Fig. 1). A tight correlation between alleles at the rs12585014 and rs7988338 SNPs (r^2 =0.99) was observed. The regression analysis revealed no association of the haplotypes with heel QUS (not shown).

DISCUSSION

The role of the RANKL/RANK/OPG pathway in bone remodelling processes is well established (Trouvin & Goëb, 2010). Variations in bone formation–resorption mechanisms controlled by these molecules determine bone mass and could thus influence QUS measurements. Therefore, in the present study we considered genes of the *RANKL/RANK/OPG* pathway as interesting candidates for testing any association they may have with the QUS parameter. To our knowledge, this is the first study in which the possible implication of SNPs in *RANKL, RANK* and *OPG* as genetic factors of the heel quantitative ultrasound parameter (BUA) has been investigated. Our results revealed no significant association of any of the ten tested SNPs, pointing to the fact that genes of the *RANKL/RANK/OPG* pathway play no significant role in the genetics of heel QUS in young Caucasian adults. These findings are in line with previous studies that found no association of rs4355801, rs3102735, rs2073618, rs9594759, rs12585014, rs7988338, rs2148073, rs1805034, rs12458117 and rs3018362 SNPs with different BMD phenotypes (Brändström et al., 2003; Kim et al., 2007; Koh et al., 2007; Tu et al., 2015).

In contrast, in previous association studies, GWAS and meta-analysis a significant genetic association of the *RANKL*, *RANK* and *OPG* genes with bone phenotypes has been demonstrated as determined by DXA (Paternoster, Ohlsson et al., 2010; Richards et al., 2008; Rivadeneira et al., 2009; Roshandel et al., 2011). However, most of these studies were conducted on postmenopausal women and older men. As our cohort comprised only young adults, the differences in age range with previous studies could be responsible for the lack of agreement in association results. Thus, it is possible that *RANKL*, *RANK* and *OPG* genes contribute to bone

phenotypes in the elderly but not in early bone mineralisation process. Further meta-analysis studies, including stratification by age range, would be of interest for investigating this possibility.

On the other hand, a significant association with SNPs in the *RANKL/RANK/OPG* pathway has been observed in studies in which bone mass was assessed by DXA (Paternoster,Ohlsson et al., 2010; Richards et al., 2008; Rivadeneira et al., 2009; Roshandel et al., 2011; Styrkarsdottir et al., 2008). Interestingly, in line with our findings in the Osteoporotic Fractures in Men (MrOS) study, in which volumetric bone mineral density (vBMD) was assessed by quantitative computed tomography (QTC), no associations of SNPs in *RANKL, RANK* and *OPG* with vBMD were observed (Yerges et al., 2009; Yerges et al., 2010). It could therefore be suggested that *RANKL, RANK* and *OPG* are genetic markers for bone phenotypes estimated by DXA, but not for those determined with other techniques, such as QUS and QTC.

Considering this, and according to previous evidence, this raises the possibility that some loci for heel QUS and BMD may be distinct, while others would be the same. Similarly to that which occurs with the *RANKL, RANK* and *OPG* genes, the vitamin D receptor (*VDR*) gene has been considered an interesting candidate for osteoporosis and many studies have focused on the association of different *VDR* polymorphisms and BMD, reporting significant associations (Canto-Cetina et al., 2015; Wang et al., 2013). However, with regard to QUS, many studies have shown that *VDR* polymorphisms are not related to heel ultrasound parameters (Correa-Rodríguez et al., 2015b; Moayyeri et al., 2014; Omasu et al., 2004). In contrast, another osteoporosis-related candidate gene, *WNT16*, has been previously associated with both, BMD (Medina-Gomez et al., 2012; Zheng et al., 2012) and QUS (Correa-Rodríguez et al., 2015a; García-Ibarbia et al., 2013; Moayyeri et al., 2014).

It must be taken into account that due to the technical characteristics of the ultrasound bone densitometer used we only obtained information on BUA, this being a limitation of the study. Thus, any possible associations of the selected SNPs with other ultrasound parameters, such as speed of sound (SOS), stiffness index (SI), and Quantitative ultrasound index (QUI), have not been evaluated. Another limitation is a relatively low statistical power (50%) for detecting differences below 0.3 SD as a result of the sample size. Therefore, further independent replication studies would be of interest for confirming the findings of the present study.

In summary, our preliminary results suggest that the *RANKL/RANK/OPG* signalling pathway is not associated with calcaneal QUS in young adults. Further studies with a larger sample size and including functional assays

are needed to clarify the implication of SNPs in *RANKL, RANK* and *OPG* as genetic factors influencing changes in the QUS parameter.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Table 1. The basic characteristic of all SNPs for OPG, RANK and RANKL genes.

CHR	Gene	SNP	Allele	Function	MAF in this study	MAF 1000 Genomes Project	HWE (p)
8	*OPG*	rs4355801	A>G	Upstream of OPG	0.39	0.28	0.17
8	*OPG*	rs3102735	T>C	Downstream of OPG	0.12	0.17	0.35
8	*OPG*	rs2073618	G>C	Exon of OPG	0.46	0.33	0.63
13	*RANKL*	rs9594759	T>C	Intron of RANKL	0.48	0.44	0.40
13	*RANKL*	rs12585014	G>A	Intron of RANKL	0.18	0.20	0.11
13	*RANKL*	rs7988338	G>A	Intron of RANKL	0.19	0.20	0.40
13	*RANKL*	rs2148073	C>G	Intron of RANKL	0.18	0.23	0.60
18	*RANK*	rs1805034	C>T	Exon of RANK	0.41	0.41	0.26
18	*RANK*	rs12458117	G>A	Intron of RANK	0.19	0.14	0.35
18	*RANK*	rs3018362	A/G	Downstream of RANK	0.32	0.38	0.18

MAF the minor allele frequency. HWD p value for Hardy-Weinberg equilibrium

Table 2. Association results for the SNPs in the RANKL/RANK/OPG pathway.

Gene	SNP	Heel BUA (dB/MHz) β (95% CI)	p
OPG	rs4355801	-0.016(-0.623, 0.408)	0.682
OPG	rs3102735	0.032(-0.269, 0.636)	0.426
OPG	rs2073618	0.049(-0.165, 0.692)	0.228
RANKL	rs9594759	-0.075(-0.960, 0.028)	0.064
RANKL	rs12585014	-0.053(-0.720, 0.138)	0.184
RANKL	rs7988338	0.016(-0.211, 0.308)	0.716
RANKL	rs2148073	0.026(-0.576, 1.150)	0.514
RANK	rs1805034	0.036(-0.235, 0.612)	0.382
RANK	rs12458117	-0.008(-0.359, 0.292)	0.841
RANK	rs3018362	-0.047(-0.632, 0.158)	0.239

Beta represents the regression coefficient.
The genotype of each SNP was encoded as 0, 1, 2, where 0 and 2 denotes homozygotes with major allele and minor allele respectively and 1 codes heterozygotes.

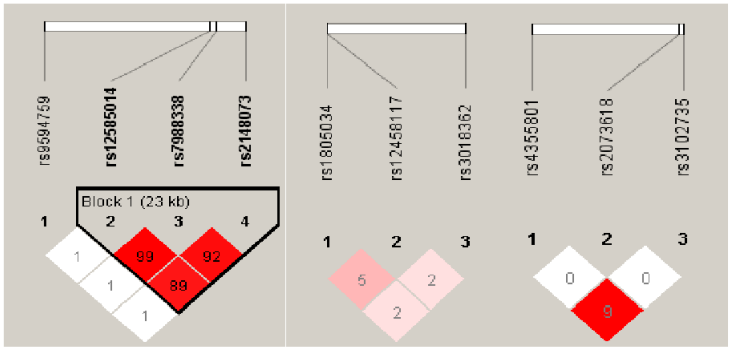

Figure 1. LD plots with R^2 values of the RANKL, RANK and OPG genes using the SNPs selected in this study. D´ values are indicated by dark depth. The R^2 values are shown as numbers in the diamonds.

6. *RSPO3* gene polymorphism is associated with ultrasound bone measurement in young adults

[Manuscrito en proceso de revisión]

RSPO3 gene polymorphism is associated with ultrasound bone measurement in young adults

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Abstract

Objective: Ultrasound bone measurement has been postulated as a valuable bone health assessment tool in primary care. The purpose of the present study was to determine whether genetic polymorphisms in *SPTBN1, RSPO3, CCDC170, DKK1, GPATCH1* and *TMEM135* genes were associated with calcaneus ultrasound measurement in an age-specific populations of young adults.

Methods: A cross-sectional study was conducted on 575 individuals (mean age 20.41±2.69). Bone mass at the right calcaneus was estimated by quantitative ultrasound (QUS). Six SNPs in *SPTBN1* (rs11898505), *RSPO3* (rs7741021), *CCDC170* (rs4869739), *MBL2/DKK1* (rs7902708), *TMEM135* (rs597319) and *GPATCH1* (rs10416265) were selected as genetic markers based on their previous association with calcaneal QUS and were genotyped using an Open Array technology. Association of the tested SNPs with ultrasound bone measurement variations was determined using linear regression analysis.

Results: A significant association of the rs7741021 SNP in *RSPO3* gene with calcaneal ultrasound parameter under both an additive and recessive model of heritance after adjusting for multiple confounding factors was observed (p=0.03 and p=0.006 respectively). For the rest of the tested SNPs, the regression analysis did not reveal a significant association with QUS parameter in our population.

Conclusions: Our results reveal a significant association between rs7741021 polymorphism and heel QUS suggesting the possible implication of the *RSPO3* gene in bone mass acquisition during early adulthood.

Key words: calcaneal quantitative ultrasound*; RSPO3;* polymorphisms; young adults.

Introduction

Bone mass later in life is determined by the equilibrium between bone formation and resorption mechanisms. The misbalance in favour of the latest leads to low bone mineral density (BMD), deterioration in bone microarchitecture and increased fracture risk, main characteristics of osteoporosis (Cosman et al., 2014), a common complex disease resulting from the interaction between genetic and environmental factors (Peacock et al., 2002). It is well established that genetic influence accounts for 70–80% of the variation in bone mass and the remaining 30-20% correspond to environmental factors including nutrition and physical activity (Sigurdsson et al., 2008). Due to the high prevalence and economic burden of osteoporosis worldwide, novel preventive strategies are needed to reduce the risk of developing osteoporosis (Harvey et al., 2010). Given the increasing understanding of the genetic of osteoporosis, the use of genetic tests to identify individuals who are at risk will become a common routine for primary care professionals over the next years. Genetic screening programs, as public health programs, will allow a risk estimation for the purpose of disease prevention or early treatment (Andermann and Blancquaert, 2010). Thus, identifying genetic markers that contribute to an increased risk of osteoporosis is of particular interest since could determine the implementation of effective prevention strategies based on genetic testing.

In the assessment of bone mass, quantitative ultrasound (QUS) has been postulated as a bone health assessment technique that is gaining much popularity. In contrast to dual X-ray absorptiometry (DXA), QUS has several advantages such as being smaller, transportable, cheaper and free of ionising radiation. Calcaneus is the most studied skeletal side for QUS assessment because of its high percentage of trabecular bone and its easy accessibility that facilitates the movement of ultrasound through it (Töyräs, et

al., 2002). Besides, a recent meta-analysis indicates that QUS is a valuable tool for fracture risk assessment independent of DXA (Huopio et al., 2004; Moayyeri et al., 2012). Thus, the clinical usefulness of QUS for measuring bone status in the community make QUS an optimal tool for population screening in clinical practice.

Genetic factors are important determinants of calcaneus ultrasound parameters. Familial studies have shown a 74% heritability for QUS parameters (Howard et al., 1998; Karasik et al., 2002). However, most of studies examining genetic influence on skeletal traits have focused on BMD measured by DXA in elderly populations. To identify genetic factors contributing to ultrasonography bone parameters, few candidate gene association studies have been conducted (Correa-Rodríguez et al. 2016a; Zajickova et al. 2005; Omasu et al. 2004; Correa-Rodríguez et al. 2016b; García-Ibarbia et al. 2013; Elfassihi et al. 2010; Holliday et al. 2011; Limer et al. 2009). Most of them, selected as candidates genes previously associated with BMD phenotypes such as *VDR* (Boroń et al., 2015; Canto-Cetina et al., 2015), *ESR1* (Koller et al., 2013; Massart et al., 2009) and *WNT16* (Koller et al., 2013; Zheng et al., 2012) since there were no data available from genome-wide association (GWA) studies focusing in characterisation of genetic factors underlying QUS variability. In 2014 the results of a meta-analysis of GWAS conducted by GEFOS/GENOMOS (Genetic Markers of Osteoporosis) consortium to assess the genetic determinants of heel bone properties in European subjects were published. Among the 9 single nucleotide polymorphisms that reached genome wide significance, rs11898505, rs7741021, rs7902708, rs4869739, rs10416265 and rs597319 mapped within or near the genes *spectrin beta non- erythrocytic 1 (STPBN1), R-spondin 3 (RSPO3), dickkopf1 (DKK1), coiled-coil domain containing 170 (CCDC170), G patch domain containing 1 (GPATCH1)* and transmembrane protein 135 *(TMEM135)* were

identified as genetic factors for heel bone properties for the first time (Moayyeri et al., 2014).

On the other hand, peak bone mass (PBM), defined as the amount of bone tissue present at the end of the skeletal maturation, has been shown to be a significant predictor of risk for osteoporosis since is a major determinant of bone mass later in life (Berger et al., 2010). However, most of previous studies aiming to investigate genetic factors associated with QUS have been conducted in mixed populations with samples of premenopausal, postmenopausal women and men considering wide age ranges (25-83 years) (Elfassihi et al., 2010; García-Ibarbia et al., 2013; Holliday et al., 2011; Limer et al., 2009; Zajickova et al., 2005).Thus, little is known about genetic markers associated with bone gain during early adulthood, a period that corresponds to the most crucial years of PBM attainment.

Taking into consideration all these evidence, we aimed to investigate whether genetic polymorphisms in *SPTBN1, RSPO3, CCDC170, DKK1, GPATCH1* and *TMEM135* genes were associated with calcaneal QUS in population of young adults.

Methods

Subject characteristics

A cross-sectional study was conducted on 575 healthy individuals of Caucasian ancestry (400 females and 175 males, median age 20.41±2.69). All the subjects who participated in this study were of European descent. A detailed medical history was obtained for all subjects. In particular, subjects with a history of bone disease, metabolic or endocrine diseases, hormonal contraceptive therapy or medication that could affect bone mass were excluded from the study. Informed consent was obtained from all individual

participants and the study was approved by local ethics committees and conducted in accordance with the Declaration of Helsinki.

Bone assessment

Bone mass was assessed by quantitative ultrasound measurement (QUS) at the right calcaneus. The CUBA clinical ultrasound bone densitometer (McCue Ultrasonic Limited, Compton, Winchester, UK) was used to perform the QUS measurements. Output included broadband ultrasound attenuation (BUA) that is referred to the slope between attenuation of sound signals and its frequency, and the unit used is dB/MHz Daily calibrations were made with a physical phantom to control the long-term stability of the apparatus.

Covariates

Weight to the nearest 0.1 kg was recorded using a Body Composition Analyzer (TANITA BC-418MA), and height was measured to the nearest 0.1 cm using a Harpenden stadiometer. Both measurements were taken without shoes. The same trained research assistant performed all the measurements. Physical activity (PA) and its frequency were determined using the self-administered International Physical Activity Questionnaire (IPAQ) (Craig et al., 2003). Dietary calcium intake (DCI) was assessed using the 72-hour recall method, considering intakes on Thursday, Friday and Saturday. Food records were converted to nutrient intake using a computerized nutrient analysis program (Nutriber 1.1.5).

SNPs selection and genotyping

Saliva samples for DNA extraction were collected from the study participants using the OG-500 Collection Kit (DNA Genotek Inc, Ontario, Canada). The DNA was isolated from the saliva samples according to the manufacturer's protocol. We selected six SNPs in *SPTBN1* (rs11898505), *RSPO3* (rs7741021), *CCDC170* (rs4869739), *MBL2/DKK1* (rs7902708), *TMEM135* (rs597319) and *GPATCH1* (rs10416265) as genetic markers based on their previous association with calcaneal QUS in the meta-analysis of the GEFOS/GENOMOS consortium (Moayyeri et al., 2014). Genotyping was performed at the Genomic and Genotyping Unit in the GENyO Center (Pfizer-University of Granada-Junta de Andalucía Centre for Genomics and Oncological Research) using OpenArray technology (Life Technologies, Carlsbad, CA, USA). A TaqMan OpenArray genotyping plate was custom designed including eight predesigned genotyping assays for each of the selected SNPs. Standard cycling conditions were used, as recommended by the manufacturer. Thermal cycling and fluorescence detection were performed using the QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). The average missing rate for the genotyping assays was 1.57%, with a range from 1.05% to 2.09%. To guarantee the accuracy of genotyping, duplicate samples and negative controls were included in all genotyping arrays, presenting 100% identical genotypes.

Statistical analysis

The Haploview software (Broad Institute of MIT and Harvard) was used to calculate allele frequencies and verify that the genotype data were in Hardy-Weinberg equilibrium (HWE). Fixed-effects designs of analysis of variance were used for comparisons of means. Differences in calcaneal quantitative ultrasound variable and genotypes of selected SNPs were tested using linear regression analyses with adjustment for the following covariates: age, sex, weight, height, physical activity and

calcium intake. Analyses of association between genotype and ultrasound bone measurement assumed both an additive and recessive model of inheritance. The results are reported as a mean±SD or frequencies. Statistical analyses were carried out using SPSS software, version 20.0 (SPSS, Chicago, IL, USA). The most conservative method (Bonferroni correction) was applied to correct for multiple comparisons. The cutoff value for significance was set at 0.05/6=0.008.

The statistical power of the study was estimated using Quanto version 1.2 software (Department of Preventive Medicine, University of Southern California, CA) considering a ultrasound bone measurement mean of 82.03 standard deviation (SD) 26.72, 5% type I error, MAFs of 0.11-0.39, 575 individuals and an additive genetic model.

Results

The characteristics of the 575 study subjects have been published previously (Correa-Rodríguez et al., 2016). The mean and SD or range for 575 subjects´ descriptive characteristics are as follows: age 20.41 ± 2.69 years; weight 63.72 ± 12.82 kg; height 1.67 ± 0.08 m; calcium intake 804.42 ± 356.49 mg/day; and PA 1779.00 (0-56640) MET/min. The mean for the total population was 82.03 ± 26.72 (dB/Mhz), similar to that previously observed for young adults (Babaroutsi et al., 2005; Scheffler et al., 2014).

Table 1 shows the location, function, genotype and allele frequency of the SNPs selected as genetic markers. All the SNPs tested were observed to be in HWE.

The association analysis of the genotypes of the polymorphism in the *SPTBN1*, *RSPO3, CCDC170, MBL2/DKK1*, *TMEM135* and *GPATCH1* genes with calcaneus ultrasound parameter adjusting for age, weight, height, physical activity and calcium intake are

shown in Table 2. The rs7741021 SNP in *RSPO3* gene showed a significant association with ultrasound bone parameter under both an additive and recessive model of heritance (p=0.03 and p=0.006 respectively). Considering the cutoff value for significance after applying the Bonferroni correction for multiple testing (p=0.008), only the p value for the recessive model remained significant.

The regression analysis did not reveal a significant association for the rest of the tested SNPs with QUS parameter after adjustment for covariates (Table 2). Of note, subjects with the CC homozygote genotype for the rs7902708 polymorphisms in *DKK1* gene showed the lowest calcaneus ultrasound measurement values although this difference did not reach statistical significance (Table 2).

Discussion

In the present study we analysed the possible implication of SNPs in *SPTBN1, RSPO3, CCDC170, DKK1, GPATCH1* and *TMEM135* genes as genetic factors of calcaneal QUS in a population of young adults. Our findings revealed a significant association of the rs7741021 SNP with calcaneus ultrasound parameter suggesting that the in *RSPO3* gene could be a genetic marker implicated in bone mass regulation during youth. These results are in accordance with those obtained by the GEFOS/GENOMOS consortium that previously detected the implication of *RSPO3* as genetic marker for calcaneal QUS. In addition, this gene has been also associated with bone mineral density assessed by DXA and fracture risk in a recent GWAS study (Duncan et al., 2011).

RSPO3 codifies for a member of the R-spondin (RSPO) family of proteins that activate the canonical WNT (wingless-type MMTV integration site family)/β-catenin signaling pathway at the receptor level (Wei et al., 2007). The Wnt/β-catenin signaling pathway is fundamental for osteogenesis, promoting bone formation by functioning as a positive

regulator of osteoblasts (Hill et al., 2005; Hu et al., 2005). Then, an impaired signalling trough this receptor due to genetic variation of *RSPO3* gene might cause lower bone bass accrual. However, the *RSPO3* gene function is still poor understood as well as the functional relevance of SNPs in this region. The rs7741021 SNP associated with QUS parameter maps in an intronic region within *RSPO3* gene between exons 1 and 2 and probably this SNP would not be the real causing variant affecting *RSPO3* gene function. Thus, future research work involving linkage disequilibrium analysis of this region and fine mapping are needed to characterise causal variants in this region. In addition, functional studies are necessary to elucidate the exact molecular mechanisms by which RSPO3 is implicated in the bone mass acquisition process and more precisely, how this receptor could cause the presence of lower levels of quantitative ultrasound parameter in young adults.

On the other hand, our results revealed no significant association of SNPs in *SPTBN1, CCDC170, DKK1, GPATCH1* and *TMEM135* genes with calcaneal QUS, pointing these genes might not play a relevant role in bone gain during early adulthood. The differences in population age range between GEFOS/GENOMOS study (25-83 years) and our study cohort (18-25 years) may account for the discordance in association results and point to the possibility that these genes could be genetic markers for ultrasound bone parameter later in life but not for bone mass accrual that occurs in early stages.

Regarding the limitation of the study, one potential limitation is its cross-sectional design that could not infer causality. Furthermore, it must be taken into account that we did not fully cover all genetic variation in the selected genes since only six SNPs significantly associated with QUS in a previous study were analysed. Thus, further

studies in these regions should be of interest to confirm the preliminary findings of the present study.

In summary, we investigated the possible influence of SNPs in *SPTBN1, RSPO3, CCDC170, DKK1, GPATCH1* and *TMEM135* genes on calcaneus quantitative ultrasound in Caucasian young adults. Our results reveal the significant association between rs7741021 polymorphism and provide evidence for the possible implication of the *RSPO3* gene in bone mass during early adulthood.

Authorship

Correa-Rodríguez M monitored data collection, wrote the statistical analysis plan, cleaned and analyzed the data, and drafted and revised the paper. Schmidt-RioValle Jacqueline and Rueda-Medina B analyzed the data, and drafted and revised the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Table 1. Details of single nucleotide polymorphisms analysed in this study.

Locus	SNP	Closest gene	Genetic function	Genotype	N (frequency)	Allele (frequency)	HWE p value
2p16.2	rs11898505	*SPTBN1*	intronic	AA	79 (14.03)	A (0.37)	0.75
				AG	259 (46.00)	G (0.62)	
				GG	225 (39.96)		
6q22.33	rs7741021	*RSPO3*	intronic	AA	206 (36.39)	A (0.60)	0.84
				AC	269 (47.52)	C (0.39)	
				CC	91 (16.07)		
6q25.1	rs4869739	*CCDC170*	intronic	AA	243 (42.85)	A (0.65)	0.22
				AT	246 (43.38)	T (0.35)	
				TT	78 (13.75)		
10q21.1	rs7902708	*MBL2/DKK1*	intronic	CC	7 (1.24)	C (0.11)	0.77
				CG	118 (20.92)	G (0.88)	
				GG	439 (77.83)		
11q14.2	rs597319	*TMEM135*	intronic	AA	274 (48.66)	A (0.68)	0.19
				AG	228 (40.49)	G (0.31)	
				GG	61 (10.83)		
19q13.11	rs10416265	*GPATCH1*	Non-synonymous coding	AA	252 (44.28)	A (0.67)	0.06
				AG	268 (47.10)	G (0.32)	
				GG	49 (8.61)		

HWD p value for Hardy-Weinberg equilibrium

Table 2. Linear regression analysis of tested SNPs genotypes and bone ultrasound parameter adjusting for age, sex, height, weight, physical activity and calcium intake.

Gene	Genotype	BUA (dB/MHz)		p value	p value
SNP (rs)		mean	SD	(additive model)	(recessive model)
SPTBN1	AA	84.56	25.849	0.980	0.742
rs11898505	AG	81.71	26.959		
	GG	81.71	27.260		
RSPO3	AA	78.22	30.334	0.03	0.006
rs7741021	AC	84.46	23.991		
	CC	83.73	25.965		
CCDC170	AA	81.23	28.226	0.673	0.750
rs4869739	AT	83.93	25.533		
	TT	78.69	26.244		
MBL2/DKK1	CC	72.29	36.732	0.770	0.526
rs7902708	CG	84.62	26.625		
	GG	81.57	26.778		
TMEM135	AA	83.76	26.244	0.361	0.070
rs597319	AG	80.83	27.382		
	GG	80.51	25.940		
GPATCH1	AA	79.69	27.679	0.338	0.096
rs10416265	AG	83.56	26.063		
	GG	85.69	26.912		

Data are shown as mean±SD and are adjusted for age, sex, weight, height, physical activity and calcium intake; p values were determined by ANCOVA; BUA broadband ultrasound attenuation.

7. The effects of body composition, dietary intake and physical activity on calcaneus quantitative ultrasound in Spanish young adults

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The Effects of Body Composition, Dietary Intake, and Physical Activity on Calcaneus Quantitative Ultrasound in Spanish Young Adults

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Abstract

Identifying modifiable factors that influence bone gain during early adulthood in order to maximize peak bone mass (PBM) is a potential primary strategy in the prevention of osteoporosis in later life. The present study examined the relationships between body composition, dietary intake and physical activity (PA), and bone health measured by quantitative ultrasound (QUS) at the right calcaneus. The study population consisted of 781 Spanish men and women (age 19.1 $\pm$ 3.6). Body composition, dietary intake, PA, and bone strength were assessed. Calcaneus QUS was significantly correlated with age, height, weight, body mass index, lean mass, fat mass, protein intake, and moderate and high PA. No significant correlation between calcium intake and broadband ultrasound attenuation (BUA, dB/MHz) was detected. Linear regression analyses revealed that independent variables accounted for 18.8% of the total variance of calcaneus BUA ($p = .000$). Lean mass and high PA were significant predictors of BUA variance in young adults ($p = .000$ and $p = .045$, respectively). Results indicate that lifestyle choices and their consequences during early adulthood could influence bone mass, particularly PA and lean mass. Furthermore, this study provides novel data about bone mass as indicated by the QUS measurements at the time of PBM acquisition.

Keywords

calcaneus quantitative ultrasound, nutrition, physical activity, body composition, young adults, peak bone mass

Osteoporosis is a disease characterized by decreased bone mass and deterioration of bone tissue (Ferrari, 2005). The risk of osteoporosis may be reduced by maximizing the peak bone mass (PBM), defined as the amount of bone present in the skeleton at the end of its maturation process, during early adulthood (Matkovic & Weaver, 2000). Acquiring a high PBM decreases the risk of osteoporotic fractures in later life by 50% (Rizzoli, Bianchi, Garabedian, McKay, & Moreno, 2010). Bone mineral accrual is determined by the interaction of genetic (40–80% of the variance) and environmental factors such as physical activity (PA), nutrient intake, and body mass (Pollitzer & Anderson, 1989; Ralston & Uitterlinden, 2010).

PA has been associated with bone mineral accrual and maintenance, having an important osteogenic effect (Vicente-Rodriguez, 2006). Weight-bearing exercise changes trabecular orientation and density, resulting in beneficial effects on bone mass (Cassell, Benedict, & Specker, 1996; Daly, Saxon, Turner, Robling, & Bass, 2004). Previous studies have utilized quantitative ultrasound (QUS) measurements to detect the positive effect of PA on bone mass in children and adolescents (De Smet et al., 2015; Robinson, Winters-Stone, Gabel, & Dolny, 2007). Similarly, association studies have found a positive relationship between PA and bone mineral density (BMD) in young adults and adolescents (Gracia-Marco et al., 2011; Neville et al., 2002).

Authors have postulated that calcium is an important nutrient that influences skeletal calcium retention during growth and plays a significant role in the PBM achieved in early adulthood (Heaney et al., 2000). However, findings regarding the effects of calcium intake on bone gain have been inconclusive, indicating both positive (Nieves et al., 2010; Peters, Verly, Marchioni, Fisberg, & Martinini, 2012) and negative effects (De Smet et al., 2015; Lanou, Berkow, & Barnard, 2005; Winzenberg,

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Shaw, Fryer, & Jones, 2006) depending on variations in measurement site and the average calcium intake.

It is well established that low body mass index (BMI) is a risk factor for osteoporosis and fracture (Kanis, Johnell, Oden, Johannson, & McCloskey, 2008). However, when considering the independent effects of BMI components such as fat mass (FM), previous studies have shown conflicting results (Janicka et al., 2007; Mosca, Da Silva, & Goldberg, 2013; Rocher, Chappard, Jaffré, Benhamou, & Courteix, 2008; Sayers & Tobias, 2010; Wetzsteon et al., 2008). The role of the lean mass (LM) component of BMI in bone accrual is well known, however, with LM contributing to the mechanical loading of the skeleton (Courteix et al., 1998; Gracia-Marco et al., 2012; Rauch, Bailey, Baxter-Jones, Mirwald, & Faulkner, 2004).

The tool traditionally used to measure BMD is dual energy X-ray absorptiometry (DXA). BMD measured via QUS demonstrates a high correlation with BMD measured by DXA (Moayyeri et al., 2012) and also reflects additional information on microstructure, elasticity of bone, and connectivity (Toyras, Nieminen, Kroger, & Jurvelin, 2002). In addition, its portability, noninvasiveness, lack of radiation, and low cost makes QUS an excellent alternative technique for assessing bone mass in healthy populations (Gluer, 1999).

Identifying modifiable factors that influence QUS measures during early adulthood in order to maximize PBM is a potential primary strategy in the prevention of osteoporosis in later life. However, in contrast to DXA, evidence of the influence of lifestyle factors on bone gain measured by calcaneus QUS during early adulthood is limited. Therefore, the purpose of this study was to investigate the relationships between body composition, dietary intake, and PA and bone health measured by calcaneus QUS in a large population of healthy young Spanish adults.

Method

Participants

We recruited 781 healthy individuals of Caucasian ancestry (519 females and 262 males, median age 19.11 $\pm$ 3.57 years) from different public academic centers in Granada, Spain. The local ethics committee of the University of Granada approved the study, which was conducted in accordance with the Declaration of Helsinki, and we obtained written informed consent from all participants. Young adults excluded from participation included those with a history of bone, metabolic, or endocrine disease or who were currently undergoing hormone-replacement therapy or taking medications that could affect bone mass. A member of the research team visited young adults at their academic centers to collect data from October 2013 to March 2014.

Anthropometric Measurements

We collected body weight, FM, and LM measurements, with participants wearing light clothing and no shoes, using a body composition analyzer (TANITA BC-418MA) to the nearest 0.11 kg. We used a Harpenden stadiometer to measure height to the nearest 0.5 cm, with participants again not wearing shoes. We calculated BMI as weight divided by height square (kg/m^2). The same trained research assistant performed all the measurements.

Dietary Intake

We assessed daily intake of nutrients using a self-administered 72-hr recall method that covered intake on a Thursday, Friday, and Saturday (Nelson & Bingham, 1997). The food record, which is the gold standard, provides quantitatively precise information on food intake during a specific period of time. We instructed the participants about measurements of food before they completed the records. We analyzed completed food records using a computerized nutrient analysis program (Nutriber 1.1.5), calculating the daily intakes of energy and four nutrients including carbohydrates (g), proteins (g), fat (g), and calcium (mg). We did not include the intake of nutritional supplements in the analysis because only three participants had regularly taken calcium supplements.

PA

To asses PA over the previous 7 days, we used the self-administered short version of the International Physical Activity Questionnaire (IPAQ). The questionnaire is a valid and reliable instrument to measure PA in European adult populations (Craig et al., 2003). It calculates total hours spent performing vigorous and moderate PA and walking. We derived the metabolic equivalent (MET)-hours by multiplying the total hours spent performing each level of activity with the MET value for vigorous PA (MET = 8.0), moderate PA (MET = 4.0), and walking (MET = 3.3) and then summing three, as described on the IPAQ website (http://www.ipaq.ki.se). We classified participants as having high PA when they had performed either vigorous activity on at least 3 of the 7 days and achieved a minimum total score of 1,500 MET-min/week or PA of any intensity on each of the 7 days to achieve a minimum total score of 3,000 MET-min/week. We classified participants as having moderate PA when they had performed vigorous activity for at least 20 min/day for at least 3 days, moderate-intensity activity for at least 30 min/day for at least 5 days, or performed activity of any intensity level on at least 5 days to achieve a total score of at least 600 MET-min/week. We classified participants as having low PA when they failed to meet the criteria for high or moderate PA.

QUS Measurement

We assessed bone quality via ultrasound measurements at the right calcaneus using the CUBA clinical ultrasound bone densitometer (McCue Ultrasonics Limited, Compton, Winchester, United Kingdom). Calcaneus ultrasonography provides information about microstructure, elasticity, and connectivity of bone through different parameters such as broadband

Table 1. Descriptive Characteristics of Study Subjects by Gender.

Characteristic	Females ($n = 519$)	Males ($n = 262$)	Overall ($n = 781$)	p Value
Anthropometric				
Age (years)	19.32 ± 3.51	18.69 ± 3.66	19.11 ± 3.57	.020
Height (m)	1.63 ± .06	1.73 ± .09	1.66 ± .08	.000
Weight (kg)	59.69 ± 11.32	70.98 ± 14.97	63.48 ± 13.73	.000
BMI (kg/m^2)	22.44 ± 4.11	23.57 ± 4.24	22.82 ± 4.19	.000
Fat mass (kg)	16.34 ± 9.09	12.71 ± 9.17	15.12 ± 9.27	.000
Lean mass (kg)	43.79 ± 4.64	58.22 ± 9.88	48.63 ± 9.67	.000
Dietary				
Energy (kcal/day)	1952.96 ± 1268.48	2171.95 ± 755.60	2026.80 ± 1126.29	.010
Protein (g/day)	67.93 ± 34.96	69.41 ± 44.64	68.43 ± 38.48	.612
Carbohydrate (g/day)	222.70 ± 226.95	210.96 ± 154.27	218.74 ± 205.31	.452
Fat (g/day)	65.24 ± 49.51	63.15 ± 30.86	64.54 ± 44.10	.532
Calcium intake (mg/day)	815.84 ± 368.59	880.51 ± 390.09	837.85 ± 377.04	.024
Physical activity[a]				
Low	238.27 ± 216.92	318.47 ± 237.59	251.74 ± 221.62	.122
Moderate	1548.62 ± 679.21	1729.37 ± 662.02	1600.08 ± 678.15	.041
High	6086.69 ± 5513.63	6691.65 ± 3958.89	6350.78 ± 4893.73	.391
Heel ultrasound				
BUA (dB/MHz)	83.34 ± 16.12	93.42 ± 18.38	86.73 ± 17.56	.000

Note. Data are shown as mean ± *SD*. BMI = body mass index; BUA = broadband ultrasound attenuation; MET = metabolic equivalent.
[a]The calculation of MET-hours is described in the Methods section.

ultrasound attenuation (BUA), speed of sound, stiffness index, and QUS index (Krieg et al., 2008). Due to the technical characteristics of the ultrasound bone densitometer we used in this study, we obtained information about BUA only. BUA (dB/MHz) measures the attenuation of sound waves as they pass from the transmitting transducer to the receiving transducer. We used the calcaneus bone for QUS assessment because it contains a large percentage of trabecular bone, which has a high metabolic turnover rate (Toyras et al., 2002). The same trained research assistant performed all calcaneus QUS measurements using the same instrument. Daily calibrations were made with physical phantom to control the long-term stability of the apparatus.

Statistical Analysis

We used SPSS Version 21.0 (SPSS, Chicago, Illinois) for performing all the analyses; p values < .05 were considered to be statistically significant. We express results as the mean ± *SD*. We assessed differences according to gender by independent t-test and determined the univariate relationships between BUA and selected variables by computation of the partial correlation coefficients (r) after adjustment for the confounding factors of sex, age, and BMI.

Results

Descriptive Characteristics

Table 1 shows the descriptive characteristics of the study population by gender and as a whole. We observed significant differences between males and females in height, weight, FM, LM, energy intake, calcium intake, moderate PA, and BUA values. The mean calcaneus BUA for the population was 86.73 ± 17.56 dB/MHz, and males had significantly higher BUA than females ($p = .000$). As expected, males had significantly greater body height, weight, BMI, and LM than females ($p = .000$), whereas females had significantly greater FM than males ($p = .000$).

Although average calcium intake (837.85 ± 377.04 mg/d) was below the recommended intake level in both genders, males had significantly higher calcium consumption ($p = .024$) and energy intake ($p = .010$) than females. We observed no significant differences by gender concerning the intake of macronutrients (Table 1).

Effect of PA, Body Composition, and Dietary Intake on BUA

Moderate and high levels of PA showed significant positive correlations with BUA values. We also observed significant positive correlations between height, weight, BMI, FM and LM, and BUA after adjusting for sex, age, and BMI (Table 2). We detected no significant correlation between calcium intake (mg/day) and BUA; however, protein intake (g/day) showed a significant positive correlation ($p = .002$) with heel QUS measurement. Statistically significant correlations between height, weight, BMI, LM, and BUA were only moderate (correlation coefficients 0.32–0.43). For moderate and high levels of PA, protein intake, and FM, we observed low correlations with BUA (correlation coefficients < .18).

Determinants of BUA Parameter

We performed a linear regression model to determine the influence of each factor on calcaneus BUA and revealed that

Table 2. Correlations Between BUA and Lifestyle Variables.

Lifestyle Variable	*r*	*p* Value
Age (years)	.107	.004
Height (m)	.326	.000
Weight (kg)	.436	.000
BMI (kg/m^2)	.334	.000
Fat mass (kg)	.141	.000
Lean mass (kg)	.435	.000
Energy (kcal/day)	.013	.730
Protein (%)	.113	.002
Carbohydrate (%)	.037	.314
Fat (%)	.003	.940
Calcium intake (mg/day)	.010	.792
Low PA	.162	.138
Moderate PA	.122	.003
High PA	.180	.000

Note. Correlation calculations were adjusted for sex, age, and BMI. BMI = body mass index; BUA = broadband ultrasound attenuation; PA = physical activity.

Table 3. Linear Regression Model.

Model	Coefficient	*SE*	Standard coefficient	*t*	*p* Value
Intercept	47,285	3,937		12.01	.000
Lean mass (kg)	688	73	.382	9.48	.000
Fat mass (kg)	133	74	.068	1.80	.071
High PA (IPAQ-score)	1	0	.079	1.97	.049
Moderate PA (IPAQ-score)	0	1	.017	.431	.666
Protein intake (g)	38	22	.068	1.76	.079

Note. Model summary: $F = 26.287$; $p = .000$; $R^2 = 18.8$; SEE = 15.605 Db/MHz. IPAQ = International Physical Activity Questionnaire; PA = physical activity.

available independent variables accounted for 18.8% of the total variance of calcaneus BUA (Table 3). The linear regression model included five independent variables: LM, FM, high and moderate PA, and protein intake. LM and high PA contributed significantly to BUA variance in young adults ($p = .000$ and $p = .045$, respectively). Conversely, FM, moderate PA, and protein intake did not contribute significantly to the variance in BUA values.

Discussion

Identifying youth with low bone accrual at the time of PBM acquisition, using a technique such as QUS, and optimizing the environmental factors that increase bone gain could be a preventive strategy for osteoporosis. Accordingly, the present study involved a population of young healthy individuals characterized for lifestyle factors and provides novel data about bone strength as indicated by QUS measurements. The results show that LM and high levels of PA were consistent independent predictors of calcaneus BUA in young adults.

Our finding that high and moderate PA were positively correlated with BUA values is in accordance with previous studies that have reported the positive effect of PA on bone mass using QUS (De Smet et al., 2015; Robinson et al., 2007). However, only high PA showed a significant contribution to bone mass in linear regression analysis. Similarly, researchers have identified the influence of an intense level of PA on BMD among Northern Irish young adults (Neville et al., 2002) and in an independent group of Spanish adolescents (Gracia-Marco et al., 2011).

Our finding of a lack of effect of calcium intake on bone gain is also in agreement with previous studies. De Smet et al. (2015) showed a lack of association between calcium consumption and BUA using QUS. In addition, Winzenberg et al. (2006) reported in a systematic review that there was no effect of calcium supplementation on BMD in healthy children. Finally, in a review of 22 cross-sectional studies in youth, Lanou, Berkow, and Barnard (2005) concluded that weight, age, height, pubertal status, and PA, but not calcium intake, are the most consistent predictors of higher bone mass, using DXA and QUS. To our knowledge, only two studies have reported a significant association of calcium intake with BUA, one in postpubertal adolescents and young adults (Peters et al., 2012) and the other in young female runners (Nieves et al., 2010). This discordance might be attributable to the differences in mean daily calcium intake in study subjects.

Interestingly, we found a significant positive correlation between protein intake and calcaneus BUA. Authors have postulated that dietary proteins provide the amino acids for building bone matrix and are also influential factors for bone growth (Bonjour, Ammann, Chevalley, & Rizzoli, 2001). Along these lines, investigators have previously described the positive effect of proteins on bone remolding during childhood and adolescence (Alexy, Remer, Manz, Neu, & Schoenau, 2005; Chevalley, Bonjour, Ferrari, & Rizzoli, 2008).

Regarding body composition, in the present study we found that calcaneus BUA was positively related to body weight, height, BMI, LM, and FM in young adults, which is in accordance with prior studies using QUS measurements in children and adults (Adami et al., 2004; Babaroutsi, Magkos, Manios, & Sidossis, 2005; Cvijetic, Baric, Bolanca, Juresa, & Ozegovic, 2003; Mosca et al., 2013; Rocher et al., 2008; Sayers & Tobias, 2010). Nevertheless, in the linear regression model in the present study, only LM contributed significantly to the variance in BUA values, similar to the findings described previously (Courteix et al., 1998; Gracia-Marco et al., 2012; Rauch et al., 2004). This finding may imply that the positive relationship between body weight and bone mass is primarily due to the association with higher LM. The mechanostat theory, which predicts that the increase in muscle mass during development creates the stimulus for the increase in bone mass, provides a possible explanation for the significant correlation of LM with bone mass (Rauch et al., 2004). Data from the present and previous studies suggest that the promotion of high levels of PA in children and young adults in order to increase LM could be an effective preventive strategy to maximize PBM and reduce the risk of osteoporosis in later life.

This study has some limitations inherent to the assessment of dietary intake and PA using self-administered questionnaires. Investigators have previously described evidence of underreporting of food intake in self-reported questionnaires (Dodd, 2007). In addition, though calcaneus ultrasonography can provide information about microstructure, elasticity, and connectivity of bone through different parameters such as BUA, speed of sound, stiffness index, and QUS index, due to the technical characteristics of the ultrasound bone densitometer we used in this study, we were only able to obtain information about BUA. Thus, we did not evaluate the influence of lifestyle factors on other ultrasound parameters. Also, our participants were young adults with nondiverse demographic characteristics, which may limit the generalizability of the results to other populations. However, the main significant findings of our study have been confirmed in independent populations across different geographical areas.

In summary, our findings suggest that lifestyle choices during early adulthood could influence bone mass as determined by QUS. In particular, we observed that a high level of PA and LM are significant positive contributors to QUS parameters of the calcaneus. Future studies and meta-analyses are needed to clearly define the roles of lifestyle factors that influence QUS measurements in young adults.

Human and Animal Rights and Informed Consent

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained for all participants.

Authors' Contribution

Correa-Rodríguez M. contributed to conception and design, contributed to acquisition, drafted the manuscript, gave final approval, and agrees to be accountable for all aspects of work ensuring integrity and accuracy. Schmidt Rio-Valle Jacqueline contributed to acquisition, critically revised the manuscript, gave final approval, and agrees to be accountable for all aspects of work ensuring integrity and accuracy. González-Jiménez Emilio contributed to acquisition, critically revised the manuscript, gave final approval, and agrees to be accountable for all aspects of work ensuring integrity and accuracy. Rueda-Medina B contributed to conception and design, drafted the manuscript, critically revised the manuscript, gave final approval, and agrees to be accountable for all aspects of work ensuring integrity and accuracy.

Declaration of Conflicting Interests

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8. Dietary antioxidant quality score (DAQs) is associated with calcaneal quantitative ultrasound in young women

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Dietary Antioxidant Quality Score (DAQs) is associated with bone mass assessed by calcaneal quantitative ultrasound in young women

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Abstract

Introduction: Evidence suggests that intake of antioxidants could positively influence bone mass by preventing bone metabolism against oxidative stress.

Objective: We aimed to investigate the possible influence of single antioxidant intakes and Dietary Antioxidant Quality score (DAQs) on calcaneal Quantitative Ultrasound (QUS) in a population of young adults.

Methods: A total of 605 young Spanish adults participated in this study (median age 20.38±2.67). Bone mass was measured by calcaneal QUS to determine Broadband Ultrasound Attenuation (BUA, dB/MHz) parameter. Body composition was assessed by bioelectrical impedance analysis and dietary intakes were determined using a 72-hour diet recall interview. DAQs was applied to calculate antioxidant nutrients intake. Linear regression analyses were performed to investigate the possible influence of DAQs on calcaneal QUS.

Results: Most of young adults showed a low-quality antioxidant intake (only 17.6% of women and 20.3% of men had a score of 4 or 5 in DAQs). A positive correlation between DAQs and BUA was observed in women (r=0.117; p=0.024). Linear regression analysis revealed that DAQs was significantly associated with BUA parameter in women after adjusting by body weight, height, calcium intake and physical activity (p= 0.035). No significant associations between single antioxidant and calcaneus QUS measurement were found.

Conclusion: Our findings suggest that high-quality antioxidant intakes could influence bone health in young women. Future studies should further investigate the protective role of antioxidant nutrients against osteoporosis.

Key words: antioxidants; calcaneus quantitative ultrasound; nutrition; young adults.

El Índice de Calidad Antioxidante de la Dieta (DAQS) está asociado con la masa ósea evaluada mediante ultrasonido cuantitativo en el calcáneo en mujeres jóvenes

Resumen

Introducción: La evidencia sugiere que la ingesta de antioxidantes podría influir positivamente en la masa ósea mediante la prevención contra el estrés oxidativo del metabolismo óseo.

Objetivo: El objetivo fue investigar la posible influencia del consumo de antioxidantes y del Índice de Calidad Antioxidante de la Dieta (DAQs) en la masa ósea evaluada mediante ultrasonido cuantitativo (QUS) en el calcáneo en una población de adultos jóvenes.

Métodos: Un total de 605 adultos jóvenes españoles participaron en este estudio (mediana 20,38 ± 2,67 años). La masa ósea se evaluó mediante QUS en el calcáneo para determinar el parámetro de Atenuación de Ultrasonido de banda ancha (BUA, dB / MHz). La composición corporal se determinó mediante bioimpedancia eléctrica y la ingesta dietética se determinó a través del recordatorio de 72 horas. El DAQs se aplicó para calcular la ingesta total de nutrientes antioxidantes. Se realizaron análisis de regresión lineal para investigar la posible influencia del DAQs en QUS en el calcáneo.

Resultados: La mayoría de los adultos jóvenes mostraron una ingesta de antioxidantes de baja calidad (sólo el 17,6% de las mujeres y el 20,3% de los hombres tenían una puntuación de 4 o 5 en DAQs). Se observó una correlación positiva entre DAQs y BUA en las mujeres ($r = 0,117$; $p = 0,024$). El análisis de regresión lineal reveló que DAQs se asoció significativamente con el parámetro BUA en las mujeres después de ajustar por el peso corporal, la altura, la ingesta de calcio y la actividad física ($p = 0,035$). No se encontraron asociaciones significativas entre la ingesta de antioxidantes individuales y QUS en el calcáneo.

Conclusión: Nuestros resultados sugieren que una ingesta de antioxidantes de alta calidad podría influir en la salud ósea en mujeres jóvenes. Futuros estudios deben profundizar en el papel protector de los nutrientes antioxidantes contra la osteoporosis.

Palabras clave: antioxidantes; ultrasonido cuantitativo de calcáneo; nutrición; adultos jóvenes.

Introduction

Osteoporosis is considered a public health problem characterized by low bone density and reduced bone quality through the deterioration of bone microarchitecture [1]. As a consequence, sufferers have an increased susceptibility to osteoporotic fractures [2]. Osteoporosis is a multifactorial and complex disease determined by both genetic and environmental factors [3].

Oxidative stress and low serum levels of antioxidants have been proposed to be contributors to osteoporosis. In vitro and animal studies have shown that oxidative stress could induce bone loss by modulating osteoclast activation and osteoblast suppression [4–7]. In this line, a number of epidemiologic studies have reported positive associations between oxidative stress and bone mineral density (BMD) [8,9].

Evidence suggests that intake of antioxidants could positively influence bone mass by preventing bone metabolism against oxidative stress. Previous studies have investigated a relationship between antioxidants intake and BMD, fracture risk and osteoporosis reporting contradictory results [10–14]. Most studies generally analyzed the association between single antioxidant intake and bone status. Little is yet known regarding diet quality indexes of antioxidant intakes and their potential relation with bone status. However, people consume foods with complex combinations of antioxidant nutrients [15] and therefore, this traditional approach misses information regarding interactions between different antioxidant contained in food.

Quantitative ultrasound (QUS) have been proposed as an alternative method to assess bone mass and provide parameter of bone structure (microstructure, elasticity and connectivity) [16]. The QUS have been valued for its highly correlation with BMD measured by DXA [17]. Its portability, non-invasiveness, radiation-free and low cost has made it as an useful method for assessing bone status [18]. Until now, no studies have examined the relationship between antioxidant intakes and bone mass assessed by QUS. Therefore, the aim of the current study was to investigate the influence of single antioxidant intakes and Dietary Antioxidant Quality score (DAQs) on calcaneal QUS in young adults. We hypothesized that high-quality antioxidant intake would be associated with greater calcaneal QUS parameter.

Methods

Subjects

Six hundred and five individuals aged 18 to 25 (69.3% females and 30.7% males) were agreed to participate in this study and were recruited from different academic centres of Granada (Spain). All participants were evaluated by means of a detailed medical history. Subjects with any of the following criteria were excluded from the study: history of bone disease, metabolic or endocrine diseases, hormone-replacement therapy or current treatments that could affect bone mass. Written informed consent was obtained from each participant and the study was approved by local ethics committees and conducted in accordance with the Declaration of Helsinki.

Anthropometric measurements

Body weight (kg) and fat mass (%) were measured twice (without shoes and in light clothes) to the nearest 0.11 kg by bioelectrical impedance analysis (TANITA BC-418MA®). A Harpenden stadiometer (Holtain 602VR®) was used for height measurements. Height was measured twice without shoes to the nearest 0.5 cm. The averages of the two values for each measurement were used in the analysis. Anthropometric measurements were performed in the morning after a 12-h fast and 24-h abstention from exercise. BMI was calculated as weight over height squared (kg/m^2).

Calcaneal QUS

Bone mass status was measured by ultrasonography at the right calcaneus (BUA, dB/MHz) using the CUBA clinical ultrasound bone densitometer (McCue Ultrasonics Limited, Compton, Winchester, UK). The calcaneus is used for QUS assessment because it contains a high percentage of trabecular bone and it is easily accessible [19]. Daily calibrations were made with physical phantom to control the long-term stability of the apparatus.

Daily nutrient intake

Daily nutrient intake was assessed by a 72-hour diet recall interview considering intakes on Thursday, Friday and Saturday to capture weekly variations in weekdays and weekend. In a face-to-face interview with well-trained

investigators, individuals were asked to recall all food consumed in the preceding 72 hours, including foods eaten outside the home, nutrition supplements and beverages. In order to improve the accuracy of the descriptions of meals, pictorial food models were employed. A computerized food analysis program (Nutriber 1.1.5) was used to assess completed food records [20]. The food composition table reported by Mataix et al. was used for conversion of food into nutrients [21].

Physical activity

Physical activity was assessed using a self-administered questionnaire (International Physical Activity Questionnaire-IPAQ*).* The questionnaire has proven to be a valid instrument for measuring PA in the European adult population [22]. It was used to calculate the total hours of vigorous PA, moderate PA and walking over the last 7 days. A MET-h was derived by multiplying the respective total hours by the Metabolic Equivalent of Task (MET) value for vigorous PA (MET = 8.0), moderate PA (4.0) and walking (3.3), and then adding all three [22].

Antioxidant nutrient intake

A DAQs was used to calculate antioxidant nutrient [23]. The test score assessed the consumption of vitamin C, vitamin E, vitamin A, selenium and zinc. Daily nutrient intake was compared to that of the Daily Recommended Intake for Spanish population (RDI) [24]. When the intake of nutrient was below 2/3 of the RDI, a value of 0 was obtained and when the intake was above 2/3 of the RDI, it was obtained a value of 1. DAQs is scored with a final score range from 0 (very poor quality) to 5 (high quality).

Statistical analysis

SPSS Statistic version 21.0 (SPSS, Chicago, IL, USA) was used for all the analyses. Mean and standard deviation (SD) where normally distributed and as median (interquartile range) where skewed are given as descriptive statistics. Sex-specific differences were assessed by independent t-test. Sperman´s correlation coefficient (r) was used to test the correlation between antioxidants nutrient, DAQs and calcaneus QUS adjusted by age, body weight, height,

calcium intake and physical activity. To analyze the associations between single antioxidants intake, DAQs and calcaneus QUS, multiple regression analysis were performed after adjusting by age, body weight, height, calcium intake and physical activity. Results are reported as standardized β-coefficient, R, R^2, adjusted R^2, t and p value. P-values < 0.05 were considered to be statistically significant.

Results

The basic characteristics were summarized separately for men and women in table 1. The mean age for the study population was 20.4±2.7 and the mean BMI was 22.6 ± 3.7 kg/m^2. Significant differences between men and women were observed in height, weight, BMI, fat mass, physical activity and BUA values. Men had significantly higher body height, weight, and BMI than women ($p<0.001$), whereas women had significantly higher fat mass than men ($p<0.001$). Reported energy intake was higher in men than women, but there was no evidence of any significant differences. Average calcium intake was below the recommended intake level (RDA) in both genders. The mean calcaneus BUA for the sample was 86.7±17.6 (dB/MHz) and males had significant higher BUA than females ($p<0.001$). Regarding the intake of antioxidant nutrients, the averages intakes of vitamin E, vitamin A and zinc were lower than the recommended in both sexes. By contrast, the intake of vitamin C and selenium reached the dietary goals. Considering gender, a significant difference has been observed concerning the intake of vitamin C ($p=0.036$).

The percentages of young adults that are below 2/3 of the RDI for antioxidant nutrients are shown in table 2. Regarding vitamin E, a higher percentage of women and men had inadequate antioxidant intake (defined as intakes below 2/3 of the DRI). As can be observed, most of women were low antioxidant consumers (only 17.6% of women had a score of 4 or 5 in DAQs). In this line, only 20.3% of men showed a high-quality antioxidant intake (DAQs 4 or 5).

Sperman´s correlation revealed a positive relationship between DAQs and calcaneus BUA in women ($r=0.117$; $p=0.024$) (Table 3). In order to analyse the influence of DAQs and each antioxidants intake on calcaneal QUS, multiple regression models were applied after adjusting by body weight, height, calcium

intake and physical activity (Table 4). Interestingly, the multiple regression analysis revealed that DAQs was significantly associated with BUA parameter in women (p=0.035). No significant associations between single antioxidant nutrient and calcaneus QUS measurement were found.

Discussion

The present study explores the associations between DAQs and single antioxidant intakes on calcaneal QUS measurement in a sample of 605 young adults. Our findings provide evidence for the influence of DAQs on calcaneal BUA parameter in young women supporting the hypothesis that high-quality antioxidant intake could positively influence bone mass in young women. To our knowledge, there has been no previous study that investigates the association of DAQs on bone mass assessed by calcaneal QUS measurement.

To date, only two studies have investigated the association of DAQs with bone status [11,12]. In agreement with our findings, Rivas et al. reported a significant positive association between DAQs and BMD among 280 healthy women aged 18 to >45 (p= 0.021) [12]. On the other hand, in the study of De França et al. 150 postmenopausal women over 45 years old with osteoporosis were included [11]. In contrast to Rivas et al. and with our findings, they did not find relationship between DAQs and BMD in any skeletal sites. One possible reason for this discrepancy may be attributed to limited sample size or sample consisted of osteoporotic women. It would be possible that DAQs was not suitable for assessing the association of antioxidant dietary intakes and bone mass in osteoporotic subjects since the antioxidant considered in this score could have a minimum effect on low BMD values. In addition, in this study they have applied an adaption of the original DAQs since they used Estimated Averages Requirements (EAR) instead of RDI. Note that both previous studies used DXA for measurements of bone mass, none of them used calcaneal ultrasound, and hence, we could not compare our effect sizes for BUA.

This study is the first study to explore the association of DAQs with bone mass in a population of men. Although our study reported a lack of association, we cannot completely discard an association of DAQs with bone mass in men since the relatively small sample size compared to women. Further studies including

larges sample sizes are required to assess the relationship between DAQs and bone mass in men.

In this study, when antioxidant nutrient intakes were analyzed separately, we did not observe any association between single antioxidants and calcaneus QUS. Previous studies have assessed the role of select dietary antioxidants, vitamin C [10,14,25–28], vitamin E [14,29], vitamin A [14,30–32], zinc [33] and selenium [14,29,34] and bone mass revealing inconsistent findings. One possible cause of inconclusive results could be differences in samples sizes and characteristics, study designs and dietary assessments among studies. Note that these studies have focused on the effects of single antioxidant on bone health. By using this approach, potential interactions among different antioxidant dietary intakes have been ignored because people consume food with a complex combination of antioxidants rather than single antioxidant. Consequently, in order to analyze the effect of overall diet and detect possible interactions, recent studies are using diet quality indexes as an alternative method [10,35,36].

The current study has a larger sample size than previous studies exploring the association of DAQs and bone mass. Moreover, this study provides the first investigation of DAQs and bone health in both men and women. Furthermore, all analyses were adjusted for relevant covariates known to affect bone mass. One limitation of our study was its cross-sectional design, which could not infer causality. Another limitation is inherent to the assessment of dietary intake using a self-administered questionnaire. The literature supports the use of 72-hour recall as a pertinent method for assessing nutrient intake since it collects better data on the typical or average diet [37]. However, evidence of underreporting of food intake in self-administered questionnaires has been reported previously [37,38]. In our study the 72-hour recall was interviewer-driven. Additionally, well-trained investigators asked study subjects to recall all food intakes and, in order to improve the accuracy of the descriptions of meals, pictorial food models were employed. Note that another potential limitation is the lack of data regarding the culinary treatments that might influence on the reported intake of antioxidants. Finally, the effect of other antioxidants such as flavonoids was not considered.

In summary, our findings of significant associations between DAQs and calcaneal ultrasound in young women reflect the protective role of high-quality

antioxidant intakes as environmental factor contributing to bone health. Future studies should further explore the potential influence of antioxidant nutrients against osteoporosis.

Conflict of interest: The authors declare no conflicts of interest.

Human and Animal Rights and Informed Consent

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Written informed consent was obtained for all participants.

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Table 1. Basic characteristics of the study population (n= 605).

	Females	*Males*
N (%)	419 (69.3)	186 (30.7)
Age	20.4±2.7	20.5±2.6
Height (m)	1.6±0.1**	1.7±0.1
Weight (kg)	59.4±10.1**	73.1±13.0
BMI (kg/mg²)	22.1±3.6**	23.6±3.7
Fat mass (%)	24.4±7.3**	15.1±5.4
Intake		
Daily energy intake (kcal/day	1990±1275**	2139±716
Calcium intake (mg/day)	799.7±346.4	855.2±377.1
Vitamin C (mg/day)	79.6±65.5*	93.2±76.9
Vitamin E (mg/day)	5.4±3.4	5.9±3.9
Vitamin A (ug/day)	595.2±489.8	627.7±447.8
Zn (mg/day)	8.9±3.9	9.9±4.3
Se (ug/day)	132.8±89.0	143.9±79.3
Total physical activity (MET-hrs) [a]	38.7 (0-246.2) **	61.7 (0- 243.5)
BUA (dB/MHz)	83.3±15.8**	96.31±16.8

Data are shown as mean±SD.
*p < 0.05 between females and males **, p < 0.001 between females and males.
All the nutrients were adjusted for energy intake.
BMI: Body Mass Index mineral; MET: metabolic equivalent of a task. representing energy expenditure per day.
[a] MET-hrs are expressed as mean and range.

Table 2. Daily intake of the antioxidant nutrients in the study population.

	Females	*Males*
	% sample 2/3 RDI	*% sample 2/3 RDI*
Vitamin C (mg/day)	34.2	32.3
Vitamin E (mg/day)	84.8	78.9
Vitamin A (ug/day)	57.7	66.7
Zn (mg/day)	67.1	61.3
Se (ug/day)	12.5	6.4

Table 3. Spearman correlation coefficients (r) between antioxidant nutrients, DAQs and calcaneal QUS.

	Calcaneal BUA			
	Females		*Males*	
	r	*p-value*	*r*	*p-value*
Vitamin C (mg/day)	0.083	0.107	0.089	0.251
Vitamin E (mg/day)	0.047	0.343	0.047	0.547
Vitamin A (ug/day)	0.036	0.487	0.036	0.487
Zn (mg/day)	0.041	0.431	0.111	0.151
Se (ug/day)	0.079	0.128	-0.048	0.533
DAQs	0.117	0.024	0.049	0.531

DAQs: Dietary Antioxidant Quality Score

Adjusted by age, weight, height, calcium intake and physical activity

Table 4. Association between antioxidant nutrients and DAQs on calcaneal QUS measurement (dB/MHz).

	Females							*Males*						
Variables	*R*	*R²*	*Adjusted R²*	*SE*	*Coefficient*	*t*	*p*	*R*	*R²*	*Adjusted R²*	*SE*	*Coefficient*	*t*	*p*
Vitamin C (mg)	0.335	0.112	0.100	14.988	0.080	1.618	0.106	0.259	0.067	0.040	16.485	0.090	1.194	0.234
Vitamin E (mg)	0.329	0.108	0.096	15.003	0.045	0.912	0.362	0.240	0.058	0.029	16.630	0.057	0.743	0.458
Vitamin A (ug)	0.327	0.107	0.095	15.011	0.033	0.683	0.495	0.251	0.063	0.035	16.648	0.064	0.838	0.403
Zn (mg)	0.328	0.107	0.095	15.009	0.036	0.733	0.464	0.276	0.076	0.049	16.525	0.136	1.813	0.072
Se (ug)	0.334	0.112	0.100	14.973	0.075	1.531	0.127	0.246	0.060	0.033	16.626	-0.040	-0.535	0.593
DAQs	0.341	0.117	0.105	14.951	0.104	2.114	0.035	0.248	0.062	0.033	16.556	0.087	1.123	0.263

DAQs: Dietary Antioxidant Quality Score

Adjusted by age, weight, height, calcium intake and physical activity

9. Identifying SNP-SNP interactions associated with bone quantitative ultrasound parameter in early adulthood

[Manuscrito en proceso de revisión]

Identifying SNP-SNP interactions associated with bone quantitative ultrasound parameter in early adulthood

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Purpose: The aim of this study was to perform SNP-SNP interaction analyses in selected candidate genes influencing heel quantitative ultrasound (QUS) parameter, a screening tool for osteoporosis, in a population of young Caucasian adults to identify non additive effects and, potentially, novel insights into the mechanism of disease.

Methods: The study population comprised a total of 575 individuals (mean age 20.41; SD 2.36) whose bone mass was assessed through QUS to determine Broadband Ultrasound Attenuation (BUA, dB/MHz). A total of thirty-two SNPs were included as genetic markers in this study on the basis of their previous association with QUS and/or bone mineral density (BMD) parameters. The association of all possible SNP pairs with QUS was assessed by linear regression and a SNP-SNP interaction was defined as a significant departure from additive effects.

Results: The pairwise SNP-SNP analysis revealed several significant interactions. The interaction involving SNPs rs9340799 and rs3736228 located in the *ESR1* and *LRP5* genes, respectively, showed the strongest association after adjusting for BMI, physical activity and calcium intake (p-value = 0.001, β(95% CI) = 14.289 (5.548, 23.029). In addition, our model reported others such as *TMEM135-WNT16* (p= 0.007, β(95%CI) = 9.101 (2.498, 15.704), *ESR1-MBL2/DKK1* (p= 0.012, β(95%CI) = 13.641 (2.959, 24.322) or *OPG-LRP5* (p= 0.012, β(95%CI) = 8.724 (1.936, 15.512).

Conclusion: Our findings provide new insights into the genetic architecture of QUS traits supporting that several SNP-SNP interactions, especially that between *ESR1* and *LRP5* genes, influence heel QUS in Caucasian young adults.

Key words: Gene interaction; *ESR1*; *LRP5*; Quantitative Ultrasound.

Mini abstract

Osteoporosis is a complex disease determined by genetic and environmental factors. We investigated SNP-SNP interactions in candidate genes influencing heel quantitative ultrasound (QUS) parameter in young adults. Our findings provide new insights into the genetic architecture of QUS traits supporting that several SNP-SNP interactions, especially that between *ESR1* and *LRP5* genes.

Introduction

Osteoporosis is considered a serious public health concern that causes more than 8.9 million fractures annually worldwide [1]. Due to the ageing population, its prevalence is expected to further increase [2]. Osteoporosis is a skeletal disease characterized by low bone mineral density (BMD) and microarchitecture deterioration of bone tissue leading to an increased risk of bone fragility fractures [3]. It is widely accepted that osteoporosis is a complex disease determined by multiple genetic variants that interact with each other and with the environment modulating individual susceptibility [4, 5].

Peak bone mass (PBM) is an important determinant in osteoporosis risk later in life [6]. Enhancing bone mass accrual to maximize PBM, which is attained by early adulthood, could help reduce the risk of fracture in the elderly [6]. Current evidence suggests that genetic factors are major contributors to regulation of PBM, accounting for 50% and 80% of the variance in BMD [7, 8]. Therefore, identifying genetic factors affecting bone mass in early adulthood would be worthwhile.

In recent years, quantitative ultrasound (QUS) has gained much popularity as an alternative and non-invasive technique to evaluate bone status [9]. QUS provides not only information on bone mass but also the quality aspects of bone including microstructure, elasticity and connectivity which may be important in determining the fracture risk [10, 11]. Besides this, previous studies have shown the ability of heel QUS to predict independently fracture risk [10, 12]. Compared to dual-energy x-ray absorptiometry (DXA), QUS is more easily accessible, low-cost, non-invasive and non-ionizing [13].

In order to identify the genetic factors involved in osteoporosis, extensive genetic studies have reported association of several genetic variants with different bone phenotypes [14–17]. However, these explain little of the heritability in complex phenotypes [18]. The missing heritability problem refers to the observation that the number of significant associations discovered does not form a substantial proportion of heritability for most traits. Thus, SNP-SNP interactions could help explain the missing heritability of common complex traits and therefore, their identification is considered of relevance. To date, only a few studies have been performed to identify SNP-SNP interactions influencing osteoporosis-related traits [19–25]. Most of them have focused on BMD measured by DXA, and therefore the possible influence of genetic interactions on QUS parameters is still unknown [24–26].

The independent association between single nucleotide polymorphisms (SNPs) in *WNT16* (rs2908007, rs2908004, and rs2707466) [17, 27, 28], *RSPO3* (rs774121) [17] and *LRP5* (rs3736228) genes and QUS parameters have been well established. In previous replication studies from our group we investigated the implication of 32 genetic markers reported to influence QUS and/or BMD parameters, and confirmed that *WTN16*, *RSPO3* and *LRP5* are genetic factors that determine bone mineralization in young adults [27].

Taking into consideration all this evidence, and bearing in mind that it is likely that common variants at different loci interact to influence QUS traits, the aim of this study was to identify SNP-SNP interactions between SNPs independently associated with QUS, that could contribute to QUS traits variation in a population of young Caucasian adults.

Methods

Study subjects

The population study comprised five hundred and seventy-five healthy individuals of Caucasian ancestry (400 females and 175 males, median age 20,41±2,69 recruited from different centres of Granada (Spain). Written informed consent was obtained from all individuals. The study was approved by local ethics committees and conducted in accordance with the Declaration of Helsinki. Subjects with a history of bone disease, metabolic or endocrine diseases, and hormonal contraceptive therapy that could affect bone mass were excluded.

Covariates

Body weight, body fat mass and lean mass measurements were estimated using body composition analyzer (TANITA BC-418MA) to the nearest 0,11 Kg. Height was measured using a Harpenden stadiometer (Holtain 602VR®) to the nearest 0,1 cm. Body mass index (BMI) was calculated as weight divided by height squared (kg/m^2). Physical activity (PA) were determined using the self-administered International Physical Activity Questionnaire (IPAQ) [29]. Dietary calcium intake (DCI) was assessed using 72h recall method considering intakes on Thursday, Friday, and Saturday. To improve the accuracy of the food descriptions, standard household measures and pictorial food models were employed during the interviews to define amounts when requested. Food records were converted to nutrient intake using a computerized nutrient analysis program (Nutriber 1.1.5).

QUS of the heel

Bone mass status was assessed by ultrasound measurements at the right calcaneus (BUA, dB/MHz) using the CUBA clinical ultrasound bone densitometer (McCue Ultrasonic Limited, Compton, Winchester, UK). The calcaneus is used for QUS assessment because it contains a high percentage of trabecular bone and its easy accessibility [30]. Daily calibrations were made with physical phantom to control the long-term stability of the apparatus.

Selection criteria for SNPs and genotyping

Saliva samples for DNA extraction were collected from study participants using the OG-500 Collection Kit (DNA Genotek Inc, Ontario, Canada). DNA was isolated from saliva samples according to manufacturer´s protocol.

A total of thirty-two SNPs mapping to genes identified as important in candidate gene and/or genome wide association studies (GWAS) for osteoporosis related phenotypes were included as genetic markers in this study on the basis of their previous association with QUS and/or BMD parameters.

Genotyping was performed at the Genomic and Genotyping unit of GENYO centre (Pfizer-University of Granada-Junta de Andalucía Centre for Genomics and Oncological Research) using the Open Array technology (Life Technologies, Carlsbad, CA, USA). A TaqMan OpenArray genotyping plate was custom designed including thirty-two predesigned TaqMan genotyping assays for each of the selected SNPs. Standard cycling conditions were used as recommended by the manufacturer. Thermal cycling and fluorescence detection were performed using QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems). Genotyping call rate for the thirty-two Taqman assays included in the array was higher than 95%. To guarantee accuracy of genotyping negative controls and duplicate samples were included in all genotyping arrays. We observed 100% reproducibility.

Statistical analysis

For all analyses the genotype of each SNP was encoded as 0, 1, 2, where 0 and 2 denotes homozygotes with major allele and minor allele respectively and 1 codes heterozygotes.

For each SNP, the Hardy-Weinberg equilibrium (HWE) was assessed by Pearson's goodness-of-fit χ^2 statistic. The first part of this discovery screening consisted in reducing redundancy between SNPs by keeping only one SNP out of all SNPs in strong pairwise linkage disequilibrium ($r^2 > 0.90$) that led to the final selection of 30 SNPs (rs2707466 and rs7988338 were excluded) (Table 1). All possible pairwise SNP-SNP interactions between the selected thirty SNPs (435 possible interactions) were assessed by bivariate linear regression modelling of QUS measurements. Linear regression analyses were adjusted for the following covariates: BMI, physical activity and calcium intake. The interaction between SNP_1 and SNP_2 was defined as the additional effect of their concomitant carriage on QUS over the addition of their independent effects (departure from additivity): $\beta_{interaction} = \beta_{observed} - \beta_{expected}$ with $\beta_{expected} = \beta_{SNP1}+\beta_{SNP2}$, where $\beta_{observed}$ is the observed effect of the concomitant carriage of SNP_1 and SNP_2 (versus no carriage) in a bivariate linear regression, β_{SNP1} is the effect (β coefficient) of SNP_1 in the absence of SNP_2, β_{SNP2} is the effect of SNP_2 in the absence of SNP_1 in the same bivariate regression framework (bivariate refers only to the number of genetic markers (SNPs), as non-genetic covariates have systematically been included for all analyses). The results were reported as $\beta_{interaction}$ (change of the outcome variable QUS with 95% confidence intervals (CIs). To all significant associations, the highly conservative Bonferroni correction considering the number of of tested SNPs was applied. The ut-off value for significance was set at $p<0.0001$ (0.05/435). Statistical analyses were performed using the STATA software (version 11.0; STATA Corporation, College Station, TX).

Results

The characteristics of the 575 study subjects have been published previously [27]. Table 1 shows marker information, including rsID and minor allele frequency (MAF) for thirty-two SNPs genotyped. None of the SNPs failed the missingness test (genotyping >0.05) or the frequency test (MAF<0.01) and were observed to be in HWE.

The pairwise SNP-SNP interactions tested by linear regression demonstrated multiple interactions that

reached statistical significance ($p < 0.05$). The interaction involving SNPs rs9340799 and rs3736228 located in the *ESR1* and *LRP5* genes, respectively, showed the strongest significant association after adjusting for BMI, physical activity and calcium intake. This interaction amounted to a β (95% CI) = 14.289 (5.548, 23.029) and p-value = 0.001. Interestingly, we found several significant interactions between different polymorphisms in these genes, further supporting the presence of epistasis between these two loci (rs556442 in *LRP5* and rs9340799 in *ESR1;* rs2234693 in *ESR1* and rs3736228 in *LRP5;* rs2306862 in *LRP5* and rs9340799 in *ESR1*; rs556442 in *LRP5* and rs2234693 in *ESR1*). In addition, an interaction between rs597319 in *TMEM135* and rs2908004 in *WNT16* genes also reached a high level of significance (p= 0.007, β(95%CI) = 9.101 (2.498, 15.704).

Among the significant SNP-SNP interactions, four had both SNPs within the same genes (rs2908004 and rs3801387 in *WNT16;* rs3736228 and rs2306862 in *LRP5;* rs2982552 and rs3020331 in *ESR1*; rs2982552 and rs9340799 in *ESR1*).

Discussion

The aim of this study was to obtain a more comprehensive view of the genetic basis of heel QUS by testing for two-way interactions between common variants in candidate genes. A number of SNPs in candidate genes were analysed considering potentially important covariates for bone mineralization process such as, calcium intake, physical activity and BMI.

Our findings provide evidence for several SNP-SNP significant interactions, highlighting the level of significance reached by the interaction between rs9340799 in *ESR1* and rs3736228 in *LRP5*. To our knowledge, no such SNP-SNP interaction has been demonstrated so far.

ERα is the major mediator of oestrogen action in bone and its gene (*ESR1*) has been previously associated with osteoporosis-related phenotypes [31]. The positive effects of the oestrogens on the skeleton have been well established; oestrogens play a major role in the aetiology of osteoporosis by the regulation of bone turnover and inhibition of bone loss [32]. These biological effects are mediated by binding and activation of specific oestrogen receptors (ERα and ERβ) [33]. The rs9340799 variant is located in the first intron of the *ESR1* gene and although this intron may contain regulatory elements, the functional implication of this genetic variant remains unknown. In addition, low-density lipoprotein receptor-related protein 5 (*LRP5*) encodes a cell surface receptor in the Wnt canonical signal, which acts as a regulator of osteoblast growth and as inhibitor of osteoblast and osteocyte apoptosis [34]. Similarly, the functional implication of the rs3736228 SNP located in *LRP5* is still unknown [35, 36].

The results of this study may reflect a well-regulated cross-talk between ESR1 and LRP5 genes in bone physiology. Although *ESR1* and *LRP5* have different roles in skeletal maintenance, they might interact in bone cell biology with either synergistic or antagonistic roles. It could be hypothesised that individuals

carrying risk alleles at rs9340799 and rs3736228 SNPs have a lower expression of both *ESR1* and *LRP5,* which could lead to an impaired bone mass. As we have analysed a cohort including only young adults, our findings also may suggest that the interaction between rs9340799 and rs3736228 SNPs might be implicated in early mineralization mechanisms that lead to bone mass accrual. Although previous studies have reported the individual contribution of these polymorphisms to osteoporosis-related phenotypes in early adulthood [36–39], the mechanisms through which they might interact are in the preliminary stages. Future studies are required to elucidate the molecular mechanisms by this interaction is implicated in the bone mass acquisition process during early adulthood.

Besides the significant interaction between *LRP5* and *ESR1*, our model reported others such as *TMEM135-WNT16*, *ESR1-MBL2/DKK1* or OPG-LRP5 that were nominally significant. Interestingly, all of these genes belong to well characterize pathways implicated in the complex mechanisms that regulate bone formation. Thus, a novel line of research would be very interesting to elucidate how they are interacting at the molecular level.

On the other hand, we identified some SNP-SNP interactions between variants that were not in LD within the same gene. That was the case of genes *WTN16, LPR5* and *ESR1*. This finding suggests that beyond the individual effect of SNPs in a gene, epistatic SNP-SNP interactions in the same gene occur and could be a potential factor contributing to the unexplained heritability of bone mass acquisition.

Our study is the first to report the association of these SNP-SNP interactions with heel QUS trait. However, the positive findings would not remain significant if conservative Bonferroni correction was applied. Our results should be regarded as preliminary and interpreted with caution since the population size included reached a relatively limited statistical power. On the other hand, the current approach considered only two-locus interactions and therefore more complex interactions between three or more genetic markers have evaded detection. Larger studies are needed to confirm our findings and to elucidate mechanisms by which the genetic interaction between these genes influences quantitative bone phenotypes in early adulthood.

In conclusion, our findings provide new insights into the genetic architecture of QUS traits supporting that several SNP-SNP interactions, especially that between *ESR1* and *LRP5* genes, influence heel QUS in Caucasian young adults.

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Conflict of Interest

The authors declare that they have no conflict of interest.

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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Table 1. General information for the studied single nucleotide polymorphisms (SNPs).

Chromosome	Gene	SNP	Allele	MAF in this study	HWE (p)
2	*SPTBN1*	rs11898505	G>A	0.37	0.75
6	*RSPO3*	rs7741021	A>C	0.39	0.84
6	*CCDC170*	rs4869739	A>T	0.35	0.22
6	*ESR1*	rs3020331	C>T	0.43	0.10
6	*ESR1*	rs2982552	C>T	0.49	0.19
6	*ESR1*	rs2234693	T>C	0.44	0.19
6	*ESR1*	rs9340799	A>G	0.34	0.75
7	*WNT16*	rs2908007	T>C	0.18	0.33
7	*WNT16*	rs2908004	T>C	0.22	0.25
7	*WNT16*	rs3801387	T>C	0.10	0.78
7	*WNT16*	rs3801385	A>G	0.09	0.33
7	*WNT16*	rs2707466	G>A	0.22	0.39
7	*WNT16*	rs2536184	G>A	0.03	0.09
8	*OPG*	rs4355801	A>G	0.39	0.17
8	*OPG*	rs3102735	T>C	0.12	0.35
8	*OPG*	rs2073618	G>C	0.46	0.63
10	*MBL2/DKK1*	rs7902708	G>C	0.11	0.77
11	*TMEM135*	rs597319	A>G	0.31	0.19
11	*LRP5*	rs2306862	C>T	0.19	0.32
11	*LRP5*	rs556442	A>G	0.42	0.88
11	*LRP5*	rs3736228	C>T	0.17	0.46
13	*RANKL*	rs9594759	T>C	0.48	0.40
13	*RANKL*	rs12585014	G>A	0.18	0.11
13	*RANKL*	rs7988338	G>A	0.19	0.40
13	*RANKL*	rs2148073	C>G	0.18	0.60
17	*SOST*	rs4792909	G>T	0.42	0.31
17	*SOST*	rs851054	A>G	0.38	0.06
17	*SOST*	rs2023794	T>C	0.05	0.18
18	*RANK*	rs1805034	C>T	0.41	0.26
18	*RANK*	rs12458117	G>A	0.19	0.35
18	*RANK*	rs3018362	A>G	0.32	0.18
19	*GPATCH1*	rs10416265	A>G	0.32	0.06

MAF minor allele frequency, HWD p value for Hardy-Weinberg equilibrium

Table 2. Results of gene-gene interaction analysis (p < 0.05).

SNP1	Gene 1	SNP2	Gene 2	β (95% CI)	p-value
rs9340799	*ESR1*	rs3736228	*LRP5*	14.289 (5.548, 23.029)	0.001
rs2908004	*WNT16*	rs3801387	*WNT16*	77.350 (25.890, 128.808)	0.003
rs597319	*TMEM135*	rs2908004	*WNT16*	9.101 (2.498, 15.704)	0.007
rs3736228	*LRP5*	rs2306862	*LRP5*	23.476 (5.623, 41.327)	0.010
rs9340799	*ESR1*	rs7902708	*MBL2/DKK1*	13.641 (2.959, 24.322)	0.012
rs4355801	*OPG*	rs556442	*LRP5*	8.724 (1.936, 15.512)	0.012
rs3801387	*WNT16*	rs2982552	*ESR1*	-8.705 (-15.554, -1.855)	0.013
rs556442	*LRP5*	rs9340799	*ESR1*	9.859 (2.045, 17.673)	0.013
rs11898505	*SPTBN1*	rs10416265	*GPATCH1*	-9.200 (-16.751, -1.649)	0.017
rs2982552	*ESR1*	rs3020331	*ESR1*	13.312 (2.242, 24.381)	0.018
rs2148073	*RANKL*	rs1805034	*RANK*	8.443 (1.127, 15.758)	0.023
rs4869739	*CCDC170*	rs7741021	*RSPO3*	-7.748 (-14.501, -0.995)	0.024
rs2234693	*ESR1*	rs3736228	*LRP5*	10.606 (1.361, 19.851)	0.024
rs556442	*LRP5*	rs12458117	*RANK*	-9.760 (-18.261, -1.258)	0.024
rs3801387	*WNT16*	rs4355801	*OPG*	7.333 (0.887, 13.777)	0.025
rs2306862	*LRP5*	rs9340799	*ESR1*	8.239 (0.973, 15.504)	0.026
rs2982552	*ESR1*	rs9340799	*ESR1*	9.322 (1.037, 17.606)	0.027
rs7902708	*MBL2/DKK1*	rs4355801	*OPG*	-8.159 (-15.383, -0.934)	0.027
rs2306862	*LRP5*	rs4355801	*OPG*	7.779 (0.888, 14.668)	0.027
rs2306862	*LRP5*	rs11898505	*SPTBN1*	7.989 (0.793, 15.183)	0.029
rs556442	*LRP5*	rs2234693	*ESR1*	7.449 (0.664, 14.233)	0.031
rs3801385	*WNT16*	rs2982552	*ESR1*	-11.658 (-22.351, -0.965)	0.032
rs9340799	*ESR1*	rs7741021	*RSPO3*	7.191 (0.409, 13.973)	0.037
rs597319	*TMEM135*	rs851054	*SOST*	6.828 (0.405, 13.25)	0.037
rs2148073	*RANKL*	rs10416265	*GPATCH1*	-7.879 (-15.478, -0.278)	0.042
rs597319	*TMEM135*	rs2908007	*WNT16*	6.889 (0.197, 13.579)	0.043
rs3801387	*WNT16*	rs12458117	*RANK*	7.949 (0.192, 15.704)	0.044
rs2234693	*ESR1*	rs3801385	*WNT16*	13.418 (0.273, 26.562)	0.045
rs3018362	*RANK*	rs10416265	*GPATCH1*	7.359 (0.114, 14.604)	0.046
rs12458117	*RANK*	rs7741021	*RSPO3*	-7.388 (-14.744, -0.031)	0.048
rs2908004	*WNT16*	rs4355801	*OPG*	7.273 (0.014, 14.530)	0.049

SNP1 and SNP2 indicate the individual SNPs within a given SNP-SNP interaction model. Beta represents the regression coefficient. P values are shown adjusted for the covariates BMI, physical activity and calcium intake.

IV. RESUMEN DE RESULTADOS Y DISCUSIÓN

El trabajo de esta Tesis Doctoral se ha centrado en la caracterización de marcadores genéticos y factores ambientales implicados en el nivel de masa ósea, evaluado mediante ultrasonografía a nivel del calcáneo en una población de adultos jóvenes. A pesar de que se han producido relevantes avances en los últimos años, destacando la publicación del primer estudio GWAS en parámetros óseos de QUS, existe un número reducido de trabajos que hayan valorado los factores genéticos y ambientales que influyen en los parámetros de ultrasonografía en edades tempranas. Por lo tanto, su conocimiento continúa siendo muy limitado.

El avance en el conocimiento de las bases genéticas que determinan la adquisición de la masa ósea en edades temprana podría facilitar la comprensión de las bases moleculares subyacentes en este proceso. Además, la caracterización de nuevos marcadores genéticos podría ayudar al desarrollo de nuevas herramientas que permitirían un diagnóstico precoz de los individuos con mayor riesgo de desarrollar osteoporosis en la edad adulta. Por otra parte, el establecimiento de los factores ambientales determinantes en los niveles de mineralización ósea facilitaría la implantación de estrategias preventivas y terapéuticas en individuos en riesgo de padecer la enfermedad. A continuación, se resumen y se discuten los resultados más relevantes de los nueve trabajos que conforman la presente Tesis Doctoral, los cuales han sido agrupados en base a los objetivos previamente planteados.

1. Caracterización de nuevos marcadores genéticos de masa ósea en adultos jóvenes

Mediante la estrategia de genes candidatos se investigó la posible implicación de una serie de genes en la adquisición de masa ósea en edades tempranas (publicaciones 1, 2, 3, 4, 5 y 6).

El gen **VDR** ha sido ampliamente estudiado por su papel regulador tanto en la formación como en la resorción ósea, promoviendo la diferenciación de los osteoblastos y la producción de proteínas implicadas en el proceso de regulación ósea (St-Arnaud, 2008; Yamamoto et al., 2013). En el estudio del gen *VDR,* nuestros resultados no evidenciaron diferencias significativas entre los polimorfismos seleccionados (*FokI*, rs9729 y *TaqI*) y los valores de masa ósea evaluados mediante ultrasonografía en adultos jóvenes tras el ajuste por variables de confusión (publicación 1, Tablas 4, 5 y 6). En línea con nuestro trabajo se encuentran los resultados obtenidos en dos estudios anteriores en los que no se hallaron asociaciones significativas entre marcadores genéticos del gen *VDR* y parámetros óseos determinados con QUS (Omasu et al., 2004; Zajickova et al., 2005). Además, en el único meta-análisis de GWAS realizado hasta la fecha en ultrasonido cuantitativo a nivel del calcáneo, tampoco se detectaron asociaciones con variantes genéticas del *VDR* (Moayyeri et al., 2014). Por el contrario, un estudio realizado en una cohorte de 124 mujeres y hombres adolescentes identificó una asociación significativa entre el SNP *FokI* y parámetros QUS únicamente en el grupo de hombres (Laaksonen et al., 2004). No obstante, la discrepancia respecto a nuestro trabajo podría deberse al bajo poder estadístico ocasionado por el limitado tamaño de la muestra.

Es importante destacar que los trabajos previos en los que se determinó la masa ósea mediante DXA también están en línea con nuestros resultados (Macdonald et al., 2006; Uitterlinden et al., 2006). En un estudio llevado a cabo por el consorcio GEFOS no se describió ninguna asociación entre variantes genéticas del gen *VDR,* incluyendo *FokI* y *TaqI,* y la DMO en una población de 25.000 individuos europeos (Uitterlinden et al., 2006). Por otro lado, en otro trabajo llevado a cabo con 3000 mujeres británicas tampoco se identificaron asociaciones significativas entre polimorfismos del *VDR* y la DMO (Macdonald et al., 2006). En definitiva, en concordancia con estudios previos, nuestros resultados descartan un papel relevante del gen *VDR* en los niveles de masa ósea determinados mediante ultrasonografía.

Uno de los hallazgos más relevantes aportados por el presente trabajo fue la descripción por primera vez de la asociación significativa entre los polimorfismos rs2908007, rs2908004 y rs2707466 del gen ***WNT16*** y la masa ósea, la cual fue determinada mediante ultrasonografía en adultos jóvenes tras el ajuste por covariables de confusión (publicación 2, Tabla 3). Dichas asociaciones mantuvieron el nivel de significación estadística tras la corrección de Bonferroni por comparaciones múltiples. Estos resultados concuerdan con los obtenidos en trabajos previos (García-Ibarbia et al., 2013; Moayyeri et al., 2014). En un meta-análisis de GWAS llevado a cabo por el consorcio GEFOS, se observó la asociación significativa entre el SNP rs2908004 y varios parámetros de QUS incluyendo el BUA (Moayyeri et al., 2014). Por otro lado, en otro estudio realizado en una cohorte de población española, los polimorfismos rs2908004 y rs270766 también se asociaron con las medidas de QUS a nivel del calcáneo (García-Ibarbia et al., 2013). Además, los tres polimorfismos del gen *WNT16* identificados en nuestro estudio también se asociaron previamente con otros fenotipos relacionados con la osteoporosis (DMO y riesgo de fracturas) en meta-análisis de GWAS (Koller et al., 2013; Zheng et al., 2012), así como en un estudio previo de genes candidatos (Hendrickx et al., 2014). Estos resultados sustentan la hipótesis de que determinados SNPs del gen *WNT16* sean marcadores genéticos tanto para fenotipos óseos determinados con DXA como con QUS.

Por otro lado, es interesante destacar que, en un meta-análisis llevado a cabo en dos cohortes bien diferenciadas de adultos mayores y niños, el nivel de significación alcanzado tras la asociación entre marcadores genéticos del *WNT16* y la DMO fue mayor en la cohorte de niños (Medina-Gomez et al., 2012). Los resultados de este meta-análisis, junto con nuestros resultados, apuntan hacia un papel relevante del gen *WNT16* en la adquisición de la masa ósea en edades tempranas.

El gen *WNT16* codifica una proteína perteneciente a la familia Wnt implicada en la regulación de la homeostasis ósea (Logan & Nusse, 2004). Wnt16 es un ligando que

regula la resorción ósea inhibiendo la osteoclastogénesis a través de la vía no-canónica (Kobayashi et al., 2015). A pesar de que el papel en el metabolismo óseo de diferentes proteínas WNT de la vía no-canónica es menos conocido, la importancia de Wnt16 sí se ha confirmado previamente en modelos de ratón (Wnt16 -/- knockout) (Medina-Gomez et al., 2012; Zheng et al., 2012).

Otro de los genes candidatos seleccionados fue el ***LRP5***, implicado en la regulación del metabolismo óseo a través de la vía de señalización beta-catenina. De los SNPs seleccionados como marcadores genéticos, se identificó una asociación significativa entre la variante rs3736228 y el parámetro QUS en nuestra cohorte de adultos jóvenes tras el ajuste por covariables (publicación 3, Tabla 3). Sin embargo, cuando se realizaron los análisis estratificando la población de estudio en función del género, en mujeres sólo se observó una tendencia a la asociación, mientras que en el caso de los hombres no se encontraron evidencias de asociación (publicación 3, Tabla 3). No obstante, hay que tener en cuenta que, posiblemente, la pérdida del nivel de significancia estadística sea consecuencia de la reducción del poder estadístico al estratificar la población en diferentes subgrupos.

El gen *LRP5* ha sido identificado como un gen clave en la adquisición de la masa ósea determinada mediante DXA durante el crecimiento (Gong et al., 2001). De acuerdo con nuestros resultados, algunos trabajos previos han descrito asociaciones entre el polimorfismo rs3736228 y diversos fenotipos óseos determinados mediante DXA en poblaciones de niños y adultos jóvenes (Cheung, Huang, Chan, & Kung, 2008; Ferrari et al., 2004; Saarinen et al., 2007). Por otra parte, estudios de genes candidatos (Canto-Cetina et al., 2013; Markatseli et al., 2011), GWAS (Richards et al., 2008) y meta-análisis de GWAS (Estrada et al., 2012; Tran, Nguyen, Eisman, & Nguyen, 2008) han coincidido en identificar asociaciones entre esta variante del gen *LRP5* y la DMO y/o el riesgo de fracturas en poblaciones de adultos mayores. El hecho de que los resultados sean consistentes en cohortes poblacionales con distintos rangos de edad, sustenta la

posibilidad de que el gen *LRP5* sea un factor genético determinante en la mineralización ósea, tanto en las etapas avanzadas de la vida como en las etapas tempranas.

Sin embargo, en los dos estudios realizados hasta el momento en los que se ha analizado el papel del gen *LRP5* con los parámetros de QUS, no se han identificado asociaciones estadísticamente significativas con ninguno de los marcadores genéticos analizados, incluido el SNP rs3736228 (Kumar et al., 2011; Saarinen et al., 2007). La falta de concordancia entre estos estudios y nuestro trabajo, podría explicarse por varias razones. Por un lado, debido al limitado poder estadístico del estudio de Saarinen et al., consecuencia del tamaño muestral reducido (235 jóvenes finlandeses) y la baja frecuencia de la variante del polimorfismo rs3736228 en población caucásica (MAF= 0.138). Por otro lado, en relación al estudio de Kumar et al., las discrepancias observadas podrían deberse a las diferencias en las características poblacionales. Otro hecho a considerar es que en el estudio de Kumar et al. sólo se mostraron resultados parciales, presentándose únicamente los valores de significancia alcanzados sin especificar la magnitud del efecto. No obstante, es importante destacar que la significación estadística alcanzada por el SNP rs3738228 no se mantuvo tras la corrección de Bonferroni por comparaciones múltiples, debido posiblemente al limitado poder estadístico. Por lo tanto, para clarificar la implicación de la variante genética rs3736228 del gen *LPR5* en los parámetros óseos evaluados mediante QUS, serán necesarios futuros estudios de replicación en otras cohortes independientes de adultos jóvenes.

En relación al gen ***SOST***, otro de los genes implicados en la regulación ósea a través de la inhibición de la vía canónica de señalización Wnt, los resultados obtenidos en nuestro estudio no muestran asociación significativa con el nivel de masa ósea determinado mediante ultrasonido cuantitativo (publicación 3, Tabla 3). No obstante, dado que es el primer trabajo en el que se ha investigado el posible papel del gen *SOST* en la mineralización ósea en edades tempranas, es necesario realizar futuros estudios de

replicación en otras poblaciones para confirmar que este gen no es juega un papel relevante en el parámetro óseo QUS.

Teniendo en cuenta la extensa caracterización de nuestra cohorte de estudio y en base a la evidencia disponible que sostiene la implicación de los genes de la vía Wnt en la adipogénesis (Bennett et al., 2002; Ross et al., 2000), se planteó analizar también la posible contribución de los polimorfismos seleccionados de los genes *LRP5* y *SOST* en fenotipos asociados a la obesidad (IMC, masa grasa y masa magra). El análisis de los marcadores genéticos no reveló ninguna asociación estadísticamente significativa en nuestra población (publicación 3, Tablas 4, 5 y 6). Hasta el momento, los estudios que han investigado la implicación de estos genes de la vía Wnt en relación a la obesidad son escasos, mostrando además resultados contradictorios. En línea con nuestros resultados, en un estudio anterior también se observó una falta de asociación entre variantes del gen *LRP5* y fenotipos de la obesidad en una cohorte de 1244 sujetos (Yu et al., 2010). Por el contrario, otros trabajos han sugerido una implicación de los genes *LRP5 y SOST* en la obesidad (Ashouri et al., 2015; Guo et al., 2006; Piters et al., 2012). Es importante tener en cuenta que estos estudios son muy heterogéneos en cuanto a las poblaciones incluidas (cohortes de individuos chinos, iraníes y europeos), el diseño de los estudios (tamaño de la muestra, género o edad) y metodología utilizada (estudios familiares, estudios caso-control, longitudinal y transversal). Por lo tanto, es necesario clarificar la implicación de estos genes en la obesidad mediante futuros estudios en cohortes poblacionales similares.

El gen ***ESR1*** se ha estudiado ampliamente como gen candidato de la osteoporosis ya que juega un papel clave en el proceso de remodelación ósea (Gennari et al., 2005). En el modelo de regresión lineal sin ajuste por variables de confusión, se identificó una asociación significativa entre el SNP rs2982575 y el parámetro QUS en nuestra cohorte de adultos jóvenes (publicación 4, Tabla 3). No obstante, tras el ajuste por múltiples covariables, no se observaron asociaciones significativas entre los SNPs del gen *ESR1*

seleccionados en esta Tesis Doctoral como marcadores genéticos y la masa ósea evaluada mediante ultrasonografía (publicación 4, Tabla 4).

Respecto a los polimorfismos *XbaI* y *PvuII*, en trabajos anteriores en los que se determinó la DMO como fenotipo relacionado con la osteoporosis, también se evidenció una falta de asociación estadística (Gennari et al., 1998; Sowers, Jannausch, Liang, & Willing, 2004; Valero et al., 2005). De forma similar y en conformidad con los resultados obtenidos en nuestro estudio, en un meta-análisis de GWAS realizado en una cohorte de 18917 individuos pertenecientes a ocho centros europeos tampoco se mostraron resultados positivos (Ioannidis et al., 2004). Por el contrario, Albagha et al. (2005) describieron una asociación significativa entre el parámetro QUS y los haplotipos *XbaI-PvuII*. Del mismo modo, en un estudio llevado a cabo en una población de mujeres postmenopáusicas se identificó una asociación entre los polimorfismos *XbaI-PvuII* y el parámetro SOS (Binh et al., 2006). No obstante, es importante considerar que, aunque BUA y SOS son parámetros de ultrasonografía, no reflejan las mismas características óseas (Krieg et al., 2008). BUA está influenciado por la conectividad ósea mientras que SOS está directamente relacionado con la elasticidad y la densidad del hueso (Krieg et al., 2008). Dado que en nuestro estudio la masa ósea fue determinada mediante el parámetro BUA, la razón de las discrepancias observadas podría deberse a la estimación de diferentes parámetros. Además, es especialmente relevante destacar que ambos estudios se realizaron en cohortes de mujeres postmenopáusicas. Por lo tanto, futuros estudios en los que se evalúe la masa ósea mediante QUS en edades tempranas son necesarios para confirmar estos hallazgos.

De forma similar, nuestros resultados no evidenciaron asociaciones estadísticamente significativas entre el resto de polimorfismos del gen *ESR1* incluidos en el presente trabajo y el parámetro QUS, sugiriendo que estas variantes genéticas no desempeñan un papel relevante en la ganancia de hueso que se produce en etapas tempranas. Los polimorfismos rs3020331, rs2982552, rs2982575 y rs2504063 alcanzaron un nivel de significación

estadística para parámetros DXA y/o QUS en previos meta-análisis de GWAS (Koller et al., 2013; Moayyeri et al., 2014; Rivadeneira et al., 2009). No obstante, hasta la fecha no hay datos funcionales que apoyen una función reguladora de estas variantes genéticas en la expresión del gen *ESR1*. Además, con el objetivo de maximizar el tamaño de la muestra y el poder estadístico, los estudios anteriormente mencionados se llevaron a cabo de forma conjunta en poblaciones con un amplio rango de edad (18-96 años), sin realizar análisis estratificados. El hecho de que la cohorte de nuestro estudio estuvo constituida únicamente por adultos jóvenes (18-25 años), a diferencia de los trabajos anteriores, podría ser la causa de la falta de concordancia en los resultados. Por lo tanto, de acuerdo con la evidencia disponible, nuestros resultados sugieren que estos SNPs podrían ser marcadores genéticos que influyen en la masa ósea en etapas avanzadas de la vida, aunque no en etapas tempranas. No obstante, dada la limitada evidencia sobre el papel potencial de los polimorfismos del gen *ESR1* en la adquisición de masa ósea, no se puede descartar la posibilidad de que estos marcadores genéticos sean variantes causales implicados en la ganancia ósea. Por lo tanto, son necesarios estudios en adultos jóvenes para confirmar los hallazgos preliminares del presente estudio.

Dado el papel de los genes del sistema **RANKL/RANK/OPG** en el proceso de regulación y remodelado óseo (Boyle et al., 2003), en el desarrollo de este trabajo de Tesis Doctoral se consideró analizar por primera vez la posible influencia de diez polimorfismos del sistema de genes *RANKL/RANK/OPG* en los niveles de masa ósea en el calcáneo, determinados mediante ultrasonido cuantitativo. Nuestros resultados no revelaron ninguna asociación significativa entre las variantes genéticas analizadas del sistema *RANKL/RANK/OPG* con el parámetro QUS en el calcáneo, identificándose únicamente una tendencia a la significación en el polimorfismo rs9594759 del gen *RANKL* tras ajustar por variables de confusión (publicación 5, Tabla 2). Estos hallazgos podrían ser indicativos de la falta de implicación del sistema de genes *RANK/RANKL/OPG* en la adquisición del nivel de masa ósea en adultos jóvenes. No obstante, dado que nuestro trabajo es el único que por el momento ha analizado la implicación del sistema

RANKL/RANK/OPG en el parámetro QUS, no se puede descartar completamente la influencia de estos marcadores genéticos en el proceso de adquisición de masa ósea. Luego, al igual que en el caso del gen *SOST*, la consistencia de estos resultados no se demostrará hasta que no se realicen estudios de replicación en otras cohortes, ya que los resultados de un trabajo individual o en una única población no son suficientes para dar por descartada la implicación de un gen en un determinado fenotipo o patología.

Por otro lado, aunque los resultados obtenidos en este trabajo están en línea con estudios previos que no evidenciaron resultados positivos utilizando el DXA para la evaluación ósea (Brändström et al., 2003; Dong et al., 2009), numerosos estudios de asociación, GWAS y meta-análisis han identificado al sistema de genes *RANKL/RANK/OPG* como un factor genético determinante para diversos fenotipos óseos determinados mediante DXA (Paternoster et al., 2010; Rivadeneira et al., 2009; Roshandel et al., 2011; Styrkarsdottir et al., 2008). Es interesante destacar que la mayoría de estos estudios se han llevado a cabo en individuos de edades avanzadas. Concretamente, los meta-análisis de GWAS se realizan en la totalidad de la cohorte poblacional sin estratificar por edad, con objeto de maximizar el tamaño de la muestra y, por consiguiente, el poder estadístico. En este sentido, las diferencias en los rangos de edad entre las cohortes poblaciones de trabajos previos y nuestra cohorte, constituida únicamente por adultos jóvenes, podrían ser la causa de la falta de concordancia en los resultados. Por lo tanto, cabe la posibilidad de que determinadas variantes genéticas del sistema *RANKL/RANK/OPG* estén relacionadas con fenotipos óseos en edades avanzadas, pero no en el proceso de mineralización que se lleva a cabo en edades tempranas.

Por otro lado, debe tenerse en cuenta que las asociaciones significativas entre SNPs de los genes *RANKL/RANK/OPG* se observaron en estudios que determinaron la masa ósea mediante DXA (Paternoster et al., 2010; Richards et al., 2008; Rivadeneira et al., 2009; Roshandel et al., 2011; Styrkarsdottir et al., 2008). En línea con nuestro trabajo, en el estudio MROS (*Osteoporotic fractures in men*) en el que la densidad ósea volumétrica

(vDMO) se determinó con tomografía cuantitativa computarizada (QTC), no se observaron diferencias significativas (Yerges et al., 2009, 2010). Este hallazgo podría indicar que los genes del sistema *RANKL/RANK/OPG* son marcadores genéticos para fenotipos óseos determinados con DXA, pero no para aquellos estimados con diferentes técnicas como QTC o QUS.

Los genes ***SPTBN1*** (rs11898505), ***RSPO3*** (rs7741021), ***CCDC170*** (rs4869739), ***MBL2/DKK1*** (rs7902708), ***TMEM135*** (rs597319) y ***GPATCH1*** (rs10416265) se analizaron en el presente trabajo en base a su identificación como marcadores genéticos de parámetros QUS a nivel del calcáneo en el único estudio GWAS publicado hasta la fecha (Moayyeri et al., 2014). En los resultados obtenidos en nuestra cohorte de adultos jóvenes, tan sólo el SNP rs7741021 del gen *RSPO3* mostró asociación significativa con los niveles de BUA tras ajustar por factores de confusión bajo el modelo dominante y recesivo (publicación 6, Tabla 2). Sin embargo, tras la corrección de Bonferroni por comparaciones múltiples, el nivel de significación se mantuvo estadísticamente significativo únicamente en el modelo recesivo.

RSPO3 codifica un miembro de la familia de proteínas R-espondina (RSPO), que activa la vía canónica de señalización WNT/β-catenina (Wei et al., 2007). Como se ha mencionado anteriormente, esta vía de señalización es fundamental para la formación ósea (Hill, Später, Taketo, Birchmeier, & Hartmann, 2005; Hu et al., 2005). Por lo tanto, una alteración de la vía canónica a través de este receptor, originada por la variación genética del gen *RSPO3,* podría ser la causante de una disminución de la formación de hueso. Sin embargo, la función del gen *RSPO3,* así como la relevancia funcional de SNPs en esta región, aún es poco conocida. El SNP rs7741021 se encuentra en una región intrónica del gen *RSPO3*. Luego, es probable que este polimorfismo no sea la variante causante real que afecte a la función del gen *RSPO3*. Por lo tanto, futuros trabajos de investigación que realicen un mapeo genético mediante equilibrio de ligamiento en esta región serán necesarios para caracterizar las posibles variantes causales.

Además, en el futuro será imprescindible realizar estudios funcionales para investigar los mecanismos moleculares exactos por los cuales *RSPO3* está implicado en el proceso de adquisición de la masa ósea y, más concretamente, el mecanismo por el cuál este receptor podría ser el causante de unos niveles más bajos de masa ósea en adultos jóvenes.

Con respecto a los genes *SPTBN1*, *CCDC170*, *MBL2/DKK1*, *TMEM135* y *GPATCH1*, a diferencia de los hallazgos del estudio GWAS en QUS, nuestros datos siguieren que no son marcadores genéticos determinantes en los parámetros QUS en etapas tempranas. Es importante tener en cuenta la notable diferencia en cuánto a la edad poblacional de nuestro trabajo en comparación con este estudio (Moayyeri et al., 2014). El estudio GWAS se llevó a cabo en una cohorte mixta que incluyó mujeres premenopáusicas, postmenopáusicas y hombres con un amplio rango de edad (25-83 años), mientras que nuestro estudio se realizó en una población bien definida de adultos jóvenes de edades comprendidas entre 18 y 25 años. Luego, se podría hipotetizar que, mientras que el gen *RSPO3* podría determinar genéticamente la mineralización ósea a lo largo de toda la vida, el resto de genes sólo lo harían ya en edades más avanzadas, en las cuales empiezan a predominar los procesos de resorción ósea. Para confirmar esta posibilidad, serán necesarios futuros estudios de replicación.

En resumen, los resultados del presente trabajo revelaron asociaciones independientes entre polimorfismos de los genes *WNT16* (rs2908007, rs2908004 y rs2707466), *LRP5* (rs3736228) y *RSPO3* (rs7741021) con el parámetro QUS a nivel del calcáneo en adultos jóvenes. Por otro lado, no se detectó una asociación significativa entre variantes de los genes *VDR, SOST, ESR1, RANKL, RANK, OPG, SPTBN1*, *CCDC170*, *MBL2/DKK1*, *TMEM135* y *GPATCH1* y los niveles de masa ósea en nuestra población. Considerando globalmente las evidencias encontradas en el presente trabajo de Tesis Doctoral, se puede plantear la hipótesis de que existan marcadores genéticos comunes y otros específicos de cada fenotipo (QUS o DXA). Tal y como se mencionó anteriormente, numerosos trabajos previos han demostrado la influencia de los genes

RANKL/RANK/OPG y *VDR* en la DMO determinada con DXA (Canto-Cetina et al., 2015; Wang et al., 2013). Sin embargo, en relación con la valoración ósea mediante ultrasonido cuantitativo, nuestros resultados, junto con los de trabajos previos, han descartado la implicación de estos mismos genes en parámetros de ultrasonido cuantitativo (Moayyeri et al., 2014; Omasu et al., 2004; Zajickova et al., 2005). Por el contrario, en el caso del gen *WNT16,* se ha evidenciado sólidamente su asociación con los niveles de masa ósea, evaluándose tanto con DXA (Medina-Gomez et al., 2012; Zheng et al., 2012) como con QUS (García-Ibarbia et al., 2013; Moayyeri et al., 2014). De igual forma, nuestros resultados, junto con los obtenidos en estudios previos, apuntarían a la existencia de marcadores genéticos que ejercen un papel fundamental ya desde edades tempranas, como los polimorfismos de los genes *WNT16, LRP5* o *RSPO3*. Por el contrario, otros genes como *RANKL, RANK, OPG, ESR1, SPTBN1*, *CCDC170*, *MBL2/DKK1*, *TMEM135* y *GPATCH,* parecen tener un papel relevante en los mecanismos de remodelado óseo que ocurren a edades avanzadas.

2. Influencia de la antropometría, la actividad física y la dieta en la masa ósea en adultos jóvenes

En la presente Tesis Doctoral se investigaron variables antropométricas, nutricionales y de actividad física como posibles factores modificables que podían influir en los niveles de masa ósea en adultos jóvenes. La identificación de los factores ambientales más determinantes en la adquisición del PMO podría guiar futuras estrategias de prevención primaria frente a la osteoporosis, centradas en maximizar la ganancia ósea en edades tempranas.

El ejercicio físico ha sido propuesto como un factor determinante en la adquisición de la masa ósea en edades tempranas (Nilsson et al., 2012). En línea con otros trabajos, nuestros resultados revelaron una correlación positiva entre los niveles moderados y

vigorosos de actividad física y el parámetro de ultrasonido cuantitativo (publicación 7, Tabla 2) (De Smet et al., 2015; Robinson, Winters-Stone, Gabel, & Dolny, 2007). Tras el análisis de regresión lineal, tan sólo el nivel de actividad física vigoroso alcanzó un nivel de significación estadística (publicación 7, Tabla 3). Estos resultados, junto con los observados en cohortes independientes de adultos jóvenes, avalan el papel clave de un alto nivel de actividad física en la adquisición de la masa ósea (Bielemann, Martinez-Mesa, & Gigante, 2013; Luis Gracia-Marco et al., 2011; Neville et al., 2002).

La posible relación entre variables antropométricas y el nivel de masa ósea ha sido investigada ampliamente, evidenciando una falta de consistencia en los resultados (Weiler et al., 2000; Zhu et al., 2014). En nuestro trabajo, aunque se detectaron correlaciones positivas entre la altura, el peso, el IMC, la masa grasa, la masa magra y el parámetro QUS; en el análisis de regresión lineal tan sólo la masa magra alcanzó el nivel de significación estadística (publicación 7, Tabla 3). Estos resultados están en línea con los descritos previamente en trabajos anteriores (Chen et al., 2015; Gracia-Marco et al., 2012; Ho-Pham et al., 2014). Probablemente, la aparente relación del peso con la masa ósea es un artefacto ocasionado por la fuerte correlación entre éste y la masa magra. Los resultados obtenidos aportan evidencia a favor de la hipótesis de que la masa ósea está más determinada por cargas dinámicas sobre el hueso como la que aporta la masa magra, y no por cargas estáticas como la aportada por la masa grasa (Rauch, Bailey, Baxter-Jones, Mirwald, & Faulkner, 2004). En definitiva, un nivel alto de actividad física que promueva el incremento de la masa magra en adultos jóvenes podría ser una estrategia efectiva para maximizar la mineralización ósea y reducir así el riesgo de padecer osteoporosis en edades avanzadas.

En las últimas décadas se han publicado numerosos trabajos que han investigado la influencia de diversos factores nutricionales en la mineralización ósea en edades tempranas, mostrando resultados no concluyentes. En nuestro trabajo no se observó una relación estadísticamente significativa entre la ingesta de calcio y el nivel masa ósea

determinado con QUS (publicación 7, Tabla 2). A pesar de que el binomio ingesta de calcio-masa ósea ha sido ampliamente investigado, siguen publicándose tanto resultados positivos como negativos (De Smet et al., 2015; Lanou, 2005; Winzenberg, Shaw, Fryer, & Jones, 2006). Es probable que las diferencias en las ingestas medias de calcio en las distintas poblaciones estudiadas sean las causantes de estas discrepancias.

Otro hallazgo a resaltar es la correlación positiva que se identificó entre la ingesta proteica y el parámetro óseo QUS (publicación 7, Tabla 2). Estos resultados, en consonancia con estudios previos, sugieren un papel clave de las proteínas en el proceso de remodelación ósea que se produce en edades tempranas (Alexy et al., 2005; Chevalley et al., 2008). En esta línea, se ha descrito que las proteínas ingeridas en la dieta aportan los aminoácidos necesarios para la formación de la matriz ósea y, por lo tanto, son determinantes en el crecimiento óseo (Bonjour, 2011).

Para profundizar más en el conocimiento de los factores nutricionales, que podían estar influenciando en el parámetro de ultrasonido cuantitativo en adultos jóvenes, se analizó la ingesta de antioxidantes a través del Índice de calidad antioxidante de la dieta (DAQs). La evidencia disponible sugiere que la ingesta de antioxidantes puede influir positivamente en la masa ósea, actuando frente al estrés oxidativo del metabolismo óseo (Wauquier, Leotoing, Coxam, Guicheux, & Wittrant, 2009). Nuestros resultados detectaron una asociación significativa entre el DAQs y el parámetro QUS tras el ajuste por múltiples variables de confusión en mujeres (publicación 8, Tabla 4). En línea con nuestro trabajo, estudios *in vitro* y estudios animales han mostrado como el estrés oxidativo puede modular la pérdida ósea mediante la activación de los osteoclastos y la supresión de los osteoblastos (Bai et al., 2004; Garrett et al., 1990; Lean et al., 2003).

Hasta la fecha, tan sólo dos trabajos han investigado la relación entre el DAQs y la mineralización ósea. Nuestros resultados están en línea con los obtenidos en un estudio realizado en 280 mujeres con diferentes rangos de edad (Rivas et al., 2012), mientras que

difieren de los descritos en una cohorte de 150 mujeres postmenopáusicas (De França et al., 2013). Es importante resaltar que nuestro trabajo tiene un tamaño muestral considerablemente mayor al de los estudios anteriormente mencionados. Además, ha sido el primero en examinar la posible asociación entre la ingesta de antioxidantes y la masa ósea determinada con QUS, ya que en los estudios previos fue determinada mediante DXA.

Por otra parte, el presente trabajo ha sido el primero en investigar la posible influencia del DAQs en la masa ósea en una cohorte de hombres. A pesar de que en nuestro estudio no se observó una asociación significativa, no se puede descartar el papel determinante de la ingesta de antioxidantes de alta calidad en la masa ósea en hombres. Probablemente, la razón principal de la falta de resultados positivos sea el bajo poder estadístico debido al considerable menor tamaño de la muestra en comparación con el grupo de mujeres. En resumen, nuestros resultados sugieren que la ingesta de antioxidantes de alta calidad puede ser un factor importante para la salud ósea en mujeres jóvenes. Futuros estudios serán necesarios para investigar en profundidad el papel de los nutrientes antioxidantes frente a la osteoporosis.

3. Determinación de posibles interacciones genéticas implicadas en la adquisición de la masa ósea en la adultez temprana

En el caso de enfermedades y fenotipos complejos como el QUS, la identificación de marcadores genéticos aislados sólo explica una pequeña parte del efecto genético que determina su aparición (Manolio et al., 2009). Una importante fracción de su heredabilidad continúa siendo desconocida y se ha hipotetizado que las interacciones entre polimorfismos podrían explicar parte de la heredabilidad no identificada en fenotipos y/o enfermedades complejas (Zuk, Hechter, Sunyaev, & Lander, 2012). Por esta razón, en el

presente trabajo se planteó realizar un análisis para detectar posibles interacciones SNP-SNP que pudieran influir en el parámetro de ultrasonido cuantitativo.

Inicialmente, se incluyeron un total de 32 SNPs de 13 genes en los análisis de interacción génica, sin embargo, tras excluir dos SNPs (rs2707466 y rs7988338) que se encontraban en desequilibrio de ligamiento con otros polimorfismos seleccionados ($r^2 >$ 90) para disminuir la redundancia, el número de SNPs se redujo a 30 SNPs. Nuestros resultados revelaron una asociación significativa entre varias interacciones genéticas y el parámetro QUS tras el ajuste por variables de confusión (publicación 9, Tabla 2). Uno de los hallazgos más interesantes fue el alto grado de significación estadística alcanzado por la interacción entre el SNP rs9340799 del gen *ESR1* y el SNP rs3736228 del gen *LRP5*. Es importante destacar que un número considerable de las interacciones SNP-SNP detectadas se produjeron entre variantes genéticas de estos genes, evidenciando la posible implicación de la interacción génica *ESR1-LRP5* en los niveles de masa ósea. La caracterización de esta interacción plantea la posibilidad de que, aquellos adultos jóvenes portadores de alelos de riesgo en los polimorfismos rs9340799 y rs3736228, podrían estar expuestos a una alteración en los niveles de expresión de ambos genes, que provocaría una alteración del proceso de mineralización ósea.

Teniendo en cuenta que nuestra cohorte estuvo constituida únicamente por adultos jóvenes, estos resultados podrían ser indicativos de un posible papel determinante de la interacción rs9340799-rs3736228 en el proceso de mineralización ósea que tiene lugar en la adultez temprana. Estudios en modelos de ratón con la supresión específica del *ESR1*, han revelado que los efectos de los estrógenos sobre la masa ósea están mediados por efectos directos sobre los osteoclastos y los osteoblastos (Manolagas et al., 2013). De este modo, el *ESR1* en los osteoblastos estimula la señalización de Wnt, promoviendo la formación ósea (Manolagas et al., 2013). De forma similar, estudios en ratones han evidenciado un papel directo del *LRP5* en la regulación ósea a través de las células osteoblásticas (Babij et al., 2003; Cui et al., 2011). En la misma línea, trabajos previos

identificaron una asociación significativa individual de estos SNPs con fenotipos óseos relacionados con la osteoporosis en edades tempranas (Cheung et al., 2008; Koller et al., 2013; Saarinen et al., 2007). No obstante, los mecanismos por los cuáles los polimorfismos rs9340799 y rs3736228 de ambos genes podrían interaccionar son desconocidos, por lo que en el futuro será necesario realizar estudios funcionales para investigar el significado biológico de estas asociaciones.

Los resultados presentados a cerca de las interacciones SNP-SNP deben evaluarse con cautela, ya que la significancia estadística de estas interacciones no se mantuvo tras la corrección por Bonferroni. Probablemente, la pérdida de significación estadística se deba al limitado poder estadístico consecuencia del tamaño muestral. Por lo tanto, estos resultados preliminares necesitan ser validados en futuros estudios de replicación con mayores cohortes poblaciones.

Consideraciones finales

La osteoporosis es un problema de salud pública con un enorme impacto socio-sanitario debido a su elevada incidencia, sus consecuencias y los importantes gastos sanitarios que genera. El avance en el conocimiento de los factores genéticos y ambientales implicados en los fenotipos asociados con el nivel de masa ósea en diferentes etapas de la vida, contribuirá tanto al mejor conocimiento de la fisiopatología de la enfermedad como al desarrollo de nuevas herramientas terapéuticas y de prevención que permitan frenar la progresión de la osteoporosis.

Durante el desarrollo de la presente Tesis Doctoral se ha investigado la influencia de diferentes factores genéticos y ambientales en los niveles de masa ósea en edades tempranas. Por primera vez, se ha identificado una asociación significativa entre variantes de los genes *WNT16*, *LRP5* y *RSPO3* y el nivel de masa ósea evaluado mediante ultrasonografía en adultos jóvenes. Con respecto a los factores ambientales, en este trabajo se ha descrito que una mayor masa magra y un nivel alto de actividad física son predictores positivos de mayores niveles de masa ósea. En relación a los factores dietéticos, el índice de calidad antioxidante de la dieta (DAQS) se asoció con el nivel de masa ósea en el calcáneo. Por último, en este trabajo se observaron asociaciones significativas entre diversas interacciones SNP-SNP y el parámetro QUS, destacando la interacción entre el polimorfismo rs9340799 del gen *ESR1* y el SNP rs3736228 del gen *LRP5*. Este hallazgo sugiere que, aquellos adultos jóvenes portadores de alelos de riesgo en los polimorfismos rs9340799 y rs3736228, podrían estar condicionados por una baja expresión de ambos genes que provocaría una alteración del proceso de mineralización ósea. No obstante, al tratarse de resultados preliminares, son necesarios futuros estudios de replicación en cohortes independientes para validar los hallazgos aquí presentados.

Con el conocimiento actual se puede comenzar a esbozar un modelo de factores implicados en los niveles de masa ósea en adultos jóvenes (Figura 13). La identificación y

caracterización de los marcadores genéticos y factores ambientales involucrados facilitará el desarrollo de nuevas estrategias terapéuticas y actuaciones preventivas frente a la osteoporosis en esta etapa.

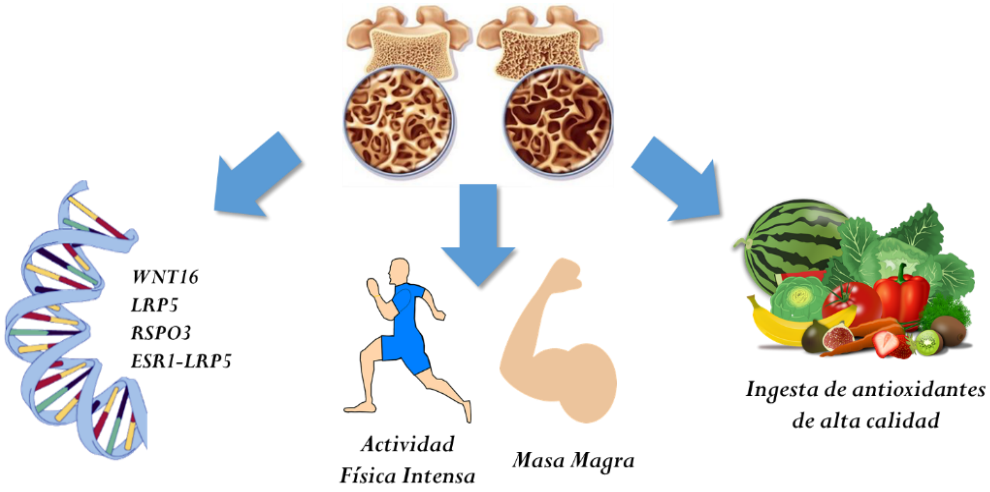

Figura 13. Factores determinantes del nivel de masa ósea en adultos jóvenes.

Figura tejido óseo: https://jafer1309.wordpress.com/2015/03/11/anatomia-de-un-hueso-2/

V. PERSPECTIVAS FUTURAS

Con la realización de la presente Tesis Doctoral se ha colaborado en la caracterización de marcadores genéticos y factores ambientales implicados en el parámetro de ultrasonido cuantitativo a nivel del calcáneo en adultos jóvenes. En la actualidad, existe un número limitado de estudios que valoran los factores genéticos que influyen en el parámetro de QUS, habiéndose publicado hasta la fecha un único estudio GWAS. No obstante, al tratarse de un fenotipo poligénico, quedarían aún numerosos marcadores genéticos por identificar.

Dado que los equipos de QUS se han propuesto como un sustituto potencial al DXA, facilitando una mayor accesibilidad a la valoración ósea en la población general, se impone la necesidad de establecer colaboraciones en un futuro próximo para aumentar la cohorte de estudio y buscar nuevos marcadores genéticos. Un aumento del tamaño de la muestra podría plantear la posibilidad de realizar un estudio GWAS ya que, como se ha mencionado anteriormente, hasta la fecha sólo se ha publicado un GWAS referente a la evaluación ósea mediante ultrasonido cuantitativo en el calcáneo. Esta estrategia podría tener como consecuencia la identificación de nuevas asociaciones a nivel genómico, permitiendo establecer qué marcadores genéticos son claves en los parámetros QUS en edades tempranas. Además, la realización de estudios de replicación a nivel internacional, será de gran interés para confirmar los hallazgos obtenidos en esta Tesis Doctoral, así como para determinar las posibles diferencias entre distintas poblaciones. Por último, con el objetivo de continuar avanzando en el conocimiento de la arquitectura que determina el proceso de mineralización ósea a edades tempranas, otro de los retos futuros es la realización de estudios funcionales para desentrañar la relevancia funcional de los marcadores genéticos asociados y concretar su influencia sobre la regulación de la expresión génica.

Por otro lado, es necesario seguir profundizando en el estudio de la influencia de los factores modificables sobre la masa ósea en la adultez temprana, puesto que el conocimiento actual no ha permitido establecer conclusiones definitivas. Por lo tanto, en

futuros trabajos de investigación, sería muy interesante investigar la influencia de otros factores ambientales como la ingesta de fósforo, la vitamina D o el consumo de hábitos tóxicos en los niveles de masa ósea en adultos jóvenes.

Asimismo, son necesarios estudios longitudinales con el objeto de investigar cómo influyen determinados factores modificables y genéticos en el proceso de adquisición de la masa ósea a lo largo de los años.

Finalmente, resulta indispensable trasladar los resultados de investigación obtenidos sobre los factores de riesgo asociados a fenotipos relacionados con la masa ósea en edades tempranas a la práctica clínica. En este sentido, sería de gran interés la creación de algoritmos que incluyan tanto los factores ambientales (dieta, actividad física, etc.) como las variantes genéticas asociadas a fenotipos de la osteoporosis, con el objetivo de identificar de forma precoz a los individuos con mayor riesgo de padecer la enfermedad. Esto facilitaría el diseño y el desarrollo de estrategias preventivas y terapéuticas más eficaces, las cuales podrían implantarse antes de la aparición de los primeros síntomas de osteoporosis y así contribuir a frenar la progresión de esta patología tan prevalente.

VI. CONCLUSIONES

CONCLUSIONES

1. Variantes genéticas de los genes *WNT16*, *LRP5* y *RSPO3* se asocian significativamente con los niveles de masa ósea determinados mediante ultrasonografía en una población de adultos jóvenes, sugiriendo que estos genes podrían jugar un papel relevante en la adquisición del pico de masa óseo en edades tempranas.

2. Los genes *VDR, SOST, ESR1, RANKL, RANK, OPG, SPTBN1*, *CCDC170*, *MBL2/DKK1*, *TMEM135* y *GPATCH1* parecen no tener un papel determinante como marcadores genéticos de la mineralización ósea en adultos jóvenes.

3. Factores modificables como la masa magra y la actividad física intensa influyen en el nivel de masa ósea en adultos jóvenes. Por lo tanto, un nivel elevado de actividad física que incremente la masa magra sería una estrategia efectiva para maximizar la masa ósea en la adultez temprana.

4. Nuestros resultados sugieren que la ingesta en la dieta de antioxidantes de alta calidad influye en la salud ósea en mujeres jóvenes.

5. Se ha identificado una interacción entre el SNP rs9340799 del gen *ESR1* y el SNP rs3736228 del gen *LRP5* que alcanzó un alto grado de significación estadística con el parámetro óseo QUS en adultos jóvenes. La caracterización de esta interacción plantea la posibilidad de que aquellos adultos jóvenes portadores de alelos de riesgo en ambos polimorfismos podrían estar expuestos a una alteración en los niveles de expresión de ambos genes que provocaría una alteración del proceso de mineralización ósea.

CONCLUSIONS

1. Genetic variants of the *WNT16, LRP5* and *RSPO3* genes are significantly associated with levels of bone mass determined by ultrasonography in a population of young adults, suggesting that these genes may play a relevant role in the acquisition of peak bone mass at early ages.

2. *VDR, SOST, ESR1, RANKL, RANK, OPG, SPTBN1, CCDC170, MBL2/DKK1, TMEM135* and *GPATCH1* genes might not have a determinant role as genetic markers of bone mineralization in young adults.

3. Modifiable factors such as lean mass and intense physical activity influence on bone mass levels in young adults. Therefore, a high level of physical activity that increases lean mass may be an effective strategy to maximise bone mass in early adulthood.

4. Our results suggest that dietary intake of high-quality antioxidants influences bone health in young women.

5. An interaction between rs9340799 SNP of the *ESR1* gene and rs3736228 SNP of the *LRP5* gene that reached a high level of statistical significance for QUS parameter in young adults was identified. The identification of this interaction raises the possibility that those young adults with risk alleles in rs9340799 and rs3736228 polymorphisms could be exposed to an alteration in the levels of expression of both genes that might cause an alteration of the process of bone mineralization.

VII. BIBLIOGRAFÍA

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VIII. ANEXOS

ANEXO 1. OTRAS PUBLICACIONES DEL DOCTORANDO

Correa Rodríguez M, Rueda Medina B, González Jiménez E, Schmidt Rio-Valle J. Associations between body composition, nutrition and physical activity in young adults. *American Journal of Human Biology* 2016. doi: 10.1002/ajhb.22903.

Navarro-Pérez CF, González-Jiménez E, Schmidt-RioValle J, Meneses-Echávez JF, Correa-Bautista JE, **Correa-Rodríguez M**, Ramírez-Vélez R. Nivel y estado nutricional en niños y adolescentes de Bogotá, Colombia: Estudio FUPRECOL. *Nutrición Hospitalaria* 2016, 33(4), 915–922. doi: 10.20960/nh.392.

Correa Rodríguez M, Rueda Medina B, González Jiménez E, Navarro-Pérez CF, Schmidt Rio-Valle J. The levels of bone mineralization are influenced by body composition in children and adolescents. *Nutrición Hospitalaria* 2014;30(4),763–8. doi: 10.3305/nh.2014.30.4.7683.

ANEXO 2. COMUNICACIONES A CONGRESOS

Association study of polymorphism in the *LRP5* gene region and calcaneal ultrasound parameter in young adults. **Correa-Rodríguez M**, Schmidt Rio-Valle J, González-Jiménez E, Rueda-Medina B. World Congress on Osteoporosis, Osteoarthritis and Musculoskeletal Diseases. Málaga, 14-17 abril 2016.

Replication study of polymorphisms associated with quantitative ultrasound in a cohort of Spanish young adults. **Correa-Rodríguez M**, Schmidt Rio-Valle J, Rueda-Medina B. World Congress on Osteoporosis, Osteoarthritis and Musculoskeletal Diseases. Málaga, 14-17 abril 2016.

Association study of *RANKL/RANK/OPG* polymorphisms with heel quantitative ultrasound in young adults. **Correa-Rodríguez M**, Schmidt Rio-Valle J, Rueda-Medina B. World Congress on Osteoporosis, Osteoarthritis and Musculoskeletal Diseases. Málaga, 14-17 abril 2016.

Analysis of the effect of calcium and *VDR* gene in Young adults bone strength indicated by heel quantitative ultrasound. **Correa-Rodríguez M**, Schmidt Rio-Valle J, González-Jiménez E, Rueda-Medina B. II Congreso Internacional de Investigación en Salud y Envejecimiento. Almería, 2-3 julio 2015.

ANEXO 3. CERTIFICADO COMITÉ DE ÉTICA

Universidad de Granada
Vicerrectorado de Política
Científica e Investigación

COMISIÓN DE ÉTICA EN INVESTIGACIÓN
DE LA UNIVERSIDAD DE GRANADA

La Comisión de Ética en Investigación de la Universidad de Granada, oído el informe preliminar del Presidente del Comité en Investigación Humana, emite informe favorable a la metodología en la investigación titulada "ANÁLISIS DE LA INFLUENCIA DE VARIABLES GENÉTICAS EN EL NIVEL DE MINERALIZACIÓN ÓSEA DURANTE LA ADOLESCENCIA" que dirige D./Dña. Blanca María Rueda Medina, quedando registrada con el nº: 834.

Granada a 02 de diciembre de 2013

LA PRESIDENTA
Fdo: Mª Dolores Suárez Ortega

LA SECRETARIA
Fdo: Irene Luque Fernández

ANEXO 4. CONSENTIMIENTO INFORMADO - INFORMACIÓN AL PARTICIPANTE

CONSENTIMIENTO INFORMADO – INFORMACIÓN AL PARTICIPANTE

Antes de proceder a la firma de este consentimiento informado, lea atentamente la información que a continuación se le facilita y realice las preguntas que considere oportunas.

Naturaleza del estudio:
Se le invita a participar en un estudio de investigación en el que se analizará la forma en que los genes y factores ambientales se relacionan con la aparición y el tratamiento de las enfermedades. Debe decidir si desea tomar parte en él. Tómese el tiempo necesario para decidir. Lea detenidamente el texto que sigue y pregunte al investigador cualquier duda que pueda tener.

¿En qué consiste el estudio?
El estudio que vamos a llevar a cabo trata de descubrir los genes que contribuyen a que el nivel de mineralización ósea (fortaleza de los huesos) sea el adecuado en la etapa más importante para lograrlo, que es hasta los 25 años.

Para poder medir el nivel de mineralización de sus huesos se le tomarán una serie de medidas, ninguna de las cuales es invasiva, ni dolorosa:

- Bioimpedancia: para ello se tendrá que poner de pie descalzo en una especia de báscula que determinará su peso y calculará la cantidad de grasa y músculo que tiene en el cuerpo.
- Densitometría ósea por ultrasonografía: consiste en poner el pie (sin calcetín) en una especie de "bota" que mide en unos segundos cuanto calcio tiene en los huesos, o lo que es lo mismo lo fuerte que los tiene. Al someterse a esta prueba, usted no sufrirá ningún tipo de radiación ni efecto dañino para su organismo.

Además, deberá cumplimentar varios cuestionarios relacionados con el ejercicio que hace y lo que come normalmente.

¿Por qué se me pide una muestra de saliva para estos análisis?
Se le pide que done saliva para analizar sus genes, o ADN. Su autorización para donar esta muestra de saliva es optativa y totalmente voluntaria. Si decide retirar su consentimiento, la muestra que se donó será destruida y no se utilizará en el estudio. Con el fin de garantizar la confidencialidad de todos sus datos se utilizarán procedimientos diseñados para impedir que los resultados del estudio puedan vincularse directamente con usted. Una vez finalice el proyecto, su muestra de ADN será incluida en una colección de muestras para que pueda ser utilizada por los investigadores en futuros proyectos de la misma línea de investigación.

Importancia de su participación:
Gracias a su colaboración en el presente estudio los investigadores podrán analizar la influencia de los factores genéticos en el nivel de mineralización ósea en la adolescencia. Alcanzar un buen nivel de mineralización ósea en esta etapa es fundamental para prevenir en el futuro el desarrollo de osteoporosis y depende en gran medida de ciertos factores genéticos.

Implicaciones para el participante:

- La donación/participación es totalmente voluntaria.
- El donante puede retirarse del estudio cuando así lo manifieste y sin tener que dar explicaciones.
- Todos los datos de carácter personal, obtenidos en este estudio son confidenciales y se tratarán conforme a la Ley Orgánica de Protección de Datos de Carácter Personal 15/99.
- La donación/información obtenida se utilizará exclusivamente para los fines específicos de este estudio.
- Cuando finalice el estudio, su muestra será conservada por el equipo investigador en una "Colección de muestras" para poder desarrollar en el futuro otros estudios relacionados con esta temática.

Riesgos de la investigación para el participante:
La donación de una muestra de saliva es un procedimiento no invasivo, que no causa dolor ni molestia alguna y que solo dura un par de minutos. No existe ningún riesgo derivado del procedimiento de donación de saliva ya que consiste en verter la saliva en un recipiente que le facilitarán los investigadores.

Si requiere información adicional se puede poner en contacto con los investigadores Blanca Mª Rueda Medina y Jacqueline Schmidt Rio-Valle de la Facultad de Ciencias de la Salud de la Universidad de Granada en el teléfono: 958 24 34 58 o en el correo electrónico: blarume@ugr.es y jschmidt@ugr.es

CONSENTIMIENTO INFORMADO – CONSENTIMIENTO POR ESCRITO DEL PARTICIPANTE

Detección precoz de osteoporosis: identificación de factores genéticos implicados en mineralización en adultos jóvenes

Yo (Nombre y Apellidos):.....

- He leído el documento informativo que acompaña a este consentimiento (Información al Paciente)
- He podido hacer preguntas sobre el estudio "*Análisis de la influencia de variables genéticas y factores ambientales en la mineralización ósea durante la adolescencia".*
- He recibido suficiente información sobre el estudio "*Análisis de la influencia de variables genéticas y factores ambientales en la mineralización ósea durante la adolescencia".*
- He hablado con el investigador informador: …….
- Comprendo que mi participación es voluntaria y soy libre de participar o no en el estudio.
- Se me ha informado que todos los datos obtenidos en este estudio serán confidenciales y se tratarán conforme establece la Ley Orgánica de Protección de Datos de Carácter Personal 15/99.
- Se me ha informado de que la donación/información obtenida sólo se utilizará para los fines específicos del estudio.
- Se me ha informado de que una vez concluido el presente proyecto la donación será depositada en una colección de muestras, para poder desarrollar futuros proyectos de la misma línea de investigación y llevados a cabo por los mismos investigadores de este estudio.
- **Deseo** ser informado/a de mis datos genéticos y otros de carácter personal que se obtengan en el curso de la investigación, incluidos los descubrimientos inesperados que se puedan producir, siempre que esta información sea necesaria para evitar un grave perjuicio para mi salud o la de mis familiares biológicos.

  Sí No

Comprendo que puedo retirarme del estudio:

- Cuando quiera
- Sin tener que dar explicaciones

Presto libremente mi conformidad para participar en el *proyecto titulado* "*Análisis de la influencia de variables genéticas en la mineralización ósea durante la adolescencia".*

Firma del paciente	Firma del investigador informad
Nombre y apellidos:…….	Nombre y apellidos: ……..
Fecha: ……	Fecha: …….

ANEXO 5. RECORDATORIO DE 72 HORAS

DÍA 1	Lugar	Alimento y tecnología culinaria	Cantidad (g) o medida casera
DESAYUNO Hora:			
MEDIA MAÑANA Hora:			
COMIDA Hora:			
MERIENDA Hora:			
CENA Hora:			
RECENA Hora:			
ENTRE HORAS Hora:			

DÍA 2	**Lugar**	**Alimento y tecnología culinaria**	**Cantidad (g) o medida casera**
DESAYUNO Hora:			
MEDIA MAÑANA Hora:			
COMIDA Hora:			
MERIENDA Hora:			
CENA Hora:			
RECENA Hora:			
ENTRE HORAS Hora:			

DÍA 3	Lugar	Alimento y tecnología culinaria	Cantidad (g) o medida casera
DESAYUNO Hora:			
MEDIA MAÑANA Hora:			
COMIDA Hora:			
MERIENDA Hora:			
CENA Hora:			
RECENA Hora:			
ENTRE HORAS Hora:			

ANEXO 6. CUESTIONARIO DE ACTIVIDAD FÍSICA

Imprimir formulario | Enviar por correo electrónico

JUNTA DE ANDALUCIA
CONSEJERÍA DE SALUD

PROMOCIÓN DE LA SALUD EN EL LUGAR DE TRABAJO

VERSIÓN PARA LOS USUARIOS/AS DE LA EMPRESA

CUESTIONARIO INTERNACIONAL DE ACTIVIDAD FÍSICA (IPAQ)

Nos interesa conocer el tipo de actividad física que usted realiza en su vida cotidiana. Las preguntas se referirán al tiempo que destinó a estar activo/a en los últimos 7 días. Le informamos que este cuestionario es totalmente anónimo.

Muchas gracias por su colaboración

1.- Durante los últimos 7 días, ¿en cuántos realizo actividades físicas intensas tales como levantar pesos pesados, cavar, ejercicios hacer aeróbicos o andar rápido en bicicleta?	
Días por semana (indique el número)	
Ninguna actividad física intensa (pase a la pregunta 3)	☐
2.- Habitualmente, ¿cuánto tiempo en total dedicó a una actividad física intensa en uno de esos días?	
Indique cuántas horas por día	
Indique cuántos minutos por día	
No sabe/no está seguro	☐
3- Durante los últimos 7 días, ¿en cuántos días hizo actividades físicas moderadas tales como transportar pesos livianos, o andar en bicicleta a velocidad regular? No incluya caminar	
Días por semana (indicar el número)	
Ninguna actividad física moderada (pase a la pregunta 5)	☐
4.- Habitualmente, ¿cuánto tiempo en total dedicó a una actividad física moderada en uno de esos días?	
Indique cuántas horas por día	
Indique cuántos minutos por día	
No sabe/no está seguro	☐
5.- Durante los últimos 7 días, ¿en cuántos días caminó por lo menos 10 minutos seguidos?	
Días por semana (indique el número)	
Ninguna caminata (pase a la pregunta 7)	☐
6.- Habitualmente, ¿cuánto tiempo en total dedicó a caminar en uno de esos días?	
Indique cuántas horas por día	
Indique cuántos minutos por día	
No sabe/no está seguro	☐
7.- Durante los últimos 7 días, ¿cuánto tiempo pasó sentado durante un día hábil?	
Indique cuántas horas por día	
Indique cuántos minutos por día	
No sabe/no está seguro	☐

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PROGRAMA DE DOCTORADO EN MEDICINA CLÍNICA Y SALUD PÚBLICA