HAROKOPIO UNIVERSITY

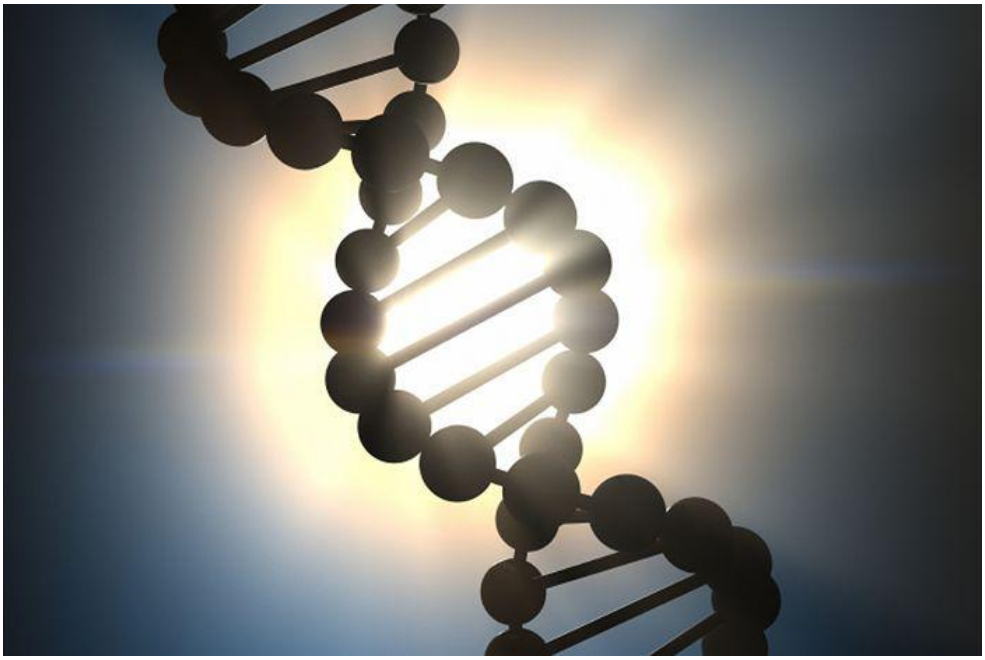
SCHOOL OF HEALTH SCIENCE AND EDUCATION

DEPARTMENT OF NUTRITION AND DIETETICS

Genes and Lifestyle Interactions on bone metabolism indices

PhD Thesis

EFFIMIA V. GRIGORIOU



Athens, 2019

ΧΑΡΟΚΟΠΕΙΟ ΠΑΝΕΠΙΣΤΗΜΙΟ

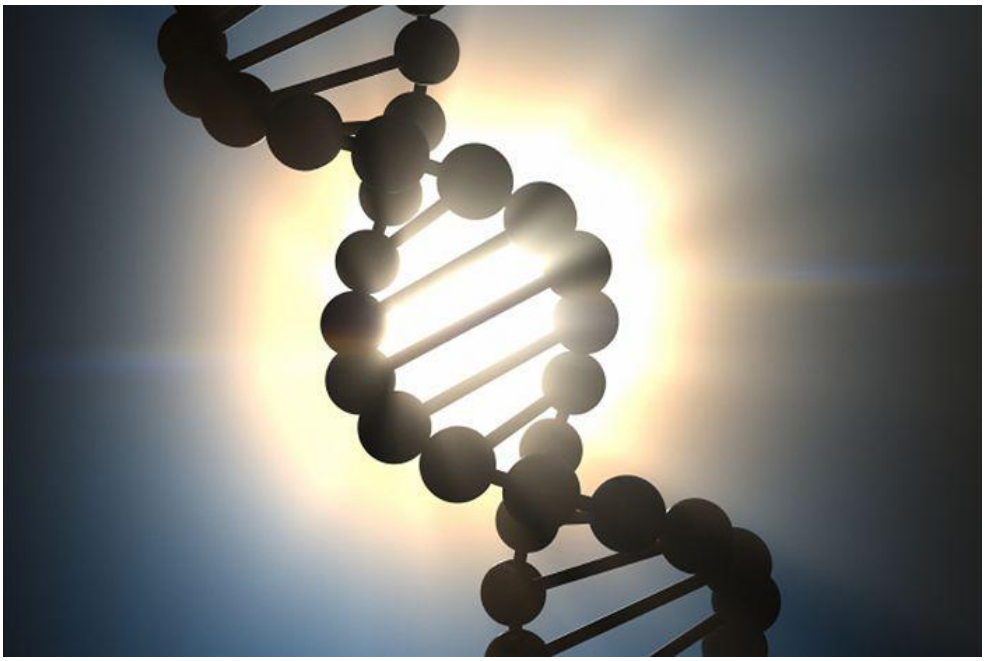
ΣΧΟΛΗ ΕΠΙΣΤΗΜΩΝ ΥΓΕΙΑΣ ΚΑΙ ΑΓΩΓΗΣ

ΤΜΗΜΑ ΕΠΙΣΤΗΜΗΣ ΔΙΑΙΤΟΛΟΓΙΑΣ-ΔΙΑΤΡΟΦΗΣ

Αλληλεπιδράσεις γονιδίων και παραγόντων του τρόπου ζωής σε δείκτες οστικού μεταβολισμού

Διδακτορική Διατριβή

ΕΥΦΗΜΙΑ Β. ΓΡΗΓΟΡΙΟΥ



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To my kids Aggeliki and Vasilis

Στα παιδιά μου, Αγγελική και Βασίλη

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CONTENTS

ΠΕΡΙΛΗΨΗ

Εισαγωγή: Η έλλειψη βιταμίνης D καθώς και οι παράμετροι της οστικής υπερηχοτομογραφίας (QUS), αποτελούν τους πιο σημαντικούς κλινικούς παράγοντες κινδύνου ευθραυστότητας των οστών. Πρόσφατα στοιχεία υποδεικνύουν ότι ο επιπολασμός της έλλειψης βιταμίνης D μπορεί να είναι κοινός σε χώρες που θεωρούνταν ως χαμηλού κινδύνου (π.χ. Μεσογειακές χώρες). Η γενετική συμβολή στην οστεοπόρωση είναι καλά προσδιορισμένη. Πρόσφατες μελέτες σάρωσης του γονιδιώματος (GWAS) αναγνώρισαν νέους γενετικούς τόπους που σχετίζονται με τους φαινοτύπους της σκελετικής υγείας.

Σκοποί: Οι στόχοι της παρούσας διδακτορικής διατριβής είναι α) να αξιολογήσει την έλλειψη βιταμίνης D στον ελληνικό πληθυσμό, β) να αξιολογήσει τις τιμές των παραμέτρων QUS, γ) να προσδιορίσει την επίδραση των παραγόντων του τρόπου ζωής στα επίπεδα της βιταμίνης D και τις τιμές QUS, δ) να ταυτοποιήσει διατροφικά μοντέλα στον ελληνικό πληθυσμό και πως αυτά σχετίζονται με τα επίπεδα βιταμίνης D και τις παραμέτρους QUS και τέλος ε) η αξιολόγηση της επίδρασης μίας σειράς γενετικών δεικτών στους παραπάνω φαινοτύπους.

Μέθοδοι: Η διατριβή έγινε στα πλαίσια της συγχρονικής μελέτης παρατήρησης OSTEOS. Η στρατολόγηση του πληθυσμού διεξήχθη από τον Ιούνιο του 2010 έως τον Ιούλιο του 2012. Στη μελέτη πήραν μέρος 970 ενήλικες από διάφορες περιοχές της Ελλάδας και συμπλήρωσαν το κατάλληλο ερωτηματολόγιο. Μετρήθηκε η 25(OH)D στον ορό με μέτρηση ενζυμικού ανοσοπροσδιορισμού (ELISA). Οι παράμετροι QUS, η εξασθένηση υπερήχων ευρείας ζώνης (BUA), η ταχύτητα του ήχου (SOS) και ο δείκτης ακαμψίας (SI) αξιολογήθηκαν με συσκευή "Achilles". Ένα έγκυρο Ερωτηματολόγιο Συχνότητας Κατανάλωσης Τροφίμων (FFQ) χρησιμοποιήθηκε για την αξιολόγηση των διατροφικών συνηθειών του πληθυσμού. Για την εκχύλιση DNA, χρησιμοποιήθηκαν η συσκευή DNA iPrepTM και το κιτ αίματος iPrepTMPureLinkTMgDNA και η γονοτύπιση πραγματοποιήθηκε με την αλυσιδωτή αντίδραση πολυμεράσης πραγματικού χρόνου (RT-PCR). Για στατιστική ανάλυση χρησιμοποιήθηκε το IBM SPSS Statistics 21.0. Χρησιμοποιήθηκε ανάλυση της διακύμανσης για την εκτίμηση των παραγόντων που καθορίζουν την 25(OH)D ορού. Η ανάλυση κύριων συνιστωσών (PCA) διεξήχθη για τον προσδιορισμό των διατροφικών μοντέλων.

Αποτελέσματα: Ο Μέσος όρος των επιπέδων της 25(OH)D ορού του συνολικού πληθυσμού ήταν 20,00± 8,00 ng/mL. Η PCA ανάλυση ανέδειξε έξι διατροφικά μοντέλα να εξηγούν το 52,2

% της μεταβλητότητας των διατροφικών συνηθειών των συμμετεχόντων. Τα άτομα με 25(OH)D ≥ 20 ng/mL είχαν υψηλότερες τιμές SOS σε σχέση με αυτούς που είχαν 25(OH)D < 20 ng/mL. Ταυτοποιήθηκαν οι παράγοντες που καθόριζαν τα επίπεδα της 25(OH)D και βρέθηκαν να είναι ο Δείκτης Μάζας Σώματος (ΔΜΣ), η γήρανση (>65 ετών), η έκθεση στον ήλιο το καλοκαίρι, η οργανωμένη φυσική δραστηριότητα και η συμμόρφωση με το «γλυκό» διατροφικό μοντέλο. Επιπρόσθετα το «υγιεινό» διατροφικό μοντέλο προστάτευε τα επίπεδα 25(OH)D κατά τους χειμερινούς μήνες. Οι παράγοντες που καθορίζουν τις παραμέτρους QUS βρεθήκαν να είναι η ηλικία, ο ΔΜΣ, οι ώρες καθιστικών δραστηριοτήτων, η οργανωμένη φυσική δραστηριότητα και η συμμόρφωση με το «υγιεινό» διατροφικό μοντέλο. Από τη συμμετοχή τη μελέτης OSTEOS στην σύμπραξη GEFOS-GENOMOS αποκαλύφθηκαν, στις συνδυαστικές τυχαίες επιδράσεις, και τη μετα-ανάλυση των πληθυσμιακών μελετών, εννέα μονο-νουκλεοτιδικοί πολυμορφισμοί (SNPs) να ξεπερνάνε το κατώφλι στατιστικής σημαντικότητας ($P < 5 \times 10^{-8}$) σε συσχέτιση με τους δείκτες ποσοτικής υπερηχοτομογραφίας στην πτέρνα. Παράλληλα με τους πολυμορφισμούς στα γονίδια που έχουν ήδη συσχετιστεί με την οστεοπόρωση, ESR1 (6q25.1: rs4869739, rs3020331, rs2982552), SPTBN1 (2p16.2: rs11898505), RSPO3 (6q22.33: rs7741021), WNT16 (7q31.31: rs2908007), DKK1 (10q21.1: rs7902708) και GPATCH1 (19q13.11: rs10416265), βρέθηκε και ένας καινούργιος πολυμορφισμός (rs597319) στο γονίδιο TMEM135, που πρόσφατα συνδέθηκε με την οστεοβλαστογένεση και την μακροβιότητα, να σχετίζεται σημαντικά με τις τιμές BUA και SOS. ($P < 8.23 \times 10^{-14}$). Στην μελέτη μας οι ομοζυγώτες για το A αλληλόμορφο για τον πολυμορφισμό rs11520772 έχουν υψηλότερες τιμές SOS, σε σχέση με τους ομοζυγώτες στο ελλάσων αλληλόμορφο (T), λαμβάνοντας υπ'όψιν παράγοντες του τρόπου ζωής και βιοχημικές παραμέτρους. Οι ομοζυγώτες για το συχνό αλληλόμορφο (A) για τον πολυμορφισμό rs597319 έχουν υψηλότερες τιμές SI συγκρινόμενοι με τους ομοζυγώτες για το σπάνιο αλληλόμορφο (G).

Συζήτηση: Η μελέτη αυτή αναφέρει ότι η έλλειψη της βιταμίνης D είναι ιδιαίτερα διαδεδομένη στους υγιείς Έλληνες, άνδρες και γυναίκες. Αναλύονται επίσης τα διατροφικά μοντέλα του ελληνικού υγιούς πληθυσμού, καθώς και την επίδρασή τους στην συγκένρωση 25(OH)D ορού . Η συσχέτιση των διατροφικών μοντέλων με τις παραμέτρους QUS δεν έχει ερευνηθεί ποτέ πριν. Ερευνήθηκε επίσης η επίδραση ενός νέου πολυμορφισμού κοντά στο γονίδιο TAX1BP1. Τέλος εκτιμήθηκε η επίδραση που έχει ο πολυμορφισμός rs597319 ο οποίος βρίσκεται σε ένα νέο γενετικό τόπο κοντά στο γονίδιο TMEM135 στους συμμετέχοντες της μελέτης OSTEOS. Απαιτούνται περισσότερες μελέτες για τη διερεύνηση του μηχανισμού δράσης αυτών των

πολυμορφισμών στις παραμέτρους της σκελετικής υγείας. Η μελέτη καταδεικνύει την πολυπαραγοντική αιτιότητα των επιπέδων της 25(OH)D και των παραμέτρων QUS και επισημαίνει ότι απαιτείται περαιτέρω έρευνα για τον προσδιορισμό περισσότερων παραγόντων που σχετίζονται με την κατάσταση της βιταμίνης D και την υγεία των οστών.

Λέξεις κλειδιά: βιταμίνη D, οστεοπόρωση, παράμετροι οστικής ποσοτικής υπερηχοτομογραφίας, διατροφικά πρότυπα, τρόπος ζωής, πολυμορφισμός ενός νουκλεοτιδίου

ABSTRACT

Introduction: Vitamin D deficiency and quantitative ultrasound (QUS) parameters are among the most important clinical risk factors of bone fragility. Recent data indicate that prevalence of vitamin D deficiency may be common in countries previously considered as low risk (e.g., Mediterranean countries). Few data are available for Greek population. The genetic contribution to osteoporosis is well determined. Genome Wide association Study (GWAS) identified new locus were associated with bone health phenotypes.

Aims and Objectives: The objectives of the current PhD thesis were a) to evaluate the prevalance of vitamin D deficiency in greek population, b) To evaluate quantitative ultrasound (QUS) parameters' values, c) to identify the effect of lifestyle factors on vitamin D status and QUS values, d) to identify dietary patterns of greek population and how they are associated with vitamin D status and quantitative ultrasound parameters and finally e) to evaluate the effect of the eight polymorphisms, were identified from a recent GWAS, associated with QUS indices, on the above phenotypes. The polymorphisms are the rs11520772 of the TAX1BP1 gene, the rs2908007 of the WNT16 gene, the rs2982552 of ESR1 gene, rs3000634 of USPL1 gene, the rs3020331 of ESR1 gene, the rs597319 of TMEM135 gene, rs7741021 of RSPO3 gene and the rs9292469 of NPR3 gene.

Methods: OSTEOS is an observational cross-sectional study. The subject's recruitment conducted from June 2010 to July 2012. 970 adults were recruited throughout Greece and they completed the appropriate questionnaire. Serum 25(OH)D measured by enzyme immunoassay; QUS parameters, broadband ultrasound attenuation (BUA), speed of sound (SOS) and stiffness index (SI), were assessed with an Achilles device. A validated Food Frequency Questionnaire (FFQ) was used for assessment of population's dietary habits. For the DNA extraction the DNA iPrepTM device and the iPrepTMPureLinkTMgDNA Blood Kit was used, while the genotyping was performed by Real Time-Polymarase Chain Reaction (RT-PCR). For statistical analysis, IBM SPSS Statistics 21.0, was used. Univariate Analysis of Variance was used for the assessment of serum 25(OH)D determinants. Principal Component Analysis (PCA) was carried out for dietary patterns determination.

Results: Mean serum 25(OH)D of the total population was 20,00 ± 8,00 ng/mL while six dietary patterns explained 52.2% of variability of the population nutritional habits. Individuals with

25(OH)D ≥ 20 ng/mL had higher SOS than those with 25(OH)D < 20 ng/mL. The determinants of serum 25(OH)D were Body Mass Index (BMI), elderly (>65 years), summer sun exposure, organized physical activity and adherence to 'sweet' pattern. Additionaly, a 'healthy' pattern protected Vitamin D levels in winter months. The Determinants of QUS parameters were age, BMI, hours of sedentary activities, organized physical activity participation and adherence to 'healthy' pattern. Our participation from the GEFOS/GENOMOS consortium revealed that, in combined random effects, meta-analysis of the discovery and replication cohorts, nine single nucleotide polymorphisms (SNPs) had genome-wide significant ($P < 5 \times 10^{-8}$) associations with heel bone properties. Alongside SNPs within or near previously identified osteoporosis susceptibility genes including ESR1 (6q25.1: rs4869739, rs3020331, rs2982552), SPTBN1 (2p16.2: rs11898505), RSPO3 (6q22.33: rs7741021), WNT16 (7q31.31: rs2908007), DKK1 (10q21.1: rs7902708) and GPATCH1 (19q13.11: rs10416265), we identified a new locus on chromosome 11q14.2 (rs597319 close to TMEM135, a gene recently linked to osteoblastogenesis and longevity) significantly associated with both BUA and SOS ($P < 8.23 \times 10^{-14}$). In our cohort 'AA' genotype of rs11520772 polymorphism on TAX1BP1 gene have higher SOS values comparing with (T) homozygotes for the minor allele, taking into account lifestyle and biochemical parameters. AA homozygotes of rs597319 close to TMEM135 gene have higher SI values comparing with (G) homozygotes.

Conclusion: This study reports that vitamin D deficiency is highly prevalent among OSTEOS participants. The dietary patterns revealed by PCA were related for the first time with QUS parameters. The effect of a novel polymorphism close to the TAX1BP1 gene on the above phenotypes also investigated. The effect of the rs597319 polymorphism on a new locus on the TMEM135 gene is also identified for the participants of the current study. More studies are needed for the investigation of the mechanism of action of this novel genetic variant on bone health parameters. The study demonstrates the multifactorial causation of 25(OH)D levels and QUS parameters and points out that further research is required to determine more genetic, environment and biochemical factors related to vitamin D status and bone health.

Key words: vitamin D, osteoporosis, bone quantitative ultrasound parameters, dietary patterns, lifestyle, single-nucleotide polymorphism

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ABBREVIATIONS AND ACRONYMS

QUS:	Quantitative Ultrasound
GWAS:	Genome Wide Association Study
25(OH)D:	25-hydroxyvitamin D
BUA:	Broadband Ultrasound Attenuation
SOS:	Speed of Sound
SI:	Stiffness Index
RT-PCR:	Real Time-Polymarase Chain Reaction
PCA:	Principal Component Analysis
BMI:	Body Mass Index
SNPs:	Single Nucleotide Polymorphisms
ESR1:	Estrogen Receptor 1
SPTBN1:	Spectrin Beta, Non-erythrocytic 1
WNT16:	Wnt family member 16
DKK1:	Dickkopf 1
GPATCH1:	G-patch domain containing 1
TMEM135:	Transmembrane Protein 135
TAX1BP1:	Tax1 Binding Protein 1
RSPO3:	R-Spondin 3
NPR3:	Natriuretic Peptide Receptor 3
DALYs:	Disability-Adjusted Life Years
CI:	Confidence Intervals
SD:	Standard Deviations
DXA:	Dual X-ray Absorptiometry
BMD:	Body Mass Density
FRAX™:	World Health Organization Fracture Risk Algorithm
RANK:	Receptor Activator of Nuclear Factor κB (also known as TNFRSF11A)
RANKL:	RANK Ligand (also known as TNFSF11)
OPG:	Osteoprotegerin (also known as TNFRSF11B),
LRP5:	Lipoprotein Receptor-related Protein 5
sFRP:	Frizzled Related Proteins

SOST:	Sclerostin
PTHrP:	Parathyroid Hormone-related Peptide
PTHLH:	Parathormone -Like Hormone
SOX6:	SRY-box transcription factor 6
SOX9:	SRY-box transcription factor 9
COL2A1:	Type II Collagen A1
IBSP:	catenin-binding sialoproteine
BNSP2:	Sialoprotein 2
OPN:	Osteopontin
FN:	Femoral Neck,
LS:	lumbar Spine,
H:	Hip
7-DHC:	7-dehydrocholesterol
25-OHase:	25-hydroxylase enzyme (also known as CYP27A1, CYP2R1)
1-OHase:	1,25 hydroxyvitamin-D-1α hydroxylase (also known as CYP27B1).
VDR:	Vitamin D Receptor
FGF23:	Fibroblast Growth Factor 23
FFQ:	Food Frequency Questionnaire
IPAQ:	International Physical Activity Questionnaire
iPTH:	Intact Parathyroid Hormone
ECLIA:	Electrochemilluminescense Immunoassay technology
CVs:	Coefficients of Variation
VDSP:	Vitamin D Standardization Program
SD:	Standard Deviation
Ca:	Calcium
P:	Phosphorus
GLM:	General Linear Model
EAR:	Estimated Average Requirement

1. INTRODUCTION

1.1 Osteoporosis Definition and epidemiology

Osteoporosis is a systemic skeletal multifactorial disease characterized by reduced bone mass and microarchitectural deterioration of the structure of bone tissue leading to enhanced bone fragility and increased susceptibility to fractures [1]. Despite the fact that low bone mass retains a significant role in the diagnosis of osteoporosis, clinical significance in the diagnosis of the disease, but also in the determination of its severity has now been definitively transferred to the clinical consequence, which is the easy fracture that has already happened, or there is an increased risk of happening [2]. It could be more simply defined as a systemic disease characterized by an increased risk of easy fracture, thus limiting the necessity of low bone mass and considering this parameter as a strong causative factor in fracture induction but with other endogenous (e.g. deterioration of bone structure, sarcopenia etc.) or exogenous factors (e.g. increased fall frequency, severe co-morbidities etc.) that will eventually increase the fracture risk significantly [2].

Osteoporosis has been recognized as an established and well-defined disease that affects more than 75 million people in the United States, Europe and Japan. The Osteoporotic fractures reach annually at 8.9 million worldwide, of which more than 4.5 million occur in the Americas and Europe. The lifetime risk for a wrist, hip or vertebral fracture has been estimated to be in the order of 30% to 40% in developed countries , very close to that for coronary heart disease. Osteoporosis is not only a major cause of fractures, it also ranks high among diseases that cause people to become bedridden with serious complications. These complications may be life-threatening in elderly people. In the Americas and Europe osteoporotic fractures account for 2.8 million disability-adjusted life years (DALYs) annually, somewhat more than accounted for by hypertension and rheumatoid arthritis, but less than diabetes mellitus or chronic obstructive pulmonary diseases. Collectively, osteoporotic fractures account for approximately 1% of the DALYs attributable to noncommunicable diseases [3].

The incidence of hip fractures in Greece doubled during 30 years (1977-2007), among people aged ≥50 years, although a mild decrease was observed in almost all age groups after 2002. The most affected group is 80 and over. The incidence of hip fractures increased approximately 100

% throughout the study; it progressively increased from 1977 to 2002 and exhibited a mild significant decrease thereafter. The relative risk of hip fractures among subjects aged 60-69 in 2007 has declined compared with 1977 (0.85, 95 % confidence intervals (CI) 0.79-0.92, $p < 0.0005$). Among people aged 70-79, an increased relative fracture risk (1.53, 95 % CI 1.45-1.61, $p < 0.0005$) was estimated in 2007 compared with 1977. People ≥80 years old were responsible for half of the hip fractures in 2007 but only for the 22.5 % of fractures in 1977. The relative fracture risk in people aged ≥80 was 2.81 times higher (95 % CI 2.64-2.98, $p < 0.0005$) in 2007 than in 1977. [4]. At another Greek study which took place in seventeen primary health care institutions and four prefectures of Thessaly, investigators collected a population of 740,115 residents that represents approximately 8% of the total population of Greece. The total and the estimated prevalence of osteoporosis per 10000 residences were 500.7 (95%CI 403.3 - 598.00). The most prominent difference is the higher prevalence of the diseases of musculoskeletal system in women. This prevalence is being more than 10-fold higher for osteoporosis than other diseases of musculoskeletal system [5]. The prevalence of osteoporosis in the EU is estimated at 27.6 million in 2010 (Figure 1) [6].

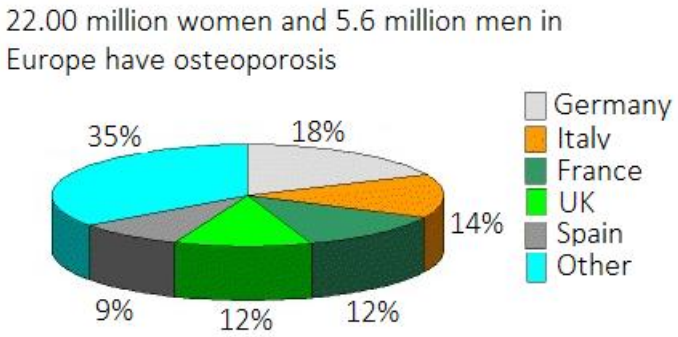


Figure1. *The prevalence distribution of osteoporosis in Europe (EU) and the 5 countries with the highest populations in 2010 [6]*

1.2 Diagnosis of Osteoporosis

The diagnostic criterion of osteoporosis as defined by the World Health Organization is that the bone density is less than 2.5 standard deviations (SD) than the mean T-score of young women, measured by the dual X-ray absorptiometry (DXA) method. [7] (table 1). These criteria refer to postmenopausal Caucasian women. For premenopausal women, the diagnosis is based on the Z-score and the evaluation for secondary osteoporosis is required. In elderly men (> 65 years),

the diagnostic criteria are the same as for postmenopausal women. For younger men, at least one risk factor is required [2,7,8,9]. According to National Bone Health Alliance Working Group, position statement for the clinical diagnosis of osteoporosis postmenopausal women and also men over the age of 50 years should be diagnosed as having osteoporosis if it is demonstrated that the individual is at an elevated risk for future fractures. The use of BMD testing and the finding of a T-score of ≤−2.5 at the spine or hip also constitute the one way to make the diagnosis. The term osteoporosis is also used for individuals who have experienced a low-trauma hip fracture and for those who have osteopenia by BMD who sustain a low-trauma vertebral, proximal humerus, pelvis, or, in some cases, distal forearm fracture. Finally, according to National Bone Health Alliance Working Group, diagnosis for osteoporosis can be used for individuals who have an elevated fracture risk based on the World Health Organization Fracture Risk Algorithm, FRAX™ [10]. FRAX™ have been developed for the prediction of hip fracture and other osteoporotic fractures, based on clinical risk factors alone, or the combination of clinical risk factors plus BMD, available at www.shef.ac.uk/FRAX. The FRAX algorithms are suitable for men and women.

Table 1: *World Health Organization Criteria for Classification of Osteopenia and Osteoporosis.*

Diagnostic Category	**BMD T-score**
Normal	T-score > -1SD
Osteopenia	-1SD ≥ T-score > -2.5SD
Osteoporosis	T-score ≤ -2.5SD
Established osteoporosis	T-score ≤ -2.5 SD + 1 at least fragility fracture

The DXA technique allows accurate bone mineral density (BMD) measurement but does not provide information on structural and qualitative bone characteristics that have an important role in determining the fracture risk.

Although BMD is the main predictive risk factor for an osteoporotic fracture, measurement of quantitative ultrasound (QUS) has been found to be associated with bone fragility and increased fracture risk [11]. The QUS measuring at the heel is an alternative, ionizing radiation–free and relatively inexpensive, portable screening technique that is able to identify women at high risk of bone fragility and fracture [12] and may be used by general practitioners in primary

care. The ultrasound attenuation (BUA) is due to its absorption and dispersion into the bones and soft tissues. It is affected by both bone density and structural parameters. Wave velocity (SOS) is affected by bone density and tissue elasticity. Therefore, the method evaluates bone density (BMD) and bone structure. These measurements are correlated with bone density (as measured by the DXA method) and can be used to categorize individuals in osteoporotic and healthy, while it seems that the QUS technique can predict fracture risk, especially in older women. The lower the QUS parameters values, the higher the fracture risk [13, 14]. The method is chosen because it gives information on density as well as on the quality and architecture of the bone tissue. In addition, the method is economical and fast, while the measuring device is portable and the subject is not exposed to ionizing radiation [15].

1.3 Clinical manifestations of osteoporosis

Osteoporosis is asymptomatic and is called a silent disease. It is clinically manifested with osteoporotic fractures [2]. Clinically, the osteoporotic fractures (fragility fractures) are those caused by low-intensity trauma e.g. fall from a standing position or those caused automatically [16]. Fractures in the spine, hip and forearm are considered to be classically osteoporotic, but all kinds of fractures can occur in people with bone quality disorders [17]. Vertebral fractures cause severe back pain and progressive loss of stature, accompanied by kyphosis. Kyphosis, in addition to malformation, can cause cardiac and respiratory failure. Distal radius fractures cause disability and malformation. Hip fractures are considered the most serious osteoporotic fractures. They are associated with increased morbidity and mortality ranging from 20-30% during the first year after the fracture [16]. Quality of life is significantly affected, with only 50% of patients able to regain their ability to walk and self-service. When a fracture occurs, the probability of a new fracture is extremely high and approaches 86% [2]. However, two-thirds of the individuals who have suffered a fracture have no osteoporosis as defined by BMD. This means there are other factors other than BMD that increase the fracture risk [17].

Osteoporosis may be idiopathic or secondary. Idiopathic osteoporosis occurs in two clinical types, type I and type II. These two clinical types have pathogenic and clinical differences [2]. Type I osteoporosis occurs in relatively young women in the first 15 years after menopause. Type II osteoporosis occurs in the elderly (after the age of 70) in both sexes, with female predominance 3 to 1 relative to men. Secondary osteoporosis is caused when one of the risk

factors is predominant and almost exclusively responsible for causing osteoporosis. It can be caused by drugs, by diseases, by systemic immobilization and by related diseases [2]. Idiopathic osteoporosis can be treated with various pharmacological and non-interventions but can not be cured. On the contrary, removing the cause of secondary osteoporosis can result in significant improvement or even cure [2].

Idiopathic osteoporosis is a chronic disease with significant effects on the health, functionality and quality of life of patients [18]. The World Health Organization recognizes osteoporosis as a major public health problem [16]. Its growing incidence due to increased life expectancy leads to increased social and financial costs for patients, their families and health systems [19].

1.4 Bone Mass

The bone mass presents a rapid linear growth during early childhood and adolescence and until the 15th to 20th year so and completes bone integration [20]. In this phase there is a superiority of osteoblast activity over osteoclasts and bone production precedes bone resorption [21]. Development is completed in girls from 16th to 18th year and in boys between 18th and 20th year of age [22]. The bone mass continues to grow by depositing new layers of bone tissue. In this phase, osteoblastic activity excels in osteoclasting [23]. The increase in bone mass ceases when the peak bone density is reached [2, 22].

Peak bone mass is the maximum bone density achieved during lifespan. The duration of peak bone mass achievement depends on genetic, racial and hormonal factors. In Greece, the peak bone mass was found to be achieved in men in the 30th and in women on their 25th birthday [2].

After reaching its maximum value, the bone mass remains constant for years and then begins to decrease [24]. Men have a reduction in bone density of 4% every decade since the third decade of their life. There is no reduction in bone mineral density until women menopause. With menopause, women lose bone at a relatively fast rate from 0.5-1.5% / year [2, 25]. This rapid loss continues for 5-10 years after menopause and gradually restrains itself [17]. After the age of 65, the rate of bone loss is not very different between the two genders [25]. As a result, the bone mass at any given time of adulthood depends on the achieved peak bone mass and the rate of loss. Figure 2 shows the course of bone mass during their lifespan.

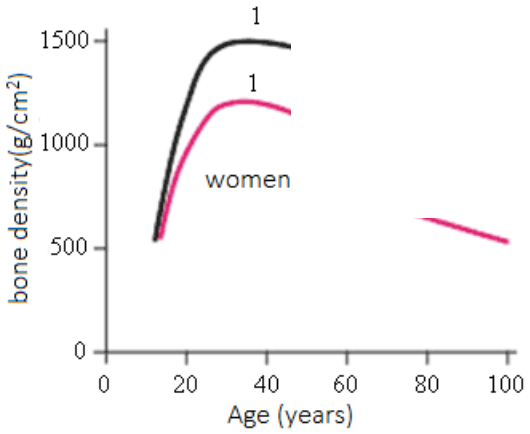


Figure 2. *Bone density during life 1) Peak bone density, 2) Postmenopausal bone loss, 3) Bone loss in the elderly.*

1.5 Pathophysiology of osteoporosis

The main pathogenetic mechanisms are the following:

A. Failure to achieve normal peak bone mass. During development, there is a positive balance in bone remodeling resulting in increased bone mass. The interaction of hormonal, environmental and genetic factors has a key role in this procedure. The conditions that affect bone production or enhance bone resorption, hormonal disorders and exposure to risk factors, during this period, may lead to low peak bone density and increased fractures risk in older ages [26].

B. Uncoupling phenomenon. The balance between osteoclastic and osteoblastic activity is of great importance for bone health as its disorder leads to a reduction in bone mass, alteration of the bone structure architecture and increased bone fragility [26]. In the osteoblast - osteoclast uncoupling phenomenon, both increased bone resorption and reduced bone production lead to loss of bone mass.

- Increased bone resorption. Factors which trigger the osteoclastic activity, such as corticosteroids, estrogen deficiency (e.g. after menopause), hyperparathyroidism and hyperthyroidism, lead to increased bone resorption thus resulting in a negative balance of bone remodelling and loss of bone mass [2, 23].
- Reduced bone formation. Bone formation is reduced by various factors, such as age, resulting in less bone formation in each bone reconstruction cycle and bone mass reduction [2, 26].

C. Increased bone turnover rate. Regardless of the balance of osteoclastic - osteoblastic activity, its increased rate leads to new bone mineralization disorders and to collagen production and maturation disorders. The result is reduced stiffness and poor quality-bone production which also leads to increased fragility and increased risk of fractures regardless of bone mass [26].

1.6 Risk Factors of osteoporosis

The etiology of osteoporosis, as well as most chronic diseases, is multifactorial. Many risk factors have been identified that interact with each other and are involved in the etiopathogenicity of the disease, increasing the risk of osteoporosis and osteoporotic fractures. The main risk factors for osteoporosis are categorized into genetic, clinical and environmental [27].

1.1.6.1 Genetic Factors

The genetic contribution to osteoporosis has been known for several years. Gender and race are key genetic risk factors. Studies of twin siblings and families have shown high inheritance. Genetic factors determine the 50-85% of the peak bone density variation [28]. The bone loss that happens with age is also inherited. 40% of the bone loss variation in the wrist and in the spine is genetically determined, although the genetic contribution is lower than in the peak bone density. The age of menopause in women, which is one of the most important factors of bone loss due to estrogen deficiency, is genetically determined [29]. Heredity also affects hip geometry by 70-85%, bone turnover markers by 40-70%, and bone microarchitecture by 50-60% [28]. Heritability affects 54% and 68% the fracture of the wrist and the hip, respectively, in menopausal women; this effect decreases rapidly with age and reaches 3% in hip fractures in women 79 years of age [30, 31].

Regulation of bone mass and bone remodelling: The regulation of bone resorption, bone formation, and bone mass, depends on several factors that act in a paracrine or autocrine manner to regulate bone cell activity, under the control of circulating calcium-regulating hormones. The predisposition for osteoporosis is due to inherited polymorphisms in genes encoding many of these regulators.

Bone absorption is initially regulated by the signalling pathway of the receptor activator of nuclear factor κB, RANK), which plays a major role in the differentiation and function of osteoclasts. The RANK receptor (known as TNFRSF11A) is found in osteoclast cells and is activated by the RANK ligand (RANKL, also known as TNFSF11), which causes osteoclast activation by positive regulation of the nuclear factor κB and other intracellular signals. This process is inhibited by osteoprotegerin (OPG also known as TNFRSF11B), which acts as a decoy receptor for RANKL [32,33]

Bone formation is also regulated by the Wnt signaling pathway. The members of the family of Wnt proteins bound to and activate the lipoprotein receptor-related protein 5 (LRP5), to regulate bone formation, bone resorption and bone mass. There are at least 19 members in the Wnt family but it has not yet been determined which are the most important in regulating bone metabolism. The soluble frizzled related proteins (sFRP), Dickkopf1 (Dkk1) and sclerostin (SOST), are the LRP5 signal inhibitors. It is possible that the regulation of bone formation depends on the balance between levels of Wnt activate molecules and levels of inhibitors such as sFRP and the sclerostin. Sclerostin is of particular interest because it is produced by osteocytes in response to mechanical loading and is likely to play a key role in mechanical transduction. According to recent research, some neural pathways are also playing a role in the regulation of bone metabolism. These include the sympathetic nervous system through the production of catecholamines, nitric acid and endocavidoids system. [32,33]

The mature bone is formed by calcification of the articular cartilage of the bone. Parathyroid hormone related peptide (PTHrP), bound in receptor 1 of parahthyroid hormone which is encoded by the parathormone -like hormone (PTHLH). The PTHrP is important for the development of cartilage plate and the transcriptional factor SOX6 is responsible for its establishment, as a result these proteins play an essential role in chondrocyte differentiation and endochondral ossification. The SOX9 transcription factor regulates the expression of Type II Collagen A1 (COL2A1), which encodes the major cartilage protein. After its formation, the cartilage is ossified by metal deposition. This raises the possibility that variation in this gene might affect bone density by playing a role in skeletal development. Osteoblast-specific transcription factor (osterix) may suppress RUNX and is necessary for the complete differentiation of osteoblasts. The catenin-binding sialoproteine (IBSP) encodes the sialoprotein 2 (BNSP2) and the phosphoprotein 1 (SPP1), which encodes the osteopontin (OPN), bind strongly to calcium and hydroxyapatite and may play a role in the attachment of osteoclasts to

the surface of the bone. According to the above, it can be concluded that genetic variability is due to a wide range of candidate genes expected to affect bone metabolism, including genes not expressed in bone tissue. [32,33].

Polymorphism or Mutation: Genetic variants in the human genome are divided into 2 main categories depending on their frequency in the population and the functional effects they have on the target gene.

A) Polymorphism. The term is used to express common genetic variants that occur frequently in the population (1%). The most common type of polymorphism is the single nucleotide polymorphism (SNP) in which a nucleotide in DNA is substituted for another but deletions and duplications also occur. Another type of polymorphism is the copy number variants (CNVs), where deletions or multiplications occur.

B) Mutation. The term is used to describe a rare genetic variant (frequency much less than 1%) that has a major effect on gene function.

Polymorphisms have been found to have modest effects on gene function either by altering the protein structure of the gene product or by altering gene expression. Although the resulting changes in expression or function of an individual gene are small, it is thought that common diseases like osteoporosis are attributable to a substantial extent to the combined effects of many hundreds to thousands of these polymorphisms.

Most mutations directly affect the protein coding sequence of the target gene, causing profound changes in protein structure and function, but some act by regulating gene-expression. Mutations typically cause monogenic “Mendelian” disorders that segregate in pedigrees according to a predictable pattern, such as osteogenesis imperfecta.

The BMD and other osteoporosis-related phenotypes regulation is primarily determined by the effects of polymorphisms in multiple genes, each with relatively small effects, rather than the effects of mutations in a few genes. This is also confirmed by recent genome-wide association studies (GWAS), where small effects from dozens of common variants near or in genes were observed [34, 35-39].

Single Gene Disorders of Relevance to Osteoporosis

Several rare diseases have been identified where profound effects on bone mass, bone fragility, and bone turnover occur as the result of mutations in single genes. Severe osteoporosis, bone fragility, or excessively high bone mass, are called single gene disorders and they are related to osteoporosis. These diseases have provided important insights into the molecular pathways that regulate bone mass, bone cell function, and bone quality.

Osteogenesis imperfecta: The disease is most often caused by mutations in the COL1A1 and COL1A2 genes, but recent work has shown that mutations in the CRTAP, LEPRE, and PPIB genes, which form a protein complex necessary for prolyl-3-hydroxylation of collagen, can cause recessive forms of the disease.

Mendelian osteoporosis syndromes: The osteoporosis-pseudoglioma syndrome is a rare recessive disorder characterized by low bone mass and increased bone fragility that has been found to be caused by inactivating mutations in the LRP5 gene. Severe osteoporosis in males can also form part of the phenotypein patients within activating mutations in the CYP17 gene encoding aromatase and the ESR1 gene encoding the estrogen receptor α, conditions that both illustrate the importance of estrogen in the regulation of bone mass in men.

High bone mass syndromes: The autosomal dominant high bone mass syndromes is associated with activating mutations in the LRP5 gene. The recessive syndromes of sclerosteosis and Van Buchem disease are caused by inactivating mutations in the sclerostin (SOST) gene. Some instances of unusually high BMD in the normal population may be due to heterozygosity for SOST mutations.

Osteopetrosis: When the disease is caused by defects in osteoclast function it is called osteoclast-rich osteopetrosis. When it is caused by defects in osteoclast differentiation it is called osteoclast-poor osteopetrosis. In the first case, many different gene mutations have been identified, all of which impair the ability of osteoclasts to resorb bone. The second case is caused usually by inactivating mutations in the TNFRSF11A gene that encodes RANK or the TNFSF11 gene that encodes RANKL [32].

Camurati-Engelmann disease: It is caused by mutations that cluster in the latency associated peptide region of TGF1, which prevent or inhibit binding of latency-associated peptide to the mature TGF1 molecule and increase levels of bioactive TGF1, which presumably is the cause of the increased bone turnover that is characteristic of the disease.

In all of the examples listed above, the consequences of the gene mutation on bone cell function or bone matrix are so profound as to overwhelm the effects of the many other genes that contribute to regulation of bone fragility and bone mass. Although the above disorders are caused by rare mutations with large effects, common polymorphic variations in some of these genes have also been described that regulate BMD in the normal population, albeit with much smaller effects [32].

Methods for Identifying Osteoporosis Susceptibility Genes

In recent years a number of genes and polymorphisms associated with bone density, bone size, the risk of fractures and other skeletal parameters [33, 40, 41] have been identified.

The investigation of genetic risk factors aims at identifying genetic markers (polymorphisms) associated with several skeletal phenotypes (peak bone density, bone mass, osteoporosis and fractures, bone metabolism markers, bone loss). The purpose of these studies is to identify risk or protective alleles among individuals of a particular population which may be useful in the clinical practice to detect individuals with an increased genetic risk of developing the disease [42].

Several approaches have been used for the polymorphisms identification in genes responsible for common form of osteoporosis: The linkage analysis, the candidate genes association studies, the genome-wide association studies (GWAS) and the meta-analysis of GWAS.

The linkage analysis has identified genetic locus for bone mineral density, but these have not been confirmed in other studies or in meta-analysis. This led scientists to identify genetic locus of osteoporosis by the candidate gene approach. According to this procedure, the genotyping was performed in candidate genes, which have a role in bone biology and the relative effect of a specific allele or haplotype in quantitative trait or disease of interest was studied.

However, studies investigating genetic polymorphisms in relation to bone phenotypes have not yet revealed genetic markers of high significance in large populations that can be used as prognostic tools in clinical practice. The heterogeneity and the small sample size in combination

with the limited number of identified polymorphisms leads to a limitation of the strength of the findings. For this reason, the use of stronger study techniques such as the Genome Wide Association studies (GWA) [35]. The GWA studies allow thousands of polymorphic sites to be investigated over a short period of time. The aim of GWAS is the quantification of the genetic variability of a population and the association between genetic markers and specific phenotypes [36]. A significant number of GWA studies regarding bone phenotypes, have been already published [37-39, 43]. The data of GWAS in osteoporosis have highlighted genetic markers with significant influence on bone phenotypes and new genetic loci that affect the skeleton [37-39, 43-47]. The discovery of novel «candidate genes» contributes to the investigation of molecular and cellular mechanisms which are intended to clarify the pathogenesis of the disease and the development of new effective pharmacological treatments. However, these studies also show conflicting results, which makes it imperative to confirm their results on specific populations, by conducting replication studies and meta-analysis of GWAS [48].

The genes that have been investigated by recent GWAS associated with bone mineral density (BMD) at the lumpar spine and/or femoral neck as well as with fracture incidence, are presented in Table 2 [37, 49]. Despite these advances, common and rare variants explain only 5.8% of the total variance in BMD [49].

Table 2: *Genes associated with BMD and fracture from GWAS.*

Genetic loci	Gene	Polymorphism (SNP)	Risk allele/ frequency	β/ associated phenotype	Fractureodss ratio	Biochemical pathway	Reference
1q24.3	DNM3	rs479336	G/ 0.3727	-0,04/ FN		Unspecified	49
1p31.3	GPR177 (WLS)	rs1430742	C/ 0.1951	0,100/ FN 0,105/LS		WNT-β-catenin	37, 50, 51
1p31.3	GPR177 (WLS)	rs2566755	C/ 0.1978	0,100/ FN 0,104/ LS		WNT-β-catenin	37, 50, 51
1p36	ZBTB40/ WNT4	rs6426749	T/ 0.1804	0,082/ FN 0,107/ LS	1,09	WNT-β-catenin	37, 49, 52, 50
1p36	ZBTB40	rs7524102	G/ 0.2253	0,079/ FN 0,094/ LS	1,07	WNT-β-catenin	37, 49, 52, 50
2q13	ANAPC1	rs17040773	C/ 0.1543	0,03/ FN		Unspecified	49
2q14.2	INSIG2	rs1878526	A/ 0.2605	0,04/ LS		Unspecified	49
2p16	SPTBN1	rs11898505	A/ 0.1987	0,067/ LS	1.06	Encodes bone protein	37, 49
2p21	PKDCC	rs7584262	T/ 0.1841	0,03/ FN		Unspecified	49
2q24	GALNT3	rs6710518	T/ 0.3599	-0,064/ FN		Unspecified	53
3q13.2	KIAA2018	rs1026364	T/ 0.391	0,03/ FN		Unspecified	49
3p22	CTNNB1	rs87938	A/ 0.4652	-0,07/ FN	1,06	WNT-β-catenin	37, 49
3q25.31	LEKR1	rs344081	C/ 0.1905	0,06/ LS		Unspecified	49
4p16.3	IDUA	rs3755955	A/ 0.1502	-0,05/ FN -0,05/ LS		Unspecified	49
4q21.1	MEPE (BNSP2)	rs1471403	T/ 0.3425	0,068/ LS		endochondral ossification (IBSP expression on osteoblasts)	49, 50
4q22.1	MEPE (BNSP2)/ SPP1 (OPN)	rs6532023	T/ 0.3411		1,06	endochondral ossification	49, 50
5q14	MEF2C	rs1366594	A/ 0.3745	-0,085/ FN		WNT (SOST regulation)	37, 49, 50

5q31	ALDH7A1	rs13182402	G/ 0.1094		2,25	Inhibition of osteoblast differentiation, reduce bone formation	43
6p21.1	SUPT3H /RUNX2	rs11755164	T/ 0.3329	-0,03/ LS		endochondral ossification	49
6q22	RSPO3	rs13204965	C/ 0.1108	-0,067/ FN		WNT-β-catenin	50
6q25	ESR1	rs2504063	G/ 0.4199	-0,066/ FN -0,078/ LS		Expression of estrogen receptor	37, 49, 52
6q25	C6orf97	rs2941740	G/ 0.57	0,073/FN 0,070/ LS		Unspecified	37
6p22.3	SOX4	rs9466056	A/ 0.4006	-0,03/ FN -0,03 /LS		endochondral ossification	49
7p14.1	STARD3NL	rs1524058	T/ 0.418	-0,07/ LS	1,05	Unspecified	37, 49
7q21.3	FLJ42280	rs4729260	G/0.2495	-0,085/ FN -0,081/ LS		Unspecified	37, 49
7q21.3	FLJ42280	rs7781370	C/ 0.1978	-0,083/ FN -0,074/ LS		Unspecified	
7q21.3	SLC25A13	rs4727338	G/0.32		1.08		
7q31.31	WNT16	rs3801387	G/ 0.2514	-0,08/ FN -0,10/ LS	1,06	WNT-β-catenin	49, 51
7q31.31	C7orf58	rs13245690	G/ 0.2546	0,03/ LS		Unspecified	49
7q36.1	ABCF2	rs7812088	A/0.0609	0,04/ FN		Unspecified	49
8q13.3	XKR9/ LACTB2	rs7017914	G/ 0.3013	0,02/ FN		Unspecified	49
8q24.12	TNFRSF11B (OPG)	rs2062377	T/ 0.3091	0,094/ S		RANK-RANKL-OPG	49
8q24.12	TNFRSF11B (OPG)	rs4355801	A/ 0.53	-0.09/ LS (effect)		RANK-RANKL-OPG	38
8q24.12	TNFRSF11B (OPG)	rs11995824	C/ 0.3503	-0,06/ FN		RANK-RANKL-OPG	37

				-0,093/ S			
9q34.11	FUBP3	rs7851693	G/ 0.37	0,05/ FN	1,05	Unspecified	49
10p11.23	MPP7	rs3905706	T/0.3887	0,05/ S		Unspecified	40
10q21.1	MBL2/ DKK1	rs1373004	T/ 0.251	-0,04/ FN -0,05/ S	1,1	WNT-β-catenin (inhibitor)	49
10q22.3	KCNMA1	rs7071206	C/ 0.2669	-0,05/ S		Unspecified	49
10q24.2	CPN1	rs7084921	T/ 0.3974	0,03/ FN		Unspecified	49
11p11.2	LRP4/ ARHGAP	rs7932354	C/ 0.451	0.073/ FN		WNT-β-catenin	37, 49
11q13.4	LRP5	rs599083	G/ 0.3429	-0,067/ S		WNT-β-catenin interaction with Dkk1	38, 49
11q13.4	LRP5	rs3736228	T/ 0.14	0,13/ S	1,09	WNT-β-catenin	37
11p14.1	LIN7C	rs10835187	T/ 0.3951	-0,02/ S	1,05	Unspecified	37, 49
11p14.1	DCDC5	rs16921914	A/ 0.2308	0,077/ S		Unspecified	20
11p15	SOX6	rs7117858	G/ 0.261	0,088/ FN		endochondral ossification	20, 29, 32
12p11.2	KLHDC5/PTHLH (κωδικοποιεί την PTHRP)	rs7953528	A/ 0.1456	0,04/ FN		endochondral ossification	49
12q13	SP7 (osterix)	rs2016266	G/ 0.3626	0,012/ S		endochondral ossification osteoblastes differentiation	37, 49, 52
12p13.12	DHH	rs12281008	A/ 0.0751	0,05/ S		Unspecified	49
12p13.33	ERC1/ WNT5B	rs2887571	G/ 0.2129	-0,03/ FN -0,04/ S		WNT-β-catenin	49
12q23.3	C12orf23	rs1053051	T/ 0.4066	-0,03/ FN		Unspecified	49
13q14	TNFSF11 (RUNKL)	rs9533090	T/ 0.3178	-0,120/ S		RANK-RANKL-OPG	37, 49, 52
14q32.12	RPS6KA5	rs1286083	C/ 0.2418	-0,05/ FN -0,04/ S	1.05	Unspecified	49
16q12.1	CYLD	rs1564981	G/ 0.3338	-0,03/ S		Unspecified	49
16q12.1	SALL1/ CYLD	rs1566045	C/ 0.1415	-0,06/ FN		Unspecified	49

16p13.3	AXIN1	rs9921222	T/ 0.418	-0,03/ FN -0,04/ S		WNT-β-catenin	49
16p13.3	C16orf38/ CLCN7	rs13336428	G/ 0.4176	-0,04/ FN -0,04/ S		Unspecified	49
16p13.11	NTAN1	rs4985155	G/ 0.4116	-0,03/ FN -0,03/ S		Unspecified	49
16q23	ADAMTS18	rs16945612 rs11859065 rs11864477	C/ 0.1168 A/ 0.1163 C/ 0.1021	0,04 /H 0,04/ H 0,04/ H		osteoblastes differentiation, bone remodelling	47
16q24	FOXL1	rs10048146	G/ 0.1914	-0,193/ S		osteoblastes differentiation WNT-β-catenin	37, 49
17q12	CRHR1	rs9303521	G/ 0.4849	-0,068/ S		NFκB activation	37
17p13.3	SMG6	rs4790881	C/ 0.315	0,05/ FN 0,04/ S		Unspecified	49
17q21	HDAC5	rs228769	G/ 0.4185	0,081/ FN		Transcriptional regulation, role in cell cycle and muscle differentiation	37
17q21	SOST	rs4792909	T/ 0.4881		1.07	WNT	52
17q24.3	SOX9	rs7217932	A/ 0.456	0,03/ FN		endochondral ossification	49
18p11.21	C18orf19/ FAM210A	rs4796995	G/ 0.4551	0,02/ FN	1.08	Unspecified	49
18q21	TNFRS11A (RUNK)	rs884205	A/ 0.1882	-0,078/S		RANK-RANKL-OPG	37, 49, 52
19q13.11	GPATCH1	rs10416218	C/ 0.4331	-0,03/ S		Unspecified	49
20p12	JAG1	rs2273061	G/ 0.4831	0,072/ S	1.42	WNT-β-catenin	53
Xp22.31	FAM9B/ KAL1	rs5934507	G/ 0.2712	-0,09/ S		Unspecified	49

* FN: femoral neck, S: lumbar spine, H: hip

Low values of QUS parameters have been associated with high fracture risk. Although the BMD has been documented by various studies that are under strong genetic control, studies that evaluate genetic determinants of QUS parameter are limited.

Recent GWA, de novo replication and meta-analysis study in the GEFOS – GENOMOS consortium highlighted 9 polymorphisms associated with heel QUS phenotypes. The genome-wide association (GWA) studies assess the genetic determinants of heel broadband ultrasound attenuation (BUA; n=514260), velocity of sound (VOS; n=515514) in 13 discovery cohorts. Independent replication involved seven cohorts with GWA data (in silico n=511452) and new genotyping in 15 cohorts (de novo n=524902). In combined random effects, meta-analysis of the discovery and replication cohorts, nine single nucleotide polymorphisms (SNPs) had genome-wide significant ($p < 5 \times 10^{-8}$) associations with heel bone properties. Alongside SNPs within or near previously identified osteoporosis susceptibility genes including ESR1 (6q25.1: rs4869739, rs3020331, rs2982552), SPTBN1 (2p16.2: rs11898505), RSPO3 (6q22.33: rs7741021), WNT16 (7q31.31: rs2908007), DKK1 (10q21.1: rs7902708) and GPATCH1 (19q13.11: rs10416265), the study identifies a new locus on chromosome 11q14.2 (rs597319 close to TMEM135, a gene recently linked to osteoblastogenesis and longevity) significantly associated with both BUA and VOS ($p < 8.23 \times 10^{-14}$) [54]. GEFOS – GENOMOS identified polymorphisms near or on five genes, associated with BUA and SOS in discovery phase of the GWAS ($p < 5 \times 10^{-6}$) but didn't meet statistical significance in the replication study. Genes associated with BUA are NPR3 (5p13.3: rs9292469) and TAX1BP1 (7p15.2: rs11520772) and genes associated with SOS are UPK3B (7q11.23: rs38664), USPL1(13q12.3: rs3000634) and FOXL1 (16q24.1: rs7188801) [54].

Since many of the genetic loci have been investigated from GWAS, encode proteins involved in biochemical pathways with known function as the WNT, the regulatory system RANK- RANKL-OPG and endochondral ossification system, it is too early to understand the function of the new proteins derived from GWAS and how they are involved in the pathophysiology of the disease. Figures 3, 4 and 5 demonstrate the main genes identified by GWAS that are implicated in these paths [modified by Richards J. et al, 33].

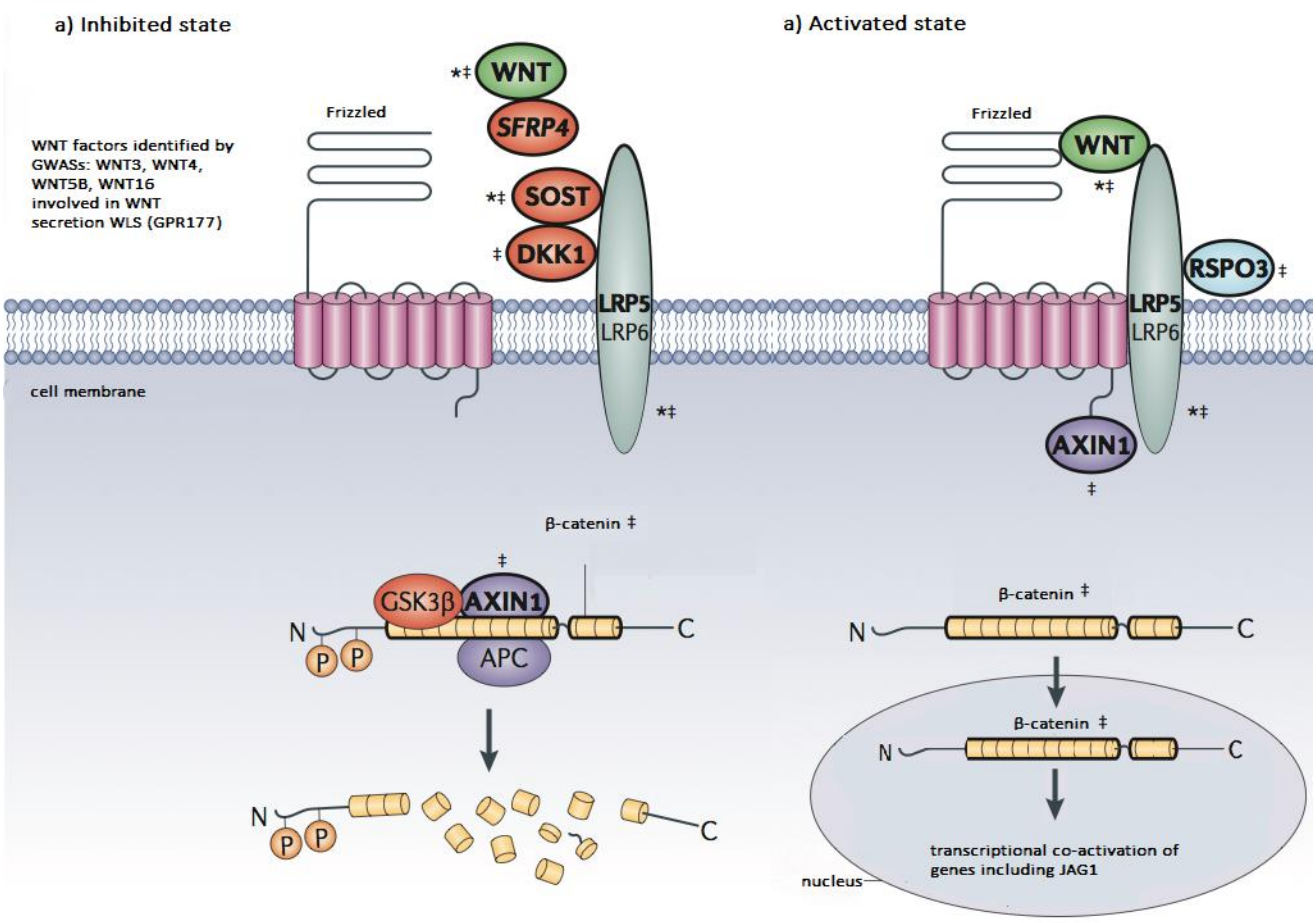


Figure 3: Simplified depiction of members of the canonical WNT signalling pathway identified through genome-wide association studies for bone mineral density.

*Bold font and bold outline: proteins identified through genome-wide association studies (GWASs). Red: inhibitory proteins of the WNT pathway. Green: activators of the WNT pathway. * Gene related to human monogenic skeletal disease. ‡ genes with evidence arising from mouse knockouts. The main role of the WNT signalling pathway is to control the stability and subsequent abundance of β-catenin, the role of which is to activate gene transcription in the nucleus.* ***a)*** *In the absence of WNT factors, β-catenin is phosphorylated by glycogen synthase kinase 3β (GSK3β), preventing the translocation of β-catenin to the nucleus. Sclerostin (SOST), dickkopf 1 (DKK1) and secreted Frizzled-related protein (SFRP4) act by inhibiting the interaction between Frizzled family members, low-density lipoprotein receptor-related protein 5 (LRP5) and WNT.* ***b)*** *WNT proteins bind to the G-protein-coupled receptor Frizzled and LRP5 to form a complex that ultimately leads to the recruitment of AXIN1 to the LRP5 co-receptor. R-spondin 3 (RSPO3) acts to disrupt DKK1 association to LRP6. This inhibits the degradation of the AXIN1–β-*

catenin complex and promotes the translocation of β-catenin to the nucleus. Jagged 1 (JAG1) has been reported to be a target of β-catenin transcriptional control and is also an important component of the NOTCH signalling pathway [modified, Richards J. et al, 33].

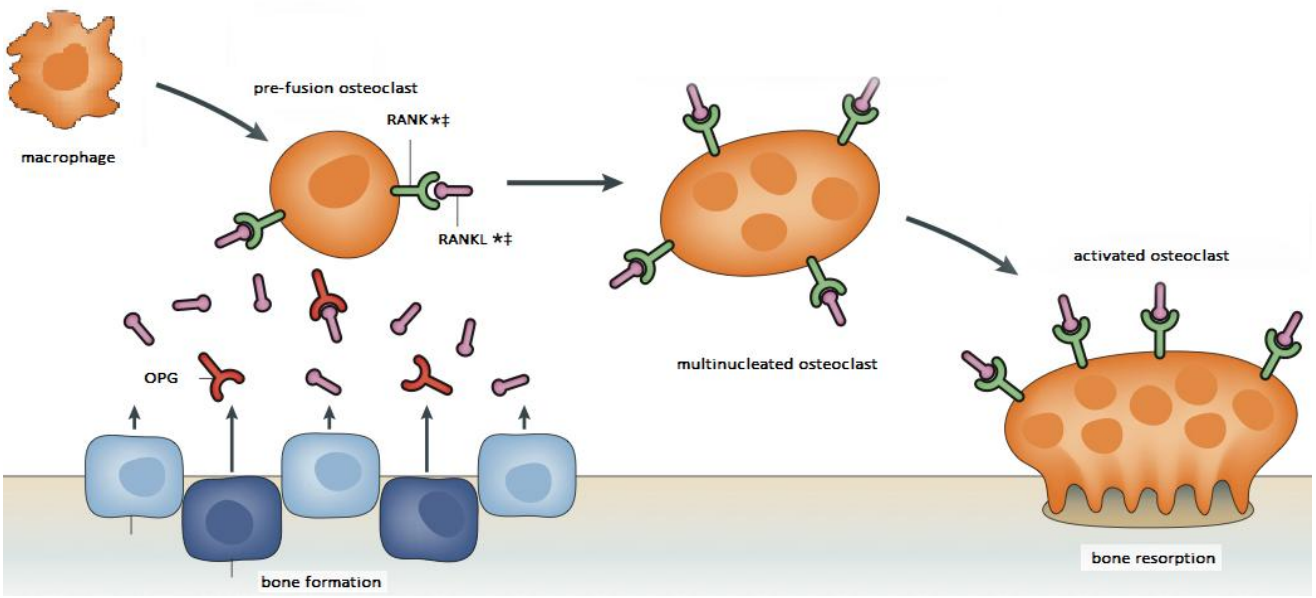


Figure 4: Simplified depictions of members of the RANK–RANKL–OPG signalling pathway identified through genome-wide association studies for bone mineral density.

*Bold font and bold outline: proteins identified through genome-wide association studies (GWASs). * Gene related to human monogenic skeletal disease. ‡ Genes with evidence arising from mouse knockouts. RANK is encoded by tumour necrosis factor receptor superfamily, member 11a (TNFRSF11A), its ligand RANKL is encoded by TNFSF11, and the decoy receptor OPG is encoded by TNFRSF11B. To generate activated osteoclasts, RANKL is secreted by osteoblasts and osteocytes in bone, and these bind to its natural receptor, RANK, on the surface of pre-fusion osteoclasts. To fine-balance this activation system, osteoblasts and osteocytes also secrete OPG, which is a natural decoy receptor for RANKL and prevents binding of RANKL to RANK [modified, Richards J. et al, 33].*

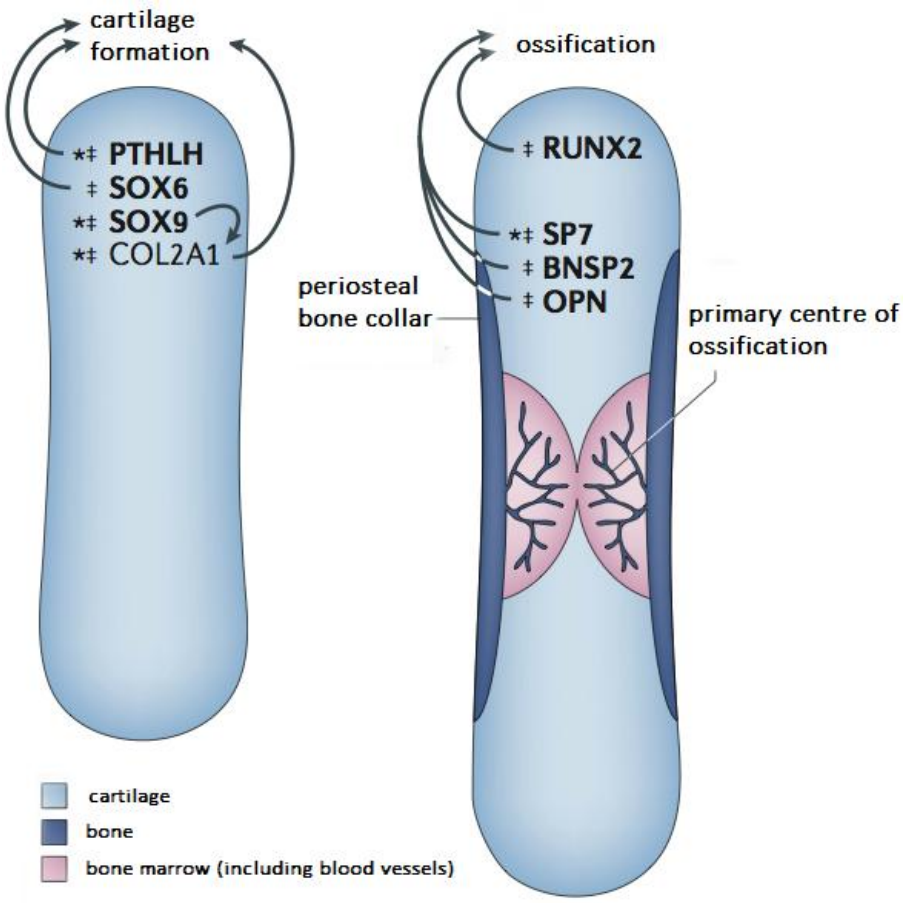


Figure 5: Simplified depiction of members of the endochondral ossification pathway identified through genome-wide association studies for bone mineral density.

*Bold font and bold outline: proteins identified through genome-wide association studies (GWASs). * Gene related to human monogenic skeletal disease. ‡ Genes with evidence arising from mouse knockouts. Bone is generated in the developing skeleton by first forming cartilage, which is then ossified, starting at the primary centre of ossification and moving outwards to the peripheral bone. Arrows indicate genes that are involved in the promotion of a process. Genes involved in the formation of cartilage are indicated in the left panel. Parathyroid-hormone-related protein (PTHrP), which is encoded by the gene PTHLH, binds to the PTHRP receptor to promote development of the cartilage growth plate. The transcription factor SOX6 is involved in the establishment of cartilage growth plate, allowing for the development of endochondral bone. The transcription factor SOX9 regulates collagen, type II, alpha 1 (COL2A1) expression, the product of which is a structural protein that is the main component of cartilage. Genes involved in ossification are depicted in the right-hand panel. RUNX2 is a transcription factor that is a regulator of ossification of cartilaginous skeleton, SP7 is a transcription factor that permits differentiation of osteoblasts, and BNSP2 is a major noncollagenous structural protein.*

Osteopontin (OPN) is a secreted protein that permits the attachment of osteoclasts to mineralized bone [modified,Richards J. et al, 33].

Genetic Score: The goal of genetic identification of individuals is to find those who, due to their genotypes, are at high risk of osteoporosis. Consequently, the effect of each allele on bone density should be counted and summed up. For this purpose, genetic scores are evaluated.

The Prospective Epidemiological Risk Factor (PERF) study, carried out in postmenopausal women 55 to 86 years of age, evaluated the cumulative effect of 63 polymorphisms, resulting from the meta-analysis of the GEFOS, on the bone mineral density and the osteoporosis development [49]. The risk alleles in the score were weighted by their individual effect on bone mineral density, and grouped into 5 categories. The difference between the average bone mineral density of the femoral neck among individuals of the category with the highest risk scores (9% of the population, n = 244) and the category with moderate risk score (34% of the population, n = 978) was 0.33 standard deviations.

From this genetic score, it was also possible to predict the risk of osteoporosis (T-score <-2,5), using the moderate risk score category as a reference (OR=1). Women with the highest score were 1.56 times (95% CI (1.12-2.18) more likely to develop osteoporosis (figure 6), while women in the lower risk category are 62% less likely to develop osteoporosis (OR=0.38 (95% CI (0.23-0.63)). [49]

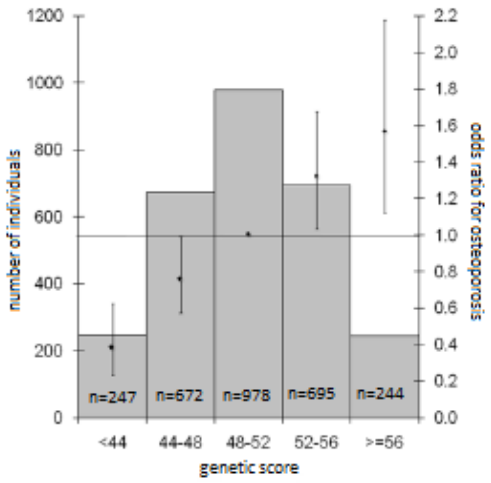


Figure 6. Combined effect of osteoporosis risk- alleles based on BMD in the Prospective Epidemiological Risk Factor (PERF) study (n=2,836 women). *The genetic score of each individual for was based on the 63 SNPs displaying genome-wide significant association with*

*BMD (55 main and 8 secondary signals). Both genetic scores are weighted for relative effect sizes estimated without the PERF study. Weighted allele counts summed for each individual were divided by the mean effect size making them equivalent to the percent of alleles carried by each individual and binned into 5 categories. Histograms describe counts of individuals in each genetic score category (left axis scale). Diamonds (right axis scale) represent risk estimates in the form of odds ratio for osteoporosis (defined as NHANES T-score<−2.5) using the middle category as reference (OR=1). Vertical lines represent 95% confidence limits [modified,*Estrada K. et al, *49].*

The observation of genetic loci associated with osteoporosis, serves the investigation of new proteins and biochemical pathways, crucial for the disease pathophysiology. As a result, new treatment goals could be set and people that are at increased risk of fracture due to their genotype could be identified.

Studies that investigate the effect of genetic markers on bone phenotypes in the Greek population are few and involve a small number of genes and polymorphisms, specific age groups and include a small number of volunteers. [55-61].

***1.1.6.2 Clinical Factors*:**

These include: low peak bone density achievement, age, premature menopause (<40 years), primary or secondary amenorrhoea, primary or secondary hypogonadism in men, low body weight, loss >10% of body weight in age <25 years, vitamin D deficiency, diseases and medications associated with secondary osteoporosis [27, 62].

Also, evaluation of bone metabolism (bone turnover) is extremely crucial as, as mentioned above, the disorders between bone resorption (osteoclestic activity) and bone production (osteoblastic activity) are a major pathogenetic factor of osteoporosis. The rate of bone remodelling evaluated with the use of specific markers (bone turnover markers). These indicators refer either to products of osteoblast enzyme activity or bone resorption products (osteoclastic activity) [63]. They are useful in clinical practice for 1) detecting people with an increased rate of bone loss and increased fracture risk [64], 2) the prediction of the therapy benefit [65], 3) the evaluation of the effectiveness of the treatment [66] and 4) improving the compliance with the treatment [67]. The utility of markers of bone turnover requires its

investigation into studies to evaluate the impact of clinical risk factors on bone density and the risk of osteoporosis.

1.1.6.3 Environmental Factors:

These include: smoking, low physical activity, long-term immobilization, high intake of alcohol and caffeine, low calcium intake, low intake of vitamin k, high phosphorus and sodium intake, low vitamin D intake [27, 62].

Nutrition (nutrient intake and dietary habits) undoubtedly plays an important role in bone health during lifespan and especially during the achievement of peak bone density but also for its maintenance during adulthood. Nutrients may have a beneficial or aggressive action on the skeleton [68]. They affect bones in various ways including possible changes in bone structure, at the rate of bone metabolism, endocrine and/or paracrine system and homeostasis of calcium and other minerals or trace elements [69].

As mentioned above, a special position hold calcium, magnesium, phosphorus, sodium, vitamins D, K and A, as well as trace elements such as iron, zinc and copper [70]. The intake of macronutrients, especially proteins and lipids (mainly of polyunsaturated ω-3 and ω-6) associated significantly with bone mineral density [71, 72]. In addition, ingredients such as alcohol, caffeine and phytoestrogens are factors that have been associated with bone health [73-75].

However, humans consume complex combination of nutrients that have a cumulative and interactive effect in their meals and related to several phenotypes or health outcomes. The need of a more holistic approach of nutrition led to the investigation of statistical methods from which overall dietary patterns can be derived, in which multiple related dietary characteristics are considered as a single exposure for a specific population. There are two main categories of dietary pattern approaches, (i) the data driven or posteriori dietary pattern approach, that includes PCA and (ii) the a priori dietary pattern approach, which uses dietary indexes created using existing nutritional knowledge [76, 77]. Apart from the individual nutrients, food groups and dietary patterns seem to impact on bone health, although studies are very limited. For example, the increased consumption of fruit and vegetables is associated with bone mineral density, bone size and bone turnover [78-80]. Also nutritional schemes, such

as the Mediterranean Diet and DASH diet have been studied with regard to potential benefits to skeletal health [81, 82].

The above nutritional factors have not been investigated in a large sample of Greek population in relation to bone mass and markers of bone turnover. For example, nutrient intake and its correlation with bone health has been investigated in several studies in small populations and in limited age range [83-88]. The study of individual risk factors without exploring and understanding the interactions between them has little practical and clinical significance. However, understanding the interactions between them may be the key to more effective and more personalized disease prevention and cure [89]. However, the number of studies investigating how risk factors interact are very limited.

Dietary factors play, as mentioned, an important role as risk factors for osteoporosis. The nutritional intake of individual nutrients, such as calcium, affects skeletal health at various stages of life [48]. However, interactions of nutrients in foods significantly alter the action of individual components. Also, significant bone action may have food components that are not individually associated with bone health. Therefore, the study of nutrition science has shifted from the exploration of individual nutrients to the investigation of eating habits and patterns, studying the human diet from a holistic view. This approach prevails internationally in chronic disease studies, and recent data are available for osteoporosis [55, 56].

Risk factors vary with age. Studies have demonstrated that polymorphisms affecting peak bone mineral density may be different from those affecting it at the post-menopausal period. [90, 91]. The markers of bone turnover vary according to the normal change in bone density with age [22]. Vitamin D levels change with age due to decreased sun exposure and reduced skin composition [92]. Dietary factors also change significantly. Eating patterns of teenagers may differ significantly from those of adults, while the diet of the elderly varies significantly due to diseases that require special dietary regimens, difficulties in eating, digestion and absorption of food, weaknesses in meal preparation etc [93].

The investigation of risk factors in specific populations is important for the design and implementation of health policies related to the prevention and treatment of osteoporosis. The stratification of a large and well- characterized population is crucial for the extraction of results widely accepted.

1.7 Vitamin D

Between the most important risk factors of bone fragility, vitamin D deficiency is a subject of extensive research. Vitamin D is unique among hormones because it can be made in the skin from exposure to sunlight. Vitamin D comes in two forms. Vitamin D_2 is manufactured through the ultraviolet irradiation of ergosterol from yeast, and vitamin D_3 through the ultraviolet irradiation of 7-dehydrocholesterol from lanolin. Vitamin D_2 is found naturally in sun-exposed mushrooms. Both are used in over-the-counter vitamin D supplements, but the form available by prescription in the United States is vitamin D_2. Vitamin D_3 is synthesized in the skin and is present in oil-rich fish such as salmon, mackerel, and herring; commercially available vitamin D_3 is synthesized from the cholesterol precursor 7-dehydrocholesterol naturally present in the skin or obtained from lanolin. Both vitamin D_2 and vitamin D_3 are used for food fortification and in vitamin D supplements. [94]

1.7.1 Metabolism of Vitamin D

Vitamin D (D represents D_2, or D_3, or both) that is ingested is incorporated into chylomicrons, which are absorbed into the lymphatic system and enter the venous blood. Vitamin D is formed in the skin from 7-dehydrocholesterol (7-DHC) via the intermediate previtamin D_3 and with the help of sunlight (UVB: 290–315 nm). Previtamin D_3 is converted by body heat to vitamin D_3 (cholecalciferol). Excessive sunlight exposure degrades previtamin D_3 and vitamin D_3 into inactive photoproducts, thus preventing excessive production of the sunshine vitamin in the skin. Vitamin D that comes from the skin or diet is biologically inert and requires its first hydroxylation in the liver. The liver converts vitamin D_3 via the enzyme 25-hydroxylase (25-OHase: CYP27A1, CYP2R1) into 25-hydroxyvitamin D (25(OH)D), also known as calcidiol. The mitochondrial CYP27A1 and microsomal CYP2R1 are the two major enzymes involved in the hydroxylation at C-25, although there are several CYP enzymes that show 25-hydroxylase (25-OHase) activity but with higher Km and lower Vmax. Serum 25(OH)D (1 ng/mL = 2,5 nmol/L) is used for the medical laboratory evaluation of the vitamin D status. 25(OH)D is then converted in the kidneys via the enzyme 25-hydroxyvitamin D-1-α-hydroxylase also known as cytochrome p450 27B1 (1-OHase: CYP27B1) into the metabolically active vitamin D hormone [1α,25(OH)2D]. This enzyme is also called renal 1-α-hydroxylase – since it occurs in the kidneys (endocrine effect). The renal synthesis of $1,25(OH)_2D$ is regulated by several factors including

serum phosphorus, calcium, fibroblast growth factor 23 (FGF-23), parathyroid hormone (PTH) and itself.

$1,25(OH)_2D$ interacts with its vitamin D nuclear receptor, which is present in the small intestine, kidneys, and other tissues. $1,25(OH)_2D$ stimulates intestinal calcium absorption. Without vitamin D, only 10 to 15% of dietary calcium and about 60% of phosphorus are absorbed. Vitamin D sufficiency enhances calcium and phosphorus absorption by 30–40% and 80%, respectively. $1,25(OH)_2D$ interacts with its vitamin D receptor in the osteoblast to stimulate the expression of receptor activator of nuclear factor κB ligand; this, in turn, interacts with receptor activator of nuclear factor κB to induce immature monocytes to become mature osteoclasts, which dissolve the matrix and mobilize calcium and other minerals from the skeleton. In the kidney, $1,25(OH)_2D$ stimulates calcium reabsorption from the glomerular filtrate.

The vitamin D receptor is present in most body tissues and cells. These include endothelial cells, isle cells of pancreas, hematopoietic cells and t-lymphocytes. As a result $1,25(OH)_2D$ has a wide range of biological actions, including inhibiting cellular proliferation and inducing terminal differentiation, inhibiting angiogenesis, stimulating insulin production, inhibiting renin production, and stimulating macrophage cathelicidin production

There are several tissues and cells that possess 1-α-hydroxylase (1-OHase) activity. Besides the kidneys, a multitude of tissues have a local 1-OHase including bone, placenta, prostate, keratinocytes, macrophages, T-lymphocytes, dendritic cells, several cancer cells, and the parathyroid gland. Depending on the availability of 25(OH)D and the amounts required, these cells can produce the biologically active vitamin D hormone with the help of their local 1-OHase (autocrine and paracrine effect). $1\alpha,25(OH)_2D$ is like the sex hormones (e.g. estradiol) and corticosteroids (e.g. cortisone), which are all steroid hormones. Via a feedback mechanism, the $1\alpha,25(OH)_2D$ level regulates the synthesis of 1α,25(OH)2D and reduces the synthesis and secretion of parathyroid hormone in the parathyroid

glands. $1\alpha,25(OH)_2D$ induces its own destruction by activating the 25-hydroxyvitamin D-24-hydroxylase (24-OHase: CYP24A1), which leads to the multistep catabolism of both 25(OH)D and $1\alpha,25(OH)_2D$ into biologically inactive, water-soluble metabolites including calcitroic acid.

The local production of $1,25(OH)_2D$ may be responsible for regulating up to 200 genes that may facilitate many of the pleiotropic health benefits that have been reported for vitamin D.

[94, 95]. In figure 7 the synthesis and metabolism of vitamin D concerning its skeletal actions is presented.

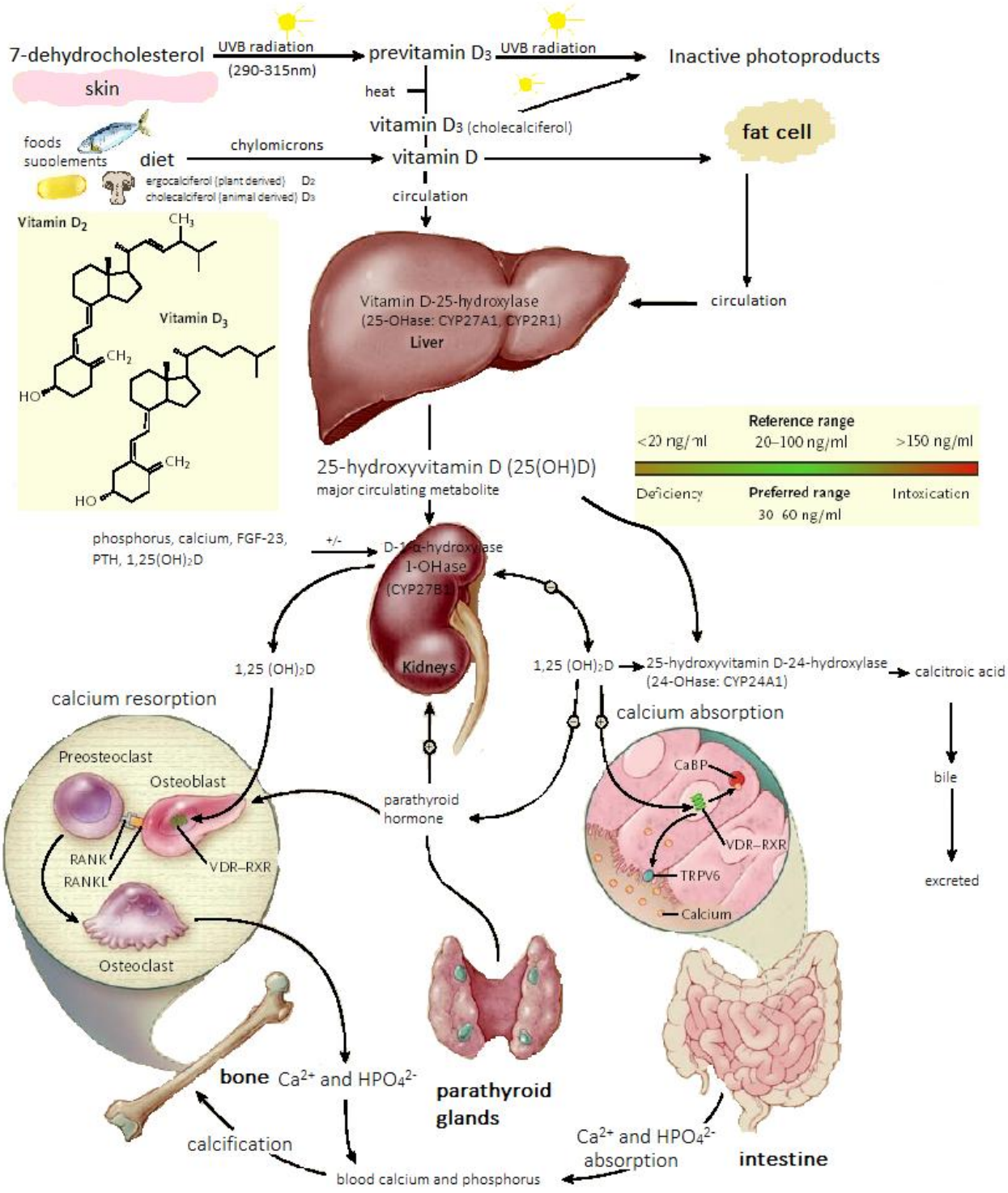


Figure 7. *The synthesis and metabolism of vitamin D for the regulation of calcium, phosphorus and bone metabolism* [91].

1.7.2 Vitamin D deficiency

There have been several recent consensus statements or guidelines that have included definitions of Vitamin D deficiency. It is generally agreed that the serum concentration of 25(OH)D is the best marker of an individual's Vitamin D status because it is the major circulating form and reflects the combination of dietary intake and cutaneous skin synthesis. However, different thresholds for the level of 25(OH)D that is considered to reflect efficiency are used. For example, the Institute of Medicine's report on the dietary reference intake for Vitamin D published in 2010 [96] defined a level of 50 nmol/l (<20 ng/mL) as meeting the needs of 97.5% of the population, whereas the Endocrine Society Clinical Practice Guideline published in 2011 defined Vitamin D deficiency as a level <50 nmol/l, with levels of between 52.5 nmol/l (21 ng/mL) and 72.5 nmol/l (29ng/mL) regarded as Vitamin D insufficiency, and levels of greater than 72.5 nmol/l being regarded as optimal [97] . In the current study, we decided to use the following definitions: severe deficiency: <25 nmol/L (<10 ng/mL), deficiency: 25-50 nmol/L (10-19.9 ng/mL), insufficiency: 50 -75 nmol/L (20-29.9 ng/mL), sufficiency: ≥75 nmol/L (≥30 ng/mL) and toxicity: ≥ 375nmol/L (≥150ng/mL), as it shown in Table 3.

The recommended dietary intake for vitamin D that is required to maintain the concentration of 25 (OH) D serum levels at least 20 ng/mL (50nmol/L) in 97.5% of the population, with little or no sun exposure is presented in Table 4 [96].

Table 3. *Vitamin D status according serum 25(OH)D.*

	Serum 25(OH)D
severe deficiency	25 nmol/L <10 ng/mL
deficiency	25-50 nmol/L 10-19.9 ng/mL
insufficiency	50 -75 nmol/L 20-29.9 ng/mL
sufficiency	≥75 nmol/L ≥30 ng/mL
toxicity	≥150ng/mL ≥ 375nmol/L

Table 4. *Vitamin D intakes recommended by the IOM and the Endocrine Practice Guidelines Committee*

Life stage group	**IOM recommendations**				**Committee recommendations for patients at risk for vitamin D deficiency**	
	AI	**EAR**	**RDA**	**UL**	**Daily requirement**	**UL**
Infants						
0 - 6 months	400 IU (10 µg)			1,000 IU (25 µg)	400–1,000 IU	2,000 IU
6 - 12 months	400 IU (10 µg)			1,500 IU (25 µg)	400–1,000 IU	2,000 IU
Children						
1-3 yr		400 IU (10 µg)	600 IU (15 µg)	2,500 IU (63 µg)	600–1,000 IU	4,000 IU
4-8 yr		400 IU (10 µg)	600 IU (15 µg)	3,000 IU (63 µg)	600–1,000 IU	4,000 IU
Males						
9-13 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	600–1,000 IU	4,000 IU
14-18 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	600–1,000 IU	4,000 IU
19-30 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	1,500–2,000 IU	
31-50 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	1,500–2,000 IU	
51-70 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	1,500–2,000 IU	
>70 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	1,500–2,000 IU	

Females						
9-13 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	600–1,000 IU	4,000 IU
14-18 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	600–1,000 IU	4,000 IU
19-30 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	1,500–2,000 IU	10,000 IU
31-50 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	1,500–2,000 IU	10,000 IU
51-70 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	1,500–2,000 IU	10,00o IU
>70 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	1,500–2,000 IU	10,000 IU
Pregnancy						
14-18 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	600–1,000 IU	4,000 IU
19-30 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	1,500–2,000 IU	10,00o IU
31-50 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	1,500–2,000 IU	10,000 IU
Lactation[a]						
14-18 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	600–1,000 IU	4,000 IU
19-30 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	1,500–2,000 IU	10,00o IU
31-50 yr		400 IU (10 µg)	600 IU (15 µg)	4,000 IU (100 µg)	1,500–2,000 IU	10,000 IU

AI, Adequate intake; EAR, estimated average requirement; UL, tolerable upper intake level.

a Mother's requirement, 4,000–6,000 IU/d (mother's intake for infant's requirement if infant is not receiving 400 IU/d).

1.7.3 Risk Factors of Vitamin D deficiency

Decreased synthesis in the skin

- Sunscreen wearing: Wearing a sunscreen with a sun protection factor of 30 reduces vitaminD synthesis in the skin by more than 95%.
- Skin pigmentation: UVB radiation absorbed from melanin. People with a naturally dark skin tone have natural sun protection and require at least three to five times longer exposure to make the same amount of vitamin D as a person with white skin.
- Age: The synthesis of 7-dehydrocholesterol in the skin decreased.
- Season, latitude: The number of UVB photons reaching the Earth depends on the angle of the sun. The more lateral the angle, the less radiation reaches the Earth. At around 35 degrees north latitude and above (Greece 35o -40o), little or no vitamin D can be produced from sun exposure from November to February. Vitamin D level reaches its nadir in late winter and it takes about six weeks to raise the serum levels.
- Patients with implants or skin burns: A reduction of 7-dehydrocholesterol is observed in these patients.

Decreased bioavailability

- Malabsorption: Malabsorption maybe caused by, cystic fibrosis, celiac disease, Whipple's disease, Crohn's, gastric bypass, cholesterol absorption drugs, etc.
- Obesity: Obesity is associated with vitamin D deficiency. There is an inverse association of serum 25(OH)D and body mass index (BMI) greater than 30 kg/m^2.

Increased catabolism

- Due to medication: It activates the destruction of 25(OH)D and $1,25(OH)_2D$ to inactive products.

Lactation

- Breast milk is poor in vitamin D: breast milk contains 20 IU / L)

Decreased synthesis of 25 (OH) D or increased loss in urine

- Hepatic failure: In moderate failure, the absorption decreases but in 90% failure 25(OH)D is not produced.
- Nephrotic syndrome: A loss of 25(OH)D binded with vitamin D binding protein occurs.

Decreased synthesis of $1,25(OH)_2D$

- Chronic renal failure

Hereditary disorders – rickets

- Rickets due to faulse-deficiency of vitamin D. Mutation in the 1,25 hydroxyvitamin-D-1α hydroxylase gene (CYP27B1).
- Vitamin D resistant rickets. Mutation to vitamin D receptor gene (VDR).
- Autosomal dominant hypophosphataimic rickets. A mutation in the gene that prevents or reduces FGF23 cleavage.
- X-linked hypophosphatemic rickets — mutation of the PHEX gene, leading to elevated levels of fibroblast growth factor 23 and other phosphatonins.

Acquired disorders

- Osteomalacia due to tumor.
- Primary hyperparathyroidism
- Granulomatous disorders, sarcoidosis, tuberculosis, some types of lymphoma
- Hyperthyroidism

1.7.4 Heritability of vitamin D deficiency

The heritability of vitamin D as estimated by twin studies reaches the 50-80% [13,14]. As identified by 3 recent GWAS meta-analysis, 6 genetic loci (GS: rs3755967, NADSYN1/ DHCR7: rs12785878, CYP2R1: rs10741657, CYP24A1: rs17216707, AMDHD1: rs10745742, SEC23A: rs8018720), associated with 25(OH) D levels and explain the 7.5% of heritability. The difference between twin (50-80%) and GWAS studies may be due to influence by environmental conditions [31-33]

1.7.5 Consequences of Vitamin D deficiency

The consequences of Vitamin D deficiency in adults are osteomalacia, acceleration of bone loss, muscle weakness, instability, and therefore increased risk of falling [98]. In children, lack of Vitamin D causes rickets and growth retardation. Vitamin D receptors are found in enterocytes, osteoblasts, the cells of the distal convoluted renal tubules, in the cells of the parathyroid gland, colon, pituitary, ovaries, cells of the immune system etc. Therefore, Vitamin D deficiency is associated, by many observational studies, with major diseases, such as osteoporosis, diabetes, some forms of cancer, autoimmune diseases, infectious diseases and hypertension etc but causalities have not been proven [98].

1.7.6 Prevalence of vitamin D insufficiency and deficiency

The prevalence of vitamin D insufficiency and deficiency is significant. It is estimated that about 1 billion people worldwide suffer of vitamin D insufficiency or deficiency. The elderly, children and women during pregnancy are at increased risk for development of vitamin D deficiency. The incidence of vitamin D deficiency increases depending on the distance of the country of residence from the equator. In the past, it was believed that in countries with increased sunshine, vitamin D deficiency is rare due to skin production by the photolytic conversion of 7-dihydroxycholesterol to vitamin D3 by the effect of ultraviolet radiation [98]. However, recent studies indicate that in the Mediterranean countries and younger age groups there is an increased incidence of deficiency. Studies in the Greek population showed increased vitamin D deficiency rates in the elderly (20% from a sample of 231 people aged > 60 years) [99], postpartum women (20% from a sample of 123 women) [100], children (14% from a sample of 124 children) and adolescents (47% from a sample of 45 teenagers) [35], mainly from urban areas of Greece. However, no data are available for general overlapping.

Understanding of skeletal and extraskeletal actions of vitamin D and the significant consequences of deficiency, make this an extremely important factor in the investigation of risk factors for osteoporosis and other chronic diseases (e.g. cancer, diabetes). The prevalence of vitamin D defficiency in a wide range of ages of the Greek population is considered important.

Although the development of effective therapeutic methods contributes to the problem, prevention is recognized globally as the most effective measure to reduce the disease. Prevention is based on identifying and addressing risk factors associated with the disease [16].

1.8. AIMS AND OBJECTIVES

Objectives of the study:

1. To determine the prevalence of vitamin D deficiency in the Greek population.

2. To determine the characteristics of the bone structure as derived from QUS measurement.

3. To identify dietary patterns in the population and their effect on bone phenotypes.

4. To determine the effect on the characteristics of the bone structure, polymorphisms that are derived from GWAS, in larger populations.

5. Evaluation and interpretation of the effect of the potential interaction of the aforementioned factors on serum vitamin D levels.

6. The evaluation and interpretation of the effect of the possible interaction of the aforementioned factors on the characteristics of the bone structure.

The aims of each report supporting the PhD thesis were:

Paper 1. To investigate Vitamin D status in adult women and men from northern and southern regions of Greece including several islands, in relation to QUS parameters.

Paper 2. To identify the dietary patterns that indicate the nutritional habits of healthy Greek adults and secondly to evaluate the effect of these patterns on serum 25(OH)D levels and QUS phenotypes.

Paper 3. To identify the effect of the rs11520772 polymorphism on serum 25(OH)D levels and on the QUS phenotypes, derived from a large-scale GWA, replication and metanalysis study (**paper 4**).

2. SUBJECTS AND METHODS

2.1 Population of the study

OSTEOS is an observational cross-sectional study, conducted from June 2010 to July 2012. A population of 970 community-dwelling Greek adults (133 males, 830 females) and without blood related affinity, so that the population is homogeneously racial. Also, since bone health parameters and the risk factors affecting them vary with age, the participation of individuals of a vast age group in the study was considered necessary for the complete association between bone health parameters and risk factors. Thus, the total sample consists of adults over 18 years old. Observation studies require a large number of people to achieve high statistical power.

The population was recruited at the health promotion events carried out by the Skeletal Health Association 'Butterfly' in rural and urban areas throughout Greece (Central Greece, Peloponnese, Thessaly, Aegean Islands and Macedonia), with a view to representing regions at national level, both urban and rural. The Skeletal Health Association 'Butterfly' organizes public awareness events concerning osteoporosis in the prefecture of Attica and in various prefectures of the country. These events take place in cooperation with the respective Municipalities and Communities, their duration is 2 days and the participation is free. The events are announced and advertised by the Association and the responsible local authorities. The volunteers are informed for the purposes of the study by the competent local authorities and they declare their voluntary participation. The regions where the recruitment had taken place are shown in figure 8.

Figure 8: *Geographical depiction of the study. Mean 25(OH)D levels (ng/mL) by region.*

2.2 Exclusion criteria

Subjects with a known history of bone metabolic diseases (such as Paget's disease, osteomalacia, osteogenesis imperfecta, renal osteodystrophy, etc.) are excluded from the study. Subjects with endocrinopathies (primary hyperparathyroidism, hypoparathyroidism, thyrotoxicosis, pituitary and adrenal diseases, such as Cushing's disease, Addison's disease), malignancies, connective tissue diseases, malabsorption syndrome, inflammatory bowel diseases (ulcerative colitis, Crohn's disease), liver cirrhosis, hepatic failure and renal failure, are excluded from the study. Early menopause (40 years) and 1-year amenorrhea are exclusion criteria. Also excluded from the study are people taking medication that affects bone metabolism, such as hormone replacement therapy, anabolic steroids or progesterone,

bisphosphonates, calcitonin, teriparatide, vitamin D, tibolone, glucocorticoids, anti-epileptics, tricyclic antidepressants, anticoagulants. Finally, volunteers should not abuse alcohol or have mental or psychiatric disorders.

Since the study aims at evaluating risk factors for idiopathic osteoporosis, the above criteria are used to exclude individuals with other bone diseases or agents causing secondary osteoporosis [2].

2.3. Demographic data collection

For the collection of demographic data, a questionnaire was designed which provides information concerning the date of birth, origin, educational level, marital status, employment status, place of residence and contact details (home address, phone number).

For the collection of medical history information, a questionnaire included questions about: ailments and disease history (with emphasis on those included in the exclusion criteria), menarche and menopause age, possible history of amenorrhea, surgical interventions with emphasis on interventions leading to menopause (hysterectomy), smoking habits, number of pregnancies, breastfeeding months, fracture history of the distal skeleton, inheritance for osteoporosis, fractures and other bone diseases, medication and history of medication (with emphasis on those included in the exclusion criteria) and possible history of long-term immobilization. Among the fractures of the peripheral skeleton, low-trauma fractures mentioned separately. The data collected aims to evaluate the presence of exclusion criteria and to record clinical (fractures, menstruation, menopause), environmental (smoking) and genetic (heredity) risk factors.

Dietary information collected via a 24-hour-recall questionnaire and a validated, semi-quantitative, seventy-six-item food frequency questionnaire (FFQ) [101]. These are the most valid methods of assessing dietary intake and dietary habits for population groups [102]. The 24-hour recall method records information about any food or drink consumed in the previous 24 hours. The record is detailed and includes the type of food or drink, quantity, cooking style and trade name if it is a packaged product. FFQs include a large number of foods. The serving of the proportions is done either with 3-portion pictures or with the use of estimating tools, such as 1 cup of tea (250ml). Frequency of consumption is categorized at never, rarely, 1-3 times a month, 1-2 times a week, 3-4 times a week, 5-6 times a week, daily / times a day. Record the

consumption frequency of breakfast, lunch, dinner, traditional food, fresh or non-food (1-2 / week or 3-4 / week or 5-6 / week or daily) and snacks (0-6/ day). Then the frequency of consumption was quantified on the basis of servings per week according to the dietary guidelines for adults in Greece [103]. Mixed foods were taken into account and assigned into the respective food groups.

The assessment of physical activity was obtained from The International Physical Activity Questionnaire (IPAQ, short version) that was completed under the surveillance of the investigator [29]. These methods evaluate activities during work as well as non-work activities (sports, recreation) in terms of their type, frequency and duration. The hours spent on sedentary activities (television (TV), personal computer (PC)) and in moderate or vigorous organized physical activity, are also evaluated.

To evaluate the hours of sun exposure, the subjects were asked how many hours per week on average they were exposed to the sun from 9:00-18:00, in the summer and in the winter.

At around 35 degrees north latitude and above (Greece 35^{o}-40^{o}), little or no vitamin D can be produced from sun exposure from November to February. Vitamin D level reaches its nadir in late winter and it takes about six weeks to raise the serum levels. Therefore, we defined two seasons to classify the subjects according to the blood collection day, winter-spring (December until May) and summer-autumn (June until November) [104, 105].

Data collection from questionnaires: The day of examination, during the interview, the investigator filled questionnaires of demographic data, medical history, the 24-hour recall, the FFQ and the assessment of physical activity questionnaire for the adult population. Thereafter the inconsistency with exclusion criteria was confirmed and the data of the subject was encoded. Furthermore, the volunteers signed the voluntary participation contract.

2.4. Measurement of anthropometric parameters

The selected anthropometric parameters are Height, Weight and Body Mass Index (BMI). The height is chosen as a characteristic phenotype of the skeleton size. Weight and BMI are selected as factors affecting bone mineral density at all life stages.

Body weight was measured with a calibrated scale to the nearest 0.1 Kg wearing light clothing and no shoes. The volunteer is placed on the scale upright, motionless in the center of the scale platform. He is asked to look straight without resting somewhere. The height was measured by

a mounted stadiometer to the nearest 0.5 cm. Measured from the top of the cranial vault up to the level of the feet. The volunteer is placed in stadiometer erect without shoes and socks, with heels connected, knees straight, shoulders relaxed and the palms resting on thighs. The heels, the buttocks, the thoracic bursa of the spine and the posterior surface of the skull touch the vertical axis of the instrument. The volunteer is asked to place his head in Frankfort horizontal plane (in a position in which the straight line between the lower point of the occipital rib and the cartilage projection in front of the outer opening of the ear flap is horizontal). In this position, the subject inhales deeply to stretch the vertebral column and the measurement is taken [93]. Weight and height were measured twice by the same investigator and measurements were averaged for each participant and the body mass index (BMI) was calculated with the following equation: BMI = weight (kg) / height^2 (m^2). BMI was classified according to the World Health Organization [106] into four categories: Underweight (<18.5 kg/m^2), Normal weight (18.5-24.9 kg/m^2), Overweight (25-29.9 kg/m^2) and Obesity (≥30 kg/m^2). Heel bone properties (bone structure and density) were measured using the Achilles quantitative ultrasound (QUS) device at the back of the heel, a water-bath ultrasound system into which the subject places his heel. QUS is a non-ionizing technique that uses ultrasound to determine bone density. Achilles generates a band of frequencies from 200 to 600 kHz. It measures the broadband ultrasound attenuation (BUA), expressed in dB/MHz and measures the ultrasound attenuation with the incident frequency of wave sound. The speed of sound (SOS) is expressed in m/s and means the necessary time for ultrasound waves to go through a determined distance inside the calcaneus bone. The third variable, stiffness index (SI), is a combination of normalized SOS and BUA and is considered to improve the variability of SOS and BUA. SI is automatically calculated by Achilles from the BUA and the SOS, using the equation SI= (0.67*BUA) + (0.28*SOS)-420 [107]. For normative data we used reference data for the QUS measurements of the calcaneus specific for Greek population [108]. The subject is informed about the effects of bone density and other bone parameters.

2.5. Blood sampling

Following a 12 hour fast, all subjects had a sample of venous blood withdrawn, without ligation, for serum isolation between 08:00-09:00 am. A total of 15mL of venous blood in 2 vials received from each subject. The first sample contains 5mL in a vial with EDTA (Ethylene diamine

tetra-acetic acid) and the second contains 10mL in a vial without anticoagulant. The first sample was used for DNA extraction and stored in 4°C until the DNA extraction procedure and the second sample was used for serum isolation. The serum is isolated by centrifuging the sample at 3000 rpm for 10 minutes and the serum is pipetted and stored in vials at -80 ° C.

A concentrated leukocyte preparation (buffy coat) is used for the DNA extraction, which is isolated by the following procedure: the blood sample in the vials with the anticoagulant is centrifuged at 3000 rpm for 10 minutes. After centrifugation the erythrocytes are precipitated at the bottom of the vial while the plasma is transferred to the top. Between erythrocytes and plasma there is a thin layer containing the concentrated white and the platelets. This layer is the buffy coat. Using a sterile pipette, the plasma up to 1-2 mm over the layer of buffy coat is removed and then the buffy coat along with the rest of the plasma and a small amount of erythrocytes (1-2 mm) is obtained. It is stored in a vial at -80 ° C until it is used for DNA extraction.

2.5.1. Measurement of biochemical parameters

Following a 12 hour fast, all subjects had a sample of venous blood withdrawn, for serum isolation between 08:00-09:00 am, that was stored at -80^{o}C, for further measurement of the following indexes level:

- 25(OH)D
- Intact parathormone, 1-84, iPTH
- Total calcium
- Phosphorus
- Creatinine

Intact parathyroid hormone (iPTH) measurements were performed on a Roche/Modular Analytics analyzer, which employs electrochemilluminescense immunoassay technology (ECLIA). The intra- and inter-assay coefficients of variation (CVs) of the iPTH assay were less than 7% and the analytical sensitivity was 1.2 pg/mL.

Serum 25(OH)D levels were determined by enzyme immunoassay [Immunodiagnostic Systems, 25(OH)D; Boldon, UK], The sensitivity of this assay is 5 nmol/L and intra- and interassay coefficients of variation of 5.3 % and 4.6 %, respectively. Standardization of the different Vitamin D assays is the key to achieving comparable results across different methods and

manufacturers. Furthermore, assay standardization is of critical importance for the establishment of common clinical cut-offs and their use in routine practice. Applying a common cut-off value on results generated with poorly standardized assays will inevitably lead to inconsistent patient classification and inappropriate therapeutic decisions. In 2010 the Vitamin D Standardization Program (VDSP) was established to improve the standardization of 25(OH)D assays. The aim of VDSP is that 25(OH)D measurements are accurate and comparable over time, location, and laboratory procedure to the values obtained using reference measurement procedures (RMPs) developed at the NIST [14] and Ghent University [15]. According to VDSP a routine method is considered as standardized if the CV is < 10% and the bias < 5% [26]. The method we have used fulfills the criteria to be considered standardized according to CDC website [109]. The biochemical parameters 25(OH)D_3, parathormone, total calcium, phosphorus refer to the calcium homeostasis system and their disorder is an important risk factor for the occurrence of bone disorders. Finally, the function of the kidneys (creatinine) is checked for exclusion.

2.5.2 Isolation and quantitative determination of genomic DNA

For the DNA extraction DNA iPrepTM device and the iPrepTMPureLinkTMgDNA Blood Kit were used. (concentrated leukocyte preparation). DNA was stored in -20 ^{o}C, until it was used for genotyping. This kit allows for the rapid and automated isolation of genomic DNA from fresh or frozen blood samples or from a concentrated leukocyte preparation. The above device extracts 200μL DNA solution in 30 minutes from 13 samples of 300μL buffy coat, using the technology based on magnetic microspheres. The cells are lysed using the Lysis Buffer and the proteins are digested with Proteinase K. The resulting product is mixed with the magnetic microspheres (Dynabeads), which bind to DNA and then the microsphere bound DNA is isolated from the solution using magnetic separation. The microspheres are thoroughly washed with the washing buffer to remove the impurities. Genomic DNA is then eluted into the eluate solution, to a final volume of 200 μL and stored at -20 ° C for later genotyping.

Quantitative determination of DNA concentration: Quantification of the DNA concentration is done by a fluorimetric method. A fluorescent dye capable of binding to DNA molecules is added to the samples. The samples are then photometric on a fluorescence photometer and based on

the absorption of the fluorescent substance their concentration is determined. The concentration is determined using a standard reference curve, which is generated after photometry of samples of known DNA concentration.

2.5.3. Replication study of significant associated polymorphism

Although the GWA technique is the method of screening for the extensive investigation of genetic risk factors in osteoporosis, for the acceptance of the results of this approach, it is necessary to confirm them in samples of similar populations. Part of the study population participated in GEFOS/GENOMOS consortium [54], for de novo replication of GWAS meta-analysis. Therefore 307 samples had genotyped at Kbioscience laboratory. The iPlex Gold test, which belongs to the Multiple Polymerase Chain Reaction (PCR) methods, is applied. With multiplex PCR methods it is possible to detect multiple polymorphisms in a single reaction. In the context of GEFOS/ GENOMOS consortium had de novo genotyped 8 polymorphisms (rs11520772, rs2908007, rs2982552, rs3000634, rs3020331, rs597319, rs7741021, rs9292469) where meet the GWAS statistically significance level with the QUS parameters, BUA, SOS and SI. At 307 subjects of OSTEOS study, rs11520772 meets statistical significant for association with SOS parameter and selected for further genotyping of 245 subjects, in current study.

The genotyping for the rest 245 subjects was done using the machine StepOnePlus™ of the company Applied Biosystems StepOne™ with the analysis TaqMan® SNP Genotyping. This is a complete collection of mapped primers and probes leading to the determination of mononucleotide polymorphisms in human DNA samples. A special TaqMan® reagent is used to amplify and detect specific SNPs in purified genomic DNA samples. Reagent activity is optimized by adding a special master mix. The following protocol is used for genotyping using StepOnePlus ™:

1. Dilution of the pure genomic DNA is performed so that the nucleic acid content ranges from 1-20 mg to 4.5 μl to be added. The amount of 9μg was determined from where the final concentration of the nucleic acids was 2 μg / mL.

2. Add 4.5 μL of DNA to each well.

3. At each plate, at least 2 negative markers are added, where purified water is added instead of adding DNA.

4. Two positive markers are added to each plate, each containing genomic material for both versions of the polymorphic site.

5. Add 5.5 µL of the reaction mixture (consisting of 5 µl Master mix and 0.5 Ml Genotyping assay) to each well.

6. The plate is covered with a specific plastic cover.

7. Centrifuge the plate for spin down.

8. Insert to StepOnePlus ™

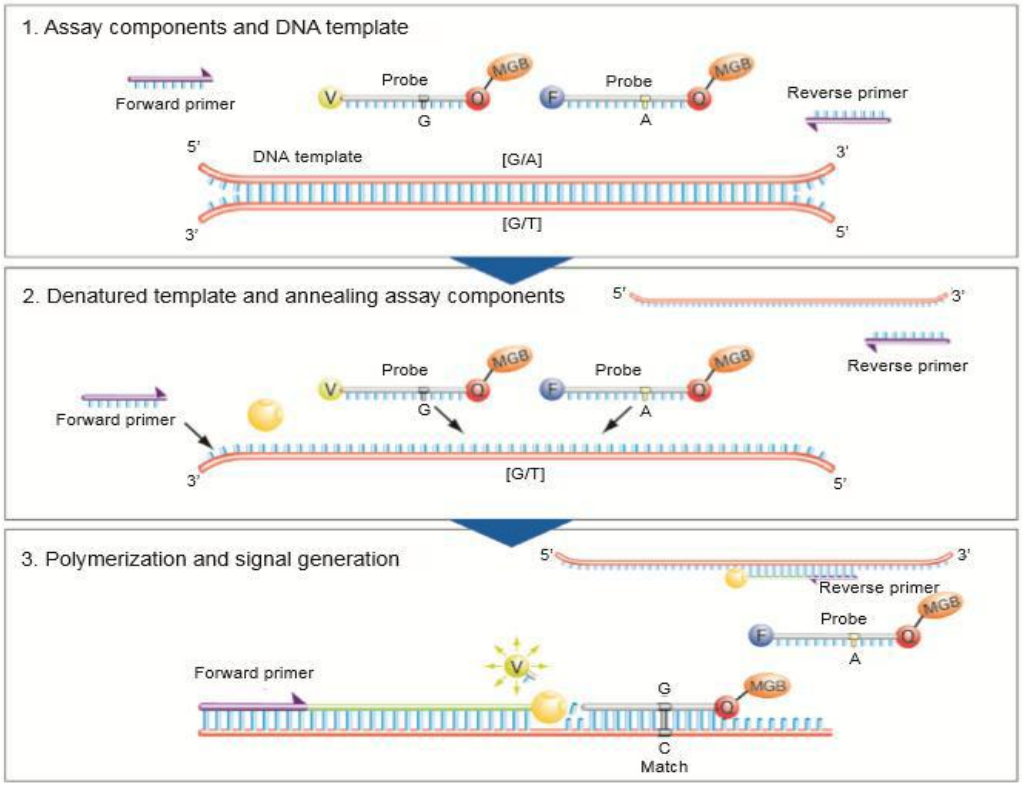


Figure 9: *The procedure of the genotypic identification method.*

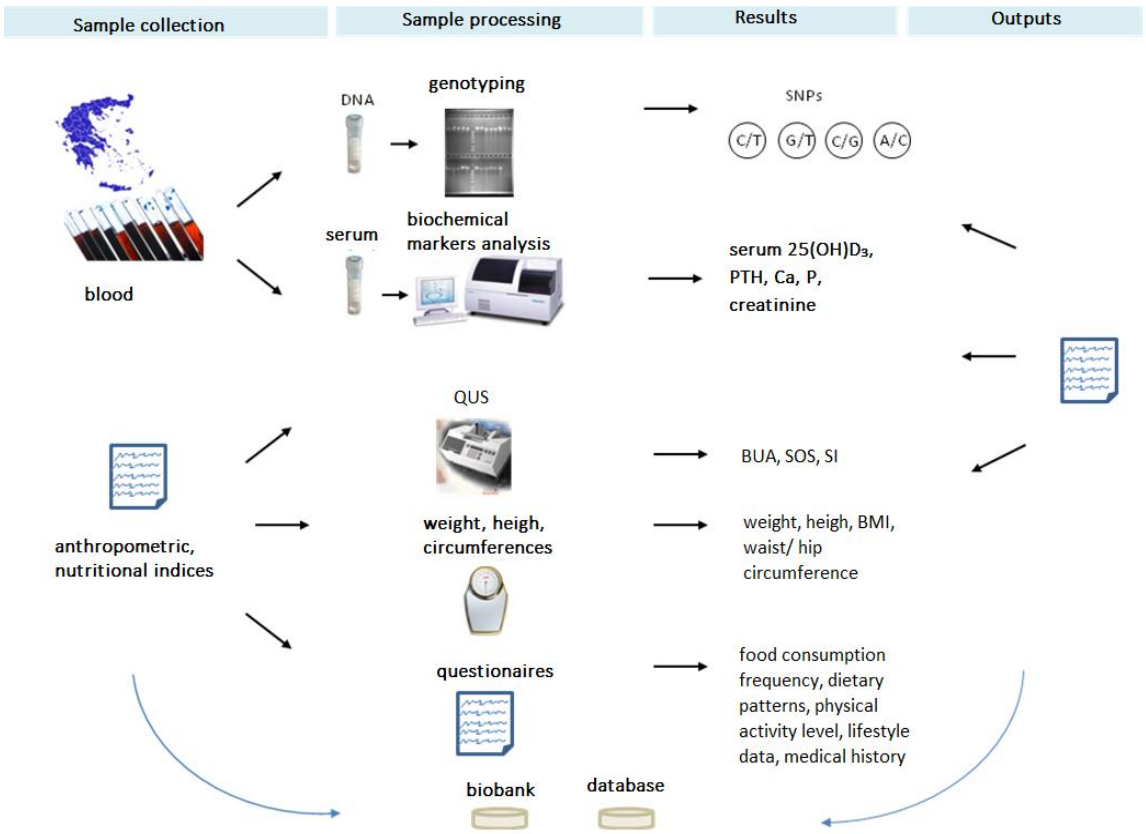


Figure 10: *Graphic Illustration of the study protocol.*

2.6. STATISTICAL ANALYSIS

Continuous variables are presented as mean values and SD, while categorical variables are presented as relative frequencies. Differences between categorical variables and groups of the study were assessed using the $\chi 2$ test. P–P plots were applied to assess the normality of the distribution of the continuous variables. Student's t test was applied to evaluate differences in continuous variables between the two study groups. Analyses were based on 2-sided tests, while statistical significance was set at $p \leq 0.05$. The statistical software packages IBM SPSS Statistics 13.0 and 21.0 (SPSS Inc., Texas, USA) were used for all statistical calculations, where appropriate.

Linear Regression and Univariate General Linear Model with a stepwise procedure (using bone structure and quality markers and serum vitamin D as dependent variables) are used to control for confounding factors (age, weight, height) and to examine possible interactions between the independent variables (risk factors). Analyses were based on 2-sided tests, while statistical significance was set at $p \leq 0.05$.

Concerning the dietary analysis, the transformations of foods and food groups from daily, weekly or monthly average intake to servings per week were calculated programmatically in R. Outliers were defined as values that exceed 5 SD above or below the mean and were removed from subsequent analyses.

Principal component analysis (PCA) was conducted to identify underlying dietary patterns [110]. In order for PCA to be effective in assessing food patterns, strong correlations between the predictive variables should exist. The correlation matrix of the food variables used in the present analysis showed that there were several correlation coefficients (r) > $|\cdot 4|$, indicating that the variables were highly correlated. Moreover, the Kaiser–Meyer–Olkin test of sphericity and Barlett's criterion was 0.76, implying high interrelationships between food variables and suitability of the data set for PCA. The orthogonal rotation (varimax option) was used to derive optimal non-correlated components (dietary patterns). From the entire database twenty food groups were used. To decide the number of components to retain the Kaiser criterion was used, according to which the number of components that should be retained from PCA is equal to the number of Eigenvalue > 1. In our analysis, six food patterns were selected. Based on the fact that factor loadings/correlation coefficients represent the correlation of each predicting variable with the dietary pattern score, higher absolute values indicate that the variable contributes more to the construction of this particular pattern. The dietary patterns were named according to scores of the predicting variables that correlated most with the component/pattern (> $|0\cdot 4|$). The PCA was performed using the statistical software package IBM SPSS Statistics for Windows, Version 19.0. Outliers were defined as values that exceed 3 SD above or below the mean and were removed from subsequent analyses.

A voluntary participation agreement was obtained from each participant. The study was approved by the Ethics Committee of Harokopio University.

3. RESULTS AND DISCUSSION

The current dissertation was divided into 4 work sections, from which four manuscripts appeared, which are presented in this section (results and the discussion).

In the first work section the serum 25(OH)D status, the quantitative ultrasound parameters as well as their determinants in the current population, was identified.

In a population of 970 subjects (134 males and 836 females), the mean serum 25(OH)D concentration was 20.00 ± 8ng/mL. The 54% of the population had Vitamin D deficiency (25(OH)D < 20ng/mL) and the 12,3 % meet the sufficient levels. The mean weight was 73.07±14.7 kilograms and the mean height was 1.63±0.08 metres.

Descriptive characteristics of the total population and according to the Vitamin D status are demonstrated in table 5. The Vitamin D status by gender and by age group is demonstrated in the figure 11 and in the figure 12, respectively. Table 6 demonstrates the mean level of 25(OH)D, PTH, BUA, SOS, SI, by age group, BMI categories, seasons of examination and area of residence. Urban residents had higher SOS values than rural residents, after adjustment for age, gender and time of participation in organized physical activity. Searching for contributors of 25(OH)D deficiency (<20 ng/mL) we revealed that obesity status increases 1.458 times (p=0.006), the risk in all age groups. Vitamin D deficiency was also related to lower SOS values in the current population in a relation to SOS values of subjects with 25(OH)D< 20ng/mL (1561.5 ± 37 and 1553.2 ± 46.6, p = 0.008, respectively) and after adjustment for age (B = 13.04, p = 0.04). The correlation among 25(OH)D, PTH and QUS parameters, and the other anthropometric, biochemical, and life-style parameters is presented in Table 7. The determinants of serum 25(OH)D levels are presented in table 8.

Table 5. Descriptive characteristics of population

	Total (mean ± SD)	Severe deficiency (mean ± SD)	Deficiency (mean ± SD)	Insufficiency (mean ± SD)	Adequacy (mean ± SD)	*p* value
N (number of subjects)	970	78	440	333	119	
Age (years)	49.58 ± 13.54	51.01 ± 15.12	50.81 ± 13.50	47.97 ± 13.17	48.59 ± 13.37	0.21
BMI (kg/m^2)	27.53 ± 5.6	28.56 ± 7.21	28.14 ± 5.67	27.07 ± 5.18	25.79 ± 4.78	0.00
PTH (pg/mL)	40.4 ± 15.6	47.8 ± 19.1	41.1 ± 15.2	38.8 ± 14.5	37.7 ± 15.8	0.00
BUA (dB/MHz)	115.1 ± 16.31	111.7 ± 17.62	114.12 ± 16.36	116.38 ± 16.06	115.4 ± 16.29	0.377
SOS (m/s)	1553.3 ± 85.73	1550.6 ± 42.78	1544.94 ± 120.24	1562.22 ± 35.76	1558.16 ± 40.22	0.098
SI	91.31 ± 19.68	90.24 ± 23.67	90.97 ± 19.20	91.68 ± 18.96	92.48 ± 20.74	0.917
Sun exposure winter (hours/day)	5.09 ± 5.5	4.14 ± 4.96	5.0 ± 5.48	5.65 ± 5.8	4.86 ± 5.0	0.195
Sun exposure summer (hours/day)	12.61 ± 11.02	10.75 ± 11.36	11.93 ± 10.46	13.52 ± 11.54	13.7 ± 11.27	0.084
TV watching or PC activity (hours/day)	3.09 ± 2.24	3.22 ± 1.94	3.15 ± 2.31	2.95 ± 2.06	3.18 ± 2.63	0.609
Organized physical activity moderate and/or vigorous (minutes/day)	13.46 ± 32.33	7.09 ± 15.68	10.16 ± 26.84	14.92 ± 37.46	24.84 ± 39.24	0.00

Severe deficiency < 10 ng/mL, deficiency 10–19.9 ng/mL, insufficiency 20–29.9 ng/mL, adequacy ≥ 30 ng/mL

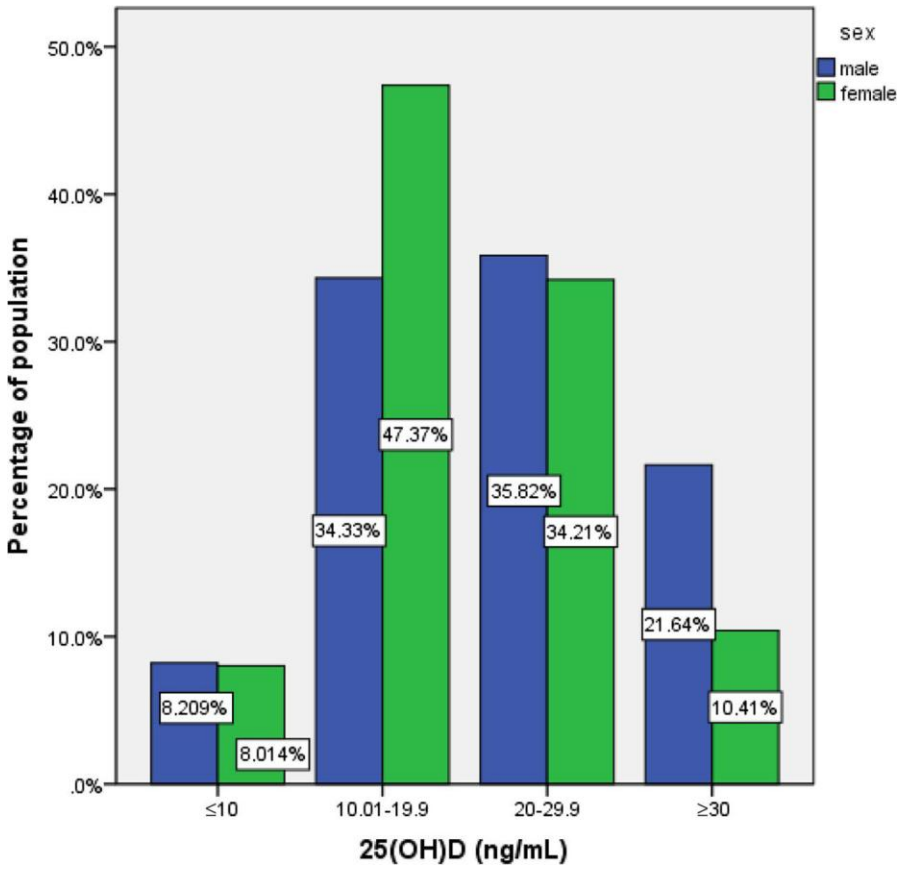


Figure 11. Percentages of males and females with severe deficiency (≤10 ng/mL), deficiency (10–19.9 ng/mL), insufficiency (20–29.9 ng/mL), and adequacy (≥30 ng/mL) of serum 25(OH)D.

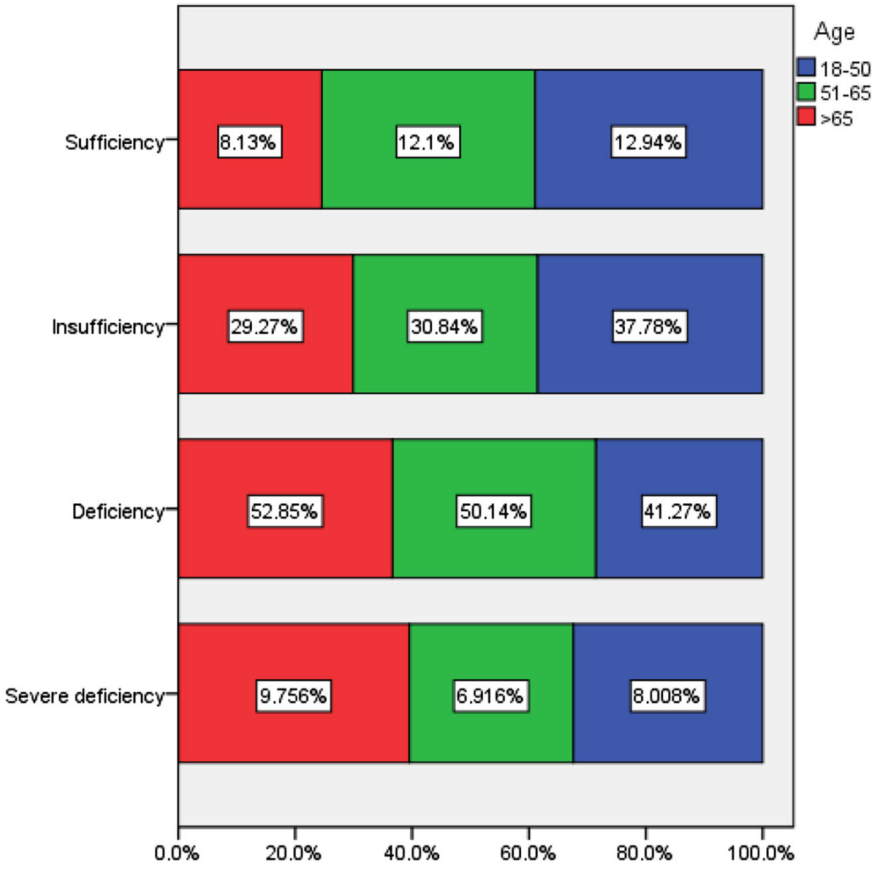


Figure12. Age-specific prevalence of different categories of vitamin D status.

Table 6. Mean level of 25(OH)D, PTH, BUA, SOS, SI, by age group, BMI categories, seasons of examination and area of residence.

	25(OH)D (ng/mL)	PTH(pg/mL)	BUA (dB/MHz)	SOS (m/s)	SI
Age group (years) (%population)					
18–50 (50.9%)	20.5 ± 8.1*	36.0 ± 14.3*	119.68 ± 15.56*	1565.46 ± 80.98*	98.78 ± 18.49*
51–65 (36.3%)	19.7 ± 7.8	43.6 ± 14.9*	110.26 ± 14.71*	1537.22 ± 101.1*	85.03 ± 17.55*
> 65 (12.8%)	18.5 ± 7.9*	47.9 ± 15.9*	104.76 ± 16.14*	1533.95 ± 32.9*	76.85 ± 16.8*
BMI category (kg/m^2) (%population)					
Underweight (< 18.5) (1.2%)	21.8 ± 8.2	30.4 ± 10.5*	109.49 ± 12.81	1555.06 ± 25.9	93.83 ± 12.69
Normal weight (18.5–24.9) (35.3%)	20.9 ± 8.6*	35.9 ± 14.1*	113.33 ± 16.18	1558 ± 35.4	90.13 ± 18.71
Overweight (25–29.9) (37.1%)	20.1 ± 7.5	41.3 ± 16.0*	115.05 ± 16.8	1557.37 ± 37.8	90.46 ± 20.25
Obesity (≥ 30) (26.3%)	18.6 ± 7.0*	44.6 ± 14.9*	117.3 ± 16.06	1541 ± 155.6	94.09 ± 20.3
Season of examination (%population)					
Winter-spring (43%)	19.2 ± 7.7*	42.7 ± 15.1*	114.69 ± 17.02	1553.66 ± 94.06	90.39 ± 19.68
Summer-autumn (57%)	20.6 ± 8.1*	38.6 ± 15.7*	115.36 ± 15.84	1553.01 ± 79.5	92.18 ± 19.66
Area of residence					
Urban (72.5%)	20.4 ± 8.4*	39.3 ± 15.9*	115.23 ± 16.31	1549.14 ± 97.33*	90.37 ± 20*
Rural (27.5%)	19.0 ± 6.5*	43.1 ± 14.4*	114.7 ± 16.35	1564.51 ± 37.8*	94.32 ± 18.4*

*p value < 0.05 from one-way ANOVA or independent-samples T test

Table 7. Correlation among 25(OH)D, PTH and QUS parameters, and the other anthropometric, biochemical, and life-style parameters.

Continuous variables	25(OH)D r (p value)	PTH r (p value)	BUA r (p value)	SOS r (p value)	SI r (p value)
Age (years)	0.086 (0.008)	0.321 (0.00)	− 0.359 (0.00)	− 0.359 (0.00)	0.437 (0.00)
25(OH)D (ng/mL)		− 0.162 (0.00)	0.042 (0.252)	0.073 (0.051)	0.029 (0.483)
PTH (pg/mL)	− 0.162 (0.001)		− 0.125 (0.001)	− 0.135 (0.00)	− 0.170 (0.00)
BMI (kg/m^2)	− 0.141 (0.00)	0.244 (0.00)	0.120 (0.001)	0.001 (0.980)	0.071 (0.081)
BUA dB/MHz	0.042 (0.252)	− 0.125 (0.00)		0.595 (0.00)	0.896 (0.00)
SOS m/s	0.073 (0.051)	− 0.135 (0.00)	0.595 (0.00)		0.71 (0.00)
SI	0.029 (0.483)	− 0.170 (0.00)	0.896 (0.00)	0.711 (0.00)	
Sun exposure winter (hours/day)	0.043 (0.191)	0.021 (0.531)	− 0.002 (0.955)	− 0.014 (0.707)	0.041 (0.321)
Sun exposure summer (hours/day)	0.122 (0.00)	− 0.014 (0.679)	0.009 (0.811)	0.036 (0.341)	0.054 (0.199)
TV watching or PC activity (hours/day)	− 0.009 (0.800)	0.034 (0.324)	0.006 (0.867)	− 0.018 (0.633)	− 0.001 (0.972)
Organized physical activity moderate and/or vigorous (minutes/day)	0.170 (0.00)	− 0.58 (0.086)	0.100 (0.007)	0.093 (0.013)	0.090 (0.029)

Table 8. Serum 25(OH)D determinants.

Variable	B (standard errors)
Intercept	20.384 (1.136)***
Sex (ref. female)	
Male	1.796 (0.805)*
Season (ref. summer-autumn)	
Winter-spring	− 1.530 (0.566)**
Age × subjects BMI < 30 kg/m^2	− 0.019 (0.021)
Age × obese subjects (BMI ≥ 30 kg/m^2)	− 0.043 (0.021)*
Summer sun exposure (hours/day)	0.063 (0.025)***
Organized physical activity moderate and/or vigorous (minutes/day)	0.037 (0.008)*
Adjusted *R* squared	0.053

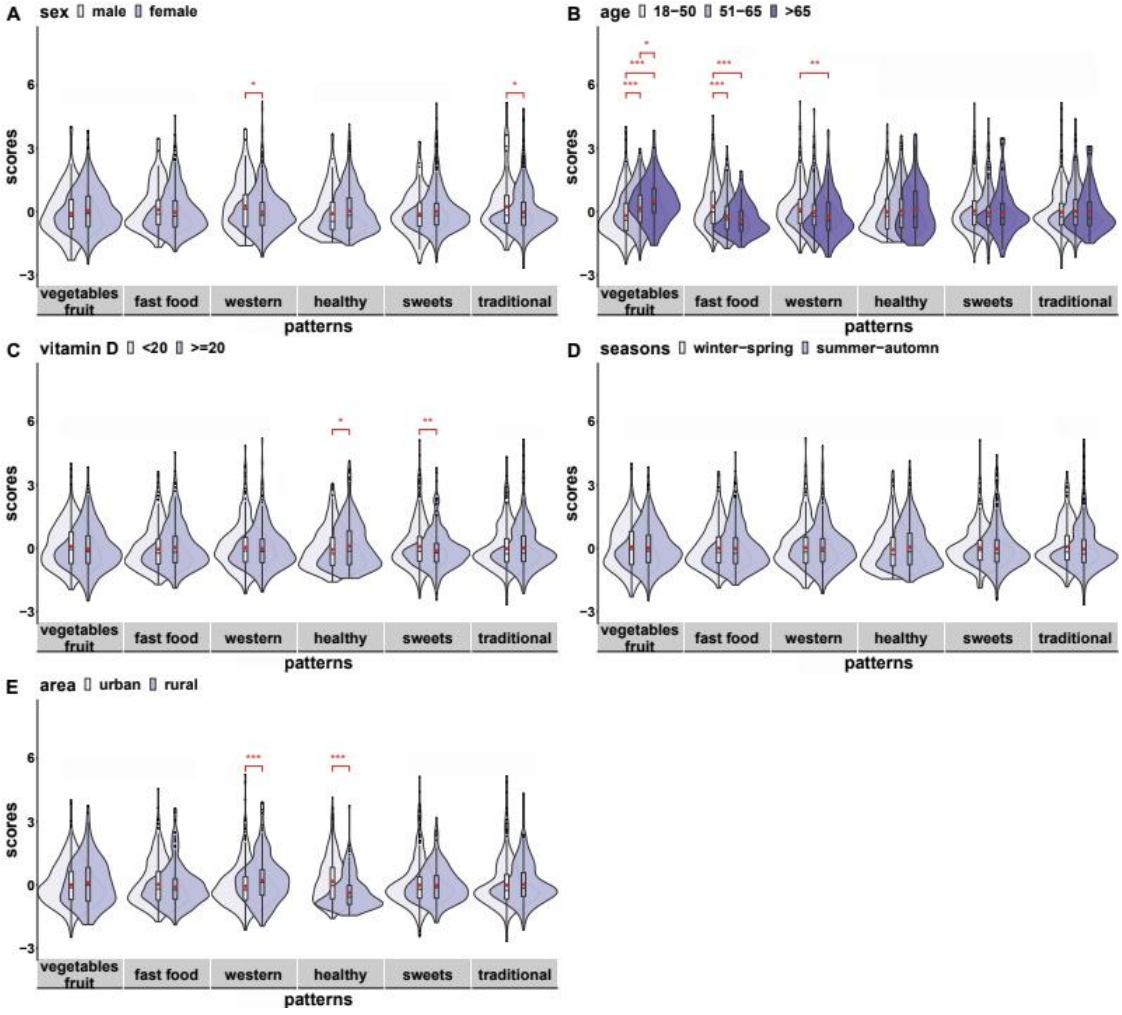
*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

In the second work section the dietary patterns of the population were identified as well as their effect on serum 25(OH)D levels and quantitative ultrasound parameters. From the PCA analysis six dietary patterns derived which explain 52.2% of the variability of Greek adults' nutritional habits. The components were defined as follows: component 1: 'vegetables-fruit' pattern, which includes cooked and raw vegetables, refined rice, fresh fruits and fish; component 2: 'fast-food' pattern, that consists of refined breads, processed meat, full fat cheese; component 3: 'western' pattern, that is characterized by red meat, refined pasta, potatoes and poultry; component 4: 'healthy' pattern that includes low fat milk and yogurt, refined breakfast cereals, non-refined breads and low fat cheese; component 5: 'sweets' pattern with milky and starchy sweets; component 6: 'traditional' pattern includes full fat dairy products as main constitute as well as legumes. The correlation between dietary patterns and continuous variables are presented in table 9. The positive association between the adherence to the "healthy" pattern and serum 25(OH)D and the negative association between the adherence to the "sweets" pattern and serum 25(OH)D is maintained after adjustment for the age, the gender, the BMI, the minutes per day of participation to organized physical activity and the hours of summer sun exposure (B=0.667, p=0.024 and B= -0.919, p=0.002). The positive effect of the adherence to the "western" dietary pattern to the BMI, is maintained after adjustment for the age, the gender, the hours spending in sedentary activities per day and the minutes per day of participation to organized physical activity (B=0.532, p=0.010). Subjects more adapted to "healthy" dietary patter have more minutes per day of participation to

organized physical activity after adjustment for the age, the gender and the BMI (B=3.704, p=0.000). The distribution of scores derived from PCA among sex, age groups, serum Vitamin D status, season of blood sampling and area of residence, is presented in figure 13. Males are more adherent to “western” and “traditional” dietary pattern after adjustment for age (B =0.255, p=0.032 and B=0.258, p=0.034). Urban residents are less adherent to “western” and more adherent to “healthy” pattern after adjustment to age, gender and BMI (B=-0.266, p=0.001 and B=0.562, p=0.000), than rural residents. Taking into account the nutritional factor, the determinants of 25(OH)D, are shaped as it shown in table 10.The determinants of BUA, SOS and SI are presented in tables 11, 12 and 13.

Table 9. Correlation between dietary patterns and continuous variables.

N=741	**1- vegetables-fruit**	**2- fast food**	**3-western**	**4- healthy**	**5-sweets**	**6- traditional**
Continuous Variables	**r (*p*-value)**	**r (*p*-value)**	**r (*p*-value)**	**r (*p*-value)**	**r (*p*-value)**	**r (*p*-value)**
Age (years)	**0.244*** (0.00)	**-0.272** (0.00)	-0.116 (0.002)	0.017 (0.65)	-0.061 (0.099)	-0.029 (0.430)
25(OH)D (ng/mL)	-0.066 (0.074)	0.017 (0.653)	-0.05 (0.173)	**0.107** (0.004)	**-0.119** (0.001)	0.018 (0.628)
PTH (pg/mL)	**0.101** (0.007)	**-0.077** (0.039)	-0.011 (0.773)	**-0.141** (0.00)	**0.086** (0.022)	-0.001 (0.974)
BMI (kg/m^2)	**0.074** (0.046)	-0.025 (0.498)	**0.094** (0.012)	-0.055 (0.144)	-0.058 (0.120)	0.004 (0.922)
BUA dB/MHz	-0.035 (0.394)	**0.102** (0.014)	0.067 (0.105)	**0.089** (0.03)	0.047 (0.256)	0.001 (0.980)
SOS m/s	-0.054 (0.199)	0.012 (0.778)	-0.009 (0.823)	0.046 (0.277)	0.075 (0.074)	-0.001 (0.974)
SI	-0.079 (0.084)	0.071 (0.121)	**0.098** (0.032)	0.046 (0.320)	-0.006 (0.893)	0.016 (0.721)
Sun exposure summer (hours/day)	0.040 (0.287)	0.068 (0.070)	0.011 (0.778)	0.05 (0.182)	0.005 (0.904)	0.050 (0.182)
TV watching or PC activity (hours/day)	-0.027 (0.481)	-0.036 (0.349)	-0.022 (0.562)	0.032(0.396)	0.033 (0.383)	-0.04(0.293)
Organized physical activity moderate and/or vigorous (minutes/day)	-0.011 (0.763)	0.027 (0.459)	0.024 (0.526)	**0.146** (0.00)	-0.021 (0.564)	0.037 (0.318)



Notes: * p<0.05, ** p<0.01, *** p<0.001, • mean, ‗ median

Figure 13: Distribution of scores derived from PCA among sex, age groups, serum Vitamin D status, season of blood sampling and area of residence

Table 10. Determinants of serum 25(OH)D.

Variable	**B (Standard errors)**
Intercept	20.993 (1.804)***
BMI (kg/m^2)	-0.157 (0.054)**
Age group (ref. >65 years) **18-50 years** **61-65 years**	 1.948 (0.959) * 1.847 (0.986)
Summer sun exposure (hours/day)	0.091(0.027)***
Organized physical activity moderate and/or vigorous (minutes/day)	0.042 (0.011)***
'Healthy' dietary pattern adherence * Winter	1.058 (0.497) *
'Healthy diet' dietary pattern adherence * Summer	0.441 (0.358)
'Sweets' dietary pattern adherence	-0.911 (0.293)**
Adjusted R Squared	0.081

Notes: *p<0.05, **p<0.01, ***p<0.001

Table 11. Determinants of BUA.

Variable	**B (Standard errors)**
Intercept	83.906 (3.816)***
Sex (ref. female) Male	 5.189 (1.877)**
BMI (kg/m^2)	0.571 (0.112)***
Age group (ref. >65 years) **18-50 years** **61-65 years**	 19.310 (2.204)*** 8.662 (2.289)***
Adherence to 'healthy' food pattern	1.878 (0.615)**
Adjusted R Squared	0.197

Notes: *p<0.05, **p<0.01, ***p<0.001

Table 12. Determinants of SOS.

Variable	B (Standard errors)
Intercept	1614.329 (14.118)***
Age (years)	-1.086 (0.271)***
TV watching or PC activity (hours/day) * 25(OH)D<20ng/mL	-5.348 (1.821)**
TV watching or PC activity (hours/day) * 25(OH)D≥20ng/mL	-0.960(2.130)
Adjusted R Squared	0.041

Notes: *p<0.05, **p<0.01, ***p<0.001

Table 13. Determinants of SI.

Variable	B (Standard errors)
Intercept	75.188 (2.416)***
Organized physical activity moderate and/or vigorous (minutes/day)	0.065 (0.029)*
Age group (ref. >65 years) **18-50 years** **61-65 years**	 22.402 (2.638)*** 8.352 (2.728)**
Adjusted R Squared	0.190

Notes: *p<0.05, **p<0.01, ***p<0.001

In the third module, the effect of 8 polymorphisms (rs11520772 on TAX1BP1, rs2908007 on WNT16, rs2982552 on ESR1, rs3000634 on USPL1, rs3020331 on ESR1, rs597319 on TMEM135, rs7741021 on RSPO3 and rs9292469 on NPR3), were derived from the GEFOS/ GENOMOS consortium, on the values of the ultrasound parameters, was evaluated. Two of these polymorphisms (rs11520772 on TAX1BP1 and rs597319 on TMEM135), had a significant effect on the SOS and SI values respectively and first associated with bone phenotypes the GEFOS/ GENOMOS consortium. The tables 14 and 15 present the effect of these polymorphisms on SOS and SI values.

Table 14. Determinants of SOS parameter.

Variable	**B (Standard errors)**
Intercept	1563.389 (24.901)***
rs11520772 (ref. TT-genotype)	
AA	47.368 (18.272)**
AT	34.414 (19.126)
Age (years)	-1.067 (0.368)*
Adjusted R Squared	0.028

Table 15. Determinants of SI parameter.

Variable	**B (Standard errors)**
Intercept	109.898 (4.412)***
rs597319 (ref. GG-genotype)	
AA	11.570 (2.971)***
AG	7.738 (2.965)*
Age (years)	-0.590 (0.073)**
Sex (ref. female)	
male	5.697 (2.889)
Adjusted R Squared	0.197

The subject we dealt with in the fourth module of work was the identification of new genetic markers of BUA and SOS parameters. It was derived from our cooperation with GEFOS/ GENOMOS consortium. In the context of the above framework, Genome Wide Association, as well as replication study was conducted, in more than 58,000 participants. Nine polymorphisms were ultimately associated with bone health markers.

The manuscripts (published or unpublished reports) are grouped in the following subsections.

- **Published reports (subsection 3.1)**

a. Paper 1. Serum 25-Hydroxyvitamin D Status, Quantitative Ultrasound Parameters And Their Determinants In Greek Population.

- **Unpublished Reports (subsection 3.2)**

a. Paper 2. Dietary patterns of Greek adults and their effects on serum Vitamin D levels and Quantitative Ultrasound parameters.

b. Paper 3. The effect of rs11520772 of the TAX1BP1 gene and the rs597319 of the TMEM135 gene polymorphisms on Quantitative Ultrasound (QUS) parameters. The OSTEOS study.

- **Consortia participation (subsection 3.2)**

a. Paper 4. Genetic determinants of heel bone properties: genome-wide association meta-analysis and replication in the GEFOS/GENOMOS consortium.

3.1. Published reports

3.1.1. Paper 1. Serum 25-Hydroxyvitamin D Status, Quantitative Ultrasound Parameters And Their Determinants In Greek Population.

This paper presents the descriptive characteristics of OSTEOS study, as well as the clinical characteristics/biochemical indices, along with the lifestyle habits of the participants. The Vitamin D Status of Greek population and its determinants are also examined.

Key points

- 54% of Greek adults had serum Vitamin D <20 ng/mL
- Serum Vitamin D <20 ng/mL was related to lower SOS values
- Obese individuals had 1.5 times increased risk to having serum Vitamin D <20 ng/mL
- Vitamin D negatively affected by female sex, winter-spring season, age in obesity
- Summer sun exposure and organized physical activity affect positively Vitamin D.

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ORIGINAL ARTICLE

Serum 25-hydroxyvitamin D status, quantitative ultrasound parameters, and their determinants in Greek population

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Abstract

Summary Vitamin D deficiency and quantitative ultrasound measurements are associated with bone fragility. We assessed these parameters and their correlates. 87.7% of the population has vitamin D inadequacy and this correlated with lifestyle factors. These results contribute to epidemiological data needed for population guidelines for bone health.

Purpose Vitamin D deficiency and quantitative ultrasound (QUS) parameters are among the most important clinical risk factors of bone fragility. Few data are available for Greek population. The aim of the study was to evaluate the serum 25-hydroxyvitamin D [25(OH)D] level and their determinants, as well as QUS parameters in Greek population.

Methods OSTEOS is an observational cross-sectional study conducted from June 2010 to July 2012. Nine hundred seventy adults were recruited from rural and urban areas throughout Greece and completed the appropriate questionnaire. Serum 25(OH)D measured by enzyme immunoassay, QUS parameters, broadband ultrasound attenuation (BUA), speed of sound (SOS) and stiffness index (SI), was assessed with an Achilles device. Univariate Analysis of Variance was used for the assessment of serum 25(OH)D determinants.

Results Mean serum 25(OH)D of the total population was 20,00 ± 8,00 ng/mL. Females had lower levels than males. The negative determinants of serum 25(OH)D in the total population were the female sex and the winter-spring season of sampling while age proved negative association solely in obese subjects. Positive determinants of vitamin D status were summer sun exposure and organized physical activity as expected. Urban had lower SOS and SI than rural residents. Individuals with 25(OH)D ≥ 20 ng/mL had higher SOS than those with 25(OH)D < 20 ng/mL. BUA, SOS, and SI are positively correlated with organized physical activity and negatively with PTH.

Conclusions This study reports that vitamin D deficiency is highly prevalent among healthy Greek men and women, demonstrates the multifactorial causation of 25(OH)D levels, and points out that further research is required to determine more factors related to vitamin D status and bone health.

Keywords Vitamin D · Life-style factors · Heel bone properties · Epidemiology

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11657-018-0526-5) contains supplementary material, which is available to authorized users.

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Introduction

Osteoporosis is a systemic skeletal multifactorial disease characterized by reduced bone mass and microarchitectural deterioration of the structure of bone tissue leading to bone fragility and increased susceptibility to fractures. Osteoporosis is a major public health concern. Women have a higher risk of osteoporotic fractures compared with men (1 in 3 women and 1 in 5 men over 50 years) [1]. Although BMD is the main predictive risk factor for an osteoporotic fracture, measurement of quantitative ultrasound (QUS) has been found to be associated with increased fracture risk [2]. The QUS measuring at the heel is an alternative, ionizing radiation-free and relatively inexpensive, portable screening technique that is able to identify women at high risk of bone fragility and fracture [3, 4] and may be used by general practitioners in primary care.

Vitamin D deficiency is also among the most important clinical risk factors of bone fragility and a subject of extensive research. The main physiological effect of vitamin D is to increase intestinal calcium absorption, as such maintaining serum calcium in order to maximize metabolic functions, signal transduction, and neuromuscular activity. The consequences of vitamin D deficiency in adults are osteomalacia, acceleration of bone loss, muscle weakness, instability, and therefore increased risk of falling [4]. In children, lack of vitamin D causes rickets and growth retardation. Vitamin D receptors are found in enterocytes, osteoblasts, the cells of the distal convoluted renal tubules, the cells of the parathyroid gland, colon, pituitary, ovaries, the cells of the immune system, etc. Therefore, vitamin D low levels were associated, by many observational studies, with major diseases, such as osteoporosis, diabetes, some forms of cancer, autoimmune diseases, infectious diseases, and hypertension but causality have not been proven [4].

Serum 25-hydroxyvitamin D (25(OH)D) is considered as the marker of vitamin D status. Guidelines about prevention of vitamin D deficiency suggest serum 25(OH)D levels above 20 ng/mL [5]. However, some experts suggest for maximal effect of vitamin D on calcium, bone, and muscle metabolism; levels of 25(OH)D should be above 30 ng/mL. Several epidemiological studies have proposed that a 25(OH)D above 30 ng/mL may have additional health benefits in reducing the risk of common cancers, autoimmune diseases, type 2 diabetes, cardiovascular disease, and infectious diseases [6].

There have been several recent consensus statements or guidelines that have included definitions of vitamin D deficiency. It is generally agreed that the serum concentration of 25(OH)D is the best marker of an individual's vitamin D status because it is the major circulating form and reflects the combination of dietary intake and cutaneous skin synthesis. However, different thresholds for the level of 25(OH)D that is considered to reflect efficiency are used. For example, the Institute of Medicine's report on the dietary reference intake for vitamin D published in 2010 [6] defined a level of 50 nmol/L (< 20 ng/mL) as meeting the needs of 97.5% of the population, whereas the Endocrine Society Clinical Practice Guideline published in 2011 defined vitamin D deficiency as a level < 50 nmol/L, with levels of between 52.5 nmol/L (21 ng/mL) and 72.5 nmol/L (29 ng/mL) regarded as vitamin D insufficiency, and levels of greater than 72.5 nmol/L being regarded as optimal [7]. In the current study, we decided to use the following definitions: severe deficiency < 25 nmol/L (< 10 ng/mL), deficiency 25–50 nmol/L (10–19.9 ng/mL), insufficiency 50–75 nmol/L (20–29.9 ng/mL), sufficiency ≥ 75 nmol/L (≥ 30 ng/mL).

Although recent data indicate that prevalence of vitamin D deficiency may be common in countries previously considered as low risk (e.g., Mediterranean countries) [4], few data are available for the Greek population. Low levels occur in elderly [8], children, adolescents [7], and adults [9] mainly from urban areas of Greece. Therefore, the objective of our study was to investigate the vitamin D status in adult women and men from northern and southern regions of Greece including several islands, in relation to QUS parameters.

Methods

Subjects

OSTEOS is an observational cross-sectional study, conducted from June 2010 to July 2012. A population of 970 community-dwelling adults (133 males, 830 females) was randomly recruited at the health promotion events carried out by the Hellenic Society for the Support of Patients with Osteoporosis in rural and urban areas throughout Greece. The regions were Central Greece, including Attica, Peloponnese, Thessaly, Aegean Islands, and Macedonia, and especially in more than 10 municipalities of Attica, Ilia, Patmos island, Viotia, Fokida, Evia, Korinthos, Halkida, Amfissa, Salamina, Fthiotida, Pella, Elassona, Volos, and Thessaloniki. Subjects were informed about events by announcements at municipalities and local authorities. Demographic data and medical history of the subjects were obtained using an appropriate questionnaire.

The International Physical Activity Questionnaire (IPAQ, short version), a self-administered tool, was used for the assessment of hours spent on sedentary activities (television, personal computer) and in moderate or vigorous organized physical activity; it was completed under the surveillance of the investigator [10].

To evaluate the hours of sun exposure, the subjects were asked how many hours per week between 9:00–18:00 on average they were exposed to the sun, in the summer, and in the winter.

At around 35° north latitude and above (Greece 35°–40°), little or no vitamin D can be produced from sun exposure from November to February. 25(OH)D level reaches its nadir in late winter and it takes about 6 weeks to raise the serum levels. Therefore, we defined two seasons to classify the subjects according to the blood collection day, winter-spring (December until May) and summer-autumn (June until November) [11, 12].

Measurements

Body weight was measured with a calibrated scale to the nearest 0.1 kg wearing light clothing and no shoes. The height was measured by a mounted stadiometer to the nearest 0.5 cm. Weight and height were measured twice by the same investigator, measurements were averaged for each participant, and the body mass index (BMI) was calculated. BMI was classified according to the World Health Organization [13] into four categories: Underweight (< 18.5 kg/m^2), normal weight (18.5–24.9 kg/m^2), overweight (25–29.9 kg/m^2), and obesity (≥ 30 kg/m^2).

Following a 12-h fast, all subjects had a sample of venous blood withdrawn for serum isolation between 08:00–09:00 am. Total calcium (Ca), phosphorus (P), parathyroid hormone (PTH), and 25(OH)D were measured. Intact parathyroid hormone (iPTH) measurements were performed on a Roche/Modular Analytics analyzer, which employs electrochemiluminescence immunoassay technology (ECLIA). The intra- and inter-assay coefficients of variation (CVs) of the iPTH assay were less than 7% and the analytical sensitivity was 1.2 pg/mL. Serum 25(OH)D levels were determined by enzyme immunoassay [Immunodiagnostic Systems, 25(OH)D; Boldon, UK]. All the assays were performed in a single batch and in the same laboratory. The sensitivity of this assay is 5 nmol/L and intra- and inter-assay coefficients of variation of 5.3% and 4.6%, respectively. Standardization of the different vitamin D assays is the key to achieving comparable results across different methods and manufacturers. Furthermore, assay standardization is of critical importance for the establishment of common clinical cutoffs and their use in routine practice. Applying a common cutoff value on results generated with poorly standardized assays will inevitably lead to inconsistent patient classification and inappropriate therapeutic decisions. In 2010, the Vitamin D Standardization Program (VDSP) was established to improve the standardization of 25(OH)D assays. The aim of VDSP is that 25(OH)D measurements are accurate and comparable over time, location, and laboratory procedure to the values obtained using reference measurement procedures (RMPs) developed at the NIST [14] and Ghent University [15]. According to VDSP, a routine method is considered as standardized if the CV is < 10% and the bias < 5% [16]. The method we have used fulfills the criteria to be considered standardized according to CDC website http://www.cdc.gov/labstandards/pdf/hs/CDC_Certified_Vitamin_D_Procedures.pdf.

Heel bone properties were measured using the Achilles quantitative ultrasound (QUS) device, a water-bath ultrasound system into which the subject places his heel. Achilles generates a band of frequencies from 200 to 600 kHz. It measures the broadband ultrasound attenuation (BUA), expressed in dB/MHz, and measures the ultrasound attenuation with the incident frequency of wave sound. The speed of sound (SOS) is expressed in metres per second and means the necessary time for ultrasound waves to go through a determined distance inside the calcaneus bone. The third variable, stiffness index (SI), is automatically calculated by Achilles from the BUA and the SOS, using the equation SI=0.67*BUA+0.28*SOS-240 [17]. The lower the QUS parameter values, the higher the fracture risk [18, 19]. For normative data, we used reference data for the QUS measurements of the calcaneus specific for Greek population [20]. A quality-control procedure using the standard phantom was performed daily before the measurements in the present study. In vivo short-term precision calculated from four repeated measurements by the same operator, with repositioning, on 15 unselected subjects and expressed as the root mean square of the coefficients of variation (CV) was 2.28% for SI. A single ultrasonometer was used throughout the study, and all measurements were carried out by specially trained technicians.

Subjects with a known history of metabolic bone diseases were excluded from the study. People with endocrine diseases, malignancies, connective tissue diseases, malabsorption syndrome, inflammatory bowel diseases (ulcerative colitis, Crohn's disease), liver cirrhosis, renal failure, and having taken drugs that affect bone metabolism were excluded from the study. Early menopause (< 40 years) and amenorrhea > 1 year were also exclusion criteria. Finally, subjects taking calcium and/or vitamin D supplements were excluded from the study.

Statistical analyses

Statistical analysis was conducted using SPSS 19 statistical software. Continuous variables are presented as mean ± standard deviation, while categorical variables are presented as relative frequencies. Analysis of variance (ANOVA) was used to examine differences among the groups for different continuous, while the chi-square test was used to evaluate associations between categorical variables. Independent relationships between vitamin D status and other variables were assessed by stepwise multiple regression. All tests are two-sided with significance level < 0.05.

A voluntary participation agreement was obtained from each participant. The study was approved by the Ethics Committee of Harokopio University.

Results

A total of 970 subjects were included in this study. The mean age of the population was 49.58 years (range, 18–86 years), while 87.2% were 18–65 years old. The mean serum 25(OH)D level of the population was 20.00 ± 8.00 ng/mL. The 57% of specimens were collected during the summer-autumn period while the rest 43% were collected during the winter-spring period. The remarkable percentage of 54% within our study population was found vitamin D deficient while only 12.3% reached the adequate level (data not shown). Figure 1 shows the percentages of men and women having severe deficiency (≤ 10 ng/mL), deficiency (10–19.9 ng/mL), insufficiency (20–29.9 ng/mL), and adequacy (≥ 30 ng/mL) of serum 25(OH)D. Descriptive characteristics of the population and their differences among vitamin D status categories are presented in Table 1.

The age-specific prevalence of different categories of vitamin D status is presented in Fig. 2. Mean levels of 25(OH)D, PTH, BUA, SOS, SI, by age group, BMI categories, seasons of examination, and area of residence are presented in Table 2. According to the age group, serum vitamin D is higher in the 18–50 age group in relation to ≥ 65 years ($p = 0.032$). Mean serum PTH is lower in subjects 18–50 years in relation to 51–65 and ≥ 65 years and the 51–65 age group had lower PTH than ≥65 years ($p = 0.00$). Mean SI and BUA are higher in the 18–50 age group in relation to 51–65 and ≥ 65 years and in 51–65 in relation to ≥ 65 years ($p = 0.00$). Mean SOS is higher in the 18–50 age group in relation to ≥ 65 years ($p = 0.00$).

According to the weight status, mean serum 25(OH)D was higher in normal-weight subjects than in obese ($p = 0.01$).

Underweight subjects had lower serum PTH than obese and normal-weight subjects had lower PTH than overweight and obese subjects ($p < 0.05$).

Mean serum 25(OH)D levels differ between individuals depending on the season of blood sampling. Those measured in winter-spring period had lower mean serum 25(OH)D than those who were measured in summer-autumn ($p = 0.007$). Serum PTH was also lower in individuals measured in summer and autumn than those measured in winter and spring ($p < 0.001$).

The 72.51% of the total population lived in urban areas. Serum 25(OH)D levels were higher in urban than in rural areas (20.39 ng/mL vs 18.96 ng/mL, $p = 0.005$) as presented in Table 2, but the difference did not exist after adjustment for BMI, season of sampling, sex, and age (data not shown). Urban residents had lower SOS (1549.14 vs 1564.51, $p = 0.031$) and SI (90.37 vs 94.32, $p = 0.037$) values than rural residents (Table 2). The relationship remained after adjustment for PTH, age, sex, and organized physical activity, only for SOS parameter (B = 17.881, $p = 0.016$) (data not shown).

The binary logistic regression with serum 25(OH)D, < 20 ng/mL and ≥ 20 ng/mL as dependent variable and obesity and age groups (18–50, 51–65, and ≥ 65) as independent

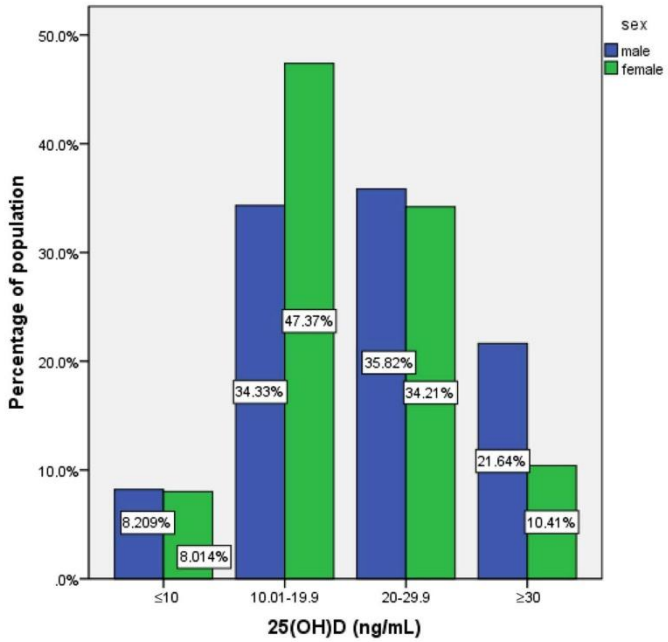

Fig. 1 Percentages of males and females with severe deficiency (≤ 10 ng/mL), deficiency (10–19.9 ng/mL), insufficiency (20–29.9 ng/mL), and adequacy (≥ 30 ng/mL) of serum 25(OH)D

Table 1 Anthropometric, biochemical, ultrasound, and lifestyle parameters in the total population and according to serum 25(OH)D categories

	Total (mean ± SD)	Severe deficiency (mean ± SD)	Deficiency (mean ± SD)	Insufficiency (mean ± SD)	Adequacy (mean ± SD)	*p* value
N (number of subjects)	970	78	440	333	119	
Age (years)	49.58 ± 13.54	51.01 ± 15.12	50.81 ± 13.50	47.97 ± 13.17	48.59 ± 13.37	0.21
BMI (kg/m^2)	27.53 ± 5.6	28.56 ± 7.21	28.14 ± 5.67	27.07 ± 5.18	25.79 ± 4.78	0.00
PTH (pg/mL)	40.4 ± 15.6	47.8 ± 19.1	41.1 ± 15.2	38.8 ± 14.5	37.7 ± 15.8	0.00
BUA (dB/MHz)	115.1 ± 16.31	111.7 ± 17.62	114.12 ± 16.36	116.38 ± 16.06	115.4 ± 16.29	0.377
SOS (m/s)	1553.3 ± 85.73	1550.6 ± 42.78	1544.94 ± 120.24	1562.22 ± 35.76	1558.16 ± 40.22	0.098
SI	91.31 ± 19.68	90.24 ± 23.67	90.97 ± 19.20	91.68 ± 18.96	92.48 ± 20.74	0.917
Sun exposure winter (hours/day)	5.09 ± 5.5	4.14 ± 4.96	5.0 ± 5.48	5.65 ± 5.8	4.86 ± 5.0	0.195
Sun exposure summer (hours/day)	12.61 ± 11.02	10.75 ± 11.36	11.93 ± 10.46	13.52 ± 11.54	13.7 ± 11.27	0.084
TV watching or PC activity (hours/day)	3.09 ± 2.24	3.22 ± 1.94	3.15 ± 2.31	2.95 ± 2.06	3.18 ± 2.63	0.609
Organized physical activity moderate and/or vigorous (minutes/day)	13.46 ± 32.33	7.09 ± 15.68	10.16 ± 26.84	14.92 ± 37.46	24.84 ± 39.24	0.00

Severe deficiency < 10 ng/mL, deficiency 10–19.9 ng/mL, insufficiency 20–29.9 ng/mL, adequacy ≥ 30 ng/mL

categorical variables, revealed that obese individuals had 1.458 times increased risk to have 25(OH)D < 20 ng/mL ($p = 0.006$). People 51–65 years had a 1.75 times increased risk of vitamin D insufficiency in relation to those of 18–50 years (data not shown).

The SOS parameter was significantly different in all categories of vitamin D, with individuals who had 25(OH)D ≥ 20 ng/mL presenting higher SOS measurements than those with 25(OH)D < 20 ng/mL (1561.5 ± 37 and 1553.2 ± 46.6, respectively, $p = 0.008$), and this significance remained after adjustment for age (B = 13.04, $p = 0.04$) (data not shown).

The correlation among different characteristics of the population and 25(OH)D, PTH, BUA, SOS, SI, is presented in Table 3.

In order to determine which factors had contributed to vitamin D status, multiple variables that could possibly

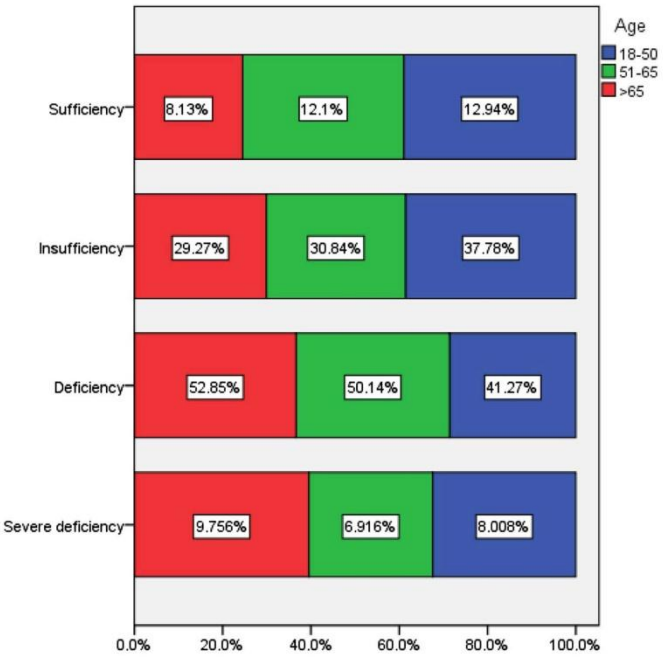

Fig. 2 Age-specific prevalence of different categories of vitamin D status

Table 2 Mean level of 25(OH)D, PTH, BUA, SOS, SI, by age group, BMI categories, and seasons of examination

	25(OH)D (ng/mL)	PTH(pg/mL)	BUA (dB/MHz)	SOS (m/s)	SI
Age group (years) (%population)					
18–50 (50.9%)	20.5 ± 8.1*	36.0 ± 14.3*	119.68 ± 15.56*	1565.46 ± 80.98*	98.78 ± 18.49*
51–65 (36.3%)	19.7 ± 7.8	43.6 ± 14.9*	110.26 ± 14.71*	1537.22 ± 101.1*	85.03 ± 17.55*
>65 (12.8%)	18.5 ± 7.9*	47.9 ± 15.9*	104.76 ± 16.14*	1533.95 ± 32.9*	76.85 ± 16.8*
BMI category (kg/m^2) (%population)					
Underweight (< 18.5) (1.2%)	21.8 ± 8.2	30.4 ± 10.5*	109.49 ± 12.81	1555.06 ± 25.9	93.83 ± 12.69
Normal weight (18.5–24.9) (35.3%)	20.9 ± 8.6*	35.9 ± 14.1*	113.33 ± 16.18	1558 ± 35.4	90.13 ± 18.71
Overweight (25–29.9) (37.1%)	20.1 ± 7.5	41.3 ± 16.0*	115.05 ± 16.8	1557.37 ± 37.8	90.46 ± 20.25
Obesity (≥ 30) (26.3%)	18.6 ± 7.0*	44.6 ± 14.9*	117.3 ± 16.06	1541 ± 155.6	94.09 ± 20.3
Season of examination (%population)					
Winter-spring (43%)	19.2 ± 7.7*	42.7 ± 15.1*	114.69 ± 17.02	1553.66 ± 94.06	90.39 ± 19.68
Summer-autumn (57%)	20.6 ± 8.1*	38.6 ± 15.7*	115.36 ± 15.84	1553.01 ± 79.5	92.18 ± 19.66
Area of residence					
Urban (72.5%)	20.4 ± 8.4*	39.3 ± 15.9*	115.23 ± 16.31	1549.14 ± 97.33*	90.37 ± 20*
Rural (27.5%)	19.0 ± 6.5*	43.1 ± 14.4*	114.7 ± 16.35	1564.51 ± 37.8*	94.32 ± 18.4*

*p value < 0.05 from one-way ANOVA or independent-samples T test

influence 25(OH)D levels were included into a stepwise multiple linear regression analysis model. Female sex, season, the hours of summer sun exposure, and participation in organized physical activity had a significant effect; Age presented a significant negative effect only among obese subjects (Table 4). This model explains the 5.3% of the variability of 25(OH)D in population.

Discussion

The present study recruited a large number of subjects from the general healthy population from several regions of Greece. Previous studies investigating vitamin D status in Greece had smaller samples from specific areas and age groups [7–9]. This is the first epidemiological study providing the association of QUS and other lifestyle parameters with vitamin D status in a large sample of Greek women and men [21, 22].

The majority of Greek adults (54%) had vitamin D deficiency (< 20 ng/mL) and only 12.3% had levels above 30 ng/mL, with males having higher serum vitamin D than females. This percentage is similar to Germany, a northern country (11.8%) [23]. Similar results were derived from other northern countries like Denmark, Poland, Bosnia [24–26], and South Australia [27]. Specifically, the 25.9% of Bosnia and Herzegovina population had severe vitamin D deficiency, greater than 8% of current population, but only 18% and 12% of Bosnia and Greek population respectively had sufficient levels of 25(OH)D. The percentage of vitamin D adequacy in our Greek healthy population is very low compared to the Bosnia and Herzegovina study which however corresponds to a patients' population. In a recent study where 1075 adults were studied from seven European countries, including Greece, about 34% of adults had 25(OH)D < 20 ng/mL [28]. In Canada, a large proportion of the population (40.8%) is reported to have 25(OH)D > 30 ng/mL [29]. This may be due to food fortification, a possible cause of the difference

Table 3 Correlation among 25(OH)D, PTH and QUS parameters, and the other anthropometric, biochemical, and life-style parameters

Continuous variables	25(OH)D *r* (*p* value)	PTH *r* (*p* value)	BUA *r* (*p* value)	SOS *r* (*p* value)	SI *r* (*p* value)
Age (years)	0.086 (0.008)	0.321 (0.00)	−0.359 (0.00)	−0.359 (0.00)	0.437 (0.00)
25(OH)D (ng/mL)		−0.162 (0.00)	0.042 (0.252)	0.073 (0.051)	0.029 (0.483)
PTH (pg/mL)	−0.162 (0.001)		−0.125 (0.001)	−0.135 (0.00)	−0.170 (0.00)
BMI (kg/m^2)	−0.141 (0.00)	0.244 (0.00)	0.120 (0.001)	0.001 (0.980)	0.071 (0.081)
BUA dB/MHz	0.042 (0.252)	−0.125 (0.00)		0.595 (0.00)	0.896 (0.00)
SOS m/s	0.073 (0.051)	−0.135 (0.00)	0.595 (0.00)		0.71 (0.00)
SI	0.029 (0.483)	−0.170 (0.00)	0.896 (0.00)	0.711 (0.00)	
Sun exposure winter (hours/day)	0.043 (0.191)	0.021 (0.531)	−0.002 (0.955)	−0.014 (0.707)	0.041 (0.321)
Sun exposure summer (hours/day)	0.122 (0.00)	−0.014 (0.679)	0.009 (0.811)	0.036 (0.341)	0.054 (0.199)
TV watching or PC activity (hours/day)	−0.009 (0.800)	0.034 (0.324)	0.006 (0.867)	−0.018 (0.633)	−0.001 (0.972)
Organized physical activity moderate and/or vigorous (minutes/day)	−0.170 (0.00)	−0.58 (0.086)	0.100 (0.007)	0.093 (0.013)	0.090 (0.029)

of vitamin D status among Canada and the other countries. In a Canadian study, fortification of milk, yogurt, and cheese at 6.75 μg (270 IU)/serving led to more than doubling of vitamin D intake across all sex and age groups and a drop in the prevalence of dietary inadequacy from > 80 to < 50% in all groups. Furthermore, no intakes approached the upper level (UL) under any fortification scenario in any sex and age group [30].

In our study, vitamin D < 20 ng/mL was related to lower SOS values. As it was showed in other studies, these two conditions may increase the risk of fracture [31, 32]. Serum 25(OH)D levels were previously reported to be an independent determinant of SOS [33]. In contrast, another study found that QUS measurements did not differ between Arabian women with serum 25(OH)D < or ≥ 30 ng/mL [34]. Although our study is lacking histomorphometric data, it is possible that the defective collagen mineralization among vitamin D-deficient patients might be a reason for the lower SOS. This finding provides evidence for clinical use of QUS in subjects with low serum 25(OH)D levels.

Vitamin D status was influenced by BMI, so obese people had lower levels from non-obese. Obese individuals had 1.458 times increased risk to having 25(OH)D < 20 ng/mL (p = 0.006), independently of the age group. In a Polish obese population, mean 25(OH)D levels were 15.8 ± 8.5 ng/mL [25]. Mean serum 25(OH)D levels of general population in Portugal was 22 ng/mL, where the 48% of population had vitamin D deficiency. In the same population, obese people had lower 25(OH)D levels than not obese [35]. Healthy Italian adults had 25(OH)D mean levels 34.3 ng/mL [36]. In another Greek study of postmenopausal non-osteoporotic women, serum 25(OH)D levels were inversely associated with body fat mass, as measured using dual-energy x-ray absorptiometry [37]. Obviously, the explanation of this expected finding resides on the fat-soluble property of vitamin D.

Hours of summer sun exposure proved to have a beneficial effect on serum 25(OH)D as also expected, and our population had higher levels in summer and autumn than winter and spring. The beneficial effect of summer sun irradiation was in agreement with the results of a Swedish study [38] and of a

Table 4 Determinants of serum 25(OH)D in total population

Variable	B (standard errors)
Intercept	20.384 (1.136)***
Sex (ref. female)	
Male	1.796 (0.805)*
Season (ref. summer-autumn)	
Winter-spring	−1.530 (0.566)**
Age × subjects BMI < 30 kg/m^2	−0.019 (0.021)
Age × obese subjects (BMI ≥ 30 kg/m^2)	−0.043 (0.021)*
Summer sun exposure (hours/day)	0.063 (0.025)***
Organized physical activity moderate and/or vigorous (minutes/day)	0.037 (0.008)*
Adjusted R squared	0.053

*p < 0.05, **p < 0.01, ***p < 0.001

Norwegian adolescent population [39]. A Danish study concludes that sunbathing and whole body sun exposure of healthy perimenopausal women leads to 13.2% and 27.6% respectively increase in serum 25(OH)D levels [24].

Participation in organized moderate or vigorous physical activity seems to have a beneficial effect on serum 25(OH)D. Similar are the results in an Australian population where those with higher levels of physical activity had less risk of 25(OH)D below 50 or 75 nmol/L [27]. As well as in a European study, engagement in ≥ 30 min per day of moderate- and vigorous-intensity physical activity was associated with higher odds for maintaining sufficient (≥ 50 nmol/L) 25(OH)D_3 concentrations [28]. In US population, an increase of 10 min of objectively measured and self-reported moderate-to-vigorous activities per day was associated with an increase in circulating 25(OH)D of 0.32 ng/mL (95% CI 0.17, 0.48) and of 0.18 ng/mL (95% CI 0.12, 0.23), respectively. Associations were not due to sun exposure during activity because it was similar for outdoor and indoor activities [40].

According to the determinants of vitamin D status, the female sex, the winter-spring season, and age only in obese subjects influence serum 25(OH)D levels negatively. Hours of summer sun exposure and organized physical activity have positive effects on serum 25(OH)D. Aging did not affect the vitamin D status, but only in obese people. Similarly, a relevant review mentioned that people with outdoor lifestyle prevented 25(OH) decline during aging [41]. In another study, vitamin D status was unrelated to age [42].

Conclusion

In conclusion, this study highlights the emerging issue of hypovitaminosis D in Greece. It also detected major determinants of serum 25(OH)D, obesity, poor exposure to sunlight, age, and physical activity. Moreover, subjects with 25OHD levels < 20 ng/mL had lower SOS values. With the evidence that vitamin D sufficiency may be linked to the prevention of multiple extra-skeletal conditions, further studies are needed to detect other environmental parameters, such as nutrition and clinical and genetic factors which might influence vitamin D status even in sunny countries, as is Greece.

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Compliance with ethical standards

Conflicts of interest None.

References

1. Report of a WHO Study Group (1994) Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. World Health Organ Tech Rep Ser 843:1–129
2. Moayyeri A, Adams JE, Adler RA, Krieg MA, Hans D, Compston J, Lewiecki EM (2012) Quantitative ultrasound of the heel and fracture risk assessment: an updated meta-analysis. Osteoporos Int 23(1):143–153
3. Marin F, González-Macías J, Díez-Pérez A, Palma S, Delgado-Rodríguez M (2006) Relationship between bone quantitative ultrasound and fractures: a meta-analysis. J Bone Miner Res 21:1457–1463
4. Holick MF (2007) Vitamin D deficiency. N Engl J Med 357:266–281
5. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, Durazo-Arvizu RA, Gallagher JC, Gallo RL, Jones G, Kovacs CS, Mayne ST, Rosen CJ, Shapses SA (2011) The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. J Clin Endocrinol Metab 96:53–58
6. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, Murad MH, Weaver CM (2011) Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab 96(7):1911–1930
7. Lapatsanis D, Moulas A, Cholevas V, Soukakos P, Papadopoulou ZL, Challa A (2005) Vitamin D: a necessity for children and adolescents in Greece. Calcif Tissue Int 77:348–355
8. Papapetrou PD, Triantaphyllopoulou M, Karga H, Zagarelos P, Aloumanis K, Kostakioti E, Vaiopoulos G (2007) Vitamin D deficiency in the elderly in Athens, Greece. J. Bone Miner Metab 25:198–203
9. Pazaitou-Panayiotou K, Papapetrou PD, Chrisoulidou A, Konstantinidou S, Doumala E, Georgiou E, Panagiotou V, Sotiriadou E, Mavroudi E, Apostolaki-Christopoulou M (2012) Height, whole body surface area, gender, working outdoors, and sunbathing in previous summer are important determinants of serum 25-hydroxyvitamin D levels. Exp Clin Endocrinol Diabetes 120(1):14–22
10. International Physical Activity Questionnaire Available at: http://www.ipaq.ki.se/. Accessed 12 Dec 2009
11. Webb AR, Kline L, Holick MF (1988) Influence of season and latitude on the cutaneous synthesis of vitamin D3: exposure to winter sunlight in Boston and Edmonton will not promote vitamin D3 synthesis in human skin. J Clin Endocrinol Metab 67:373–378
12. Heaney RP, Davies KM, Chen TC, Holick MF, Barger-Lux MJ (2003) Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. Am J Clin Nutr 77:204–210
13. World Health Organization (2000) Obesity: preventing and managing the global epidemic. Report of a WHO consultation. World Health Organ Tech Rep Ser 894:i–xii 1–253
14. Tai SS, Bedner M, Phinney KW (2011) Development of a candidate reference measurement procedure for the determination of 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 in human serum using isotope-dilution liquid chromatography-tandem mass spectrometry. Anal Chem 82:1942–1948

15. Stepman HC, Vanderroost A, Van Uytfanghe K, Thienpont LM (2011) Candidate reference measurement procedures for serum 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 by using isotope-dilution liquid chromatography-tandem mass spectrometry. Clin Chem 57:441–448
16. Stöckl D, Sluss PM, Thienpont LM (2009) Specifications for trueness and precision of a reference measurement system for serum/plasma 25-hydroxyvitamin D analysis. Clin Chim Acta 408:8–13
17. Flöter M, Bittar CK, Zabeu JL, Carneiro AC (2011) Review of comparative studies between bone densitometry and quantitative ultrasound of the calcaneus in osteoporosis. Acta Reumatol Port 36(4):327–335
18. Huopio J, Kröger H, Honkanen R, Jurvelin J, Saarikoski S, Alhava E (2004) Calcaneal ultrasound predicts early postmenopausal fractures as well as axial BMD. A prospective study of 422 women. Osteoporos Int 15(3):190–195
19. Hans D, Dargent-Molina P, Schott AM, Sebert JL, Cormier C, Kotzki PO, Delmas PD, Pouilles JM, Breart G, Meunier PJ (1996) Ultrasonographic heel measurements to predict hip fracture in elderly women: the EPIDOS prospective study. Lancet 348(9026):511–514
20. Trovas G, Tsekoura M, Galanos A, Dionyssiotis Y, Dontas I, Lyritis G, Papaioanou N (2009) Quantitative ultrasound of the calcaneus in Greek women: normative data are different from the manufacturer's normal range. J Clin Densitom 12(3):353–359
21. Babaroutsi E, Magkos F, Manios Y, Sidossis LS (2005) Lifestyle factors affecting heel ultrasound in Greek females across different life stages. Osteoporos Int 16(5):552–561
22. Babaroutsi E, Magkos F, Manios Y, Sidossis LS (2005) Body mass index, calcium intake, and physical activity affect calcaneal ultrasound in healthy Greek males in an age-dependent and parameter-specific manner. J Bone Miner Metab 23(2):157–166
23. Rabenberg M, Scheidt-Nave C, Busch MA, Rieckmann N, Hintzpeter B, Mensink GB (2010) Vitamin D status among adults in Germany–results from the German Health Interview and Examination Survey for Adults (DEGS1). BMC Public Health 15:641. https://doi.org/10.1186/s12889-015-2016-7
24. Brot C, Vestergaard P, Kolthoff N, Gram J, Hermann AP, Sørensen OH (2001) Vitamin D status and its adequacy in healthy Danish perimenopausal women: relationships to dietary intake, sun exposure and serum parathyroid hormone. Br J Nutr 86(Suppl 1):S97–S103
25. Płudowski P, Ducki C, Konstantynowicz J, Jaworski M (2016) Vitamin D status in Poland. Pol Arch Med Wewn 126(7–8):530–539
26. Sokolovic S, Alimanovic-Alagic R, Dzananovic L, Cavaljuga S, Beslic N, Ferhatbegovic-Opankovic E (2017) Vitamin D status in Bosnia and Herzegovina: the cross-sectional epidemiological analysis. Osteoporos Int 28(3):1021–1025
27. Gill TK, Hill CL, Shanahan EM, Taylor AW, Appleton SL, Grant JF, Shi Z, Dal Grande E, Price K, Adams RJ (2014) Vitamin D levels in an Australian population. BMC Public Health 14:1001
28. Manios Y, Moschonis G, Lambrinou CP, Mavrogianni C, Tsirigoti L, Hoeller U, Roos FF, Bendik I, Eggersdorfer M, Celis-Morales C, Livingstone KM, Marsaux CF, Macready AL, Fallaize R, O'Donovan CB, Woolhead C, Forster H, Walsh MC, Navas-Carretero S, San-Cristobal R, Kolossa S, Hallmann J, Jarosz M, Surwiłło A, Traczyk I, Drevon CA, van Ommen B, Grimaldi K, Matthews JN, Daniel H, Martinez JA, Lovegrove JA, Gibney ER, Brennan L, Saris WH, Gibney M, Mathers JC, Food4Me Study (2017) Associations of vitamin D status with dietary intakes and physical activity levels among adults from seven European countries: the Food4Me study. Eur J Nutr 57:1357–1368. https://doi.org/10.1007/s00394-017-1415-1
29. Greene-Finestone LS, Berger C, de Groh M, Hanley DA, Hidiroglou N, Sarafin K, Poliquin S, Krieger J, Richards JB, Goltzman D, CaMos Research Group (2011) 25-hydroxyvitamin D in Canadian adults: biological, environmental, and behavioral correlates. Osteoporos Int 22(5):1389–1399
30. Shakur YA, Lou W, L'Abbe MR (2014) Examining the effects of increased vitamin D fortification on dietary inadequacy in Canada. Can J Public Health 105(2):e127–e132
31. Gröber U, Spitz J, Reichrath J, Kisters K, Holick MF (2015) Vitamin D: update 2013 from rickets prophylaxis to general preventive healthcare. Dermatoendocrinol 5(3):331–347
32. Kauppi M, Impivaara O, Mäki J, Heliövaara M, Jula A (2013) Quantitative ultrasound measurements and vitamin D status in the assessment of hip fracture risk in a nationally representative population sample. Osteoporos Int 24(10):2611–2618
33. Gianoudis J, Bailey CA, Daly RM (2015) Associations between sedentary behavior and body composition, muscle function and sarcopenia in community-dwelling older adults. Osteoporos Int 26:571–579
34. Kauppi M, Impivaara O, Mäki J, Heliövaara M, Marniemi J, Montonen J, Jula A (2009) Vitamin D status and common risk factors for bone fragility as determinants of quantitative ultrasound variables in a nationally representative population sample. Bone 45(1):119–124
35. Bettencourt A, Boleixa D, Reis J, Oliveira JC, Mendonça D, Costa PP, Silva BM, Marinho A, Silva AM (2018) Serum 25-hydroxyvitamin D levels in a healthy population from the North of Portugal. J Steroid Biochem Mol Biol 175:97–101
36. Filoni A, Vestita M, Congedo M, Giudice G, Tafuri S, Bonamonte D (2018) Association between psoriasis and vitamin D: duration of disease correlates with decreased vitamin D serum levels an observational case-control study. Medicine 97:e11185
37. Moschonis G, Tanagra S, Koutsikas K, Nikolaidou A, Androutsos O, Manios Y (2009) Association between serum 25-hydroxyvitamin D levels and body composition in postmenopausal women: the postmenopausal health study. Menopause 16(4):701–707. https://doi.org/10.1097/gme.0b013e318199d5d5
38. Klingberg E, Oleröd G, Konar J, Petzold M, Hammarsten O (2015) Seasonal variations in serum 25-hydroxy vitamin D levels in a Swedish cohort. Endocrine 49(3):800–808. https://doi.org/10.1007/s12020-015-0548-3
39. Oberg J, Jorde R, Almås B, Emaus N, Grimnes G (2014) Vitamin D deficiency and lifestyle risk factors in a Norwegian adolescent population. Scand J Public Health 42(7):593–602. https://doi.org/10.1177/1403494814541593
40. Wanner M, Richard A, Martin B, Linseisen J, Rohrmann S (2015) Associations between objective and self-reported physical activity and vitamin D serum levels in the US population. Cancer Causes Control 26(6):881–891. https://doi.org/10.1007/s10552-015-0563-y
41. Morris HA (2005) Vitamin D: a hormone for all seasons–how much is enough? Clin Biochem Rev 26(1):21–32
42. Morris HA, Morrison GW, Burr M, Thomas DW, Nordin BE (1984) Vitamin D and femoral neck fractures in elderly South Australian women. Med J Aust 140(9):519–521

3.2. Unpublished Reports

3.2.1 Paper 2. This study indicate the nutritional habits of healthy Greek adults and their effects on serum 25(OH)D levels and QUS (Quantitative Ultrasound) parameters.

Key points

- 'Vegetables-Fruit' dietary pattern explains the biggest rate of variability.
- 'Sweet' dietary pattern adherence is a negative determinant of 25(OH)D.
- 'Healthy' dietary pattern adherence in winter is a positive determinant of 25(OH)D.
- Adherence to 'Healthy' dietary pattern is a positive determinant of BUA.

Dietary patterns of Greek adults and their effects on serum Vitamin D levels and Quantitative Ultrasound parameters

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Running Head

Dietary patterns, 25OHD and bone health in Greece

Keywords: dietary patterns, Greece, 25(OH)D, QUS parameters, lifestyle

Conflict of interest: Effimia V Grigoriou, George Trovas, Nikolaos Papaioannou, Ismene Dontas, Konstantinos Makris, Apostolou-Karampelis, George V Dedoussis, declare that they have no conflict of interest.

Abstract

Objective: The aim of this study is to investigate the dietary patterns which indicate the nutritional habits of healthy Greek adults and their effects on serum 25(OH)D levels and QUS (Quantitative Ultrasound) parameters.

Methods: This study is part of OSTEOS, an observational cross-sectional study. 741 adults from rural and urban areas throughout Greece were included in the study and completed the appropriate questionnaire. A validated Food Frequency Questionnaire (FFQ) was used for assessment of population's dietary habits. Serum 25(OH)D was measured by enzyme immunoassay; QUS parameters, broadband ultrasound attenuation (BUA), speed of sound (SOS) and stiffness index (SI), were assessed with an Achilles device. For statistical analysis SPSS Statistical software was used. Principal Component Analysis (PCA) was carried out for dietary patterns determination. Univariate Analysis of Variance was used for the assessment of serum 25(OH)D, BUA, SOS and SI determinants.

Results: Six dietary patterns explain 52.2% of variability of Greek adults' nutritional habits. 'Vegetables-Fruit' dietary pattern explains the biggest rate of variability. Determinants of serum 25(OH)D are Body Mass Index (BMI), elderly, summer sun exposure, organized physical activity, 'healthy' pattern in winter months and adherence to 'sweet' pattern. Determinants of QUS parameters are age, BMI, hours of sedentary activities, organized physical activity participation and adherence to 'healthy' pattern.

Conclusions: In this study we investigated the dietary patterns of Greek healthy population. Their effect on QUS parameters has never been researched before. The effect of dietary patterns on serum 25(OH)D was also investigated.

Abbreviations: Quantitative Ultrasound (QUS), Food Frequency Questionnaire (FFQ), Broadband ultrasound attenuation (BUA), Speed of sound (SOS), Stiffness index (SI), Principal Component Analysis (PCA), Body Mass Index (BMI), Calcium (Ca), Phosphorus (P), Parathyroid hormone (PTH), Intact parathyroid hormone (iPTH), Television (TV), Personal computer (PC), General Linear Model (GLM)

Introduction

The role of nutrition in general health and its effect on several phenotypes is widely known. Nutrients consumption and dietary habits affect bone health during life span, both in peak

bone mass achievement and its preservation during adulthood. Nutrients may have a beneficial or aggressive action on the skeleton [1].The key role of several nutrients and food items, like calcium, vitamin D, vitamin K, vitamin A, protein, polyunsaturated lipids, phosphorus, potassium, magnesium, caffeine, alcohol, phytoestrogen, as well as, dairy products, fruits and vegetables, in bone health has been thoroughly studied by the investigators [2-7]. However humans consume complex combination of nutrients that have a cumulative and interactive effect in their meals and related to several phenotypes or health outcomes. The need of a more holistic approach of nutrition led to the investigation of statistical methods from which overall dietary patterns can be derived, in which multiple related dietary characteristics are considered as a single exposure for a specific population. There are two main categories of dietary pattern approaches, (i) the data driven or posteriori dietary pattern approach, that includes PCA and (ii) the a priori dietary pattern approach, which uses dietary indexes created using existing nutritional knowledge [8,9].

Vitamin D deficiency is also an important risk factor of bone fragility. The main physiological effect of Vitamin D is to increase intestinal calcium absorption, such as maintaining serum calcium in order to maximize metabolic functions. The consequences of Vitamin D deficiency in adults' bone health are osteomalacia, acceleration of bone loss, muscle weakness, instability, and therefore increased risk of falling [10]. Serum vitamin D determinants in Greek population are, age in obese people, season of blood sampling, sex, participation in organized physical activity and sun exposure in summer months [11].

Quantitative bone ultrasound (QUS) is emerging as a low-cost , ionizing radiation–free, simple, and portable screening technique that is able to identify women at risk for osteoporosis and that may be used by general practitioners in primary care. Lower values of the QUS parameters were associated with a significant increase of any subsequent fracture at any site [12]. There are several lifestyles, nutritional and biochemical factors that affect QUS parameters. Weight status, physical activity, sedentary lifestyle affect BUA, SOS and SI in a sample of healthy Greek women of all ages [13]. Nutritional calcium, vitamin D supplement and protein are also correlated with QUS parameters [14, 15]. Although there is several data on the positive effect of adherence to specific healthy dietary patterns, on BMD and bone resorption [16-18], there is a literature gap about the effect of dietary patterns on QUS parameters.

The purpose of this study is firstly to identify the dietary patterns that indicate the nutritional habits of healthy Greek adults and secondly the effect of these patterns on serum 25(OH)D levels and QUS phenotypes. The current study is part of the OSTEOS project, an observational cross-sectional study that aims to identify the prevalence of vitamin D deficiency of healthy adults in Greece [11].

Material and methods

Study Design and population

The population of the current study was recruited during the OSTEOS study from several rural and urban areas of Greece. The design of this observational cross-sectional study, the collection of demographic data and the inclusion criteria, are detailed elsewhere [11]. Out of the initial population of 970 subjects, the dietary analysis was carried out for 741 subjects.

Anthropometric and biochemical Measurements

The anthropometric characteristics, body weight, height and BMI, are measured with appropriate methods as detailed elsewhere [11]. BMI was classified according to the World Health Organization [19], into four categories: Underweight (<18.5 kg/m^2), Normal weight (18.5-24.9 kg/m^2), Overweight (25-29.9kg/m^2) and Obesity (>=30 kg/m^2). Heel bone properties were measured using the Achilles quantitative ultrasound (QUS) device as detailed elswere [11]. It measures the broadband ultrasound attenuation (BUA), expressed in dB/MHz, the speed of sound (SOS) is expressed in m/s. The third variable, stiffness index (SI), is automatically calculated by Achilles from the BUA and the SOS. For normative data, we used reference data for the QUS measurements of the calcaneus specific for Greek population [20]. The lower the QUS parameters values, the higher the fracture risk.

Following a 12 hour fast, all subjects had a sample of venous blood withdrawn for serum isolation between 08:00-09:00 am. Total calcium (Ca), phosphorus (P), parathyroid hormone (PTH) and 25(OH)D were measured. Intact parathyroid hormone (iPTH) measurements were performed on a Roche/Modular Analytics analyzer, which employs electrochemilluminescense immunoassay technology (ECLIA). Serum 25(OH)D levels were determined by enzyme immunoassay [Immunodiagnostic Systems, 25(OH)D; Boldon, UK]. The biochemical analysis methods are analyzed in detail elsewhere [11]. According to the vitamin D status evaluation the

serum concentration of 25(OH)D is the best marker of an individual's Vitamin D status because it is the major circulating form and reflects the combination of dietary intake and cutaneous skin synthesis. Concerning serum vitamin D thresholds, used the following definitions: severe deficiency: <25 nmol/L (<10 ng/mL), deficiency: 25-50 nmol/L (10-19.9 ng/mL), insufficiency: 50 -75 nmol/L (20-29.9 ng/mL), sufficiency: ≥75 nmol/L (≥30ng/mL) [11].

Lifestyle information assessment

The assessment of hours spent on sedentary activities (television (TV), personal computer (PC)) and in moderate or vigorous organized physical activity was obtained from The International Physical Activity Questionnaire (IPAQ, short version) that was completed under the surveillance of the investigator [21]. The hours of sun exposure of the subjects were also evaluated. According to the blood collection day, the samples were divided in two seasons: winter-spring (December until May) and summer-autumn (June until November) as described in OSTEOS study [11].

Dietary assessment

Dietary information was collected via a validated, semi-quantitative, seventy-six-item FFQ [22]. All participants reported their daily, weekly or monthly average intake of several foods during the last year. Then the frequency of consumption was quantified on the basis of servings per week according to the dietary guidelines for adults in Greece [23]. Mixed foods were taken into account and assigned into the respective food groups.

Statistical Analysis

Statistical analysis was conducted using the statistical software package IBM SPSS Statistics for Windows, Version 19.0. Continuous variables are presented as mean ± standard deviation, while categorical variables are presented as relative frequencies. Analysis of variance (ANOVA) was used to examine differences among the groups for different continuous, while the Chi-square test was used to evaluate associations between categorical variables. Independent relationships between serum vitamin D levels, or QUS parameters and other variables as well as the interactions were assessed by stepwise Linear Regression and Univariate General Linear Model (GLM). All tests are two-sided with significance level <0.05.

Concerning the dietary analysis, the transformations of foods and food groups from daily, weekly or monthly average intake to servings per week were calculated programmatically in R programming language. Outliers were defined as values that exceed 5 SD above or below the mean and were removed from subsequent analyses.

Principal component analysis (PCA) was conducted to identify underlying dietary patterns [24]. In order for PCA to be effective in assessing food patterns, strong correlations between the predictive variables should exist. The correlation matrix of the food variables used in the present analysis showed that there were several correlation coefficients (r) > $|\cdot 4|$, indicating that the variables were highly correlated. Moreover, the Kaiser–Meyer–Olkin test of sphericity and Barlett's criterion was 0.76, implying high interrelationships between food variables and suitability of the data set for PCA. The orthogonal rotation (varimax option) was used to derive optimal non-correlated components (dietary patterns). From the entire database twenty food groups were used. To decide the number of components to retain the Kaiser criterion was used, according to which the number of components that should be retained from PCA is equal to the number of Eigenvalue > 1. In our analysis, six food patterns were selected. Based on the fact that factor loadings/correlation coefficients represent the correlation of each predicting variable with the dietary pattern score, higher absolute values indicate that the variable contributes more to the construction of this particular pattern. The dietary patterns were named according to the scores of the predicting variables that correlated most with the component/pattern (> $|0\cdot 4|$). The PCA was performed using the statistical software package IBM SPSS Statistics for Windows, Version 19.0. Outliers were defined as values that exceed 3 SD above or below the mean and were removed from subsequent analyses.

A voluntary participation agreement was obtained from each participant. The study was approved by the Ethics Committee of Harokopio University.

Results

The descriptive characteristics of population are detailed in Table 1. Mean age of our population is 49.8±13.4 years (range18-86years) and 89.3% are women. Concerning the nutritional habits of population, six dietary patterns were derived from PCA analysis. From the initial fifty foods and food groups, twenty were included in the PCA because of their high intercorrelation level. Six different diet components explain 52.2% of total variability of the

population nutrition. Higher absolute values of the score coefficients derived from PCA indicate that the food contributes more to the development of the component. The components were defined as follows: component 1: 'vegetables-fruit' pattern, which includes cooked and raw vegetables, refined rice, fresh fruits and fish; component 2: 'fast-food' pattern, that consists of refined breads, processed meat, full fat cheese; component 3: 'western' pattern, that is characterized by red meat, refined pasta, potatoes and poultry; component 4: 'healthy' pattern that includes low fat milk and yogurt, refined breakfast cereals, non-refined breads and low fat cheese; component 5: 'sweets' pattern with milky and starchy sweets; component 6: 'traditional' pattern includes full fat dairy products as main constitute as well as legumes (Table 2). As is shown in table 2, legumes also contributes to pattern 1, as the score coefficient is 0.419 ($> \left| 0.4 \right|$).

The correlation among the score of compliance at dietary patterns and other phenotypes is presented in Table 3.

Serum vitamin D levels associated positively with 'healthy' pattern and negatively with 'sweets' pattern (Table 3). After adjustment for age, sex and BMI, participation in physical activity and hours of summer sun exposure the statistical significance remains for both dietary patterns (B=0.667, p=0.024 and B= -0.919, p=0.002 respectively).

BMI correlated positively with adherence to 'vegetables-fruit'and 'western' pattern (Table 3). After adjustment for sex and age, hours of sedentary activities (TV watching, PC activity) and hours of participation in organized physical activities, BMI correlated positively only with adherence to 'western' (B=0.532, p=0.010).

After adjustment for age, BMI and sex, the positive effect of 'fast-food' dietary pattern on BUA is not significant (B= -0.2, p=0.754) and remains the positive effect of 'healthy' pattern (B=1.619, p=0.008).

Subjects participating more in organized physical activity have better compliance to 'healthy' pattern regardless of age, sex and BMI (B=3.704, p=0.000).

The distribution of scores derived from PCA and the compliance in specific patterns according to sex, age group, vitamin D status and area of residence are presented in Figure 1.

Males are more engaged to 'western' pattern than females. This difference exists after adjustment for age (B =0.255, p=0.032), as well. Moreover, males have better compliance to 'traditional' pattern after adjustment for age (B=0.258, p=0.034), too.

Urban living subjects have lower compliance to ‘western’ pattern than rural living subjects and after adjustment for sex, age and BMI the difference remains significant (B=-0.266, p=0.001).

Urban residents achieve higher scores in ‘healthy’ pattern than those living in rural areas, and the difference remains after adjustment for age, sex and BMI (B=0.562, p=0.000).

Variables that correlated in simple statistical tests were entered to Univariate GLM with a stepwise procedure to evaluate serum 25(OH)D and bone QUS phenotypes’ determinants. The models presented in tables 4,5,6 and 7 result from the above data. Concerning the serum 25(OH)D determinants, when all correlates are included in the model, the season of blood sampling and the adherence to ‘healthy’ pattern lose their significance. But when investigating how two determinants interact, it was derived that the adherence to a healthy dietary pattern in winter months has a positive effect on vitamin D levels. In summer months the adherence to ‘healthy’ dietary pattern doesn’t influence vitamin D levels (Table 4).

Concerning BUA determinants, when the ‘healthy’ dietary pattern is included in the regression, the participation in organized physical activity, loses statistical significance as determinant of BUA, due to its correlation with healthy diet pattern. As a result only ‘healthy’ pattern is included in the model.

Table 1: Descriptive characteristics.

	Total (mean±SD)	Males (mean±SD)	Females (mean±SD)	*p*-value
N	**741**	**79**	**662**	
Age (years)	49.8±13.36	45.26±16.07	50.40±12.85	0.003
25(OH)D (ng/mL)	20.01±7.98	22.66±8.95	19.63±7.76	0.002
PTH (pg/mL)	40.19±15.57	39.67	40.16±15.42	0.772
BMI (kg/m^2)	27.58±5.53	27.98±5.21	27.52±5.57	0.451
BUA dB/MHz	114.53±16.32	118.73±19.35	113.81±15.65	0.023
SOS m/s	1552.49±87.92	1555.29±159.71	1551.98±70.61	0.849
SI	90.94±19.62	97.5±25.7	89.96±18.32	0.019
Sun exposure summer (hours/day)	12.48±10.93	17.87±13.86	11.81±10.33	0.00
TV watching or PC activity (hours/day)	3.02±2.14	3.19±2.39	3±2.11	0.404
Organized physical activity moderate and/or vigorous (minutes/day)	12.55±27.68	16.51±30.70	12.03±27.29	0.137

Table 2. Dietary patterns derived from PCA analysis.

	Components					
Food group	**1- vegetables-fruit**	**2- fast food**	**3- western**	**4- healthy**	**5- sweets**	**6- traditional**
Non refined breads	0.204	-0.123	-0.118	**0.529**	0.131	-0.118
Refined breads	0.016	**0.848**	0.119	-0.068	0.101	0.023
Refined breakfast cereals	-0.076	-0.001	-0.022	**0.706**	0.058	0.349
Full fat cheese	0.197	**0.659**	0.106	-0.019	0.169	0.052
Low fat cheese	0.099	0.189	-0.036	**0.485**	-0.283	-0.191
Fish	**0.494***	-0.162	0.154	0.032	-0.109	-0.083
Fresh fruits	**0.521**	0.013	-0.158	0.230	0.333	0.046
Legumes	0.419	0.081	-0.029	-0.014	-0.152	**0.447**
Poultry	-0.028	0.111	**0.555**	0.364	-0.189	-0.143
Red meat	0.118	0.146	**0.767**	-0.032	-0.071	0.033
Full fat milk and yogurt	-0.049	-0.007	0.107	-0.035	0.044	**0.829**
Low fat milk and yogurt	0.077	-0.002	-0.042	**0.729**	-0.008	-0.039
Refined pasta	0.116	0.088	**0.629**	-0.140	0.158	0.148
Refined rice	**0.558**	0.072	0.299	0.072	-0.019	0.179
Milky sweets	-0.026	0.064	0.149	0.061	**0.784**	-0.083
Starchy sweets	-0.114	0.284	0.000	-0.068	**0.534**	0.055
Cooked vegetables	**0.751**	0.023	0.075	0.004	-0.039	0.083
Row vegetables	**0.669**	0.085	0.005	0.73	-0.037	-0.125
Processed meat	-0.141	**0.818**	0.120	0.110	0.010	-0.043
Potatoes	0.069	0.043	**0.612**	-0.201	0.212	0.018

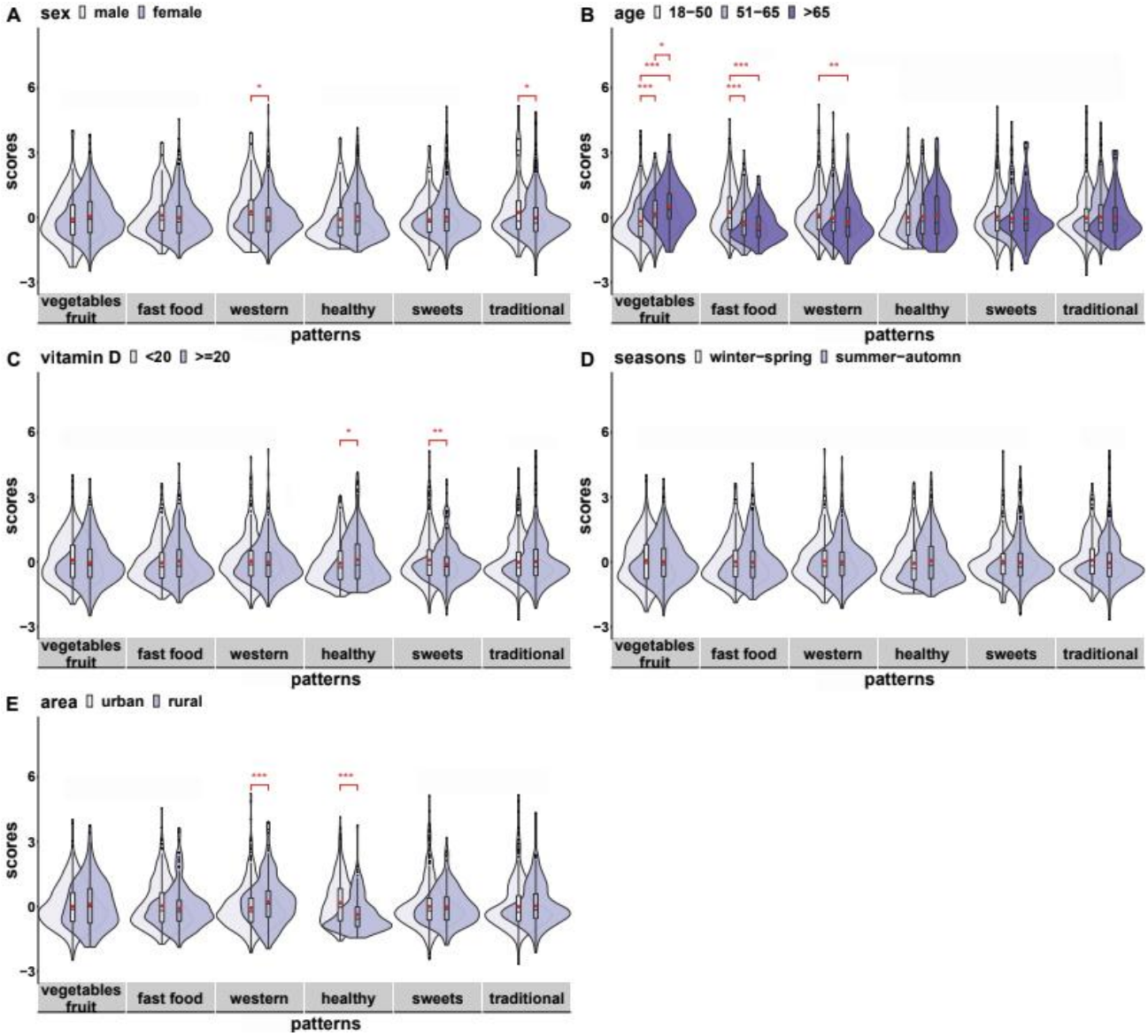
* Score coefficient in bold indicates food group contributes to pattern.

Table 3. Correlation between dietary patterns and continuous variables.

N=741	1- vegetables-fruit	2- fast food	3-western	4- healthy	5-sweets	6- traditional
Continuous Variables	**r (*p*-value)**	**r (*p*-value)**	**r (*p*-value)**	**r (*p*-value)**	**r (*p*-value)**	**r (*p*-value)**
Age (years)	**0.244*** (0.00)	**-0.272** (0.00)	-0.116 (0.002)	0.017 (0.65)	-0.061 (0.099)	-0.029 (0.430)
25(OH)D (ng/mL)	-0.066 (0.074)	0.017 (0.653)	-0.05 (0.173)	**0.107** (0.004)	**-0.119** (0.001)	0.018 (0.628)
PTH (pg/mL)	**0.101** (0.007)	**-0.077** (0.039)	-0.011 (0.773)	**-0.141** (0.00)	**0.086** (0.022)	-0.001 (0.974)
BMI (kg/m^2)	**0.074** (0.046)	-0.025 (0.498)	**0.094** (0.012)	-0.055 (0.144)	-0.058 (0.120)	0.004 (0.922)
BUA dB/MHz	-0.035 (0.394)	**0.102** (0.014)	0.067 (0.105)	**0.089** (0.03)	0.047 (0.256)	0.001 (0.980)
SOS m/s	-0.054 (0.199)	0.012 (0.778)	-0.009 (0.823)	0.046 (0.277)	0.075 (0.074)	-0.001 (0.974)
SI	-0.079 (0.084)	0.071 (0.121)	**0.098** (0.032)	0.046 (0.320)	-0.006 (0.893)	0.016 (0.721)
Sun exposure summer (hours/day)	0.040 (0.287)	0.068 (0.070)	0.011 (0.778)	0.05 (0.182)	0.005 (0.904)	0.050 (0.182)
TV watching or PC activity (hours/day)	-0.027 (0.481)	-0.036 (0.349)	-0.022 (0.562)	0.032(0.396)	0.033 (0.383)	-0.04(0.293)
Organized physical activity moderate and/or vigorous (minutes/day)	-0.011 (0.763)	0.027 (0.459)	0.024 (0.526)	**0.146** (0.00)	-0.021 (0.564)	0.037 (0.318)

*r coefficients in bold indicates statistical significance

Figure 1: Distribution of scores derived from PCA among sex, age groups, serum Vitamin D status, season of blood sampling and area of residence



Notes: * p<0.05, ** p<0.01, *** p<0.001, • mean, _ median

Table 4. Determinants of serum 25(OH)D.

Variable	B (Standard errors)
Intercept	20.993 (1.804)***
BMI (kg/m^2)	-0.157 (0.054)**
Age group (ref. >65 years)	
18-50 years	1.948 (0.959) *
61-65 years	1.847 (0.986)
Summer sun exposure (hours/day)	0.091(0.027)***
Organized physical activity moderate and/or vigorous (minutes/day)	0.042 (0.011)***
'Healthy' dietary pattern adherence * Winter	1.058 (0.497)*
'Healthy diet' dietary pattern adherence * Summer	0.441 (0.358)
'Sweets' dietary pattern adherence	-0.911 (0.293)**
Adjusted R Squared	0.081

Notes: *p<0.05, **p<0.01, ***p<0.001

Table 5. Determinants of BUA.

Variable	B (Standard errors)
Intercept	83.906 (3.816)***
Sex (ref. female)	
Male	5.189 (1.877)**
BMI (kg/m^2)	0.571 (0.112)***
Age group (ref. >65 years)	
18-50 years	19.310 (2.204)***
61-65 years	8.662 (2.289)***
Adherence to 'healthy' food pattern	1.878 (0.615)**
Adjusted R Squared	0.197

Notes: *p<0.05, **p<0.01, ***p<0.001

Table 6. Determinants of SOS.

Variable	**B (Standard errors)**
Intercept	1614.329 (14.118)***
Age (years)	-1.086 (0.271)***
TV watching or PC activity (hours/day) * 25(OH)D<20ng/mL	-5.348 (1.821)**
TV watching or PC activity (hours/day) * 25(OH)D≥20ng/mL	-0.960(2.130)
Adjusted R Squared	0.041

Notes: *p<0.05, **p<0.01, ***p<0.001

Table 7. Determinants of SI.

Variable	**B (Standard errors)**
Intercept	75.188 (2.416)***
Organized physical activity moderate and/or vigorous (minutes/day)	0.065 (0.029)*
Age group (ref. >65 years) **18-50 years** **61-65 years**	 22.402 (2.638)*** 8.352 (2.728)**
Adjusted R Squared	0.190

Notes: *p<0.05, **p<0.01, ***p<0.001

Discussion

This study investigates how nutrition affects serum Vitamin D levels, as well as bone health parameters BUA, SOS and SI, taking into consideration lifestyle parameters.

Six dietary patterns explain 52.2% of variability of Greek adults' nutritional habits. A prudent dietary pattern like 'vegetables-fruit', explains the biggest rate of variability, a positive fact for the current population. A dietary pattern with similar composition was the most dominant food pattern for another Greek adult population [25], as well. Even though in other studies [26-28] a

positive association was described between BMD and a dietary pattern was characterized by high consumption of fruit and vegetables, in the current study the adherence to a pattern rich in vegetables, fruits and fish doesn't reach the statistical significance and it doesn't correlate with heel QUS parameters. A study on older adult population suggests that better compliance to a 'prudent' diet score with fruits, vegetables and oil fish at early old age predicts bone size on average 11 years later [29].

The 'Western' pattern, as expected, related with higher BMI values and men are more susceptible to this pattern than women. In addition, males consume more full fat dairy products than females, the main constitute of the 'traditional' pattern.

Urban residents have better compliance to the 'healthy' and less to the 'western' pattern, than those living in rural areas. Other studies that have compared the dietary patterns of rural versus urban adults also indicate that rural adults have poorer dietary habits than urban adults [30]. Rural adults participate in less moderate-to-vigorous physical activity and have poorer dietary behaviors than their urban counterparts, a fact that may contribute to their higher rates of obesity [31]. Rural living usually implies healthier eating habits and more physical activity chances; nevertheless, sometimes rural residents may not have a rural lifestyle [32].

The more hours the subjects participate in organized physical activity, the higher the score they achieve in the 'healthy' pattern. Moreover, participation in organized physical activity parameter loses statistical significance as determinant of BUA, due to its correlation with the 'healthy' pattern. These two parameters together may reflect a healthier lifestyle that could be further examined by evaluation of lifestyle patterns. This beneficial effect of 'Healthy' dietary pattern on BUA may be due to low fat dairy products and breakfast cereals, included in the pattern. Dairy products contain or are fortified with significant proportions of nutrients important for bone health (calcium, protein, vitamin D etc) [33]. Breakfast cereals are also usually enriched with vitamins and minerals and they are a plant source of protein, constitutes associated with bone health [34]. SOS and SI parameters are not affected by dietary patterns.

A recently published review of 49 studies, concludes that a dietary pattern that emphasized the intake of fruit, vegetables, whole grains, poultry and fish, nuts and legumes, and low-fat dairy products and de-emphasized the intake of soft drinks, fried foods, meat and processed products, sweets and desserts, and refined grains was beneficial for bone health [35].

Serum Vitamin D level positive determinants are adulthood, hours of summer sun exposure, participation in organized physical activity and adherence to the 'healthy' pattern, that protects vitamin D levels only in winter months, when serum levels decline, maybe due to the enrichment of dairy products and breakfast cereals with vitamin D. BMI and 'Sweets' pattern are negative determinants. This study confirms the results of other studies which investigated vitamin D determinants [36-38].

BUA positive determinants are male gender, BMI, adulthood and adherence to 'healthy' pattern. SOS negative determinant is age. Vitamin D levels more than 20ng/mL have a protective effect on SOS parameter from sedentary activity. Recently published data from the OSTEOS study associate QUS parameters with vitamin D status, specifically, individuals with 25(OH)D ≥ 20 ng/mL had higher SOS than those with 25(OH)D < 20 ng/mL [11]. SI positive determinants are organized physical activity and adulthood.

A Greek study of all age group women demonstrates that adulthood, BMI, and organized physical activity are positive determinants of QUS parameters, results of which are similar to current study [39].

Many studies identify associations between individual nutrients or food items and musculoskeletal health outcomes [40]. Using individual nutrients do not consider interactions, and cumulative effects between different nutrients and foods. Dietary pattern analysis has been used to overcome these limitations by studying the overall diet rather than the intakes of individual nutrients. This is particularly important for disease prevention or treatment because the small effect of a single or a few nutrients may not be able to be detected while dietary pattern analysis considers the joint effects of nutrients and foods based on entire eating behavior. The results of dietary patterns studies can also be easy for the public to translate into dietary guidelines.

This current study has several limitations: it is an observational, cross-sectional study and it is not appropriate to draw causal effect implications or to generalize the results from this population. Residual confounding may also exist because of unmeasured variables. Another limitation of the study is the self-reported medical history and medication data. The study has also several strengths: it was contacted in a large national representative population of urban and rural citizens, with a vast age range.

Conclusions

A prudent dietary pattern consisted by vegetables, fruits, fish and rice, explains more the nutrition variability of the current population. Several determinants explain a part of variability of 25(OH)D, BUA, SOS and SI. The modifiable factors that can positively affect QUS parameters and Vitamin D levels are participation in organised physical activity, reduced hours of sedentary activities, achievement of healthy BMI, healthy dietary habits and reduced sweet consumption. More studies are needed, with larger sample, biochemical, lifestyle and genetic parameters to determine individualized guidelines and promote public health and prevention.

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References

1. Cashman KD. Diet, Nutrition, and Bone Health. J Nutr 2007;137:2507S–2512S.
2. Prentice A. Diet, nutrition and the prevention of osteoporosis. Public Health Nutr 2004;7(1A):227–243

3. Wetmore CM, Ichikawa L, LaCroix AZ, Ott SM, Scholes D. Association between caffeine intake and bone mass among young women: potential effect modification by depot medroxyprogesterone acetate use. Osteoporos Int 2008;19(4):519-527
4. Alekel DL, Van Loan MD, Koehler KJ, Hanson LN, et al. The soy isoflavones for reducing bone loss (SIRBL) study: a 3-y randomized controlled trial in postmenopausal women. Am J Clin Nutr 2010; 91(1):218-230
5. McGartland CP, Robson PJ, Murray LJ, Cran GW, et al. Fruit and vegetable consumption and bone mineral density: the Northern Ireland Young Hearts Project. Am J Clin Nutr 2004;80(4):1019-1023.
6. Tylavsky FA, Holliday K, Danish R, Womack C, et al. Fruit and vegetable intakes are an independent predictor of bone size in early pubertal children. Am J Clin Nutr 2004;79(2):311-317
7. New SA, Robins SP, Campbell MK, Martin JC, et al. Dietary influences on bone mass and bone metabolism: further evidence of a positive link between fruit and vegetable consumption and bone health?Am J Clin Nutr 2000;71(1):142-151
8. Kant AK. Dietary patterns: biomarkers and chronic disease risk. Appl Physiol Nutr Metab. 2010 Apr;35(2):199-206. doi: 10.1139/H10-005
9. Kant AK. Dietary patterns and health outcomes. J Am Diet Assoc 2004;104:615-35.
10. Holick MF. Vitamin D Deficiency. N Engl J Med 2007. 357:266-281
11. Grigoriou EV, Trovas G, Papaioannou N, Makras P, et al. Serum 25-hydroxyvitamin D status, quantitative ultrasound parameters, and their determinants in Greek population. Arch Osteoporos. 2018 15;13(1):111. doi: 10.1007/s11657-018-0526-5
12. Marín F, González-Macías J, Díez-Pérez A, Palma S, Delgado-Rodríguez M. Relationship Between Bone Quantitative Ultrasound and Fractures: A Meta-Analysis. J Bone Miner Res 2006;21:1457–1463
13. Eirini Babaroutsi, Faidon Magkos, Yannis Manios, Labros S. Sidossis. Lifestyle factors affecting heel ultrasound in Greek females across different life stages. Osteoporosis International, 2005;16(5):552–561
14. E. W. GreggA. M. KriskaL. M. SalamoneR. L. WolfM. M. et al. Correlates of Quantitative Ultrasound in the Women's Healthy Lifestyle Project Osteoporosis International 1999;10(5):416–424

15. Babaroutsi E1, Magkos F, Manios Y, Sidossis LS. Body mass index, calcium intake, and physical activity affect calcaneal ultrasound in healthy Greek males in an age-dependent and 19 parameter-specific manner. J Bone Miner Metab. 2005; 23(2):157-66
16. Yang TC, Aucott LS, Duthie GG, Macdonald HM. An application of partial least squares for identifying dietary patterns in bone health. Arch Osteoporos 2017;12:63 DOI 10.1007/s1165-7-0355-y
17. Melaku YA, Gill TK, Adams R, Shi Z. Association between dietary patterns and low bone mineral density among adults aged 50 years and above: findings from the North West Adelaide Health Study (NWAHS), British Journal of Nutrition 2016;116:1437-1446
18. Whittle CR, Woodside JV, Cardwell CR, McCourt HJ, et al. Dietary patterns and bone mineral status in young adults: The Northern Ireland Young Hearts Project. British Journal of Nutrition 2012;108:1494-1504
19. World Health Organization. (2000) Obesity: preventing and managing the global epidemic. Report of a WHO consultation. World Health Organ Tech Rep Ser. 894:i-xii, 1-253
20. Trovas G, Tsekoura M, Galanos A, Dionyssiotis Y, et al. Quantitative ultrasound of the calcaneus in greek women: normative data are different from the manufacturer's normal range. J Clin Densitom. 2009;12(3):353-9
21. International Physical Activity Questionnaire. Available at: http://www.ipaq.ki.se/. Accessed December 12, 2009
22. Bountziouka V, Bathrellou E, Giotopoulou A et al. Development, repeatability and validity regarding energy and macronutrient intake of a semi-quantitative food frequency questionnaire: methodological considerations. Nutr Metab Cardiovasc Dis 2012;22:659–667.
23. Ministry of Health and Welfare, Supreme Scientific Health Council Dietary guidelines for adults in Greece. Arch Hellenic Med 1999;16:516–524.
24. Mardia KV, Kent JT, Bibby JM. Multivariate Analysis. New York: Academic Press 1979
25. Panagiotakos DB, Pitsavos C, Skoumas Y, Stefanadis C. The association between food patterns and the metabolic syndrome using principal components analysis: The ATTICA Study. J Am Diet Assoc. 2007 Jun;107(6):979-87

26. Tucker KL, Chen H, Hannan MT, Cupples LA, et al. Bone mineral density and dietary patterns in older adults: the Framingham Osteoporosis Study. Am J Clin Nutr 2002;76:245–252
27. Ward KA, Prentice A, Kuh DL, Adams JE, Ambrosini GL Life course dietary patterns and bone health in later life in a British birth cohort study. J Bone Miner Res 2016;31:1167–1176. https ://doi. org/10.1002/jbmr.2798
28. de Jonge EA, Kiefte-de Jong JC, Hofman A, Uitterlinden AG, et al. Dietary patterns explaining differences in bone mineral density and hip structure in the elderly: the Rotterdam Study. Am J Clin Nutr 2017;105:203–211. https ://doi.org/10.3945/ajcn.116.13919 6
29. S. C. Shaw, C. M. Parsons, N. R. Fuggle, M. H. Edwards, et al. Ward. Diet Quality and Bone Measurements Using HRpQCT and pQCT in Older Community-Dwelling Adults from the Hertfordshire Cohort StudyCalcified Tissue International 2018;103:494–500
30. Trivedi, T., Liu J., Probst, J., Merchant, et al. Obesity and obesity-related behaviors among rural and urban adults in the USA. Rural Remote Health 2015;15: 3267.
31. Befort, C.A., Nazir, N., Perri, M.G. Prevalence of obesity among adults from rural and urban areas of the United States: Findings from NHANES (2005–2008). J. Rural Health 2012;28, 392–397
32. McCormack L, Wey H, Meendering J, Specker B. Differences in Physical Activity and Diet Patterns between Non-Rural and Rural Adults. Nutrients. 2018 1;10(11). pii: E1601. doi: 10.3390/nu10111601
33. Reyes-Garcia R, Mendoza N, Palacios S, Salas N, et al. Effects of Daily Intake of Calcium and Vitamin D-Enriched Milk in Healthy Postmenopausal Women: A Randomized, Controlled, Double-Blind Nutritional Study. J Womens Health (Larchmt). 2018;27(5):561-568. doi: 10.1089/jwh.2017.6655
34. Song WO, Chun OK, Kerver J, Cho S, et al. Ready-to-eat breakfast cereal consumption enhances milk and calcium intake in the US population. J Am Diet Assoc. 2006;106(11):1783-9
35. Elham Z Movassagh and Hassan Vatanparast. Current Evidence on the Association of Dietary Patterns and Bone Health: A Scoping Review American Society for Nutrition. Adv Nutr 2017;8:1–16; doi:10.3945/an.116.013326

36. Yao Y, Fu S, Li N, Hu F, et al. Residence and Fish Intake Predict Vitamin D Status in Chinese Centenarians J Nutr Health Aging. 2019;23(2):165-171. doi: 10.1007/s12603-018-1126-1
37. Ten Haaf DSM, Balvers MGJ, Timmers S, Eijsvogels TMH, et al. Determinants of vitamin D status in physically active elderly in the Netherlands. Eur J Nutr. 2018. doi: 10.1007/s00394-018-1856-1
38. Brouwer-Brolsma EM, Vaes AMM, van der Zwaluw NL, van Wijngaarden JP, et al. Relative importance of summer sun exposure, vitamin D intake, and genes to vitamin D status in Dutch older adults: The B-PROOF study. J Steroid Biochem Mol Biol. 2016;164:168-176. doi: 10.1016/j.jsbmb.2015.08.008
39. Babaroutsi E, Magkos F, Manios Y, Sidossis LS. Lifestyle factors affecting heel ultrasound in Greek females across different life stages Osteoporos Int. 2005;16(5):552-61
40. Palacios C. The role of nutrients in bone health, from A to Z.Crit Rev Food Sci Nutr 2006;46, 621–628

3.2.2 Paper 3. In this paper highlighted the genetic contribution to QUS phenotypes as well as to serum vitamin D levels.

Key points

- 'T' consist the effect allele of rs11520772 polymorphism, 8.5% of population are homozygotes (TT).
- 'G' consist the effect allele of rs597319 polymorphism, 13% of population are homozygotes (GG). '
- 'AA' (wild type) homozygotes of rs11520772 polymorphism have higher SOS value than 'TT'.
- AA' (wild type) homozygotes of rs597319 polymorphism have higher SI value than 'GG'.

The effect of rs11520772 of the TAX1BP1 gene and the rs597319 of the TMEM135 gene polymorphisms on Quantitative Ultrasound (QUS) parameters. The OSTEOS study.

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ABSTRACT

Purpose Vitamin D deficiency and quantitative ultrasound (QUS) parameters are among the most important clinical risk factors of bone fragility. The aim of the study was to evaluate the effect of SNPs, previously associated with QUS, on serum 25(OH)D levels, and QUS parameters on the OSTEOS study.

Methods OSTEOS is an observational cross-sectional study conducted from June 2010 to July 2012. The participants were adults coming from rural and urban areas throughout Greece Serum 25(OH)D was measured by enzyme immunoassay; QUS parameters, broadband

ultrasound attenuation (BUA), speed of sound (SOS) and stiffness index (SI), were assessed with an Achilles device. Genotyping was performed with the RT-PCR. Univariate Analysis of Variance was used for the assessment of serum 25(OH)D and QUS parameters determinants.

Results Mean age of our population is 49.8±13.4 years (range18-86years) and 89.5% are women. The two of eight SNPs were analyzed (rs11520772 and rs597319) met the statistical significance with the evaluated phenotypes. According to rs11520772 polymorphism, 59.5% had AA genotype, 32.0% were heterozygotes (AT) and 8.5% were homozygotes (TT) to effect allele. According to rs597319 the 42.3% of study population had AA, the 44.8% had AG and 13.0% had GG genotype. The determinants of SOS parameter were the rs11520772 polymorphism and the age. The determinants of SI were age and rs597319. There is no association between serum vitamin D levels with the evaluated genotypes.

Conclusions This study reports that the AA homozygotes of the rs11520772 associated with higher SOS values in relation to minor allele (T) homozygotes, taking into account lifestyle and biochemical parameters. Additionally AA genotype of the rs1597319 positively associated with SI comparing with to GG genotype.

Key words: single nucleotide polymorphism, vitamin D, QUS, life-style, epidemiology

INTRODUCTION

Osteoporosis is a systemic skeletal multifactorial disease characterized by reduced bone mass and microarchitectural deterioration of the structure of bone tissue leading to enhanced bone fragility and increased susceptibility to fractures [1]. Osteoporosis is a major public health concern. Women have a higher risk of osteoporotic fractures compared with men (1 in 3 women and 1 in 5 men over 50 years) [2]. Although BMD is the main predictive risk factor for an osteoporotic fracture, measurement of quantitative ultrasound (QUS) has been found to be associated with bone fragility and increased fracture risk [3]. The QUS measuring at the heel is an alternative, ionizing radiation–free and relatively inexpensive, portable screening technique that is able to identify women at high risk of bone fragility and fracture [3, 4] and may be used by general practitioners in primary care. Lower values of the QUS parameters are associated with a significant increase of any subsequent fracture at any site [4]. There are several lifestyles, nutritional and biochemical factors that affect QUS parameters. Weight status, physical activity, sedentary lifestyle affect BUA, SOS and SI in a sample of healthy Greek women

of all ages [5]. Genetic contribution to osteoporosis is well established with heritability estimates reaching 84% for central BMD [6], 74% for heel QUS [7, 8], 47% for bone loss [9] and 48% for hip fracture [10]. Recent GWAS meta-analysis highlighted 9 polymorphisms associated with heel QUS phenotypes. The rs11520772 of the TAX1BP1 gene, the rs2908007 of the WNT16 gene, the rs2982552, the rs3020331 and the rs4869739 of ESR1 gene, rs3000634 of USPL1 gene, the rs597319 of TMEM135 gene, rs7741021 of RSPO3 gene and the rs9292469 of NPR3 gene.

Vitamin D deficiency is also among the most important clinical risk factors of bone fragility and a subject of extensive research. The main physiological effect of Vitamin D is to increase intestinal calcium absorption, as such maintaining serum calcium in order to maximize metabolic functions, signal transduction and neuromuscular activity. The consequences of Vitamin D deficiency in adults are osteomalacia, acceleration of bone loss, muscle weakness, instability, and therefore increased risk of falling [12]. In children, lack of Vitamin D causes rickets and growth retardation. Vitamin D receptors are expressed by several tissues and cells as in enterocytes, osteoblasts, the cells of the distal convoluted renal tubules, in the cells of the parathyroid gland, colon, pituitary, ovaries, cells of the immune system etc. Vitamin D deficiency is associated, by many observational studies, with major diseases, such as osteoporosis, diabetes, some forms of cancer, autoimmune diseases, infectious diseases, hypertension but causalities have not been proven [12]. Serum vitamin D determinants in the Greek population are, age in obese people, season of blood sampling, gender, participation in organized physical activity and sun exposure in summer months [5]. The heritability of vitamin D as estimated by twin studies reaches the 50-80% [13, 14].

The purpose of the current study is to identify the effect of the rs11520772 polymorphism on serum 25(OH)D levels and on the QUS phenotypes. Current research is part of OSTEOS project, an observational cross-sectional study that aims to identify the prevalence of vitamin D deficiency of healthy adults in Greece [5].

METHODS

Study Design and population

The population of the current study was recruited during the OSTEOS study from several rural and urban areas of Greece. The design of this observational cross-sectional study, the collection

of demographic data and the inclusion criteria, are detailed elsewhere [5]. Out of the initial population of 970 subjects, the genetic analysis was carried out for 670 subjects.

Anthropometric and biochemical Measurements

The anthropometric characteristics, body weight, height and BMI, are measured with appropriate methods as detailed elsewhere [5]. BMI was classified according to the World Health Organization [15], into four categories: Underweight (<18.5 kg/m^2), Normal weight (18.5-24.9 kg/m^2), Overweight (25-29.9kg/m^2) and Obesity (>=30 kg/m^2). Heel bone properties were measured using the Achilles quantitative ultrasound (QUS) device [5]. It measures the broadband ultrasound attenuation (BUA), expressed in dB/MHz, and the speed of sound (SOS), expressed in m/s. The third variable, stiffness index (SI), is automatically calculated by Achilles from the BUA and the SOS. For normative data, we used reference data for the QUS measurements of the calcaneus specific for Greek population [20]. The lower the QUS parameters values, the higher the fracture risk.

Following a 12 hour fast, all subjects had a sample of venous blood withdrawn for serum isolation between 08:00-09:00 am. Total calcium (Ca), phosphorus (P), parathyroid hormone (PTH) and 25(OH)D were measured. Intact parathyroid hormone (iPTH) measurements were performed on a Roche/Modular Analytics analyzer, which employs electrochemilluminescense immunoassay technology (ECLIA). Serum 25(OH)D levels were determined by enzyme immunoassay [Immunodiagnostic Systems, 25(OH)D; Boldon, UK]. The biochemical analysis methods are analyzed in detail elsewhere [5]. According to the vitamin D status evaluation the serum concentration of 25(OH)D is the best marker of an individual's Vitamin D status because it is the major circulating form and reflects the combination of dietary intake and cutaneous skin synthesis. Concerning serum vitamin D thresholds the following definitions were used: severe deficiency: <25 nmol/L (<10 ng/mL), deficiency: 25-50 nmol/L (10-19.9 ng/mL), insufficiency: 50 -75 nmol/L (20-29.9 ng/mL), sufficiency: ≥75 nmol/L (≥30ng/mL) [17].

Lifestyle information assessment

The assessment of hours spent on sedentary activities (television (TV), personal computer (PC)) and in moderate or vigorous organized physical activity was obtained from The International Physical Activity Questionnaire (IPAQ, short version) that was completed under the surveillance

of the investigator [18]. The hours of sun exposure of the subjects were also evaluated. According to the blood collection day, the samples were divided in two seasons: winter-spring (December until May) and summer-autumn (June until November) as described in OSTEOS study [5].

DNA extraction and Genotyping

For the DNA extraction the DNA iPrepTM device and the iPrepTMPureLinkTMgDNA Blood Kit were used. The above device extract 200μL DNA solution in 30 minutes from 13 samples of 300μL buffy coat (concentrated leukocyte preparation). Buffy coat extraction is carried out when the tubes with blood samples are centrifuged for 10 minutes in 3000rpm. After centrifugation the sample is separated into 3 layers. The erythrocytes precipitate at the bottom of the tube, plasma remains at the top and the intermediate layer consists of leukocytes and platelets which is used in the DNA extraction.

The genotyping conducted for eight of nine SNPs (rs11520772, rs2908007, rs2982552, rs3000634, rs3020331, rs597319, rs7741021, rs9292469) significant associated with QUS parameters from GEFOS consortium, for 670 samples, of the total sample of OSTEOS study. It was performed with the Real Time-Polymarase Chain Reaction (RT-PCR). Genotypic identification was performed using the StepOnePlus ™ machine of Applied Biosystems StepOne ™ with TaqMan® SNP Genotyping. It is a complete collection of mapped primers and probes that lead to the determination of mononucleotide polymorphisms in human DNA samples. A special TaqMan® reagent is used to amplify and detect specific SNPs in purified genomic DNA samples. Reagent activity is optimized by adding a special Master mix.

Statistical Analysis

Statistical analysis was conducted using the statistical software package IBM SPSS Statistics for Windows, Version 19.0. Continuous variables are presented as mean ± standard deviation, while categorical variables are presented as relative frequencies. Analysis of variance (ANOVA) was used to examine differences among the groups for different continuous variables, while the Chi-square test was used to evaluate associations between categorical variables. Independent relationships between serum vitamin D levels, or QUS parameters and other variables as well as the interactions were assessed by Univariate General Linear Model with stepwise procedure. All tests are two-sided with significance level <0.05.

RESULTS

Mean age of our population is 49.8±13.4 years (range18-86years) and 89.5% are women. The two (rs11520772 and rs597319) of eight polymorphisms reach the statistical significance when associated with biochemical, anthropometric and QUS parameters, of our cohort (supplementary file, Table S1).

From total of 671 samples had genotyped for rs11520772 polymorphism, 59.5% have AA genotype, 32.0% are heterozygotes (AT) and 8.5% are homozygotes (TT) to effect allele, as it shown in. The 'A' allele frequency is 0.755 and the 'T' allele frequency is 0.245. According to rs597319 42.3% have AA genotype, 44.8% are heterozygotes (AG) and 13.0% are homozygotes (GG) to effect allele. Descriptive characteristics of population and correlation of mean serum 25(OH)D, PTH, age, BMI, BUA, SOS, SI, hours of participation in organized physical activity and hours spending in sedentary activities, by genotype are detailed in Table 1. There is no gender difference in the incidence of each genotype for the rs11520772 and the rs597319 polymorphism (p=0.897 and p=0.825, respectively) (data not shown). AA carriers have better SOS values in relation to TT carriers of rs11520772 as is shown in Table 1.

Also subjects with AA genotype have lower PTH from those with AT genotype and after adjustment for age (B= -3.446, p=0.009). SI higher values are associated to the AA genotype of rs597319 in relation to GG genotype (p=0.007) (table 1).

The mean values of serum 25(OH)D, PTH, and QUS parameters according to gender, age group, season of blood sampling and area of residence, as well as the differences between groups, are presented in table 2. After classification of population in two groups according to vitamin D status (25(OH)D< 20ng/mL and 25(OH)D≥20ng/mL), 53.7% of subjects have serum 25(OH)D< 20ng/mL and are older (p=0.013) than those who have 25(OH)D≥20ng/mL. Those who have serum 25(OH)D 20ng/mL or higher had greater values of SOS parameter (p=0.029) and lower PTH (p=0.002). After adjustment with age, the significance remain for PTH (p=0.024) but SOS does not reaches the limits of significance (p=0.054). There is no deference in prevalence of several genotypes for the rs11520772 (AA, AT, TT) and the rs597319 (AA, AG, GG) polymorphism , between age groups (18- 50 years, 51-65 years and >65 years, p=0.471 and p=0.476), seasons of blood sampling (winter-spring and summer-autumn, p=0.072 and p=0.344), serum vitamin D status (25(OH)D <20ng/mL and 25(OH)D ≥20ng/mL, p=0.655 and p=0.433) and area of residence (urban, rural, p=0.097 and p=0.825).

Among continues variables were included in this study, these were associated significant with SOS are PTH (r=-0.118, p=0.003), minutes per day of participation to organized physical activity (r=-0.173, p=0.01) and hours spending in sedentary activities per day (r=-0.093 , p= 0.019 respectively) and after adjustment for age (p= 0.049, p=0.048 and p=0.032, respectivly). Continues variables were associated with SI are age (r=-0.435, p=0.000), PTH (r=-0.181. p=0.000) and minutes per day of participation to organized physical activity (r=0.123, p=0.004). Other categorical variables associated with SI are sex (male 98.31±25.6, female 90.23±18.5, p=0.010) and area of residence (urban 90.32±20.17, rural 94.19±18.47, p=0.046). PTH lose its significance after the adjustment for age (p=0.073), participation to organized physical activity was not related to SI after adjustment for age and sex (p=0.073) as well as area of residence (p=0.159).

Variables that were correlated significant in simple statistical tests were entered in the general linear model with a stepwise procedure to evaluate the contribution of the genetic determinants of SOS and SI parameter along with the lifestyle and biochemical factors. The models presented in tables 3, 4 were derived according to the above data (tables 3,4).

Table1. Descriptive characteristics of population and mean level of biochemical, QUS and lifestyle parameters, by rs11520772 and rs597319 genotype.

	rs11520772 N=671 (%population)			**rs597319 N=478 (%population)**		
Variable Mean±SD	**AA (59.5%)**	**AT (32.0%)**	**TT (8.5%)**	**AA (42.3%)**	**AT (44.8%)**	**TT (13%)**
25(OH)D (ng/mL)	20.33±7.99	20.46±7.69	18.10±7.21	20.66±8.0	19.6±8.23	20.89±7.53
PTH(pg/mL)	**40.36±15.51***	**43.85±15.05***	39.73±2.46	43.58±15.62	43.13±14.73	42.31±16.73
Age	50.81±13.49	50.12±12.77	50.67±12.88	52.16±12.93	52.82±12.83	50.57±12.46
BMI	27.68±5.37	27.91±5.49	27.63±5.64	27.86±5.15	27.86±5.37	26.94±5.29
BUA (dB/MHz)	113.53±17.17	113.52±15.11	111.52±18.60	114.2±17.2	110.8±16.24	109.06±14.63
SOS (m/s)	**1558.56±36.49***	1546.18±125.36	**1511.07±239.14***	1558.64±38.03	1534.72±174.73	1548.01±28.9
SI	90.52±19.00	91.08±19.28	86.52±21.84	**92.2±16.67***	87.74±18.67	**82.09±16.57***
Organized physical activity moderate and/or vigorous (minutes/day)	11.57±23.33	16.14±35.32	6.5±14.20	13.71±32.27	11.67±24.20	7.39±15.3
TV watching or PC activity (hours/day)	3.05±2.10	3.15±1.96	2.98±2.17	3.09±1.91	3.25±2.163	3.0±1.75
Sun exposure summer (hours/day)	13.04±10.91	13.03±11.66	12.54±11.9	12.17±10.71	12.88±11.6	14.22±12.71

* ANOVA *p*-value<0.05

Table 2: Mean levels of 25(OH)D, PTH and QUS parameters according to sex, age group, season of blood sampling and area of residence.

	25(OH)D (ng/mL) mean±SD	**PTH (pg/mL) mean±SD**	**BUA (dB/MHz) mean**±SD	**SOS (m/s) mean**±SD	**SI mean**±SD
Vitamin D category (%population)					
25(OH)D<20ng/mL (53,7%)	-	41.63±15.91*	113.82±16.52	1545.95±115.0*	90.80±19.95
25(OH)D≥20ng/mL (46.3%)	-	38.37±14.83*	115.81±16.27	1560.86±37.31*	92.04±19.66
Sex (%population)					
male (11.97%)	22.55±9.09*	39.62±16.16	119.69±19.4*	1558.35±155.86	98.31±25.58*
female (88.03%)	19.64±7.76*	40.08±15.37	113.93±15.67*	1552.23±70.10	90.23±18.51*
Age group (years) (%population)					
18-50 (50.69%)	20.58±8.09*	35.60±14.09*	119.35±15.74*	1564.70±84.80*	98.85±18.67*
51-65 (36.95%)	19.76±7.92	43.19±14.82*	109.88±14.64*	1538.56±98.8*	84.68±17.33*
>65 (12.36%)	18.31±7.42*	48.52±16.03*	104.67±16.26*	1532.92±33.99*	76.15±17.11*
Season (%population)					
Winter-spring (38.6%)	19.18±7.73*	42.47±14.92*	113.61±17.25	1553.33±80.45	90.05±19.86
Summer - autumn (61.4%)	20.56±8.11*	38.56±15.69*	115.05±15.9	1553.05±80.45	92.35±19.73
Area of residence					
Urban (71.51%)	20.4±8.51*	38.75±15.84*	115.07±16.41	1549.44±99.44	90.32±20.17*
Rural (28.49%)	18.97±6.42*	43.43±14.12*	113.99±16.36	1562.9±38.11	94.19±18.47*

Notes: * $p<0.05$

Table 4. Determinants of SOS parameter

Variable	**B (Standard errors)**
Intercept	1563.389 (24.901)***
rs11520772 (ref. TT-genotype) AA AT	 47.368 (18.272)** 34.414 (19.126)
Age (years)	-1.067 (0.368)*
Adjusted R Squared	0.028

Notes: *p<0.05, **p<0.01, ***p<0.001

Table 5. Determinants of SI parameter

Variable	**B (Standard errors)**
Intercept	109.898 (4.412)***
rs597319 (ref. GG-genotype) AA AG	 11.570 (2.971)*** 7.738 (2.965)*
Age (years)	-0.590 (0.073)**
Sex (ref. female) male	 5.697 (2.889)
Adjusted R Squared	0.197

***p-*Value*=0.00, **p-*Value*=0.02, *p-*Value*=0.05

DISCUSSION

Current study investigates how rs11520772 and rs597319 affect bone health parameters BUA, SOS and SI as well as serum Vitamin D levels, taking into consideration lifestyle parameters.

A Greek study of all age group women demonstrates that adulthood, BMI, and organized physical activity are positive determinants of QUS parameters, these results are similar to the results derived from simple associations in current study [28]. Although when the genetic factor included in the linear model, the association of this phenotypes with QUS parameters failed to reach the statistical significance.

The rs11520772 is mapped to intronic regulatory region of TAX1BP1 gene on chromosome 7p15.2 (A→T). The rs11520772 polymorphism had been associated with BUA as derived from discovery phase of GWAS meta-analysis in GEFOS/ GENOMOS consortium [11]. TAX1BP1 gene was expressed in 27 tissues, especially in adrenal (RPKM 48.4) and thyroid (RPKM 48.2) and there's not been an identified mechanism of action on bone health. Tax1 binding protein 1 (TAX1BP1) gene encodes a HTLV-1 tax1 binding protein. The encoded protein interacts with TNFAIP3, and inhibits TNF-induced apoptosis by mediating the TNFAIP3 anti-apoptotic activity. Degradation of this protein by caspase-3-like family proteins is associated with apoptosis induced by TNF. This protein may also have a role in the inhibition of inflammatory signaling pathways [NCBI Gene ID 8887]. In ou population, wild type allele frequency is 0.755 and effect allele frequency is 0.244. The corresponding frequencies as they resulted from 1000 Genome project [18] are 0.815 for 'A' allele and 0.185 for 'T' allele for the European population and 0.898 and 0.102 for global population respectively. In the population of the OSTEOS study, rs11520772 polymorphism is related with serum with the SOS parameter. Univariate General Linear Model was used to evaluate the influence of genotype along with other statistical significant lifestyle factors, on SOS values.

The rs597319 polymorphism located in 11q14.2 a novel locus at 11q14.2 (TMEM135, rs597319) that was first identified as associated with heel QUS on GEFOS / GENOMOS consortium. The new locus near the transmembrane protein 135 (TMEM135) gene, that was genome-wide significant for both BUA and SOS. The TMEM135 gene was first identified in a human lung adenocarcinoma cell line cDNA library. It has been suggested that it is critically involved in the process of osteoblastogenesis from human multipotent adipose tissue-derived stem cells. Marrow fat cells and osteoblasts share a common stromal precursor and there is currently great interest in the role of increased marrow fat in osteoporotic conditions and the metabolic inter-relationships between these neighboring cell types. In depth protein sequence analysis showed that TMEM135 is a multi-transmembrane protein with seven transmembrane helices of high confidence. Homologies exist

between TMEM135 and the transmembrane region of frizzled-4, a known component of the Wnt signaling pathway. ENCODE project data show that two SNPs in the intronic region of TMEM135 and close to our lead signal (rs502580 and rs603140, both with high linkage disequilibrium with rs597319 [r^2 >0.92], and both highly associated with QUS outcomes in our discovery cohorts [P ~ 1.3 × 10–7 for both]) are associated with changes in MIF-1 and Cart1

motifs in osteoblastic cell lines. Interestingly, both of these transcription factors have been previously shown to be associated with skeletal development and bone density. Furthermore, TMEM135 was previously reported to be associated with longevity and walking speed in humans. In summary, the associations observed in our study might be the results of direct effects of increased osteoblastogenesis on heel bone properties, or indirect effects mediated through increased mechanical loading of the calcaneum, associated with faster movements. In our population, 'A' allele frequency is 0.646 and G allele frequency is 0.354. The corresponding frequencies as they resulted from 1000 Genome project [18] are 0.707 for 'A' allele and 0.293 for 'G' allele for the European population and 0.543 and 0.457 for global population respectively.

As identified by 3 GWAS meta-analysis, 6 genetic loci (GS: rs3755967, NADSYN1/ DHCR7: rs12785878, CYP2R1: rs10741657, CYP24A1: rs17216707, AMDHD1: rs10745742, SEC23A: rs8018720), associated with 25(OHD) levels and explain the 7.5% of heritability. The difference between twin (50-80%) and GWAS studies may be due to influence by environmental conditions [23-25]. In current study was not evaluated any association of genotypes with vitamin D status.

SOS positive determinant is AA genotype and negative determinant is age. SOS is the main QUS parameter that associated in several studies with bone quantity (BMD and bone mass) and bone quality (microarchitecture, strength and elasticity) [27]. The mechanism witch above parameters of bone may be influenced by the AA genotype of rs11520772, remains unknown. The above determinants explain the 2.8% of SOS variability.

SI positive determinants are AA genotype of rs597319 polymorphism and male gender. Age is a negative determinant. On GEFOS / GENOMOS consortium the rs597319 polymorphism was genome-wide significant for both BUA and SOS. In the current only SI was statistical associated with this SNP. SI is a combination of normalized SOS and BUA and is considered to improve the variability of SOS and BUA. The different result between two cohorts probably exists due to the small sample of our study. Gender and genotype explain the 19,7% of SI variability.

This current study has several limitations: it is an observational, cross-sectional study and it is not appropriate to draw causal effect implications or to generalize the results from this population. Residual confounding may also exist because of unmeasured variables. Another limitation of the study is the self-reported medical history and medication data. The study has

also several strengths: it was contacted in a large national representative population of urban and rural citizens, with a vast age range.

In the current study we try to evaluate the effect of several lifestyle factors as well as a genetic factors were derived from GEFOS / GENOMOS consortium on QUS parameter and 25(OH)D status. It would be more informative, for a future study, if it included the dietary habits as determinants of QUS and vitamin D status. Although Vitamin D status seems not to be significantly influenced by vitamin D intake for the European race when it is included in the same model with other lifestyles and genetic factors [29].

CONCLUSIONS

The effect of rs11520772 polymorphism on TAX1BP1 gene along with other environmental and biochemical factors on SOS parameter in a sample of Greek population has been evaluated from the current study. TAX1BP1 gene is expressed in a lot of tissues and more studies are needed for the investigation of mechanism of action of this novel polymorphism on bone health parameters. It has been also evaluated the effect of rs597319 polymorphism located close to TMEM135 a gene recently linked to osteoblastogenesis and longevity.

REFERENCES

1. NIH (2000) Osteoporosis prevention, diagnosis and therapy. NIH Consensus Statements. 17:1-45
2. Report of a WHO Study Group (1994) Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. World Health Organ Tech Rep Ser 843:1-129
3. Moayyeri A, Adams JE, Adler RA, Krieg MA, Hans D, Compston J, Lewiecki EM. (2012) Quantitative ultrasound of the heel and fracture risk assessment: an updated meta-analysis Osteoporos Int 23(1):143-53
4. Marin F. González-Macías J, Díez-Pérez A, Palma S, Delgado-Rodríguez M. (2006) Relationship between Bone Quantitative Ultrasound and Fractures: A Meta-Analysis. J Bone Miner Res 21:1457–1463
5. Grigoriou EV, Trovas G, Papaioannou N, Makras P,4, Kokkoris P,4, Dontas I, Makris K, Tournis S, Dedoussis GV. Serum 25-hydroxyvitamin D status, quantitative ultrasound

parameters, and their determinants in Greek population. Arch Osteoporos. 2018 Oct 15;13(1):111. doi: 10.1007/s11657-018-0526-5

6. Arden, N.K., Baker, J., Hogg, C., Baan, K. and Spector, T.D. (1996) The heritability of bone mineral density, ultrasound of the calcaneus and hip axis length: a study of postmenopausal twins. J. Bone Miner. Res., 11, 530–534
7. Howard, G.M., Nguyen, T.V., Harris, M., Kelly, P.J. and Eisman, J.A. (1998) Genetic and environmental contributions to the association between quantitative ultrasound and bone mineral density measurements: a twin study. J. Bone Miner. Res., 13, 1318–1327]
8. Karasik, D., Myers, R.H., Hannan, M.T., Gagnon, D., McLean, R.R., Cupples, L.A. and Kiel, D.P. (2002) Mapping of quantitative ultrasound of the calcaneus bone to chromosome 1 by genome-wide linkage analysis. Osteoporos. Int., 13, 796–802.
9. Zhai, G., Andrew, T., Kato, B.S., Blake, G.M. and Spector, T.D. (2009) Genetic and environmental determinants on bone loss in postmenopausal Caucasian women: a 14-year longitudinal twin study. Osteoporos. Int., 20, 949–953.
10. Michaelsson, K., Melhus, H., Ferm, H., Ahlbom, A. and Pedersen, N.L. (2005) Genetic liability to fractures in the elderly. Arch. Intern. Med., 165, 1825–1830
11. Alireza Moayyeri et al. Genetic determinants of heel bone properties: genome-wide association meta-analysis and replication in the GEFOS/GENOMOS consortium. Human Molecular Genetics, 2014, Vol. 23, 3054–3068, doi:10.1093/hmg/ddt675
12. Holick MF. Vitamin D Deficiency. (2007) N Engl J Med 357:266-281.
13. Karohl, C. et al. Heritability and seasonal variability of vitamin D concentrations in male twins. Am. J. Clin. Nutr. 92, 1393–1398 (2010)
14. Orton, S.-M. et al. Evidence for genetic regulation of vitamin D status in twins with multiple sclerosis. Am. J. Clin. Nutr. 88, 441–447 (2008)
15. World Health Organization. (2000) Obesity: preventing and managing the global epidemic. Report of a WHO consultation. World Health Organ Tech Rep Ser. 894:i-xii, 1-253
16. Trovas G, Tsekoura M, Galanos A, Dionyssiotis Y, et al. Quantitative ultrasound of the calcaneus in greek women: normative data are different from the manufacturer's normal range. J Clin Densitom. 2009;12(3):353-9
17. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, Murad MH, Weaver CM. (2011) Evaluation, Treatment, and Prevention of Vitamin D Deficiency:

an Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab 96(7):1911–1930

18. 1000 Genomes Project Consortium, Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, Marchini JL, McCarthy S, McVean GA, Abecasis GR. A global reference for human genetic variation. Nature. 2015 Oct 1;526(7571):68-74. doi: 10.1038/nature15393
19. Gianoudis J, Bailey C. A, Daly R. M. (2015) Associations between sedentary behavior and body composition, muscle function and sarcopenia in community-dwelling older adults. Osteoporos Int 26:571–579,
20. Kauppi M, Impivaara O, Mäki J, Heliövaara M, Marniemi J, Montonen J, Jula A. (2009) Vitamin D status and common risk factors for bone fragility as determinants of quantitative ultrasound variables in a nationally representative population sample. Bone. 45(1):119-24
21. Moschonis G, Tanagra S, Koutsikas K, Nikolaidou A, Androutsos O, Manios Y. (2009) Association between serum 25-hydroxyvitamin D levels and body composition in postmenopausal women: the postmenopausal Health Study. Menopause.16(4):701-7. doi: 10.1097/gme.0b013e318199d5d5.
22. Carlberg C. Nutrigenomics of Vitamin D. Nutrients 2019,11, 676; doi:10.3390/nu11030676
23. Wang, T. J. et al. Common genetic determinants of vitamin D insufficiency: a genome-wide association study. Lancet Lond. Engl. 376, 180–188 (2010)
24. Ahn, J. et al. Genome-wide association study of circulating vitamin D levels. Hum. Mol. Genet. 19, 2739–2745 (2010)
25. Xia Jiang et al. Genome-wide association study in 79,366 European-ancestry individuals informs the genetic architecture of 25-hydroxyvitamin D levels NATURE COMMUNICATIONS (2018) 9:260DOI: 10.1038/s41467-017-02662-22
26. Boland RL, Vitamin D physiology, Mol Cell Endocrinol. 2011;347(1-2):11-6. doi: 10.1016/j.mce.2011.05.021
27. Kok-Yong Chin and Soelaiman Ima-Nirwana. Calcaneal Quantitative Ultrasound as a Determinant of Bone Health Status: What Properties of Bone Does It Reflect? Int. J. Med. Sci. 2013; 10(12):1778-1783. doi: 10.7150/ijms.6765

28. Babaroutsi E, Magkos F, Manios Y, Sidossis LS. Lifestyle factors affecting heel ultrasound in Greek females across different life stages Osteoporos Int. 2005 May;16(5):552-61
29. Choi et al Genes and Nutrition 2018, Yao et al Am J Clin Nutr 2017

Supplementary file

Table S1. Biochemical, anthropometric and QUS parameters according to genotype of 8 polymorphisms of OSTEOS study

polymorphism Genotype (frequency %)	**25(OH)D** mean ± SD (*p-Value*)	**PTH** mean ± SD (*p-Value*)	**BMI** mean ± SD (*p-Value*)	**BUA** mean ± SD (*p-Value*)	**SOS** mean ± SD (*p-Value*)	**SI** mean ± SD (*p-Value*)
rs11520772						
AA (59.7)	20.33±7,99	**40.36±15.51***	27.68±5.37	113.53±17.17	**1558.56±36.49***	90.52±19.00
TA (31.8)	20.46±7.69	**43.85±15.05***	27.91±5.49	113.52±15.11	1546.18±125.36	91.08±19.28
TT (8.6)	18.10±7.21	39.73±2.46	27.63±5.64	111.52±18.6	**1511.07±239.14***	86.52±21.88
rs2908007						
AA (32.7%)	20.5±7.98	43.76±15.96	27.4±5.14	111.9±15.09	1539.78±153.33	88.32±18.58
GA (48.7)	19.87±8.22	43.96±15.25	28.09±5.35	112.13±18.13	1548.12±116.86	88.79±20.34
GG (18.6)	20.27±7.87	41.02±15.51	27.64±5.44	111.85±14.3	1553.37±34.51	90.74±16.99
rs2982552						
AA (37.6)	20.27±7.7	43.45±15	27.8±5.29	113.3±15.54	1558.56±35.71	90.54±19.2
GA (2.7)	20.38±8.23	42.86±15.5	27.5±5.12	111.74±16.4	1545.67±129.69	89.63±19.03
GG (19.7)	19.49±8.43	44.16±15.9	28.35v5.73	110.24±18.75	1526.15±182.55	84.34±19.28
rs3000634						
AA (73.5)	20.22±8.16	43.65±15.8	27.92±5.4	112.21±16.7	1540.71±138.81	88.05±18.78
GA (24.2)	20.08±7.72	42.8±14.16	27.54±5.15	112.33±16.5	1564.66±35.64	92.2±20.23
GG (2.3)	19.46±9.14	36.09±10.1	26.10±4.36	102.13±13.8	1538.76±32.44	83.78±19.64
rs3020331						
CC (35.2)	19.78±8.9	44.13±15.26	27.99±5.4	110.55±33.98	1550.91±33.98	86.66±18.0
TC (46.7)	20.47±7.67	42.03±15.72	27.42±5.25	113.34±17.42	1540.29±172.6	91.47±20.0
TT (18.1)	20.11±7.38	44.81±14.31	28.34±5.31	111.56±16.37	1554.14±39.18	87.02±19.06
rs597319						
AA (42.3)	20.66±8.0	43.58±15.62	27.86±5.15	114.2±17.2	1558.64±38.03	**92.2±16.67***
GA (44.8)	19.6±8.23	43.13±14.73	27.86±5.37	110.8±16.24	1534.72±174.73	87.74±18.67
GG (13.0)	20.89±7.53	42.31±16.73	26.94±5.29	109.06±14.63	1548.01±28.9	**82.09±16.57***
rs7741021						
AA (35.1)	20.24±8.4	44.03±15.0	27.57±5.24	109.82±14.99	1541.22±138.17	86.41±19.65
CA (46.8)	20.12±7.83	41.78±15.38	27.87±5.41	114.37±17.77	1557.71±37.18	91.25±19.51
CC (18.1)	20.48±7.94	45.16±16.18	27.69±5.13	110.64±15.83	1529.36±196.51	87.35±16.48
rs9292469						
CC (36.9)	19.67±8.53	41.45±14.74	27.63±5.17	110.69±20.33	1525.89±20.33	86.03±15.22
TC (48.2)	20.78±8.1	44.37±16.33	27.7±5.46	112.65±17.43	1555.1±35.89	89.72±20.39
TT (14.9)	19.60±6.9	42.74±13.18	27.93±4.86	114.0±16.77	1563.64±41.31	91.34±22.0

***p-*Value <0.05**

3.3 Consortia participation

3.3.1. Paper 4. This large- scale work extend the findings for central DXA derived BMD phenotypes by searching for single nucleotide polymorphisms (SNPs) associated with heel QUS or heel DXA measures across the human genome.

Key points

- A new locus on chromosome 11q14.2 (rs597319 close to *TMEM135),* significantly associated with both BUA and VOS
- Totaly 9 polymorphisms associated with heel QUS phenotypes.

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Genetic determinants of heel bone properties: genome-wide association meta-analysis and replication in the GEFOS/GENOMOS consortium

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Quantitative ultrasound of the heel captures heel bone properties that independently predict fracture risk and, with bone mineral density (BMD) assessed by X-ray (DXA), may be convenient alternatives for evaluating osteoporosis and fracture risk. We performed a meta-analysis of genome-wide association (GWA) studies to assess the genetic determinants of heel broadband ultrasound attenuation (BUA; $n = 14\,260$), velocity of sound (VOS; $n = 15\,514$) and BMD ($n = 4566$) in 13 discovery cohorts. Independent replication involved seven cohorts with GWA data (*in silico* $n = 11\,452$) and new genotyping in 15 cohorts (*de novo* $n = 24\,902$). In combined random effects, meta-analysis of the discovery and replication cohorts, nine single nucleotide polymorphisms (SNPs) had genome-wide significant ($P < 5 \times 10^{-8}$) associations with heel bone properties. Alongside SNPs within or near previously identified osteoporosis susceptibility genes including *ESR1* (6q25.1: rs4869739, rs3020331, rs2982552), *SPTBN1* (2p16.2: rs11898505), *RSPO3* (6q22.33: rs7741021), *WNT16* (7q31.31: rs2908007), *DKK1* (10q21.1: rs7902708) and *GPATCH1* (19q13.11: rs10416265), we identified a new locus on chromosome 11q14.2 (rs597319 close to *TMEM135*, a gene recently linked to osteoblastogenesis and longevity) significantly associated with both BUA and VOS ($P < 8.23 \times 10^{-14}$). In meta-analyses involving 25 cohorts with up to 14 985 fracture cases, six of 10 SNPs associated with heel bone properties at $P < 5 \times 10^{-6}$ also had the expected direction of association with any fracture ($P < 0.05$), including three SNPs with $P < 0.005$: 6q22.33 (rs7741021), 7q31.31 (rs2908007) and 10q21.1 (rs7902708). In conclusion, this GWA study reveals the effect of several genes common to central DXA-derived BMD and heel ultrasound/DXA measures and points to a new genetic locus with potential implications for better understanding of osteoporosis pathophysiology.

INTRODUCTION

Bone structure *in vivo* has largely been evaluated using the attenuation of a photon beam by hydroxyapatite, the principal mineral in bone. This is positively related to the mass of hydroxyapatite in the path of the beam conventionally termed bone mineral content and normalized to bone area to produce an entity termed areal bone mineral density (BMD). To allow for the reduced attenuation of the beam by overlying non-bone tissues in central areas of the body, two photon beam energies are used, resulting in a clinical technique termed dual-energy X-ray absorptiometry (DXA), which at peripheral skeletal sites is termed pDXA.

Over the past 60 years, ultrasonic material analysis has been developed as a method of determining material properties of a variety of structures. In the last 30 years, this methodology has been applied to the *in vivo* assessment of bone structure and fragility termed quantitative ultrasound (QUS). This consists of the use of two separate ultrasound measurement techniques, velocity of sound (VOS) and broadband ultrasound attenuation (BUA). While much remains to be discovered about the exact

physical determinants of QUS measures in the intact living calcaneum (1), cadaver studies have established a strong correlation of such indices with bone quantity and trabecular structure (2). Assessment of bone properties in the heel using QUS can predict the risk of prevalent osteoporotic fractures, such as those in the spinal vertebrae, comparably with DXA of the spine or hip, the so-called gold standard clinical techniques (3–5). Pearson correlation coefficients of heel QUS or pDXA with central DXA of the hip or spine in population-based studies are modest, typically in the range of 0.4–0.6 (6). Moreover, twin- and family-based studies have found genetic correlations of the order of 0.3–0.6 and environmental correlations of the order of 0.1–0.3 (7–9); yet relative risk estimates for fracture using QUS are of similar magnitude to those derived from central DXA (5,10,11). A recent meta-analysis showed that heel QUS predicts risk of various fractures (hip, vertebral and any clinical fractures) independently from hip BMD (12). Overall, these results suggest that QUS of the calcaneum might capture additional genetic determinants of bone structure beyond those associated with central DXA.

A genetic contribution to osteoporosis is well established with heritability estimates reaching 84% for central BMD (13), 74% for heel QUS (7,14), 47% for bone loss (15), and 48% for hip fracture (16). Previous genome-wide association (GWA) studies have identified several chromosomal regions associated with BMD in the hip and lumbar spine regions (17,18). The most recent meta-analysis of GWA studies, performed in the context of the Genetics Factors for Osteoporosis (GEFOS) consortium, identified 56 genome-wide significant loci (32 new) associated with hip/spine BMD (19). Fourteen out of these 56 BMD-associated loci were also associated with fracture risk in a case–control meta-analysis involving ~31 000 fracture cases among 133 000 individuals (19). Using data from the GEFOS consortium, we aimed to extend the findings for central DXA-derived BMD phenotypes by searching for single nucleotide polymorphisms (SNPs) associated with heel QUS or heel DXA measures across the human genome.

RESULTS

Participant characteristics are summarized in Table 1 and key features of the discovery and replication phases are summarized in Figure 1. In aggregate, the initial discovery phase meta-analysis in 11 cohorts (Supplementary Material, Table S1) identified 42 loci of at least suggestive significance in relation to heel bone measures, of which 9 overlapped with loci previously found to be potentially associated with hip or spine BMD in the GEFOS-BMD meta-analysis (19). Regional conditional analyses results were available for QUS measures from 9 cohorts (comprising 7 of the initial discovery cohorts and a further 2 new cohorts that joined later). Based on the results of the conditional analyses (that identified two secondary signals for the QUS measures) and final combined meta-analysis of the unconditional results from all 13 discovery cohorts, a total of 25 independent SNPs (Table 2) were selected for replication in the next phase (i.e. *in silico* studies and *de novo* genotyping). Including the two secondary signals, the selected SNPs comprise 15 SNPs that were primarily associated with either BUA or VOS, and 12 SNPs that were associated with heel DXA BMD (Table 2).

Associations between the 15 SNPs that were considered for replication primarily on the basis of their association with heel BUA or VOS are shown in Figure 2. The SNP characteristics are summarized in Table 2. In the combined meta-analysis of the discovery and replication cohorts using a random-effects model, 9 SNPs showed genome-wide significant associations, of which 7 were previously reported to be associated with central DXA BMD (19). Two of the SNPs (rs7741021 and rs2908007) also showed genome-wide significant association with heel DXA BMD (Table 2). Three SNPs on chromosome 6q25.1 (rs4869739, rs3020331 and rs2982552) mapped to intronic or regulatory regions around the *ESR1* (estrogen receptor 1) and *CCDC170* (coiled-coil domain containing 170, previously known as *C6orf97*) genes (Fig. 3), and five other SNPs mapped to loci within or near previously identified osteoporosis susceptibility genes, including 2p16.2 (*SPTBN1*, rs11898505), 6q22.33 (*RSPO3*, rs7741021), 7q31.1 (*WNT16*, rs2908007), 10q21.1 (*DKK1*, rs7902708) and 19q13.11 (*GPATCH1*, rs10416265). We identified a new locus on chromosome 11q14.2 (*TMEM135*, rs597319) significantly associated with both BUA and VOS ($P < 8.23 \times 10^{-14}$).

Subsidiary comparisons with fixed-effect meta-analysis results (Supplementary Material, Table S2 and Figs S2 and S3) suggested two additional genome-wide significant loci; one at 7p14.1 upstream of *EPDR1* (rs6974574, $P < 4.92 \times 10^{-8}$ for BUA and VOS) and the other at 13q14.11 upstream of *AKAP11* (rs9533090, $P = 5.33 \times 10^{-8}$ for VOS), although there was statistically significant between-study heterogeneity in these two loci for the respective phenotypes (Supplementary Material, Table S3), necessitating some caution in generalizing the fixed-effect meta-analysis results. Figure 4 provides a comparison of the magnitudes of association of the 25 SNPs with heel bone measures and central DXA BMD, suggesting generally concordant associations in the overlapping genome-wide significant or suggestive loci.

We further tested if the genome-wide significant or suggestive genetic loci were associated with fracture risk based on data available from 25 cohorts with up to 54 245 participants, among whom there were 14 958 cases of any fracture (excluding fractures of the skull and extremities, i.e. fingers and toes), 10 663 non-vertebral fractures and 3220 clinical vertebral fractures (Supplementary Material, Table S4). Ten of 10 SNPs associated with heel bone properties at $P < 5 \times 10^{-6}$ showed the expected directions of association with any fracture outcome based on the point estimates (Fig. 5). Furthermore, 6 of these 10 SNPs showed nominally significant ($P < 0.05$) associations with fractures, including three SNPs with $P < 0.005$ (i.e. corrected for multiple comparisons using Bonferroni method) at 6q22.33 (rs7741021), 7q31.31 (rs2908007), and 10q21.1 (rs7902708). Fixed-effect meta-analysis gave similar results (Supplementary Material, Fig. S4).

Supplementary Material, Figure S5 presents forest plots of the study-specific results and summary estimates by random-effects meta-analysis for the 15 SNPs that were considered for replication primarily on the basis of their association with heel BUA or VOS in GWA discovery meta-analysis, suggesting generally consistent results across cohorts for a majority of the SNPs. Supplementary Material, Figure S6 shows the regional association plots within a one megabase window of the top SNP in each locus in the GWA discovery meta-analysis, demonstrating

Table 1. Characteristics of studies that contributed to GWAS discovery and replication of SNP associations with heel QUS/DXA BMD measures

Stage\cohort	Country	Demographics					Heel QUS/DXA BMD outcomes					
							BUA (dB/MHz)		VOS (m/s)		Heel BMD (g/cm^2)	
		N	Females (%)	Age (years) Mean (SD)	Weight (kg) Mean (SD)	Height (cm) Mean (SD)	*N*	Mean (SD)	*N*	Mean (SD)	*N*	Mean (SD)
GWAS discovery												
EPIC	UK	2630	56	62.1 (8.6)	80.5 (15.4)	167 (9)	2630	83 (19)	2630	1632 (40)	–	–
FHS	USA	3229	58	64.6 (11.9)	76.8 (17.2)	166 (10)	3229	73 (21)	3225	1548 (38)	–	–
HKOS	China	730	100	48.7 (15.4)	54.8 (10.4)	155 (7)	730	74 (22)	730	1551 (41)	–	–
NSPHS06	Sweden	495	55	51.4 (19.1)	71.9 (12.8)	164 (10)	495	96 (21)	–	–	–	–
RSI	Netherlands	1615	54	66.5 (8.2)	74.3 (11.8)	169 (9)	1615	112 (13)	1615	1525 (37)	–	–
SHIP	Germany	1198	54	58.0 (13.5)	80.2 (15.8)	168 (9)	1198	115 (15)	1198	1565 (35)	–	–
SHIP-TREND	Germany	687	56	50.8 (13.6)	78.7 (15.1)	170 (9)	687	116 (14)	687	1571 (33)	–	–
TWINSUK1	UK	1701	100	46.2 (12.1)	65.8 (12.5)	163 (6)	1701	76 (18)	1701	1658 (49)	–	–
TWINSUK23	UK	1975	100	46.9 (12.5)	66.1 (12.2)	163 (6)	1975	76 (18)	1975	1653 (50)	–	–
H2SS	Korea	1753	53	60.8 (6.6)	61.9 (10.0)	158 (8)	–	–	1753	1591 (45)	–	–
AGES	Iceland	3179	58	76.4 (5.4)	75.8 (14.3)	167 (9)	–	–	–	–	3179	0.491 (0.152)
CroatiaKorcula	Croatia	878	64	56.3 (14.2)	79.0 (14.2)	168 (9)	–	–	–	–	878	0.443 (0.098)
CroatiaSplit	Croatia	499	57	49.3 (14.7)	80.6 (16.3)	172 (9)	–	–	–	–	499	0.459 (0.101)
Subtotal		20 569	66	60.3 (11)	73.3 (14.1)	165 (9)	14 260	86 (18)	15 514	1593 (42)	4556	0.478 (0.138)
In silico replication												
AOGC[a]	Australia/UK[b]	1955	100	69.6 (8.6)	69.6 (17.3)	158 (16)	–	–	–	–	–	–
B-PROOF	Netherlands	1092	59	74.0 (6.7)	76.0 (12.4)	168 (9)	1092	69 (17)	1091	1535 (32)	–	–
HABC	USA	1493	48	74.8 (2.9)	73.8 (14.3)	167 (9)	1493	73 (18)	1493	1541 (30)	–	–
MICROS	Italy	588	45	46.0 (16.6)	70.2 (14.9)	167 (9)	588	73 (16)	588	1544 (29)	–	–
MrOS-USA	USA	3925	0	73.9 (5.9)	83.1 (12.7)	175 (7)	3925	79 (17)	3925	1551 (30)	–	–
SOF	USA	2103	100	80.1 (4.2)	66.3 (12.5)	158 (6)	2103	59 (17)	2103	1527 (30)	–	–
YFS	Finland	1265	58	37.9 (5.0)	75.8 (15.5)	172 (9)	1265	80 (16)	1265	1559 (29)	1250	0.560 (0.110)
HCS-AUS	Australia	986	49	66.2 (7.6)	79.4 (15.5)	166 (9)	–	–	–	–	986	0.538 (0.166)
Subtotal		13 407	52	69.2 (6.9)	75.4 (14.2)	167 (9)	10 466	73 (17)	10 465	1544 (30)	2236	0.550 (0.138)
De novo replication												
AUSTRIOS-B	Austria	448	85	83.6 (5.9)	62.0 (12.3)	156 (8)	448	90 (17)	448	1496 (36)	–	–
CABRIO-C	Spain	1274	62	62.4 (9.2)	73.7 (13.1)	161 (8)	1274	70 (23)	1273	1545 (41)	–	–
CAIFOS	Australia	1113	100	80.0 (2.6)	67.5 (12.1)	157 (6)	1113	101 (9)	1113	1516 (28)	–	–
CALEX-FAM	Finland	983	79	37.0 (22.4)	64.3 (16.9)	164 (11)	983	83 (16)	–	–	–	–
EMAS	Europe[b]	2870	0	59.9 (11.0)	83.1 (13.6)	173 (7)	2870	80 (19)	2870	1550 (34)	–	–
EPICNOR	UK	5723	54	63.6 (9.2)	73.2 (12.4)	167 (9)	5723	79 (20)	5718	1638 (43)	–	–
EPOLOS	Poland	684	56	53.4 (16.0)	73.2 (13.7)	166 (10)	684	112 (13)	684	1548 (35)	–	–
FLOS	Italy	1000	84	59.8 (12.7)	64.8 (12.3)	163 (9)	1000	58 (7)	1000	1503 (83)	–	–
GEOS	Canada	5495	100	55.8 (10.3)	65.4 (11.9)	158 (6)	5495	111 (10)	5495	1546 (32)	–	–
LASA	Netherlands	894	51	75.6 (6.5)	74.2 (12.6)	166 (9)	894	71 (20)	894	1611 (44)	–	–
MrOS-SWE	Sweden	1718	0	75.4 (3.2)	80.6 (12.0)	175 (7)	1718	81 (21)	1718	1555 (38)	–	–
OPRA	Sweden	821	100	75.2 (0.1)	67.6 (11.3)	160 (6)	821	102 (10)	821	1523 (27)	–	–
OSTEOSII	Greece	307	87	50.5 (12.6)	74.1 (15.7)	163 (7)	307	112 (16)	307	1556 (36)	–	–
PEAK25	Sweden	857	100	25.5 (0.2)	64.5 (11.2)	168 (6)	857	118 (11)	857	1575 (32)	–	–
SWS	UK	715	100	29.7 (3.7)	72.4 (14.8)	163 (7)	714	72 (13)	715	1548 (27)	–	–
Subtotal		24 902	64	60.2 (10.0)	71.6 (12.7)	165 (8)	24 901	89 (16)	23 913	1568 (40)	–	–
Total		58 878	62	62.3 (9.7)	73.0 (13.6)	165 (8)	49 627	85 (17)	49 892	1570 (39)	6792	0.502 (0.138)

[a]The AOGC cohort contributed to *in silico* lookups of SNP-fracture associations only.
[b]The EMAS study comprises cohorts in Belgium, Estonia, Hungary, Italy, Poland, Spain, Sweden and UK.

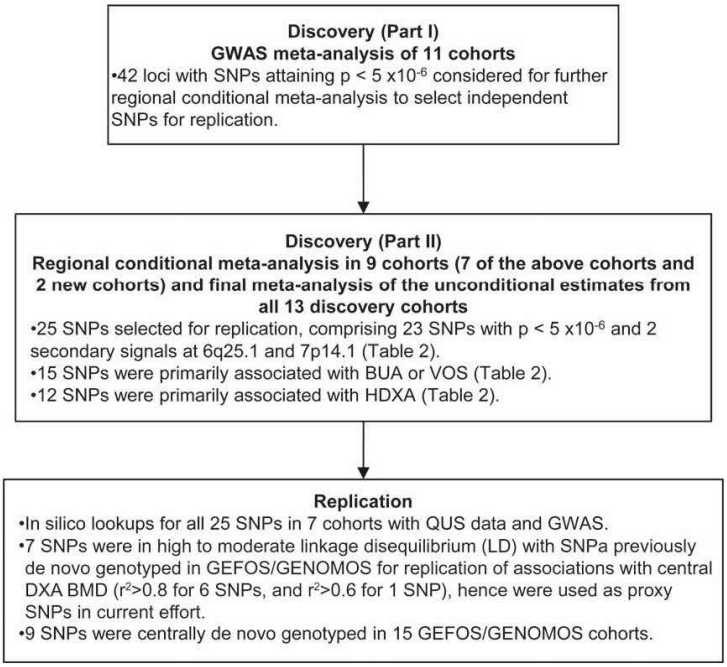

Figure 1. Flow chart summarizing key features of the discovery and replication phases.

strong credible association signals for a number of SNPs underlying the loci selected for replication.

Subsidiary investigation of potential sex differences in the association of SNPs and heel BUA or VOS measures did not reveal convincing evidence of potentially important differences, considering the secondary nature of the hypothesis and multiple comparisons done (Supplementary Material, Fig. S7).

DISCUSSION

This is the first large-scale collaborative GWA study for heel bone properties assessed by quantitative ultrasound and DXA of the heel. Its conception was inspired by the observational evidence of association of heel QUS measures and fracture risk (12), independent of central DXA BMD measures (20), demonstration of a reasonably high genetic heritability of heel QUS measures (7), and suggestions of pleiotropic effects of genes in the determination of bone phenotypes (8). Indeed, consistent with the expected similarities and differences in the physical properties of bone determined by DXA and QUS and prior evidence of moderate genetic correlations between the measures (7–9), we found evidence for some genetic loci common to heel QUS measures and central DXA BMD as well as a novel locus for heel QUS at 11q14.2 (*TMEM135*, rs597319) that had not been previously identified as associated with BMD or other bone phenotype.

Seven of nine genome-wide significant loci found in the present study were previously reported to be associated with BMD of the hip and/or spine (Fig. 4). This complements our previous findings (17–19) and lends support to the hypothesis of partially shared genetic determinants between QUS and BMD measures (7–9). A comparison of the standardized effect sizes (Fig. 4) also revealed existence of some quantitative differences for some SNPs. For example, in the 7q31.31 locus (*WNT16*), the effect of rs2908007 on heel measures was about three times as great as its effect on hip or spine BMD, supporting Karasik *et al.*'s finding that there is significant pleiotropy in the effects of genes on bone phenotypes at different measurement sites (8). Similar quantitative differences were also observed for rs7741021 at the 6q22.33 locus (*RSPO3*). In the absence of bias and assuming minimal type II errors (i.e. adequate power), such quantitative differences in effect sizes of SNPs at different skeletal sites might indicate heterogeneity in genetically mediated responses of the skeleton to environmental stimuli, including for example, ground reaction forces that are particularly high at the heel but are dampened at more proximal sites such as the lumbar spine (21,22).

Perhaps the most intriguing finding was that we identified a new locus for bone phenotypes on chromosome 11q14.2 (rs597319) near the transmembrane protein 135 (*TMEM135*) gene, that was genome-wide significant for both BUA and VOS. The *TMEM135* gene was first identified in a human lung adenocarcinoma cell line cDNA library (23). It has been

Table 2. Summary of *P*-values for association of SNPs in 25 loci with heel BUA, VOS or heel DXA BMD in GWAS discovery/replication meta-analysis

Locus	SNP	Closest gene	Genetic function	Discovery *P*-values[b]			Replication *P*-values[b]			Combined *P*-values[b]		
				BUA	VOS	DXA	BUA	VOS	DXA	BUA	VOS	DXA
Combined $P < 5 \times 10^{-8}$				9 cohorts, 14 258 participants			21 cohorts, 35 082 participants			30 cohorts, 49 335 participants		
2p16.2	rs11898505	*SPTBN1*	Intronic, regulatory region	$\mathbf{7.78\times10^{-8}}$	$\mathbf{2.92\times10^{-8}}$	7.68×10^{-1}	$\mathbf{6.66\times10^{-12}}$	1.10×10^{-4}	9.63×10^{-2}	$\mathbf{4.24\times10^{-13}}$	6.25×10^{-6}	2.65×10^{-1}
6q22.33	rs7741021	*RSPO3*	Intronic, regulatory region	$\mathbf{8.52\times10^{-7}}$	$\mathbf{1.72\times10^{-7}}$	7.69×10^{-6}	$\mathbf{1.19\times10^{-18}}$	$\mathbf{2.54\times10^{-21}}$	1.49×10^{-3}	$\mathbf{9.26\times10^{-21}}$	$\mathbf{9.58\times10^{-20}}$	$\mathbf{4.11\times10^{-8}}$
6q25.1	rs4869739	*CCDC170*	Intronic	$\mathbf{5.25\times10^{-10}}$	$\mathbf{4.75\times10^{-11}}$	$\mathbf{7.73\times10^{-10}}$	1.02×10^{-3}	$\mathbf{3.92\times10^{-8}}$	3.82×10^{-1}	$\mathbf{1.93\times10^{-9}}$	$\mathbf{2.64\times10^{-18}}$	1.21×10^{-2}
6q25.1	rs3020331[c]	*ESR1*	Intronic	1.27×10^{-2}	7.94×10^{-6}	2.01×10^{-4}	$\mathbf{3.04\times10^{-10}}$	$\mathbf{3.79\times10^{-17}}$	1.95×10^{-1}	$\mathbf{2.91\times10^{-9}}$	$\mathbf{6.64\times10^{-15}}$	1.26×10^{-3}
6q25.1	rs2982552	*ESR1*	Intronic, regulatory region	2.87×10^{-2}	$\mathbf{3.31\times10^{-6}}$	3.83×10^{-4}	$\mathbf{6.16\times10^{-17}}$	$\mathbf{1.14\times10^{-18}}$	1.00×10^{-1}	$\mathbf{1.70\times10^{-10}}$	$\mathbf{7.32\times10^{-16}}$	1.21×10^{-4}
7q31.31	rs2908007	*WNT16*	Upstream	$\mathbf{8.59\times10^{-21}}$	$\mathbf{5.02\times10^{-23}}$	$\mathbf{4.31\times10^{-11}}$	$\mathbf{1.31\times10^{-22}}$	$\mathbf{2.06\times10^{-39}}$	3.47×10^{-2}	$\mathbf{4.32\times10^{-35}}$	$\mathbf{1.62\times10^{-59}}$	$\mathbf{1.34\times10^{-9}}$
10q21.1	rs7902708	*MBL2/DKK1*	Intronic	8.23×10^{-3}	$\mathbf{1.46\times10^{-7}}$	9.51×10^{-1}	$\mathbf{1.02\times10^{-8}}$	$\mathbf{6.99\times10^{-9}}$	2.60×10^{-3}	$\mathbf{1.30\times10^{-8}}$	$\mathbf{5.29\times10^{-15}}$	2.47×10^{-1}
11q14.2	*rs597319*	*TMEM135*	*Intronic*	$\mathit{2.62\times10^{-4}}$	$\mathbf{\mathit{1.18\times10^{-8}}}$	$\mathit{5.05\times10^{-3}}$	$\mathbf{\mathit{2.01\times10^{-12}}}$	$\mathbf{\mathit{2.70\times10^{-17}}}$	$\mathit{2.20\times10^{-2}}$	$\mathbf{\mathit{8.23\times10^{-14}}}$	$\mathbf{\mathit{4.86\times10^{-26}}}$	$\mathit{3.05\times10^{-4}}$
19q13.11	rs10416265	*GPATCH1*	Non-synonymous coding	$\mathbf{8.30\times10^{-7}}$	$\mathbf{2.99\times10^{-8}}$	1.15×10^{-1}	$\mathbf{5.84\times10^{-8}}$	2.92×10^{-5}	3.45×10^{-1}	$\mathbf{2.37\times10^{-13}}$	$\mathbf{4.08\times10^{-12}}$	6.72×10^{-2}
Combined $P \geq 5\times10^{-8}$				9 cohorts, 14 258 participants			21 cohorts, 35 082 participants			30 cohorts, 49 335 participants		
5p13.3	rs9292469	*NPR3*	Upstream	$\mathbf{3.09\times10^{-6}}$	6.01×10^{-3}	9.27×10^{-1}	5.95×10^{-1}	1.69×10^{-1}	9.96×10^{-1}	1.43×10^{-1}	6.12×10^{-1}	9.42×10^{-1}
7p15.2	rs11520772	*TAX1BP1*	Intronic	$\mathbf{9.71\times10^{-7}}$	4.84×10^{-4}	6.24×10^{-1}	8.43×10^{-2}	1.32×10^{-1}	5.48×10^{-1}	2.86×10^{-4}	7.07×10^{-3}	8.79×10^{-1}
7p14.1	rs6974574[c]	*EPDR1*	Upstream	5.81×10^{-3}	1.34×10^{-5}	2.56×10^{-4}	2.51×10^{-4}	4.84×10^{-3}	7.31×10^{-1}	8.25×10^{-5}	3.89×10^{-5}	9.25×10^{-3}
7q11.23	rs38664	*UPK3B*	Intronic	9.10×10^{-4}	$\mathbf{1.52\times10^{-6}}$	6.60×10^{-1}	4.39×10^{-2}	1.58×10^{-2}	5.35×10^{-1}	3.25×10^{-4}	$\mathbf{1.02\times10^{-7}}$	8.79×10^{-1}
13q12.3	rs3000634	*USPL1*	Upstream	2.10×10^{-5}	$\mathbf{1.27\times10^{-7}}$	2.18×10^{-1}	6.80×10^{-3}	1.91×10^{-1}	5.38×10^{-1}	8.12×10^{-1}	8.00×10^{-2}	1.70×10^{-1}
13q14.11	rs9533090	*AKAP11*	Upstream	3.78×10^{-2}	5.04×10^{-3}	$\mathbf{5.05\times10^{-10}}$	7.60×10^{-3}	2.44×10^{-4}	6.44×10^{-1}	1.02×10^{-3}	1.40×10^{-5}	6.97×10^{-3}
16q24.1	rs7188801	*FOXL1*	Upstream	3.32×10^{-4}	$\mathbf{3.09\times10^{-6}}$	2.16×10^{-2}	3.91×10^{-1}	1.66×10^{-2}	5.48×10^{-1}	9.70×10^{-3}	7.62×10^{-6}	2.90×10^{-2}
				9 cohorts, 14 258 participants			6 cohorts, 10 466 participants			15 cohorts, 24 723 participants		
2p21	rs17032452	*CAMKMT*	Intronic	8.73×10^{-1}	5.30×10^{-1}	$\mathbf{1.74\times10^{-6}}$	5.49×10^{-1}	4.24×10^{-1}	3.59×10^{-1}	6.26×10^{-1}	9.67×10^{-1}	1.56×10^{-3}
3p14.2	rs6414591	*C3orf67*	Upstream	3.49×10^{-1}	2.39×10^{-1}	$\mathbf{1.72\times10^{-6}}$	1.31×10^{-1}	9.13×10^{-2}	6.83×10^{-1}	7.86×10^{-1}	8.17×10^{-1}	9.22×10^{-2}
5q31.2	rs11959305	*TGFBI*	Intronic	1.89×10^{-2}	1.82×10^{-2}	$\mathbf{6.84\times10^{-8}}$	6.52×10^{-1}	2.80×10^{-1}	8.61×10^{-1}	8.47×10^{-2}	7.74×10^{-3}	1.15×10^{-1}
7p15.3	rs7787266	*STEAP1B*	Intronic	4.08×10^{-1}	4.93×10^{-1}	$\mathbf{2.53\times10^{-6}}$	2.93×10^{-1}	3.14×10^{-1}	6.21×10^{-1}	1.97×10^{-1}	2.70×10^{-1}	9.71×10^{-3}
9q21.33	rs10868487	*GAS1*	Downstream	6.10×10^{-1}	3.81×10^{-1}	$\mathbf{2.37\times10^{-6}}$	2.61×10^{-1}	2.50×10^{-1}	7.03×10^{-1}	8.57×10^{-1}	7.46×10^{-1}	8.92×10^{-2}
13q31.1	rs9574655	*SPRY2*	Downstream	2.58×10^{-1}	1.38×10^{-1}	$\mathbf{9.09\times10^{-8}}$	8.59×10^{-1}	6.43×10^{-1}	8.67×10^{-2}	5.81×10^{-1}	4.72×10^{-1}	3.50×10^{-1}
16q12.2	rs923220	*IRX5*	Upstream	1.24×10^{-3}	7.98×10^{-3}	$\mathbf{6.05\times10^{-7}}$	7.85×10^{-1}	7.28×10^{-1}	9.34×10^{-1}	2.58×10^{-2}	3.95×10^{-2}	1.56×10^{-2}
20q11.22	rs3746429	*EDEM2*	Missense variant	4.42×10^{-1}	8.27×10^{-1}	$\mathbf{3.80\times10^{-7}}$	2.07×10^{-1}	9.14×10^{-2}	3.35×10^{-1}	7.23×10^{-1}	3.93×10^{-1}	4.35×10^{-4}
21q22.2	rs2836789	*FLJ45139*	Upstream	1.56×10^{-1}	1.36×10^{-2}	$\mathbf{1.51\times10^{-6}}$	2.09×10^{-3}	4.09×10^{-2}	5.07×10^{-1}	3.77×10^{-3}	2.27×10^{-3}	1.57×10^{-3}

P-values smaller than the genome-wide significance threshold ($P < 5 \times 10^{-8}$) or suggestive significance threshold ($P < 5 \times 10^{-6}$) are indicated in bold typeface[a].
The association statistics for a new locus at chr 11q14.2 are italicized.
[a]The *P*-values in the GWAS discovery are based on a fixed-effect meta-analysis model, while those in the replication and combined analyses are based on a random-effects meta-analysis model.
[b]The number of cohorts and participants contributing to the analysis of each SNP at each stage slightly varied depending on quality control filters as well as successful imputation or *de novo* genotyping of the particular SNP. Figure 1 and Supplementary Material, Figure S3 show the exact numbers that were available for each SNP at each stage for the confirmed loci.
[c]Secondary signals at the discovery phase following conditional analyses within the region (see Supplementary Material, Fig. S6 for the regional association plots).

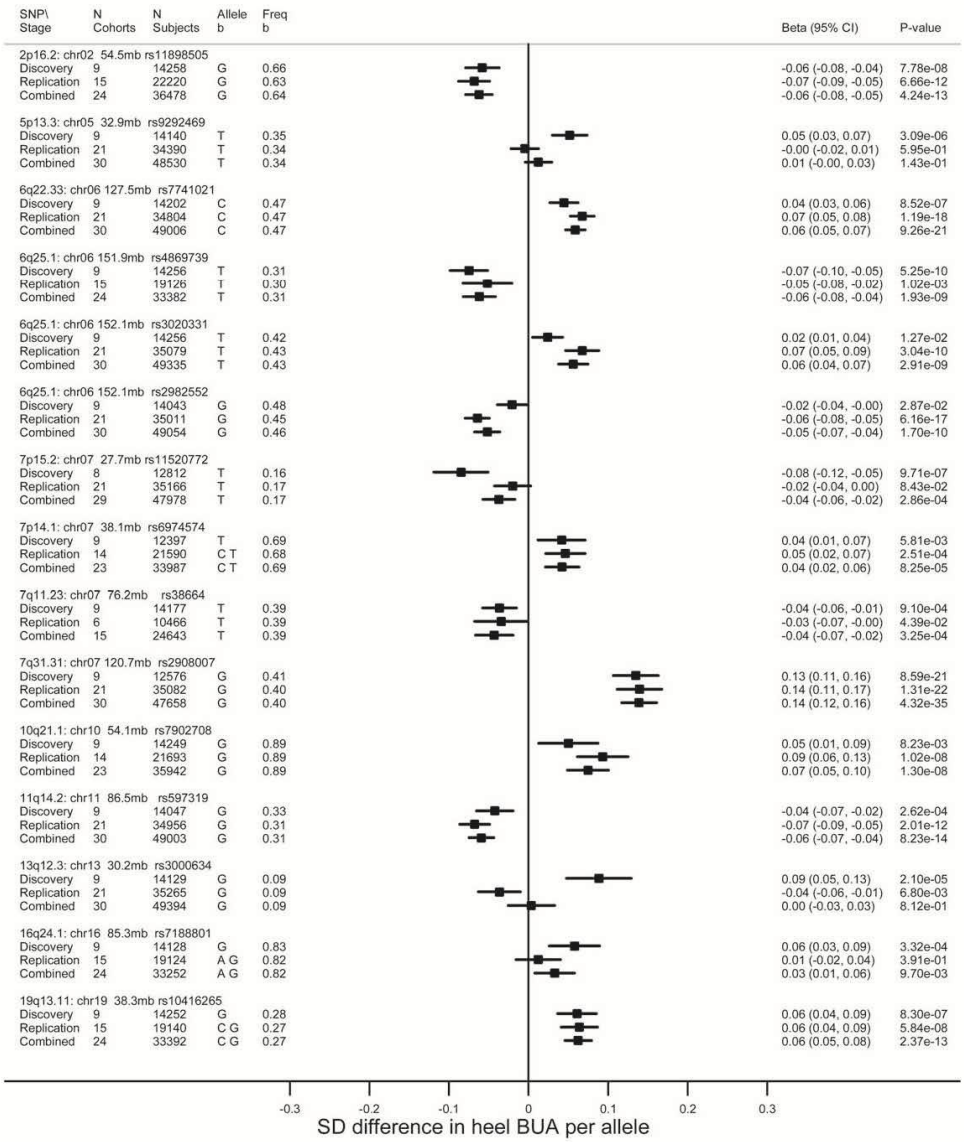

Figure 2. Summary of SNP associations with heel BUA or VOS in GWAS discovery meta-analysis and replication in independent samples of participants. The pooled estimates in the GWAS discovery are based on a fixed-effect meta-analysis model, while those in the replication and combined analyses are based on a random-effects meta-analysis model. Allele b indicates the effect allele, and the presence of two alleles in this column indicates that a proxy SNP with $r^2 > 0.8$ (except for 16q24.1 locus for which $r^2 = 0.6$) was used for the replication analyses.

suggested that it is critically involved in the process of osteoblastogenesis from human multipotent adipose tissue-derived stem cells (24). Marrow fat cells and osteoblasts share a common stromal precursor and there is currently great interest in the role of increased marrow fat in osteoporotic conditions and the metabolic inter-relationships between these neighboring cell types (25). In depth protein sequence analysis showed that *TMEM135* is a multi-transmembrane protein with seven

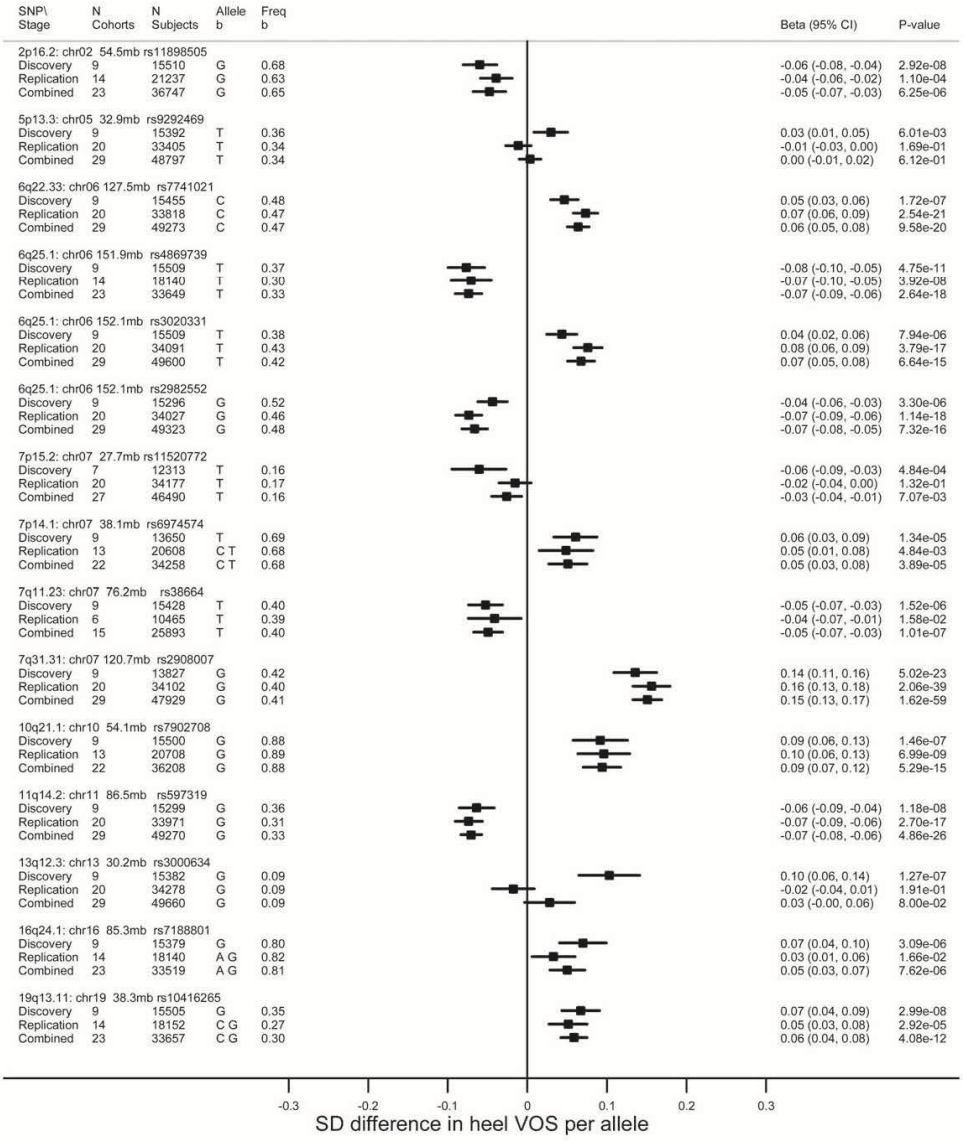

Fig. 2 *Continued*

transmembrane helices of high confidence. Homologies exist between *TMEM135* and the transmembrane region of frizzled-4 (24), a known component of the Wnt signaling pathway (26). ENCODE project (27) data show that two SNPs in the intronic region of *TMEM135* and close to our lead signal (rs502580 and rs603140, both with high linkage disequilibrium with rs597319 [$r^2 > 0.92$], and both highly associated with QUS outcomes in our discovery cohorts [$P \sim 1.3 \times 10^{-7}$ for both]) are associated with changes in MIF-1 and Cart1 motifs in osteoblastic cell lines. Interestingly, both of these transcription factors have been previously shown to be associated with skeletal development and bone density (28,29). Furthermore, *TMEM135* was

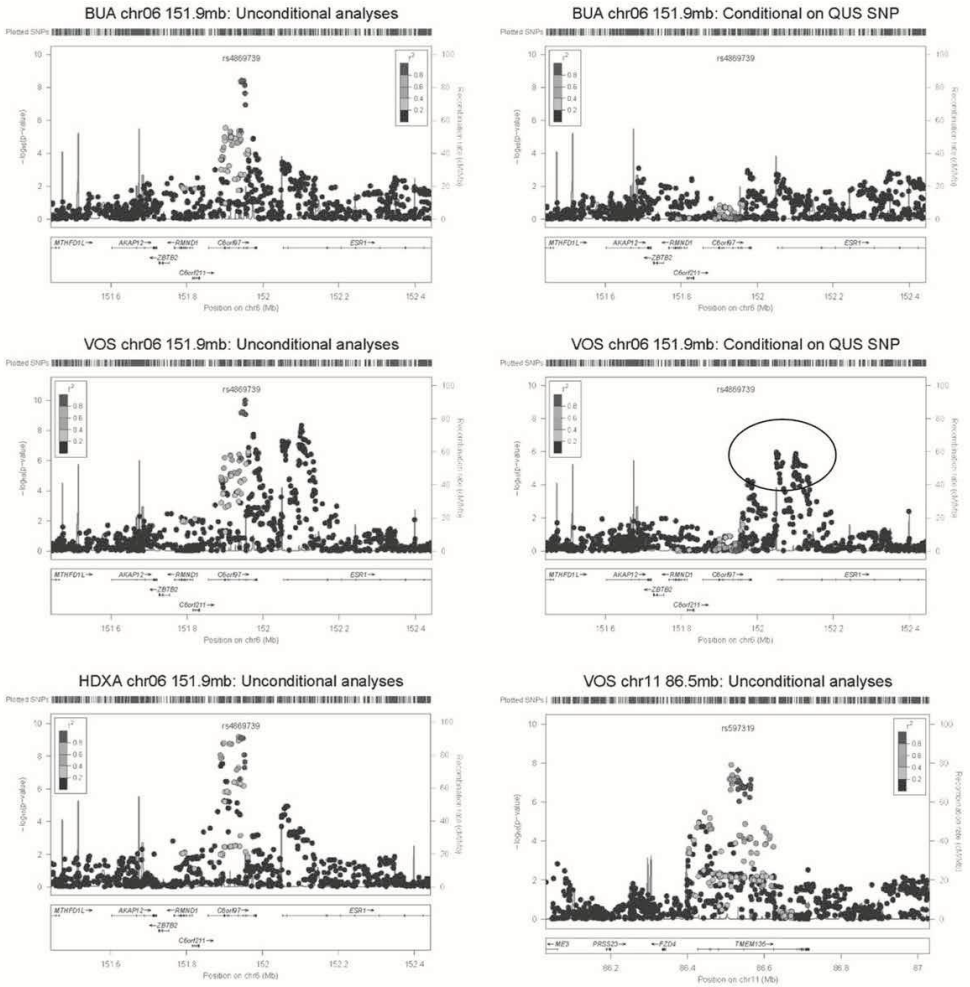

Figure 3. Association of SNPs at chromosome 6q25.1 region with heel BUA, VOS, and heel DXA BMD in meta-analysis of discovery cohorts before (left column) and after (right column) adjusting for the most significant SNP in the region (i.e. unconditional and conditional analyses respectively); as well as the unconditional results for a novel locus for heel bone properties at chromosome 11q14.2. (The conditional analyses led to the identification of the highlighted secondary signal for association of 6q25.1 with VOS. Conditional analyses results for heel DXA BMD were not available from the three relevant discovery cohorts. Color versions of the above figures have been made available in Supplementary Material, Fig. S6.).

previously reported to be associated with longevity in *Caenorhabditis elegans* models (30) as well as with longevity and walking speed in humans (31). In summary, the associations observed in our study might be the results of direct effects of increased osteoblastogenesis on heel bone properties, or indirect effects mediated through increased mechanical loading of the calcaneum, associated with faster movements.

The other genetic loci with significant associations with heel bone measures have previously been reported to be associated with BMD or fractures. The *ESR1* gene has been shown to be related to osteoporosis susceptibility in both candidate gene (32) and GWA studies (18,33). SNPs in *SPTBN1* gene were significantly associated with central DXA BMD in a previous meta-analysis of GEFOS cohorts (18), as were SNPs in *WNT16*, *DKK1*, and *GPATCH1* genes in the recent GEFOS-BMD meta-analysis (19). The *RSPO3* gene has recently been suggested as a bone-related locus by a GWA study of extreme low and high BMD populations (34). The spectrin, beta, non-erythrocytic

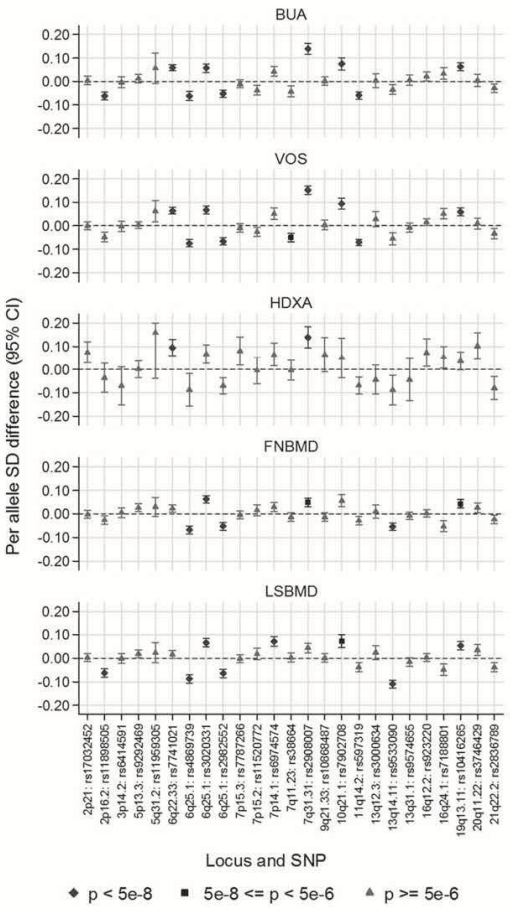

Figure 4. Comparison of magnitudes of associations of 25 SNPs with heel bone properties and central DXA BMD. The SNP associations with central DXA BMD are based on lookup of previously published results from GEFOS.

1 (*SPTBN1*) gene located at chromosome 2p16.2 codes for the β-subunit of spectrin, which is a molecular scaffold protein essential in linking plasma membrane to the actin cytoskeleton. Spectrin plays an important role in determination of cell shape, positioning of transmembrane proteins, resilience of membranes to mechanical stress, and organization of organelles and molecular traffic in cells. β-Subunits coded by *SPTBN1* are responsible for most of the spectrin-binding activity. Despite several GWA studies confirming the association between *SPTBN1* and osteoporosis (18,19,33,35), its role in bone pathophysiology is unclear.

The estrogen receptor 1 (*ESR1*) gene located at chromosome 6q25.1 codes for the estrogen receptor type 1 (also known as ER-α). Two isoforms of estrogen receptors in humans (α and β) are encoded by two different genes (*ESR1* and *ESR2*) and have distinct tissue and cell patterns of expression. Estrogen receptor is a DNA-binding transcription factor that regulates the activity of many different genes. Estrogen is well known to inhibit bone resorption through both direct and indirect actions on osteoclasts, and it is a major anabolic steroid in bone, particularly evident in the establishment of peak bone mass. Postmenopausal bone loss is complex, involving many genetically regulated processes. After menopause, bone is lost rapidly but variably for several years by most women as osteoclastic bone resorptive activity increases in association with osteocyte apoptosis (36). In an osteoporosis GWA study by deCODE Genetics in 2008 (33), several markers close to *ESR1* were reported to show association with BMD, including intronic variants and upstream SNPs close to *CCDC170* (previously known as *C6orf97*). This association was replicated in both GEFOS-BMD meta-analyses (18,19), and we found three-independent SNPs in this region associated with heel BUA and VOS. Most recently, this locus has been shown to be more associated with cortical volumetric BMD (as opposed to trabecular BMD), which implies a role of *ESR1* products in osteoblastogenesis and cortical porosity (37).

The wingless-type MMTV integration site family, member 16 (*WNT16*) gene located at chromosome 7q31.31 is part of the Wnt/LRP pathway, which is a known major anabolic pathway in bone (38). The effects of activation of this pathway include differentiation of mesenchymal precursors into osteoblasts, osteoblast proliferation, bone mineralization, and avoidance of osteoblast apoptosis, and inhibition of osteoclastogenesis through effects on expression of *OPG* and *RANKL*. Other members of this pathway such as *LRP5*, *LRP4*, *SOST*, *WLS*, *DKK1* and *CTNNB1* have previously been associated with BMD at genome-wide significance level (18,19,33,35).

The variant rs7902708 on chromosome 10q21.1 locates between the *MBL2* and *DKK1* genes and is in close linkage disequilibrium with another SNP in this locus (rs1373004, $R^2 = 0.87$ in HapMap CEU population) that was previously found to have a significant association with BMD and fracture risk in GWA meta-analyses (19). Since the *MBL2* (mannose-binding lectin 2) gene product is active in the innate immune system, it is more likely that these variants have a *cis* regulatory effects on Dickkopf-1 (*DKK1*), which is a known Wnt signaling pathway inhibitor (39). Several functional studies have showed the role of *DKK1* in osteolytic bone lesions in patients with advanced multiple myeloma (40) and its inverse relationship with bone mass has been shown in knockout mouse models (41). A similar relationship to the Wnt signaling pathway has also been proposed for the *RSPO3* gene (21). Although *GPATCH1* was also found to be associated with hip and spine BMD in a previous GEFOS meta-analysis (19), there is no functional information about it in genomic databases.

Caution must be exercised in interpreting the results of the heel DXA BMD analyses because there were less than 7000 participants contributing to the combined meta-analysis. The obtained results, however, were consistent with the work of Portero *et al.*, suggesting that heel DXA BMD and BUA measure comparable properties of the calcaneum, which reflect the amount of bone mineral in the field of view of the detector (2).

While the current study had limited statistical power in the meta-analysis of SNP associations with fracture outcomes, it was nevertheless encouraging to observe nominally statistically

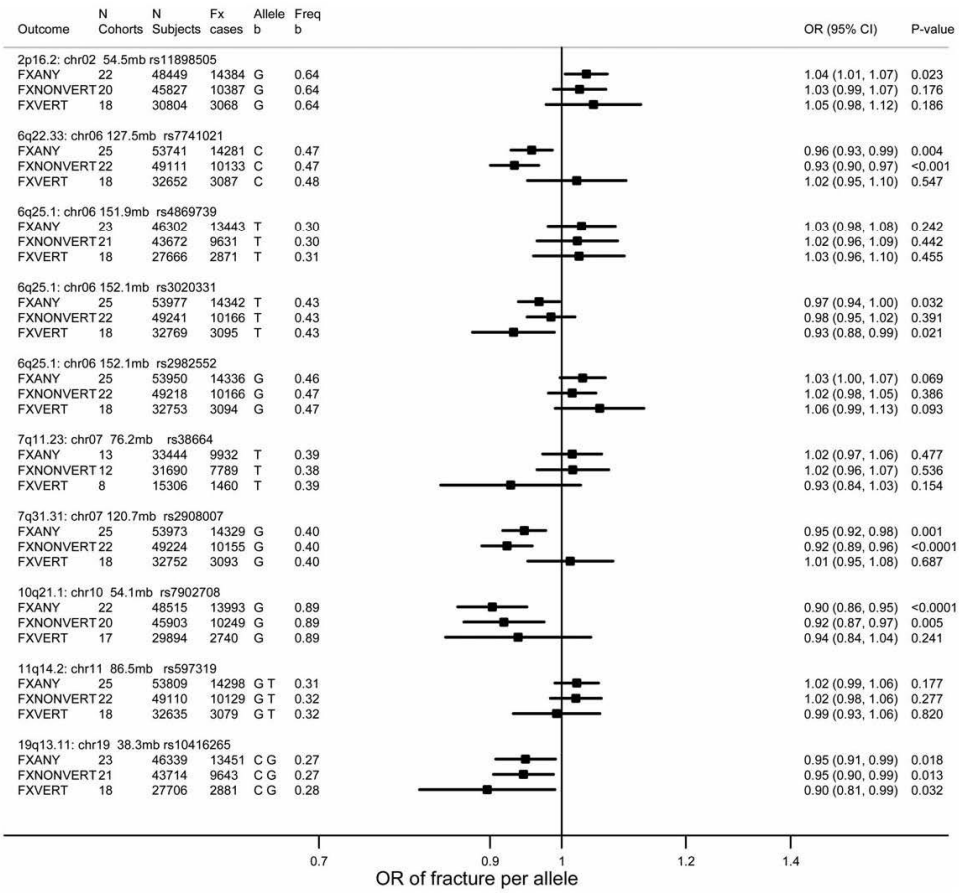

Figure 5. Per-allele odds ratios for association with fracture risk for 10 SNPs that were associated with heel BUA, VOS or heel DXA BMD at $P < 5 \times 10^{-6}$ in combined meta-analyses using a random-effects model. The pooled estimates are based on a random-effects meta-analysis model. FXANY = any fracture; FXNONVERT = non-vertebral fracture; FXVERT = vertebral fracture. Allele b indicates the effect of allele, and the presence of two alleles in this column indicates that a proxy SNP with $r^2 > 0.8$ was used for the replication analyses.

significant and expected directions of associations with fractures for six SNPs associated with heel bone measures, including three SNPs at 6q22.33 (rs7741021), 7q31.31 (rs2908007) and 10q21.1 (rs7902708) whose *P*-values for association surpassed the multiple testing chance-corrected threshold of $P < 0.005$. The concordant findings may, albeit indirectly, suggest that some of the genetic susceptibility to fracture could partly be mediated through bone properties (e.g. structural or material) captured by QUS or DXA measures; but larger well-powered studies are needed to appropriately assess such relevance.

In conclusion, the present GWA study reveals the effect of several genes common to central DXA-derived BMD and heel ultrasound/pDXA measures and points to a new genetic locus with potential implications for better understanding of osteoporosis pathophysiology. Quantitative differences seen in the standardized effect sizes of some SNPs at different skeletal sites are potentially indicative of heterogeneity in genetically mediated responses of the skeleton to environmental stimuli, including ground reaction forces that are particularly high at the heel than at central sites.

MATERIALS AND METHODS

Study subjects and measurements

The GEFOS consortium is an international collaboration of investigators dedicated to identify the genetic determinants of osteoporosis (http://www.gefos.org/). In particular, the GEFOS

consortium extended the breadth of its predecessor, the Genetic Markers for Osteoporosis (GENOMOS) consortium, into meta-analysis of GWA discovery studies. In the current GEFOS/GENOMOS project, we performed GWA discovery and replication of genetic loci associated with heel bone properties, including QUS (measures: BUA and VOS) and DXA (measure: heel BMD).

The discovery phase comprises 13 cohort studies with GWA data and relevant heel bone phenotypes (including BUA in 14 260 participants from 9 cohorts; VOS in 15 514 participants from 9 cohorts; and heel DXA BMD in 4556 participants from 3 cohorts) arising from populations across North America, Europe and East Asia. Independent replication was performed using summary results from seven cohorts with GWA data (*in silico* $n = 11\,452$) and analysis of individual-level data from 15 other cohorts in the GENOMOS consortium that were centrally genotyped for candidate polymorphisms by the Kbioscience laboratory in the UK (*de novo* $n = 24\,902$). Characteristics of the study cohorts/participants are summarized in Table 1. All studies were approved by institutional ethics review committees at the relevant organizations and all participants provided written informed consent. Further descriptive information about the participating cohorts is available from the GEFOS/GENOMOS websites (http://www.gefos.org/?q=studies and http://www.genomos.eu/index.php?page=cohorts).

Genotyping and imputation methods

All the discovery cohorts were genotyped using commercially available Affymetrix (Affymetrix Inc., Santa Clara, CA, USA) or Illumina (Illumina Inc., San Diego, CA, USA) genotyping arrays. Quality control was performed independently for each study according to standard manufacturer protocols and within study procedures. To facilitate meta-analysis, each group performed genotype imputation with IMPUTE or MACH software using genotypes from the HapMap Phase II release 22, NCBI build 36 (CEU or CHB/JPT as appropriate) as reference panels. Each imputation software estimates an overall imputation quality score for each SNP. These quality scores and minor allele frequencies for up to ~2.5 million SNPs available from each cohort were considered in the meta-analysis.

Association analyses

In the discovery phase, each cohort conducted analyses according to a standard prespecified analysis plan under an additive (i.e. per allele) genetic model. Phenotypes for the association analyses were defined as the sex-specific standardized residuals from linear regression of each outcome variable (BUA, VOS or heel BMD) on age, age-squared, weight, height and machine type (if more than one machine was used). The assumption of normality of residuals in the linear regression model was checked within each cohort for each phenotype and no deviations were reported. The SNP–phenotype associations in each study were adjusted for potential confounding by population substructure using principal components as appropriate; pedigree and twin-based studies—additionally—corrected for family structure. The final results submitted to the Coordinating Center for meta-analysis were the per-allele regression coefficients with corresponding standard errors and P-values for the associations of up to 2.5 million SNPs and standardized residuals of each outcome variable. Analysis of imputed genotypes used either the dosage information from MACH or the genotype probabilities from IMPUTE. The replication analyses used the same analytical procedures as above where applicable (e.g. using study-specific standardized residuals as outcomes).

Meta-analysis

Meta-analysis of the GWA discovery summary results was conducted in two-independent collaborating centers (Cambridge, UK and Boston, USA). Because of potentially limited power to detect sex-specific associations, we prespecified the primary analyses to involve meta-analysis of the pooled data (i.e. males and females combined). Quality control filters applied for exclusions of SNPs from the meta-analysis were: imputation quality score of <0.3 for MACH and <0.4 for IMPUTE, average minor allele frequency of $<1\%$ across studies, and SNPs missing from $>50\%$ of the cohorts contributing to each outcome. Inverse-variance fixed-effects meta-analysis (using METAL software) was conducted in the discovery set with double genomic correction (42) to control for potential inflation of the test statistics in individual studies and in the meta-analysis. The genome-wide level of statistical significance was set at $P < 5 \times 10^{-8}$ and suggestive level of significance at $5 \times 10^{-8} \leq P < 5 \times 10^{-6}$. There were no extreme genomic inflation factors noted in the discovery phase studies or in the GWA meta-analysis (Supplementary Material, Table S1). QQ plots for the combined GWAS meta-analysis results are provided in Supplementary Material, Figure S1.

To help refine the choice of SNPs to be taken forward for replication, conditional analyses were conducted within a 1 megabase window of the best-associated SNP in each locus in the discovery cohorts, if there was more than one SNP with a suggestive level of significance. These secondary analyses took the SNP in the locus with the lowest P-value and conditioned the analysis of all of the other SNPs in the locus by including it in the regression models. In addition, for loci containing SNPs previously associated with hip or spine BMD in GEFOS (19), we performed additional conditioning on the nearby "BMD SNP".

The DerSimonian and Laird random-effects model was used for meta-analysis of studies in the replication set and also in the final combined analysis of the discovery and replication studies (43). For each SNP included in the replication phase, we meta-analyzed its association with all three phenotypes, simply for completeness, but interpreted the findings while taking into account the primary outcome that the SNP was associated with in the discovery phase. Fixed-effect meta-analysis results were used for subsidiary comparison. We also conducted meta-analysis of the associations of SNPs with fracture outcomes, using only SNPs that were associated with BUA, VOS or heel DXA BMD at $P < 5 \times 10^{-6}$ in the combined analyses, to assess their potential relevance to this clinical outcome.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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REFERENCES

1. Langton, C.M. (2011) The 25th anniversary of BUA for the assessment of osteoporosis: time for a new paradigm? *Proc.* Inst. Mech. Eng. **H**, **225**, 113–125.
2. Portero, N.R., Arlot, M.E., Roux, J.P., Duboeuf, F., Chavassieux, P.M. and Meunier, P.J. (2005) Evaluation and development of automatic two-dimensional measurements of histomorphometric parameters reflecting trabecular bone connectivity: correlations with dual-energy x-ray absorptiometry and quantitative ultrasound in human calcaneum. *Calcif. Tissue Int.*, **77**, 195–204.
3. Gluer, C.C., Eastell, R., Reid, D.M., Felsenberg, D., Roux, C., Barkmann, R., Timm, W., Blenk, T., Armbrecht, G., Stewart, A. *et al.* (2004) Association of five quantitative ultrasound devices and bone densitometry with osteoporotic vertebral fractures in a population-based sample: the OPUS Study. *J. Bone Miner. Res.*, **19**, 782–793.
4. Krieg, M.A., Barkmann, R., Gonnelli, S., Stewart, A., Bauer, D.C., Del Rio, B.L., Kaufman, J.J., Lorenc, R., Miller, P.D., Olszynski, W.P. *et al.* (2008) Quantitative ultrasound in the management of osteoporosis: the 2007 ISCD Official Positions. *J. Clin. Densitom.*, **11**, 163–187.
5. Nelson, H.D., Haney, E.M., Dana, T., Bougatsos, C. and Chou, R. (2010) Screening for osteoporosis: an update for the U.S. Preventive Services Task Force. *Ann. Intern. Med.*, **153**, 99–111.
6. Gonnelli, S., Cepollaro, C., Gennari, L., Montagnani, A., Caffarelli, C., Merlotti, D., Rossi, S., Cadirni, A. and Nuti, R. (2005) Quantitative ultrasound and dual-energy X-ray absorptiometry in the prediction of fragility fracture in men. *Osteoporos. Int.*, **16**, 963–968.
7. Howard, G.M., Nguyen, T.V., Harris, M., Kelly, P.J. and Eisman, J.A. (1998) Genetic and environmental contributions to the association between quantitative ultrasound and bone mineral density measurements: a twin study. *J. Bone Miner. Res.*, **13**, 1318–1327.
8. Karasik, D., Hsu, Y.H., Zhou, Y., Cupples, L.A., Kiel, D.P. and Demissie, S. (2010) Genome-wide pleiotropy of osteoporosis-related phenotypes: the Framingham Study. *J. Bone Miner. Res.*, **25**, 1555–1563.
9. Lee, M., Czerwinski, S.A., Choh, A.C., Demerath, E.W., Sun, S.S., Chumlea, W.C., Towne, B. and Siervogel, R.M. (2006) Unique and common genetic effects between bone mineral density and calcaneal quantitative ultrasound measures: the Fels Longitudinal Study. *Osteoporos. Int.*, **17**, 865–871.
10. Bauer, D.C., Gluer, C.C., Cauley, J.A., Vogt, T.M., Ensrud, K.E., Genant, H.K. and Black, D.M. (1997) Broadband ultrasound attenuation predicts fractures strongly and independently of densitometry in older women. A prospective study. Study of Osteoporotic Fractures Research Group. *Arch. Intern. Med.*, **157**, 629–634.
11. Bauer, D.C., Ewing, S.K., Cauley, J.A., Ensrud, K.E., Cummings, S.R. and Orwoll, E.S. (2007) Quantitative ultrasound predicts hip and non-spine fracture in men: the MrOS study. *Osteoporos. Int.*, **18**, 771–777.
12. Moayyeri, A., Adams, J.E., Adler, R.A., Krieg, M.A., Hans, D., Compston, J. and Lewiecki, E.M. (2012) Quantitative ultrasound of the heel and fracture risk assessment: an updated meta-analysis. *Osteoporos. Int.*, **23**, 143–153.
13. Arden, N.K., Baker, J., Hogg, C., Baan, K. and Spector, T.D. (1996) The heritability of bone mineral density, ultrasound of the calcaneus and hip axis length: a study of postmenopausal twins. *J. Bone Miner. Res.*, **11**, 530–534.
14. Karasik, D., Myers, R.H., Hannan, M.T., Gagnon, D., McLean, R.R., Cupples, L.A. and Kiel, D.P. (2002) Mapping of quantitative ultrasound of the calcaneus bone to chromosome 1 by genome-wide linkage analysis. *Osteoporos. Int.*, **13**, 796–802.
15. Zhai, G., Andrew, T., Kato, B.S., Blake, G.M. and Spector, T.D. (2009) Genetic and environmental determinants on bone loss in postmenopausal Caucasian women: a 14-year longitudinal twin study. *Osteoporos. Int.*, **20**, 949–953.
16. Michaelsson, K., Melhus, H., Ferm, H., Ahlbom, A. and Pedersen, N.L. (2005) Genetic liability to fractures in the elderly. *Arch. Intern. Med.*, **165**, 1825–1830.
17. Ralston, S.H. and Uitterlinden, A.G. (2010) Genetics of osteoporosis. *Endocr. Rev.*, **31**, 629–662.
18. Rivadeneira, F., Styrkarsdottir, U., Estrada, K., Halldorsson, B.V., Hsu, Y.H., Richards, J.B., Zillikens, M.C., Kavvoura, F.K., Amin, N., Aulchenko, Y.S. *et al.* (2009) Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. *Nat. Genet.*, **41**, 1199–1206.
19. Estrada, K., Styrkarsdottir, U., Evangelou, E., Hsu, Y.H., Duncan, E.L., Ntzani, E.E., Oei, L., Albagha, O.M., Amin, N., Kemp, J.P. *et al.* (2012) Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. *Nat. Genet.*, **44**, 491–501.
20. Moayyeri, A., Kaptoge, S., Dalzell, N., Luben, R.N., Wareham, N.J., Bingham, S., Reeve, J. and Khaw, K.T. (2009) The effect of including quantitative heel ultrasound in models for estimation of 10-year absolute risk of fracture. *Bone*, **45**, 180–184.
21. Han, X.H., Jin, Y.R., Seto, M. and Yoon, J.K. (2011) A WNT/beta-catenin signaling activator, R-spondin, plays positive regulatory roles during skeletal myogenesis. *J. Biol. Chem.*, **286**, 10649–10659.
22. Zheng, H.F., Tobias, J.H., Duncan, E., Evans, D.M., Eriksson, J., Paternoster, L., Yerges-Armstrong, L.M., Lehtimaki, T., Bergstrom, U., Kahonen, M. *et al.* (2012) WNT16 influences bone mineral density, cortical bone thickness, bone strength, and osteoporotic fracture risk. *PLoS Genet.*, **8**, e1002745.
23. Liu, F., Li, Y., Yu, Y., Fu, S. and Li, P. (2007) Cloning of novel tumor metastasis-related genes from the highly metastatic human lung adenocarcinoma cell line Anip973. *J. Genet. Genomics*, **34**, 189–195.
24. Scheideler, M., Elabd, C., Zaragosi, L.E., Chiellini, C., Hackl, H., Sanchez-Cabo, F., Yadav, S., Duszka, K., Friedl, G., Papak, C. *et al.* (2008) Comparative transcriptomics of human multipotent stem cells during adipogenesis and osteoblastogenesis. *BMC. Genomics.*, **9**:340. doi: 10.1186/1471-2164-9-340., 340-349.
25. Fazeli, P.K., Horowitz, M.C., MacDougald, O.A., Scheller, E.L., Rodeheffer, M.S., Rosen, C.J. and Klibanski, A. (2013) Marrow fat and bone--new perspectives. *J. Clin. Endocrinol. Metab.*, **98**, 935–945.
26. Robitaille, J., MacDonald, M.L., Kaykas, A., Sheldahl, L.C., Zeisler, J., Dube, M.P., Zhang, L.H., Singaraja, R.R., Guernsey, D.L., Zheng, B. *et al.* (2002) Mutant frizzled-4 disrupts retinal angiogenesis in familial exudative vitreoretinopathy. *Nat. Genet.*, **32**, 326–330.
27. Bernstein, B.E., Birney, E., Dunham, I., Green, E.D., Gunter, C. and Snyder, M. (2012) An integrated encyclopedia of DNA elements in the human genome. *Nature*, **489**, 57–74.
28. Iioka, T., Furukawa, K., Yamaguchi, A., Shindo, H., Yamashita, S. and Tsukazaki, T. (2003) P300/CBP acts as a coactivator to cartilage homeoprotein-1 (Cart1), paired-like homeoprotein, through acetylation of the conserved lysine residue adjacent to the homeodomain. *J. Bone Miner. Res.*, **18**, 1419–1429.
29. Onodera, S., Sasaki, S., Ohshima, S., Amizuka, N., Li, M., Udagawa, N., Irie, K., Nishihira, J., Koyama, Y., Shiraishi, A. *et al.* (2006) Transgenic mice overexpressing macrophage migration inhibitory factor (MIF) exhibit high-turnover osteoporosis. *J. Bone Miner. Res.*, **21**, 876–885.
30. Exil, V.J., Silva, A.D., Benedetto, A., Exil, E.A., Adams, M.R., Au, C. and Aschner, M. (2010) Stressed-induced TMEM135 protein is part of a conserved genetic network involved in fat storage and longevity regulation in Caenorhabditis elegans. *PLoS ONE*, **5**, e14228.
31. Lunetta, K.L., D'Agostino, R.B. Sr., Karasik, D., Benjamin, E.J., Guo, C.Y., Govindaraju, R., Kiel, D.P., Kelly-Hayes, M., Massaro, J.M., Pencina, M.J. *et al.* (2007) Genetic correlates of longevity and selected age-related phenotypes: a genome-wide association study in the Framingham Study. *BMC. Med. Genet.*, **8**(Suppl. 1), S13.

32. Li, W.F., Hou, S.X., Yu, B., Li, M.M., Ferec, C. and Chen, J.M. (2010) Genetics of osteoporosis: accelerating pace in gene identification and validation. *Hum. Genet.*, **127**, 249–285.
33. Styrkarsdottir, U., Halldorsson, B.V., Gretarsdottir, S., Gudbjartsson, D.F., Walters, G.B., Ingvarsson, T., Jonsdottir, T., Saemundsdottir, J., Center, J.R., Nguyen, T.V. *et al.* (2008) Multiple genetic loci for bone mineral density and fractures. *N. Engl. J. Med.*, **358**, 2355–2365.
34. Duncan, E.L., Danoy, P., Kemp, J.P., Leo, P.J., McCloskey, E., Nicholson, G.C., Eastell, R., Prince, R.L., Eisman, J.A., Jones, G. *et al.* (2011) Genome-wide association study using extreme truncate selection identifies novel genes affecting bone mineral density and fracture risk. *PLoS Genet.*, **7**, e1001372.
35. Styrkarsdottir, U., Halldorsson, B.V., Gretarsdottir, S., Gudbjartsson, D.F., Walters, G.B., Ingvarsson, T., Jonsdottir, T., Saemundsdottir, J., Snorradottir, S., Center, J.R. *et al.* (2009) New sequence variants associated with bone mineral density. *Nat. Genet.*, **41**, 15–17.
36. Tomkinson, A., Reeve, J., Shaw, R.W. and Noble, B.S. (1997) The death of osteocytes via apoptosis accompanies estrogen withdrawal in human bone. *J. Clin. Endocrinol. Metab.*, **82**, 3128–3135.
37. Paternoster, L., Lorentzon, M., Lehtimaki, T., Eriksson, J., Kahonen, M., Raitakari, O., Laaksonen, M., Sievanen, H., Viikari, J., Lyytikainen, L.P. *et al.* (2013) Genetic determinants of trabecular and cortical volumetric bone mineral densities and bone microstructure. *PLoS Genet.*, **9**, e1003247.
38. Kubota, T., Michigami, T. and Ozono, K. (2009) Wnt signaling in bone metabolism. *J. Bone Miner. Metab.*, **27**, 265–271.
39. Fedi, P., Bafico, A., Nieto, S.A., Burgess, W.H., Miki, T., Bottaro, D.P., Kraus, M.H. and Aaronson, S.A. (1999) Isolation and biochemical characterization of the human Dkk-1 homologue, a novel inhibitor of mammalian Wnt signaling. *J. Biol. Chem.*, **274**, 19465–19472.
40. Kocemba, K.A., Groen, R.W., van Andel, H., Kersten, M.J., Mahtouk, K., Spaargaren, M. and Pals, S.T. (2012) Transcriptional silencing of the Wnt-antagonist DKK1 by promoter methylation is associated with enhanced Wnt signaling in advanced multiple myeloma. *PLoS ONE*, **7**, e30359.
41. MacDonald, B.T., Joiner, D.M., Oyserman, S.M., Sharma, P., Goldstein, S.A., He, X. and Hauschka, P.V. (2007) Bone mass is inversely proportional to Dkk1 levels in mice. *Bone*, **41**, 331–339.
42. Willer, C.J., Li, Y. and Abecasis, G.R. (2010) METAL: fast and efficient meta-analysis of genomewide association scans. *Bioinformatics*, **26**, 2190–2191.
43. DerSimonian, R. and Laird, N. (1986) Meta-analysis in clinical trials. *Control Clin. Trials*, **7**, 177–188.

4. CONCLUSIONS

The role of vitamin D in bone health is well determined. Severe vitamin D deficiency causes rickets or osteomalacia, where the new bone, the osteoid, is not mineralized. Less severe vitamin D deficiency causes an increase in serum PTH leading to bone resorption, osteoporosis and fractures. The VDR and vitamin D metabolic enzymes such as D-1-a-hydroxylase (CYP27B1) are widely expressed in many tissues and organs, except those involved in bone metabolism. Several genetic, molecular, cellular and animal studies strongly suggest that vitamin D signalling has many extra skeletal effects. These include regulation of cell proliferation, immune and muscle function, skin differentiation reproduction, as well as vascular and metabolic properties. From observational studies in humans poor vitamin D status is associated with several diseases, including autoimmune, cardiovascular and cancer, were predicted by these extra skeletal actions. Therefore, vitamin D plays a key role in several other diseases beyond osteoporosis [111].

Osteoporosis has been recognized as an established and well-defined disease that affects more than 75 million people in the United States, Europe and Japan. The fracture constitutes the clinical consequence of osteoporosis and a major cause of disability. In Greece the incidence of hip fracture in men is moderate (100-150/100,000) and in women is high (>300/ 100,000), as indicated by the IOF working group review. The incidence of hip fractures doubled during 30 years (1977-2007) among Greek people aged ≥50 years [112, 4].

Althought DXA is the gold standard method for the diagnosis of osteoporosis, the measurement of QUS parameters at the calcaneus is better utilized in a wider population, since it is conducted by a portable, low cost and ionizing radiation-free, QUS device. Additionaly QUS indices are associated with bone mass, microarchitecture and mechanical characteristics of bones as well as with fracture risk [113].

The assessment of serum vitamin D levels, and the QUS parameters, BUA, SOS and SI are generally crucial for bone health and fracture risk. On this basis, in the current study, serum 25(OH)D, was used for the evaluation of Vitamin D status. The QUS parameters, BUA, SOS and SI were selected for the evaluation of the bone health of participants.

In the framework of the OSTEOS study, a large number of subjects of different age groups were recruited from various urban and rural areas of Greece and data on biological and

anthropometric indices, medical history and eating and lifestyle habits were collected. Vitamin D status and QUS parameters were the main dependent variables analysed, so to evaluate factors like biochemical, environmental, genetic and lifestyle that affect levels of vitamin D as well as QUS parameters. This is the first study of such size in the Greek population, resulting in the extraction of valuable epidemiological data.

According to the prevalence of vitamin D deficiency and insufficiency, this study highlights the emerging issue of hypovitaminosis D in Greece. The majority of Greek adults (54%) had vitamin D deficiency (<20 ng/mL) and only 12.3% had levels above 30 ng/mL.

In a recent study of 1075 adults from seven European countries, including Greece, about 30.6% of overall Europeans and 34, 8 % of Greek adults had 25(OH)D equal to 12-20 ng/mL. The prevalence of 25(OH)D < 12ng/mL was 3.3% and 1.4% respectively [114]. In the current study there was a higher prevalence of sever deficiency of 25(OH)D since the 8% of population had 25(OH)D<10ng/mL and 46% of population had 25(OH)D levels 10-20ng/mL. Similar results regarding the concentrations of serum 25-hydroxyvitamin D [25(OH)D] are also shown in the study of Cashman et al, in more than 55,000 adults and children from several European countries. The investigators found that 13% of individuals had 25(OH)D concentrations <12 ng/mL and 40% had concentrations <20 ng/mL [115].

Searching for contributors to 25(OH)D deficiency (<20 ng/mL) we revealed that obesity status increases 1.458 times (p=0.006), the risk . A similar study that examined vitamin D status of residents of seven European countries including Greece gave contradictory results. No statistically significant differences were found in the prevalence of vitamin D < 12ng/mL and vitamin D 12ng/mL-20ng/mL, between obese and non-obese participants. In accordance with our results, another Greek study of postmenopausal nonosteoporotic women, serum 25(OH)D levels were inversely related to body fat mass, as it was measured using dual-energy x-ray absorptiometry [62] . Obviously, the explanation of this expected finding resides on the fat-soluble property of Vitamin D.

Vitamin D deficiency was also related to lower SOS values in the current population. Serum 25(OH)D levels were previously reported to be an independent determinant of SOS [116]. SOS is the main index of QUS measurement that is associated with bone quantity (BMD and bone mass) and bone quality (microarchitecture, strength and elasticity), as it derives from several studies [113]. Although our study is lacking histomorphometric data, it is possible that the defective collagen mineralization among vitamin D deficient patients might causes the lower

SOS values. This finding further supports the necessity of QUS measurement in subjects with low serum 25(OH)D levels.

According to the determinants of Vitamin D status, the results of the OSTEOS study are confirmed by the results of other similar studies [117]. The female sex, the winter-spring season and age only in obese subjects influence serum Vitamin D levels negatively, whereas the duration of exposure to sunlight in summer and the participation to organized physical activities have positive effects on serum Vitamin D. Specifically the influence of participation in organized physical activities is associated to levels of serum vitamin D independently of the sun exposure during the physical activity as confirmed by the current study and by other studies. Muscle cells contain vitamin D receptor (VDR) that it activates intracellular signalling pathways as it demonstrated by several studies. As a result $1\alpha,25(OH)_2D$ plays a role in contractility and myogenesis [118]. The effect of vitamin D on skeletal muscle through the activation of VDR may have a beneficial effect on physical performance and it may contribute to an increased activity of people with higher vitamin D levels.

Differences among European countries in the prevalence of vitamin D deficiency and insufficiency may be explained in part by the confounding effect of different methods/ assays used to measure 25(OH)D concentrations in serum or plasma, as well as by different thresholds used for the definition of deficiency and insuffiency. However, controversy exists over the most appropriate threshold for defining optimal vitamin D status, deficiency and insufficiency, concerning mainly the association with skeletal health and/or other health outcomes that are not always causal. [96, 97]

Moreover the measurement of serum 25(OH)D as a determinant of vitamin D status is under investigation. 25(OH)D is predominantly (88%), and tightly, bound to vitamin D binding protein (DBP); the remaining ;12% is loosely bound to albumin, and only 0.03% of 25(OH)D is found in its free form (8). Bioavailable 25(OH)D refers to the free and albumin-bound 25(OH)D and is thought to represent the mobile pool of vitamin D that is available for autocrine and paracrine activity in times of metabolic demand. Moreover, polymorphisms in the DBP gene and in the vitamin D receptor possibly have a strong impudence on individual need for vitamin D and on the response to supplementation [119].

Although the main source of vitamin D is the exposure to UV radiation, vitamin D intake from foods and supplements also seems to have a beneficial effect on vitamin D status. The Estimated Average Requirement (EAR) value of 10 μg/day for vitamin D as proposed by the IOM

[96] was also confirmed from the European study was mentioned above [115] which indicates that consumption of 10μg/ day vitamin D from foods and supplements decreases the risk of serum vitamin D deficiency (levels < 20ng/mL).

On the other hand in countries with low UV exposure like Canada a large proportion (40.8%) of the population is reported to have 25(OH)D >30 ng/mL [120], which may be due to food fortification. In another Canadian study, fortification of milk, yogurt and cheese at 6.75 μg (270 IU)/serving led to an increase of Vitamin D intake, more than double across all sex and age groups , and a drop in the prevalence of dietary inadequacy from >80% to <50% in all groups [121]. In the current study we evaluated how the overall nutritional habits and not only the vitamin D content of nutrition or individual foods, influences the vitamin D status.

The evaluation of the effect of nutritional intake on vitamin D levels and QUS parameter values was performed by the use of PCA procedure. PCA allows having a more holistic approach to the dietary habits of the population. From the PCA analysis six dietary patterns derived which explain 52.2% of the variability of Greek adults' nutritional habits. The major dietary pattern included fruits, vegetables, rice and fish, showing that the studied Greek population has healthy nutritional habits. However, compliance with this dietary pattern, which called 'vegetables-fruit', was not related to serum 25(OH)D or ultrasound parameters. According to the analysis described in the first manuscript , regarding compliance to 2 dietary patterns ('sweet' and 'healthy'), the 'sweet' pattern negatively affected the vitamin D levels and the 'healthy' pattern (consisted of low fat dairy products, whole grain cereals and breakfast cereals), positively affected the vitamin D levels. 'Healthy' pattern included foods with high calcium content. A high calcium intake increases the half life of 25(OH)D. Similar interactions between calcium intake and vitamin D status have been shown in rat experiments, generally indicating that a high calcium intake has a positive effect regarding vitamin D economy [121, 122]. The added value of assessing dietary factors on the variability of serum 25(OH)D levels was confirmed by an increase of 5.3% to 8.1%.

Additionally, 'healthy' dietary pattern is a positive determinant for BUA parameter along with BMI (as continuous variable) and male gender, whereas increased age-elderly is a negative determinant of BUA as expected. These parameters explain the 19.7% of variability of BUA.

Genetic contribution to osteoporosis is well established with heritability estimates reaching 74% for heel QUS [123, 124]. Estimated BMD as derived by ultrasound values is also highly

heritable (50% - 80%) and independently associated with fracture risk. Recent GWAS identified 307 polymorphisms in 203 genetic loci associated with ultrasound-derived estimated BMD [125]. The heritability of vitamin D as estimated by twin studies might reach 50-80% [126, 127].

A part of the population of OSTEOS study (n = 307) participated in the replication phase of GEFOS-GENOMOS consortium along with 14 other studies. These 15 studies were de novo genotyped for the 9 polymorphisms resulting from the GWAS meta-analysis conducted for 13 discovery cohorts. From these polymorphisms, the rs11520772 on TAX1BP1 and the rs597319 on TMEM135 gene meet statistical significance for association with SOS and SI values, at participants of OSTEOS study. . AA (wild type) carriers of rs11520772 polymorphism had an advantage on SOS values in a relation to 'T' (effect allele) homozygotes. As derived from discovery phase of GWAS meta-analysis in GEFOS/ GENOMOS consortium the rs11520772 polymorphism had been associated with BUA [11]. TAX1BP1 gene was expressed in 27 tissues, especially in adrenal (RPKM 48.4) and thyroid (RPKM 48.2) and there's not been an identified mechanism of action on bone health. Tax1 binding protein 1 (TAX1BP1) gene encodes a HTLV-1 tax1 binding protein. The encoded protein interacts with TNFAIP3, and inhibits TNF-induced apoptosis by mediating the TNFAIP3 anti-apoptotic activity. Degradation of this protein by caspase-3-like family proteins is associated with apoptosis induced by TNF. This protein may also have a role in the inhibition of inflammatory signaling pathways [NCBI Gene ID 8887]. In our population, wild type allele frequency is 0.755 and effect allele frequency is 0.244. The corresponding frequencies as they resulted from 1000 Genome project [18] are 0.815 for 'A' allele and 0.185 for 'T' allele for the European population and 0.898 and 0.102 for global population respectively.

The 'AA' genotype of rs597319 polymorphism associated to higher SI values. The rs597319 located in a novel locus at 11q14.2 (that was first identified as associated with heel QUS on GEFOS / GENOMOS consortium). The new locus near the transmembrane protein 135 (TMEM135) gene, was genome-wide significant for both BUA and SOS. It has been suggested that it is critically involved in the process of osteoblastogenesis from human multipotent adipose tissue-derived stem cells. Marrow fat cells and osteoblasts share a common stromal precursor and there is currently great interest in the role of increased marrow fat in osteoporotic conditions and the metabolic inter-relationships between these neighboring cell types. ENCODE project data show that two SNPs in the intronic region of TMEM135 and close to our lead signal (rs502580 and rs603140), both with high linkage disequilibrium with rs597319

[r2 >0.92], and both highly associated with QUS outcomes in our discovery cohorts [P ~ 1.3 × 10–7 for both]) are associated with changes in MIF-1 and Cart1 motifs in osteoblastic cell lines. Interestingly, both of these transcription factors have been previously shown to be associated with skeletal development and bone density.In depth protein sequence analysis showed that TMEM135 is a multi-transmembrane protein with seven transmembrane helices of high confidence. Homologies exist between TMEM135 and the transmembrane region of frizzled-4, a known component of the Wnt signaling pathway. Furthermore, TMEM135 was previously reported to be associated with longevity and walking speed in humans. In summary, the associations observed in our study might be the results of direct effects of increased osteoblastogenesis on heel bone properties, or indirect effects mediated through increased mechanical loading of the calcaneum, associated with faster movements. In our population, 'A' allele frequency is 0.646 and G allele frequency is 0.354. The corresponding frequencies as they resulted from 1000 Genome project [18] are 0.707 for 'A' allele and 0.293 for 'G' allele for the European population and 0.543 and 0.457 for global population respectively.

Despite the limitations, GWAs have been a valuable tool in genetic epidemiology and have been successful in identifying common variants with small or modest effect on osteoporosis disease and on vitamin D serum levels. A further GWA analysis of the study samples could reveal more genetic loci associated with bone health indicators and the vitamin D levels of the current Greek population.

This current study has several limitations: it is an observational, cross-sectional study and it is not appropriate to draw causal effect implications or to generalize the results from this population. Residual confounding may also exist because of unmeasured variables. Another limitation of the study is the self-reported medical history and medication data. The study has also several strengths: it was contacted in a large national representative population of urban and rural citizens, with a vast age range.

The above results could be important for determining the appropriate levels of serum vitamin D in the Greek population. These results are also important since they could contribute to the implementation of programs and services supporting the overall nutritional and lifestyle healthy patterns for the prevention of vitamin D deficiency and osteoporosis apart from the use of guidelines regarding specific foods or nutrients. Related health promotion policies will

contribute to the improvement of eating and lifestyle behaviours at an individual and population level on the basis of prevention of vitamin D deficiency and osteoporosis.

5. REFERENCES

1. NIH. Osteoporosis prevention, diagnosis and therapy. NIH Consensus Statements, 2000;17:1-45.
2. Λυρίτης ΓΠ. Μεταβολικά νοσήματα των οστών. 5η έκδοση. Αθήνα 2013.
3. John A Kanis on behalf of the World Health Organization Scientific Group Organized by the World Health Organization Collaborating Centre for Metabolic Bone Diseases, University of Sheffield Medical School, UK and the World Health OrganizationWHO Scientific Group Technical Report. ASSESSMENT OF OSTEOPOROSIS AT THE PRIMARY HEALTH CARE LEVEL Report of a WHO Scientific Group, Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. Report of a WHO Study Group. Geneva, World Health Organization, 1994 (WHO Technical Report Series, No. 843
4. Lyritis GP, Rizou S, Galanos A, Makras P. Incidence of hip fractures in Greece during a 30-year period: 1977-2007. Osteoporos Int. 2013;24(5):1579-85. doi: 10.1007/s00198-012-2154-z. Epub 2012 Oct 13.
5. Markos Minas, Nikolaos Koukosias, Elias Zintzaras, Konstantinos Kostikas, and Konstantinos I Gourgoulianis. Prevalence of chronic diseases and morbidity in primary health care in central Greece: An epidemiological study BMC Health Serv Res. 2010; 10: 252. doi: 10.1186/1472-6963-10-252
6. E. Hernlund, A. Svedbom, M. Ivergård, J. Compston, C. Cooper, J. Stenmark, E. V. McCloskey, B. Jönsson, and J. A. Kanis. Osteoporosis in the European Union: medical management, epidemiology and economic burden A report prepared in collaboration with the International Osteoporosis Foundation (IOF) and the European Federation of Pharmaceutical Industry Associations (EFPIA) Arch Osteoporos. 2013; 8(1-2): 136. doi: 10.1007/s11657-013-0136-1
7. Report of a WHO Study Group. Assessment of fracture risk and its application to screening for postmenopausal osteoporosis. World Health Organ Tech Rep Ser 1994;843:1-129.
8. Ebeling PR. Osteoporosis in Men. N Engl J Med 2008;358:1474-1482.

9. Haney EM, Bliziotes MM. Male osteoporosis: new insights in an understudied disease. Curr Opin Rheumatol. 2008;20:423-428.
10. E. S. Siris & R. Adler & J. Bilezikian & M. Bolognese & B. Dawson-Hughes & M. J. Favus & S. T. Harris & S. M. Jan de Beur & S. Khosla & N. E. Lane & R. Lindsay & A. D. Nana & E. S. Orwoll & K. Saag & S. Silverman & N. B. Watts The clinical diagnosis of osteoporosis: a position statement from the National Bone Health Alliance Working Group Osteoporos Int (2014) 25:1439–1443 DOI 10.1007/s00198-014-2655-z
11. Moayyeri A, Adams JE, Adler RA, Krieg MA, Hans D, Compston J, Lewiecki EM. Quantitative ultrasound of the heel and fracture risk assessment: an updated meta-analysis Osteoporos Int 2012;23(1):143-53
12. Marin F. González-Macías J, Díez-Pérez A, Palma S, Delgado-Rodríguez M. (2006) Relationship between Bone Quantitative Ultrasound and Fractures: A Meta-Analysis. J Bone Miner Res 21:1457–1463
13. Huopio J, Kröger H, Honkanen R, Jurvelin J, Saarikoski S, Alhava E. Calcaneal ultrasound predicts early postmenopausal fractures as well as axial BMD. A prospective study of 422 women. Osteoporos Int. 2004;15(3):190-5
14. Hans D, Dargent-Molina P, Schott AM, Sebert JL, Cormier C, Kotzki PO, Delmas PD, Pouilles JM, Breart G, Meunier PJ. Ultrasonographic heel measurements to predict hip fracture in elderly women: the EPIDOS prospective study. Lancet. 1996 348(9026):511-4
15. Damilakis J, Maris TG, Karantanas AH. An update on the assessment of osteoporosis using radiologic techniques. Eur Radiol 2007;17(6):1591-1602.
16. Johnell O, Kanis J. Epidemiology of osteoporotic fractures. Osteoporos Int 2005;16:S3-7.
17. Dontas IA, Yiannakopoulos CK. Risk factors and Prevention of osteoporosis-related fractures. J Musculoskelet Neuronal Interact 2007;7:268-272.
18. S. Ralston and A. Uitterlinden genetics of osteoporosis. Endocrine Reviews 2010
19. Sambrook P. Osteoporosis. Lancet 2006; 367: 2010–2018
20. International Osteoporosis Foundation, IOF: http://www.iofbonehealth.org/facts-and-statistics.html
21. Marcus R, Feldman D, Nelson DA, Rosen CJ. Osteoporosis. 3rd edition. 2008. Elsevier Academy Press

22. Seeman E. Bone quality: the material and structural basis of bone strength. J Bone Miner Metab 2008;26:1-8.
23. Andreoli TE, Carpenter CCJ., Bennett JC, Plum F. Cecil Βασική Παθολογία. 4η έκδοση. Αθήνα 2000. Ιατρικές Εκδόσεις Λίτσας
24. Ahlborg HG, Johnell O, Turner CH, Rannevik G, Karlsson MK. Bone loss and bone size after menopause. N Engl J Med 2003;349(4):327-334.
25. Rosen CJ, Bouxsein ML. Mechanisms of disease: is osteoporosis the obesity of bone? Nat Clin Pract Rheumatol. 2006;2:35-43.
26. Seeman E, Delmas PD. Bone quality-the material and structural basis of bone strength and fragility. N Engl J Med 2006;354:2250-2261.
27. Lane NE. Epidemiology, etiology, and diagnosis of osteoporosis. Am J Obstet Gynecol 2006;194:S3-11.
28. Ferrari S. Human genetics of osteoporosis. Best Pract Res Clin Endocrinol Metab 2008;22:723-735.
29. Makovey J, Nguyen TV, Naganathan V, Wark JD, Sambrook PN. Genetic effects on bone loss in peri- and postmenopausal women: a longitudinal twin study. J Bone Miner Res 2007;22:1773-1780
30. Andrew T, Antioniades L, Scurrah KJ, Macgregor AJ, Spector TD. Risk of wrist fracture in women is heritable and is influenced by genes that are largely independent of those influencing BMD. J Bone Miner Res 2005;20:67–74
31. Michaëlsson K, Melhus H, Ferm H, Ahlbom A, Pedersen NL. Genetic liability to fractures in the elderly. Arch. Intern. Med. 2005;165:1825-1830
32. Ralston SH, Uitterlinden AG. Genetics of osteoporosis Endocrine Reviews 2010;31(5):629-662
33. Richards J, Zheng F, Spector T (2012) Genetics of osteoporosis from genome-wide association studies: advances and challenges Nat Rev Genet. 18;13(8):576-88
34. Deng HW, Livshits G, Yakovenko K, Xu FH, Conway T, DaviesKM, DengH, Recker RR 2002 Evidence for a major gene for bone mineral density/content in human pedigrees identified via probands with extreme bone mineral density. Ann Hum Genet 66:61–74
35. Richards JB, Kavvoura FK, Rivadeneira F, Styrkársdóttir U, Estrada K, Halldórsson BV, Hsu YH, Zillikens MC, Wilson SG, Mullin BH, Amin N, Aulchenko YS, Cupples LA, Deloukas P,

Demissie S, Hofman A, Kong A, Karasik D, van Meurs JB, Oostra BA, Pols HA, Sigurdsson G, Thorsteinsdottir U, Soranzo N, Williams FM, Zhou Y, Ralston SH, Thorleifsson G, van Duijn CM, Kiel DP, Stefansson K, Uitterlinden AG, Ioannidis JP, Spector TD; Genetic Factors for Osteoporosis Consortium. Collaborative meta-analysis: associations of 150 candidate genes with osteoporosis and osteoporotic fracture. Ann Intern Med 2009;151(8):528-537.

36. Pearson TA, Manolio TA. How to Interpret a Genome-wide Association Study. JAMA 2008;299(11):1335-1344

37. Rivadeneira F, Styrkársdottir U, Estrada K, Halldórsson BV, Hsu YH, Richards JB, Zillikens MC, Kavvoura FK, Amin N, Aulchenko YS, Cupples LA, Deloukas P, Demissie S, Grundberg E, Hofman A, Kong A, Karasik D, van Meurs JB, Oostra B, Pastinen T, Pols HA, Sigurdsson G, Soranzo N, Thorleifsson G, Thorsteinsdottir U, Williams FM, Wilson SG, Zhou Y, Ralston SH, van Duijn CM, Spector T, Kiel DP, Stefansson K, Ioannidis JP, Uitterlinden AG; Genetic Factors for Osteoporosis (GEFOS) Consortium. Twenty bone-mineral-density loci identified by large-scale meta-analysis of genome-wide association studies. Nat Genet 2009;41(11):1199-1206.

38. Richards JB, Rivadeneira F, Inouye M, Pastinen TM, Soranzo N, Wilson SG, Andrew T, Falchi M, Gwilliam R, Ahmadi KR, Valdes AM, Arp P, Whittaker P, Verlaan DJ, Jhamai M, Kumanduri V, Moorhouse M, van Meurs JB, Hofman A, Pols HA, Hart D, Zhai G, Kato BS, Mullin BH, Zhang F, Deloukas P, Uitterlinden AG, Spector TD. Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. Lancet. 2008;371(9623):1505-1512.

39. Kiel DP, Demissie S, Dupuis J, Lunetta KL, Murabito JM, Karasik D. Genome-wide association with bone mass and geometry in the Framingham Heart Study. BMC Med Genet. 2007;8 Suppl 1:S14.

40. Williams FM, Spector TD. The genetics of osteoporosis. Acta Reumatol Port 2007;32:231-240.

41. Duncan EL, Brown MA. Genetic studies in osteoporosis--the end of the beginning. Arthritis Res Ther 2008;10:214.

42. Balding DJ. A tutorial on statistical methods for population association studies. Nat Rev Genet 2006;7:781-791.

43. Guo Y, Tan LJ, Lei SF, Yang TL, Chen XD, Zhang F, Chen Y, Pan F, Yan H, Liu X, Tian Q, Zhang ZX, Zhou Q, Qiu C, Dong SS, Xu XH, Guo YF, Zhu XZ, Liu SL, Wang XL, Li X, Luo Y, Zhang LS, Li M, Wang JT, Wen T, Drees B, Hamilton J, Papasian CJ, Recker RR, Song XP, Cheng J, Deng HW. Genome-wide association study identifies ALDH7A1 as a novel susceptibility gene for osteoporosis. PLoS Genet 2010;6(1):e1000806.

44. Chen Y, Xiong DH, Guo YF, Pan F, Zhou Q, Zhang F, Deng HW. Pathway-based genome-wide association analysis identified the importance of EphrinA-EphR pathway for femoral neck bone geometry. Bone. 2009 (in Press)

45. Karasik D, Dupuis J, Cho K, Cupples LA, Zhou Y, Kiel DP, Demissie S. Refined QTLs of osteoporosis-related traits by linkage analysis with genome-wide SNPs: Framingham SHARe. Bone. 2010 (in Press).

46. Guo Y, Zhang LS, Yang TL, Tian Q, Xiong DH, Pei YF, Deng HW. PTH and IL21R may underlie variation of femoral neck bone mineral density as revealed by a Genome-Wide association study. J Bone Miner Res 2009 (in Press).

47. Xiong DH, Liu XG, Guo YF, Tan LJ, Wang L, Sha BY, Tang ZH, Pan F, Yang TL, Chen XD, Lei SF, Yerges LM, Zhu XZ, Wheeler VW, Patrick AL, Bunker CH, Guo Y, Yan H, Pei YF, Zhang YP, Levy S, Papasian CJ, Xiao P, Lundberg YW, Recker RR, Liu YZ, Liu YJ, Zmuda JM, Deng HW. Genome-wide association and follow-up replication studies identified ADAMTS18 and TGFBR3 as bone mass candidate genes in different ethnic groups. Am J Hum Genet. 2009;84(3):388-398.

48. Zhang YP, Deng FY, Chen Y, Pei YF, Fang Y, Guo YF, Guo X, Liu XG, Zhou Q, Liu YJ, Deng HW. Replication study of candidate genes/loci associated with osteoporosis based on genome-wide screening. Osteoporos Int 2009 In Press.

49. Estrada K, Styrkarsdottir U, Evangelou E, Hsu Y, Duncan E, Ntzani E, Oei L, Albagha O, Amin N, Kemp J, Koller D, Li G, Liu C, Minster R, Moayyeri R, Vandenput L, Willner D, Xiao S, Yerges-Armstrong L, Zheng H, Alonso N, Eriksson J, Kammerer C, Kaptoge S, Leo P, Thorleifsson G, Wilson S, Wilson J, Aalto V, Alen M, Aragaki A, Aspelund T, Center J, Dailiana Z, Duggan D, Garcia M, García-Giralt N, Giroux S, Hallmans G, Hocking L, Bjerre Husted L, Jameson K, Khusainova R, Kim G, Kooperberg C, Koromila T, Kruk M, Laaksonen M, Lacroix A, Hun Lee S, Leung P, Lewis J,Masi L, Mencej-Bedrac S, Nguyen S, Nogues X, Patel M, Prezelj J, Rose L, Scollen S, Siggeirsdottir K, Smith S, Svensson O,

Trompet S, Trummer O, Schoor N, Woo J, Zhu K, Balcells S, Brandi ML, Buckley B, Cheng S, Christiansen C, Cooper C, Dedoussis G, Ford I, Frost M, Goltzman D, González-Macías J, Kähönen M, Karlsson M, Khusnutdinova E, Koh J, Kollia P, Langdah BL, LeslieW, Lips P, Ljunggren O,Lorenc R, Marc J, Mellström D, Obermayer-Pietsch B, Olmos J,Pettersson U, Reid D, Riancho J, Ridker P, Rousseau F,Slagboom89,3, Nelson L.S. Tang90,91, Roser Urreizti65, Wim Van Hul92, Jorma Viikari E, Zarrabeitia M, Aulchenko Y, Betancourt M, Grundberg E, Herrera L, Ingvarsson T, Johannsdottir H, Kwan T, Li R, Luben R,Gómez CM, Palsson S, Reppe S, Rotter J, Sigurdsson G, Meurs J, Verlaan D, Williams D,Wood D, Zhou Y, Gautvik K, Pastinen T, Raychaudhuri S, Cauley J, Chasman D, Clark G, Cummings S, Danoy P, Dennison E, Eastell R, Eisman J, Gudnason V, Hofman A, Jackson R, Jones G,Jukema W, Khaw K, Lehtimäki T, Liu Y, Lorentzon M, McCloskey E, Mitchell B, Nandakumar K, Nicholson G, Oostra B, Peacock M, Pols H, Prince R, Raitakari O, Reid I, Robbins J, Sambrook F, Sham PS, Shuldiner A, Tylavsky F, Duijn C, Wareham N, L. Cupples A, Econs M, Evans D, Harris T, Wai Chee Kung A, Psaty B, Reeve J, Spector T, Streeten A, Zillikens C, Thorsteinsdottir U, Ohlsson C, Karasik D, Richards B, Brown A, Stefansson K, Uitterlinden AG, Ralston S, Ioannidis J, Kiel D, Rivadeneira F. Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture. Nat Genet. 2012;15;44(5):491-501

50. Duncan EL, Danoy P, Kemp JP, Leo PJ, McCloskey E, Nicholson GC, Eastell R, Prince RL, Eisman JA, Jones G, Sambrook PN, Reid IR, Dennison EM, Wark J, Richards JB, Uitterlinden AG, Spector TD, Esapa C, Cox RD, Brown SD, Thakker RV, Addison KA, Bradbury LA, Center JR, Cooper C, Cremin C, Estrada K, Felsenberg D, Glüer CC, Hadler J, Henry MJ, Hofman A, Kotowicz MA, Makovey J, Nguyen SC, Nguyen TV, Pasco JA, Pryce K, Reid DM, Rivadeneira F, Roux C, Stefansson K, Styrkarsdottir U, Thorleifsson G, Tichawangana R, Evans DM, Brown MA. Genome-wide association study using extreme truncate selection identifies novel genes affecting bone mineral density and fracture risk. PLoS Genet. 2011;7(4):e1001372.

51. Hsu YH, Zillikens MC, Wilson SG, Farber CR, Demissie S, Soranzo N, Bianchi EN, Grundberg E, Liang L, Richards JB, Estrada K, Zhou Y, van Nas A, Moffatt MF, Zhai G, Hofman A, van Meurs JB, Pols HA, Price RI, Nilsson O, Pastinen T, Cupples LA, Lusis AJ, Schadt EE, Ferrari S, Uitterlinden AG, Rivadeneira F, Spector TD, Karasik D, Kiel DP. An integration of genome-wide association study and gene expression profiling to prioritize

the discovery of novel susceptibility Loci for osteoporosis-related traits. PLoS Genet. 2010;10:6(6):e1000977

52. Styrkarsdottir U, Halldorsson BV, Gretarsdottir S, Gudbjartsson DF, Walters GB, Ingvarsson T, Jonsdottir T, Saemundsdottir J, Snorradóttir S, Center JR, Nguyen TV, Alexandersen P, Gulcher JR, Eisman JA, Christiansen C, Sigurdsson G, Kong A, Thorsteinsdottir U, Stefansson K. New sequence variants associated with bone mineral density. Nat Genet. 2009;41:15–17

53. Kung AW, Xiao SM, Cherny S, Li GH, Gao Y, Tso G, Lau KS, Luk KD, Liu JM, Cui B, Zhang MJ, Zhang ZL, He JW, Yue H, Xia WB, Luo LM, He SL, Kiel DP, Karasik D, Hsu YH, Cupples LA, Demissie S, Styrkarsdottir U, Halldorsson BV, Sigurdsson G, Thorsteinsdottir U, Stefansson K, Richards JB, Zhai G, Soranzo N, Valdes A, Spector TD, Sham PC. Association of JAG1 with bone mineral density and osteoporotic fractures: a genome-wide association study and follow-up replication studies Am J Hum Genet. 2010;86(2):229-39

54. Alireza Moayyeri et al. Genetic determinants of heel bone properties: genome-wide association meta-analysis and replication in the GEFOS/GENOMOS consortium. Human Molecular Genetics, 2014, Vol. 23, 3054–3068, doi:10.1093/hmg/ddt675

55. Douroudis K, Tarassi K, Athanassiades T, Giannakopoulos F, Kominakis A, Thalassinos N, Papasteriades Ch. HLA alleles as predisposal factors for postmenopausal osteoporosis in a Greek population. Tissue Antigens. 2007;69:592-596.

56. Efstathiadou Z, Koukoulis G, Stakias N, Challa A, Zintzaras E, Tsatsoulis A. Correlation of estrogen receptor beta gene polymorphisms with spinal bone mineral density in peri- and post-menopausal Greek women. Maturitas. 2006;53:380-385.

57. Tilkeridis C, Bei T, Garantziotis S, Stratakis CA. Association of a COL1A1 polymorphism with lumbar disc disease in young military recruits. J Med Genet. 2005;42:e44.

58. Fountas L, Anapliotou M, Kominakis A, Sekeris CE, Kassi E, Moutsatsou P. Estrogen receptor alpha gene analysis in osteoporosis and familial osteoporosis. Osteoporos Int. 2004;15:948-956.

59. Douroudis K, Tarassi K, Ioannidis G, Giannakopoulos F, Moutsatsou P, Thalassinos N, Papasteriades C. Association of vitamin D receptor gene polymorphisms with bone mineral density in postmenopausal women of Hellenic origin. Maturitas. 2003;45:191-197.

60. Efstathiadou Z, Kranas V, Ioannidis JP, Georgiou I, Tsatsoulis A. The Sp1 COLIA1 gene polymorphism, and not vitamin D receptor or estrogen receptor gene polymorphisms, determines bone mineral density in postmenopausal Greek women. Osteoporos Int. 2001;12:326-331.

61. Fountas L, Moutsatsou P, Kastanias I, Tamouridis N, Tzanela M, Anapliotou M, Sekeris CE. The contribution of vitamin D receptor gene polymorphisms in osteoporosis and familial osteoporosis. Osteoporos Int. 1999;10:392-398.

62. Moschonis G, Tanagra S, Koutsikas K, Nikolaidou A, Androutsos O, Manios Y. Association between serum 25-hydroxyvitamin D levels and body composition in postmenopausal women: the postmenopausal Health Study. Menopause. 2009;16:701-707.

63. Garnero P. Bone Markers in Osteoporosis. Curr Osteoporos Rep 2009;7(3):84-90.

64. Ivaska KK, Lenora J, Gerdhem P, Akesson K, Väänänen HK, Obrant KJ. Serial assessment of serum bone metabolism markers identifies women with highest rate of bone loss and osteoporosis risk. J Clin Endocrinol Metab 2008;93:2622–2632.

65. Bauer DC, Garnero P, Hochberg MC, Santora A, Delmas P, Ewing SK, Black DM; for the Fracture Intervention Research Group. Pre-treatment bone turnover and fracture efficacy of alendronate: the Fracture Intervention Trial. J Bone Miner Res 2006;21:292–299.

66. Bauer DC, Garnero P, Bilezikian JP, Greenspan SL, Ensrud KE, Rosen CJ, Palermo L, Black DM. Short-term changes in bone turnover markers and bone mineral density response to parathyroid hormone in postmenopausal women with osteoporosis. J Clin Endocrinol Metab 2006;91:1370–1375.

67. Delmas PD, Vrijens B, Eastell R, Roux C, Pols HA, Ringe JD, Grauer A, Cahall D, Watts NB; Improving Measurements of Persistence on Actonel Treatment (IMPACT) Investigators. Effect of monitoring bone turnover markers on persistence with risedronate treatment of postmenopausal osteoporosis. J Clin Endocrinol Metab 2007;4:1296–1304.

68. Cashman KD. Diet, Nutrition, and Bone Health. J Nutr 2007;137:2507S–2512S.

69. Cashman KD. Diet and control of osteoporosis. In: Remacle C, Reusens B, editors. Functional foods, ageing and degenerative disease. Cambridge, UK: Woodhead Publishing Limited; 2004. p. 83–114.

70. Prentice A. Diet, nutrition and the prevention of osteoporosis. Public Health Nutr 2004;7(1A):227–243.

71. Calvo MS, Barton CN, Park YK. Bone mass and high dietary intake of meat and protein: analyses from data from the Third National Health and Nutrition Examination Survey (NHANES III, 1988-94). Bone 1998;23(Suppl.):S290.

72. Simopoulos AP. Essential fatty acids in health and chronic disease. Am J Clin Nutr 1999;70(Suppl.):560-569.

73. Berg KM, Kunins HV, Jackson JL, Nahvi S, Chaudhry A, Harris KA Jr, Malik R, Arnsten JH. Association between alcohol consumption and both osteoporotic fracture and bone density. Am J Med 2008;121(5):406-418.

74. Wetmore CM, Ichikawa L, LaCroix AZ, Ott SM, Scholes D. Association between caffeine intake and bone mass among young women: potential effect modification by depot medroxyprogesterone acetate use.Osteoporos Int 2008;19(4):519-527.

75. Alekel DL, Van Loan MD, Koehler KJ, Hanson LN, Stewart JW, Hanson KB, Kurzer MS, Peterson CT. The soy isoflavones for reducing bone loss (SIRBL) study: a 3-y randomized controlled trial in postmenopausal women. Am J Clin Nutr 2010; 91(1):218-230.

76. Kant AK. Dietary patterns: biomarkers and chronic disease risk. Appl Physiol Nutr Metab. 2010 Apr;35(2):199-206. doi:10.1139/H10-005,

77. Kant AK. Dietary patterns and health outcomes. J Am Diet Assoc 2004;104:615-35

78. McGartland CP, Robson PJ, Murray LJ, Cran GW, Savage MJ, Watkins DC, Rooney MM, Boreham CA. Fruit and vegetable consumption and bone mineral density: the Northern Ireland Young Hearts Project. Am J Clin Nutr 2004;80(4):1019-1023.

79. Tylavsky FA, Holliday K, Danish R, Womack C, Norwood J, Carbone L. Fruit and vegetable intakes are an independent predictor of bone size in early pubertal children. Am J Clin Nutr 2004;79(2):311-317.

80. New SA, Robins SP, Campbell MK, Martin JC, Garton MJ, Bolton-Smith C, Grubb DA, Lee SJ, Reid DM. Dietary influences on bone mass and bone metabolism: further evidence of a positive link between fruit and vegetable consumption and bone health?Am J Clin Nutr 2000;71(1):142-151.

81. Kontogianni MD, Melistas L, Yannakoulia M, Malagaris I, Panagiotakos DB, Yiannakouris N. Association between dietary patterns and indices of bone mass in a sample of Mediterranean women. Nutrition 2009;25(2):165-171.

82. Lin PH, Ginty F, Appel LJ, Aickin M, Bohannon A, Garnero P, Barclay D, Svetkey LP. The DASH diet and sodium reduction improve markers of bone turnover and calcium metabolism in adults. J Nutr 2003;133(10):3130-3136.

83. Manios Y, Moschonis G, Katsaroli I, Grammatikaki E, Tanagra S. Changes in diet quality score, macro- and micronutrients intake following a nutrition education intervention in postmenopausal women. J Hum Nutr Diet. 2007;20:126-131.

84. Kyriazopoulos P, Trovas G, Charopoulos J, Antonogiannakis E, Galanos A, Lyritis G. Lifestyle factors and forearm bone density in young Greek men. Clin Endocrinol (Oxf). 2006;65:234-238.

85. Babaroutsi E, Magkos F, Manios Y, Sidossis LS. Body mass index, calcium intake, and physical activity affect calcaneal ultrasound in healthy Greek males in an age-dependent and parameter-specific manner. J Bone Miner Metab. 2005;23(2):157-166.

86. Babaroutsi E, Magkos F, Manios Y, Sidossis LS. Lifestyle factors affecting heel ultrasound in Greek females across different life stages. Osteoporos Int. 2005;16:552-561.

87. Petridou E, Karpathios T, Dessypris N, Simou E, Trichopoulos D. The role of dairy products and non alcoholic beverages in bone fractures among schoolage children. Scand J Soc Med. 1997;25:119-125.

88. Trichopoulou A, Georgiou E, Bassiakos Y, Lipworth L, Lagiou P, Proukakis C, Trichopoulos D. Energy intake and monounsaturated fat in relation to bone mineral density among women and men in Greece. Prev Med. 1997;26:395-400.

89. Kobyliansky E, Karasik D, Belkin V, Livshits G. Bone ageing: genetics versus environment. Ann Hum Biol 2000;27:433-451.

90. Mora S, Gilsanz V. Establishment of peak bone mass. Endocrinol Metab Clin North Am 2003;32(1):39-63.

91. Holick MF. Vitamin D Deficiency. (2007) N Engl J Med 357:266-281.

92. Mithal A, Wahl D, Bonjour J,Burckhardt P, Hughes D, Eisman J, Fuleihan G, Josse R, Lips P, Torres J. Global vitamin D status and determinants of hypovitaminosis D. Osteoporos Int 2009

93. Langley-Evans S. Nutrition: A Lifespan Approach. 2009. Wiley-Blackwell

94. Michael F. Holick, Neil C. Binkley, Heike A. Bischoff-Ferrari,Catherine M. Gordon, David A. Hanley, Robert P. Heaney, M. Hassan Murad,and Connie M. Weaver. Evaluation, Treatment, and Prevention of Vitamin D Deficiency: an Endocrine Society Clinical Practice GuidelineJ Clin Endocrinol Metab, July 2011, 96(7):1911–1930

95. Uwe Gröber, Jörg Spitz, Jörg Reichrath, Klaus Kisters, and Michael F Holick. Vitamin D: Update 2013 From rickets prophylaxis to general preventive healthcare. Dermatoendocrinol. 2013 Jun 1;5(3):331-47. doi: 10.4161/derm.26738

96. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, Durazo-Arvizu RA, Gallagher JC, Gallo RL, JonesG, Kovacs CS, Mayne ST, Rosen CJ, Shapses SA (2011) The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. J Clin Endocrinol Metab 96: 53–58

97. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, Murad MH, Weaver CM. (2011) Evaluation, Treatment, and Prevention of Vitamin D Deficiency: an Endocrine Society Clinical Practice Guideline. J Clin Endocrinol Metab 96(7):1911–1930

98. Holick MF. Vitamin D Deficiency. N Engl J Med 2007;357:266-281.

99. Nicolaidou P, Hatzistamatiou Z, Papadopoulou A, Kaleyias J, Floropoulou E, Lagona E, Tsagris V, Costalos C, Antsaklis A. Low Vitamin D Status in Mother-Newborn Pairs in Greece. Calcif Tissue Int 2006;78:337-342.

100. Papapetrou PD, Triantaphyllopoulou M, Karga H, Zagarelos P, Aloumanis K, Kostakioti E, Vaiopoulos G. Vitamin D deficiency in the elderly in Athens, Greece. J Bone Miner Metab 2007;25:198–203

101. Bountziouka V, Bathrellou E, Giotopoulou A et al. Development, repeatability and validity regarding energy and macronutrient intake of a semi-quantitative food frequency questionnaire: methodological considerations. Nutr Metab Cardiovasc Dis 2012;22:659–667

102. Willet W. Nutritional epidemiology. 2nd edition. 1998. Oxford University Press.

103. Ministry of Health and Welfare, Supreme Scientific Health Council Dietary guidelines for adults in Greece. Arch Hellenic Med 1999;16:516–524

104. Webb AR, Kline L, Holick MF. Influence of season and latitude on the cutaneous synthesis of vitamin D3: exposure to winter sunlight in Boston and Edmonton will not promote vitamin D3 synthesis in human skin. J Clin Endocrinol Metab. 1988;67:373–8

105. Heaney RP, Davies KM, Chen TC, Holick MF, Barger-Lux MJ. Human serum 25-hydroxycholecalciferol response to extended oral dosing with cholecalciferol. Am J Clin Nutr. 2003;77:204–10

106. World Health Organization. Obesity: preventing and managing the global epidemic. Report of a WHO consultation. World Health Organ Tech Rep Ser. 2000;894:i-xii, 1-253

107. Flöter M, Bittar CK, Zabeu JL, Carneiro AC. Review of comparative studies between bone densitometry and quantitative ultrasound of the calcaneus in osteoporosis. Acta Reumatol Port. 2011;36(4):327-35

108. Trovas G, Tsekoura M, Galanos A, Dionyssiotis Y, et al. Quantitative ultrasound of the calcaneus in greek women: normative data are different from the manufacturer's normal range. J Clin Densitom. 2009;12(3):353-9

109. http://www.cdc.gov/labstandards/pdf/hs/CDC_Certified_Vitamin_D_Procedures.pdf.

110. Mardia KV, Kent JT, Bibby JM. Multivariate Analysis. New York: Academic Press 1979

111. Roger Bouillon, Claudio Marcocci, Geert Carmeliet, Daniel Bikle, John H. White, Bess Dawson-Hughes, Paul Lips, Craig F.Munns, Marise Lazaretti-Castro, Andrea Giustina, John Bilezikian. Skeletal and extra-skeletal actions of vitamin D: Current evidence and outstanding questions. Endocrine Reviews 2018; DOI: 10.1210/er.2018-00126

112. J. A. Kanis, A. Odén, E. V. McCloskey, H. Johansson, D. A. Wahl & C. Cooper on behalf of the IOF Working Group on Epidemiology and Quality of Life. A systematic review of hip fracture incidence and probability of fracture worldwide. Osteoporos Int 2012;23:2239–2256

113. Kok-Yong Chin and Soelaiman Ima-Nirwana. Calcaneal Quantitative Ultrasound as a Determinant of Bone Health Status: What Properties of Bone Does It Reflect? Int. J. Med. Sci. 2013; 10(12):1778-1783. doi: 10.7150/ijms.6765

114. Manios Y, Moschonis G, Lambrinou CP, Mavrogianni C, Tsirigoti L, Hoeller U, Roos FF, Bendik I, Eggersdorfer M, Celis-Morales C, Livingstone KM, Marsaux CF, Macready AL, Fallaize R, O'Donovan CB, Woolhead C, Forster H, Walsh MC, Navas-Carretero S, San-Cristobal R, Kolossa S, Hallmann J, Jarosz M, Surwiłło A, Traczyk I, Drevon CA, van

Ommen B, Grimaldi K, Matthews JN, Daniel H, Martinez JA, Lovegrove JA, Gibney ER, Brennan L, Saris WH, Gibney M, Mathers JC; Food4Me Study. (2017) Associations of vitamin D status with dietary intakes and physical activity levels among adults from seven European countries: the Food4Me study. Eur J Nutr. doi: 10.1007/s00394-017-1415-1

115. Cashman KD, Dowling KG, Skrabakova Z, Gonzalez-Gross M, Valtuena J, De Henauw S, Moreno L, Damsgaard CT, Michaelsen KF, Molgaard C, Jorde R, Grimnes G, Moschonis G, Mavrogianni C, Manios Y, Thamm M, Mensink GB, Rabenberg, M, Busch MA, Cox L, Meadows S, Goldberg G, Prentice A, Dekker JM, Nijpels G, Pilz S, Swart KM, van Schoor NM, Lips P, Eiriksdottir G, Gudnason V, Cotch MF, Koskinen S, Lamberg-Allardt C, Durazo-Arvizu RA, Sempos CT, Kiely M Vitamin D deficiency in Europe: pandemic? Am J Clin Nutr 2016;103:1033–44 doi:10.3945/ajcn.115.120873
116. Kauppi M, Impivaara O, Mäki J, Heliövaara M, Marniemi J, Montonen J, Jula A. Vitamin D status and common risk factors for bone fragility as determinants of quantitative ultrasound variables in a nationally representative population sample. Bone. 2009;45(1):119-24
117. Mathilde Touvier, Me´lanie Deschasaux, Marion Montourcy, Angela Sutton, Nathalie Charnaux, Emmanuelle Kesse-Guyot, Karen E. Assmann, Le´opold Fezeu, Paule Latino-Martel, Nathalie Druesne-Pecollo, Christiane Guinot, Julie Latreille, Denis Malvy, Pilar Galan, Serge Hercberg, Sigrid Le Clerc, Jean-Claude Souberbielle and Khaled Ezzedine. Determinants of Vitamin D Status in Caucasian Adults: Influence of Sun Exposure, Dietary Intake, Sociodemographic, Lifestyle, Anthropometric, and Genetic Factors. Journal of Investigative Dermatology 2015;135 (2): 378-388
118. Boland RL, Vitamin D physiology, Mol Cell Endocrinol. 2011;347(1-2):11-6. doi: 10.1016/j.mce.2011.05.021
119. Sadeq A Quraishi, Carlos A Camargo Jr and JoAnn E Manson. Low vitamin D status in Europe: moving from evidence to sound public health policies Am J Clin Nutr 2016;103:957–8
120. Greene-Finestone LS1, Berger C, de Groh M, Hanley DA, Hidiroglou N, Sarafin K, Poliquin S, Krieger J, Richards JB, Goltzman D; CaMos Research Group 25-hydroxyvitamin D in

Canadian adults: biological, environmental, and behavioral correlates. Osteoporos Int. 2011;22(5):1389-1399.

121. Shakur YA, Lou W, L'Abbe MR. Examining the effects of increased vitamin D fortification on dietary inadequacy in Canada. Can J Public Health. 2014;105(2):e127-32

122. Lips P. Interaction between vitamin D and calcium. Scand J Clin Lab Invest Suppl. 2012;243:60-4.doi: 0.3109/00365513.2012.681960.

123. Howard, G.M., Nguyen, T.V., Harris, M., Kelly, P.J. and Eisman, J.A. Genetic and environmental contributions to the association between quantitative ultrasound and bone mineral density measurements: a twin study. J. Bone Miner. Res. 1998;13:1318–1327.

124. Karasik, D., Myers, R.H., Hannan, M.T., Gagnon, D., McLean, R.R., Cupples, L.A. and Kiel, D.P. (2002) Mapping of quantitative ultrasound of the calcaneus bone to chromosome 1 by genome-wide linkage analysis. Osteoporos. Int., 13, 796–802

125. Kemp JP, Morris JA, Medina-Gomez C, Forgetta V, Warrington NM, Youlten SE, Zheng J, Gregson CL, Grundberg E. Identification of 153 new loci associated with heel bone mineral density and functional involvement of GPC6 in osteoporosis. Nat Genet. 2017;49(10):1468-1475. doi: 10.1038/ng.3949

126. Karohl, C. et al. Heritability and seasonal variability of vitamin D concentrations in male twins. Am. J. Clin. Nutr. 2010;92, 1393–1398

127. Orton, S.-M. et al. Evidence for genetic regulation of vitamin D status in twins with multiple sclerosis. Am. J. Clin. Nutr. 2008;88, 441–447

APPREDIX

Appredix

1

ΚΛΙΝΙΚΟ ΑΡΧΕΙΟ ΕΘΕΛΟΝΤΗ ΚΑΙ ΕΡΩΤΗΜΑΤΟΛΟΓΙΑ

ΚΩΔΙΚΟΣ ΕΘΕΛΟΝΤΗ:

ΗΜΕΡΟΜΗΝΙΑ ΕΠΙΣΚΕΨΗΣ:____ / ____ / ____

(Ημερομηνία Συμπλήρωσης Κλινικού Αρχείου Εθελοντή και Ερωτηματολογίων)

ΗΜΕΡΟΜΗΝΙΑ ΑΙΜΟΛΗΨΙΑΣ:____ / ____ / ____

Νηστικός/ή: **ΝΑΙ** ☐ **ΟΧΙ**☐

Λήψη Φαρμάκων: **ΝΑΙ** ☐ **ΟΧΙ**☐

Εάν έχει λάβει κάποιο φάρμακο πριν την αιμοληψία, παρακαλώ διευκρινίστε:

Όνομα Φαρμάκου	*Λόγος Λήψης Φαρμάκου*
1.	
2.	
3.	

<u>ΣΤΟΙΧΕΙΑ ΕΘΕΛΟΝΤΗ</u>

ΕΠΩΝΥΜΟ: …….

ΟΝΟΜΑ: …….

ΦΥΛΟ: Α☐ **Θ**☐

ΗΜΕΡΟΜΗΝΙΑ ΓΕΝΝΗΣΕΩΣ:____ / ____ / ____

ΣΤΟΙΧΕΙΑ ΕΠΙΚΟΙΝΩΝΙΑΣ ΕΘΕΛΟΝΤΗ

ΔΙΕΥΘΥΝΣΗ: ……Τ.Κ……..

ΤΗΛΕΦΩΝΟ: ……e-mail…….

ΑΠΟΤΕΛΕΣΜΑΤΑ ΥΠΕΡΗΧΩΝ

STIFFNESS INDEX …….

FOOT …….

REFERENCE …….

% YOUNG ADULT …….

T SCORE…….

% AGE MATCHED …….

Z SCORE…….

BUA…….

SOS……..

ΑΝΘΡΩΠΟΜΕΤΡΙΚΑ ΔΕΔΟΜΕΝΑ

1. **Σωματικό Βάρος**

1.1.Παρών Σωματικό Βάρος:……ΔΜΣ…….....

Νηστικός/ή: ΝΑΙ ΟΧΙ

1.2. Σύνηθες σωματικό βάρος: ……

1.3. Ιστορικό Βάρους

	Kg	**Ηλικία**	**Χρονικό διάστημα**	**Αιτία**
Απώλεια βάρους				
Αύξηση βάρους				

1.4.Σωματικό Βάρος Γεννήσεως:……..

1.5. Ηλικία κύησης:……..μήνες/εβδομάδες

2. **Ύψος**

2.1.Όρθιο Ύψος:……

2.2.Καθήμενο Ύψος: ……

2.3.Απόσταση Γονάτου από το πάτωμα:…….

3. **Περιφέρειες Σώματος**

3.1.Περιφέρεια Κεφαλής:……

3.2.Περιφέρεια Καρπού:……

3.4.Περιφέρεια Μέσου Βραχίονα:……

3.5.Περιφέρεια Μέσης: ……

3.6.Περιφέρεια Ισχίου:……

4. **Αρτηριακή Πίεση**

4.1. Συστολική Πίεση:**……**

4.2. Διαστολική Πίεση:**……**

4.3. Αριθμός Σφυγμών ανά λεπτό:**……**

5. **Ποιο χέρι χρησιμοποιείτε για να γράψετε;**

Δεξί Χέρι ☐

Αριστερό Χέρι ☐

Καμία Προτίμηση ☐

6. **Ποιο είναι το Επικρατές σας Χέρι;**

Δεξί Χέρι ☐

Αριστερό Χέρι ☐

Καμία Προτίμηση ☐

7. **Χρώμα Ματιών**

Μαύρα ☐

Καστανά ☐

Μπλε ☐

Πράσινα ☐

Ποιο ήταν το Φυσικό Χρώμα των Μαλλιών σας στην ηλικία των 15 ετών;

Ξανθό ☐

Ανοιχτό Καστανό ☐

Σκούρο Καστανό ☐

Κοκκινωπό/Καστανέρυθρο ☐

Κόκκινο ☐

Μαύρο ☐

8. **Τα Μαλλιά σας είναι:**

Ίσια ☐

Σπαστά ☐

Σγουρά ☐

9. **Χρώμα Μαλλιών**

10. **Έμμηνος Κύκλος**

10.1.Έχετε ακόμα Έμμηνο Κύκλο; ΝΑΙ ☐ ΟΧΙ☐

10.2.Ποια είναι/ήταν η διάρκεια του Έμμηνου Κύκλου σας;……

10.3.Σε ποια ηλικία, ξεκίνησε ο Έμμηνος Κύκλους σας;……

10.4.Σε ποια ηλικία, σταμάτησε ο Έμμηνος Κύκλους σας;……

10.5. Είχατε ποτέ αμηνόρροια:……ΔΙΑΡΚΕΙΑ……..ΗΛΙΚΙΑ……. ΑΙΤΙΑ……

11. **Συνολικός Αριθμός Κυήσεων**:……..

12. **Συνολικός Αριθμός Τέκνων**:……

12.1. Συνολικός Αριθμός Άρρεν:……

12.2. Συνολικός Αριθμός Θύλη:……

13. **Μήνες θηλασμού**……

ΚΛΙΝΙΚΑ ΔΕΔΟΜΕΝΑ

14. **Φαρμακευτική Αγωγή**

Παίρνετε κάποιου είδους φάρμακο ή ακολουθείτε κάποιου είδους ιατρική παρέμβαση (π.χ. χημειοθεραπεία, ραδιοθεραπεία)

 ΝΑΙ ΟΧΙ

Εάν απαντήσατε ΝΑΙ, παρακαλώ συμπληρώστε προσεκτικά τον παρακάτω πίνακα

Όνομα Φαρμακευτικής Αγωγής (Φάρμακο ή Ιατρική Παρέμβαση)	**Γιατί** παίρνετε το Φάρμακο ή ακολουθείτε την Ιατρική Παρέμβαση;	**Συχνότητα** που παίρνετε το Φάρμακο ή ακολουθείτε την Ιατρική Παρέμβαση

14.1 Λαμβάνατε στο παρελθόν (τελευταία 5 έτη) κάποια από τα ακόλουθα φάρμακα;

	ΑΙΤΙΑ	**ΔΟΣΗ**	**ΗΛΙΚΙΑ/ΔΙΑΡΚΕΙΑ**
Ορμόνες			
Ηρεμιστικά			
Αντιεπιληπτικά			
Αντιπηκτικά			
Θυροειδικά			
Διουρητικά			

Αναλγητικά			
Κορτιζόνη			
Αντιόξινα			
Ασβέστιο			
Βιταμίνη D			
Οστεοπόρωσης			
Διατροφικά συμπληρώματα			
Για άλλο νόσημα των οστών			
Άλλο			

Θεραπεία για καρκίνο: Διάρκεια/δόση Ηλικία
Ορμόνες
Εγχείρηση

15. Έχετε πάρει πότε Αντισυλληπτικό Χάπι; ΝΑΙ ☐ ΟΧΙ☐

16. Έχετε ακολουθήσει ποτέ Ορμονική Θεραπεία Αντικατάστασης; ΝΑΙ ☐ ΟΧΙ☐

17. **Ιστορικό Καταγμάτων**

ΠΕΡΙΟΧΗ	**ΑΡΙΘΜΟΣ**	**ΗΛΙΚΙΑ**	**ΑΙΤΙΟΛΟΓΙΑ**	**ΑΝΤΙΜΕΤΩΠΙΣΗ ΑΚΙΝΗΤΟΠΟΙΗΣΗ**

18. **Χειρουργική Παρέμβαση**

Έχετε υποβληθεί σε κάποιου είδους χειρουργική παρέμβαση;

 ΝΑΙ ΟΧΙ

Εάν απαντήσατε ΝΑΙ, παρακαλώ συμπληρώστε προσεκτικά τον παρακάτω πίνακα

Τύπος Χειρουργικής Παρέμβασης	**Γιατί** υποβληθήκατε στη Χειρουργική Παρέμβαση;	**Χρονολογία** που υποβληθήκατε στη Χειρουργική Παρέμβαση
Ολική Αρθροπλαστική		
Ισχίο		
Γόνατο		
Άλλο: **Διευκρινίστε:**		

19. **Έχετε κάνει Υστερεκτομή**; ΝΑΙ ΟΧΙ

12.1 Μήτρα: ΝΑΙ ΟΧΙ ΗΛΙΚΙΑ…..

12.2 Ωοθήκες: ΝΑΙ ΟΧΙ ΗΛΙΚΙΑ…..

20. **Έχετε κάποια μακροχρόνια ασθένεια ή κάποιο πρόβλημα υγείας;**

 ΝΑΙ ΟΧΙ

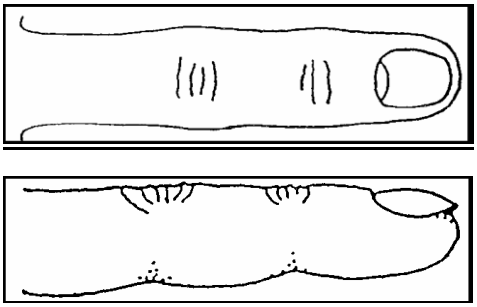
Εάν απαντήσατε ΝΑΙ, παρακαλώ διευκρινίστε:……

21. **Σας έχει πει ποτέ ο γιατρός σας πως έχετε Αρθρίτιδα;**

 ΝΑΙ ΟΧΙ

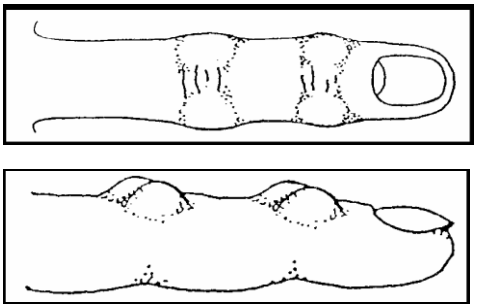
21.1. Δάχτυλα χωρίς Οζίδια

□ ΝΑΙ □ ΟΧΙ



21.2. Δάχτυλα με Οζίδια

□ ΝΑΙ □ ΟΧΙ



22. **Έχετε πάθει ποτέ:**

		ΝΑΙ	ΟΧΙ	ΗΛΙΚΙΑ
22.1.	Εγκεφαλικό επεισόδιο	□	□	□
22.2.	Έμφραγμα μυοκαρδίου	□	□	□
22.3.	Καρκίνο	□	□	□
22.4	Πνευμονία	□	□	□

23. **Σας έχει πει ποτέ ο γιατρός σας πως έχετε κάποια από τις παρακάτω ασθένειες;**

	ΟΧΙ	**ΝΑΙ**	**ΘΕΡΑΠΕΙΑ/ΔΙΑΡΚΕΙΑ**
Οστεοπόρωση			
Ανεπάρκεια - έλλειψη βιταμίνης D			
Ρευματοειδή αρθρίτιδα			
Ουρική αρθρίτιδα			
Χρόνια ηπατική νόσο			
Νεφρική ανεπάρκεια			
Νεφρολιθίαση			
Νόσο Crown			
Φλεγμονώδη νόσο εντέρου			
Χρόνια διάρροια			
Γαστροοισοφαγική Παλινδρόμηση			
Γαστρικό Έλκος			
Διαβήτης Τύπου Ι			
Διαβήτης Τύπου ΙΙ			
Υποθυρεοειδισμό			
Υπερθυρεοειδισμό			
Υποπαραθυροειδισμό			
Υπερπαραθυροειδισμό			
Νόσο επινεφριδίων			
Επιληψία			
Alzheimer			
Parkinson			
Άνοια			
Χρόνια βρογχίτιδα			
Άσθμα			
Χρόνια καρδιακή νόσο			

Καρδιακή Ανεπάρκεια			
Καρδιακή Αρρυθμία			
Στηθάγχη			
Υπερλιπιδαιμία			
Υπέρταση			
Καταρράκτης			
Γλαύκωμα			
Σκλήρυνση Οφθαλμού			
Μυωπία			
Χρόνια Βρογχίτιδα			
Χρόνιο Άγχος, Κατάθλιψη			
Ημικρανία			
Σοβαρή Δερματοπάθεια			
Αλλεργία			
Δυσλεξία			
Πλατυποδία			

24. **Το τελευταίο μήνα υποφέρατε από:**

24.1 Περιόδους ζαλάδας **NAI** **OXI**

24.2 Ξαφνικές λιποθυμίες **NAI** **OXI**

24.3 Πόνους ή δυσκαμψία στο λαιμό ή στους ώμους **NAI** **OXI**

24.4 Πόνους στις γάμπες **NAI** **OXI**

24.5 Πρησμένα πόδια **NAI** **OXI**

25. **Ενοχλείστε από πόνους στο ισχίο όταν:**

25.1 Περπατάτε ή κινείστε **NAI** **OXI**

25.2 Κάθεστε ή ξεκουράζεστε κατά τη διάρκεια της ημέρας **NAI** **OXI**

25.3 Κοιμάστε **NAI** **OXI**

26. **Ενοχλείστε από πόνους στα γόνατα:**

26.1 Περπατάτε ή κινείστε **ΝΑΙ** **ΟΧΙ**

26.2 Κάθεστε ή ξεκουράζεστε κατά τη διάρκεια της ημέρας **ΝΑΙ** **ΟΧΙ**

26.3 Κοιμάστε **ΝΑΙ** **ΟΧΙ**

27. **Ενοχλείστε από πόνους στην πλάτη:**

27.1 Περπατάτε ή κινείστε **ΝΑΙ** **ΟΧΙ**

27.2 Κάθεστε ή ξεκουράζεστε κατά τη διάρκεια της ημέρας **ΝΑΙ** **ΟΧΙ**

27.3 Κοιμάστε **ΝΑΙ** **ΟΧΙ**

28. **Έχετε πάθει κάποιο κάταγμα μετά την ηλικία των 16 ετών;** **ΝΑΙ** **ΟΧΙ**

Εάν ΝΑΙ, που πάθατε το κάταγμα;

28.1 Ισχίο

28.2 Μηριαίο

28.3 Καρπός

28.4 Σπονδυλική Στήλη

27.5 Άλλο Διευκρινίστε:……

29. **Είχατε ποτέ προσωρινή αδυναμία ή διαταραχή της ομιλίας ή της όρασης ή απώλεια μνήμης;**

ΝΑΙ **ΟΧΙ**

30. Ακινιτοποίηση **□ ΝΑΙ** **□ ΟΧΙ**

ΔΙΑΡΚΕΙΑ:

1. Λιγότερο από 1 μήνα □ 2. 1 μήνα □ 3. 2-3 μήνες □ 4. περισσότεροι μήνες……

Αιτία ……..Ηλικία…….Περιοχή…….

ΓΟΝΕΑΣ	**Εν ζωή**	**ΗΛΙΚΙΑ ΘΑΝΑΤΟΥ**	**ΑΙΤΙΑ ΘΑΝΑΤΟΥ**
Πατέρας	**□ ΝΑΙ □ ΟΧΙ**		
Μητέρα	**□ ΝΑΙ □ ΟΧΙ**		

31. Είναι οι *<u>Γονείς</u>* σας εν ζωή;

32. Είναι οι *<u>Γονείς των Γονέων</u>* σας εν ζωή;

ΓΟΝΕΑΣ ΓΟΝΕΩΝ		**Εν ζωή**	**ΗΛΙΚΙΑ ΘΑΝΑΤΟΥ**	**ΑΙΤΙΑ ΘΑΝΑΤΟΥ**
Οικογένεια Πατέρα	Πατέρας	**□ ΝΑΙ □ ΟΧΙ**		
	Μητέρα	**□ ΝΑΙ □ ΟΧΙ**		
ΓΟΝΕΑΣ ΓΟΝΕΩΝ		**Εν ζωή**	**ΗΛΙΚΙΑ ΘΑΝΑΤΟΥ**	**ΑΙΤΙΑ ΘΑΝΑΤΟΥ**
Οικογένεια Μητέρας	Πατέρας	**□ ΝΑΙ □ ΟΧΙ**		
	Μητέρα	**□ ΝΑΙ □ ΟΧΙ**		

ΑΣΘΕΝΕΙΕΣ	ΑΔΕΛΦΙΑ						ΠΑΤΕΡΑΣ	ΜΗΤΕΡΑ
Διαβήτης Τύπου Ι								
Διαβήτης Τύπου ΙΙ								
Υπερλιπιδαιμία								
Υπέρταση								
Καρδιακή Ανεπάρκεια								
Καρδιακή Αρρυθμία								
Στηθάγχη								
Άσθμα								
Χρόνια Βρογχίτιδα								
Χρόνιο Άγχος, Κατάθλιψη								
Ημικρανία								
Σοβαρή Δερματοπάθεια								
Αλλεργία								
Κάταγμα								
Οστεοπόρωση								
Άλλη νόσο των οστών								
Alzheimer								
Parkinson								
ΑΣΘΕΝΕΙΕΣ	**ΑΔΕΛΦΙΑ**						**ΠΑΤΕΡΑΣ**	**ΜΗΤΕΡΑ**
Άνοια								
Πλατυποδία								
Καταρράκτης								
Γλαύκωμα								
Σκλήρυνση Οφθαλμού								
Μυωπία								
Πρεσβυωπία								
Βαρηκοΐα								
Γαστρικό Έλκος								
Γαστροοισοφαγική Παλινδρόμηση								
Νόσο Crown								
Υποθυρεοειδισμό								
Υπερθυρεοειδισμό								
Καρκίνος: Προστάτη								
Δυσλεξία								
Άλλο – Προσδιορίστε:								
Αρθρίτιδα								

33. Οικογενειακό Ιστορικό

ΑΣΘΕΝΕΙΕΣ	ΑΔΕΛΦΙΑ						ΠΑΤΕΡΑΣ	ΜΗΤΕΡΑ
Διαβήτης Τύπου Ι								
Διαβήτης Τύπου ΙΙ								
Υπερλιπιδαιμία								
Υπέρταση								
Καρδιακή Ανεπάρκεια								
Καρδιακή Αρρυθμία								
Στηθάγχη								
Άσθμα								
Χρόνια Βρογχίτιδα								
Χρόνιο Άγχος, Κατάθλιψη								
Ημικρανία								
Σοβαρή Δερματοπάθεια								
Αλλεργία								
Κάταγμα								
Οστεοπόρωση								
Άλλη νόσο των οστών								
Alzheimer								
Parkinson								
Άνοια								
Πλατυποδία								
Καταρράκτης								
Γλαύκωμα								
Σκλήρυνση Οφθαλμού								
ΑΣΘΕΝΕΙΕΣ	ΑΔΕΛΦΙΑ						ΠΑΤΕΡΑΣ	ΜΗΤΕΡΑ
Μυωπία								
Πρεσβυωπία								
Βαρηκοΐα								
Γαστρικό Έλκος								
Γαστροοισοφαγική Παλινδρόμηση								
Νόσο Crown								
Υποθυρεοειδισμό								
Υπερθυρεοειδισμό								
Καρκίνος: Προστάτη								
Δυσλεξία								
Άλλο – Προσδιορίστε:								
Αρθρίτιδα								

ΕΡΩΤΗΜΑΤΟΛΟΓΙΟ ΤΡΟΠΟΥ ΖΩΗΣ

34. ΣΥΖΥΓΙΚΗ ΚΑΤΑΣΤΑΣΗ

Ποια είναι ή συζυγικής σας κατάσταση;

34.1 Έγγαμος/η ☐
34.2 Σταθερή σχέση ☐
34.3 Χωρισμένος/η ☐
37.4 Διαζευγμένος/η ☐
37.6 Ελεύθερος/η ☐
37.7 Χήρος/α ☐

35. ΟΙΚΟΓΕΝΕΙΑΚΗ ΚΑΤΑΣΤΑΣΗ

Ποιος μένει μαζί σας στο σπίτι;

35.1 Κανείς ☐
35.2 Σύζυγος/σύντροφος ☐
35.3 Εγγόνια ☐
35.4 Υιοί και κόρες ☐
35.5 Γαμπροί και νύφες ☐
35.6 Αδέλφια ☐
35.7 Γονείς ☐
35.8 Έμμισθα άτομα (όχι συγγενείς) ☐
35.9 Κατοικίδια ☐
35.10 Άλλοι ☐ **Διευκρινίστε:……**

36. ΜΟΡΦΩΣΗ

Ποιο είναι το μορφωτικό σας επίπεδο;

36.1 Καμία μόρφωση ☐
36.2 Πρωτοβάθμια εκπαίδευση (Δημοτικό Σχολείο) ☐
36.3 Δευτεροβάθμια εκπαίδευση (Γυμνάσιο & Λύκειο ή Εξατάξιο Γυμνάσιο) ☐
36.4 Τριτοβάθμια εκπαίδευση (Πανεπιστήμιο ή Α.Τ.Ε.Ι.) ☐
36.5 Άλλο:…….. ☐
36.6 **Συνολικά χρόνια εκπαίδευσης:……**

37. ΕΠΑΓΓΕΛΜΑ

37.1.*Ποια είναι η επαγγελματικής σας κατάσταση;*

37.1.1 Πλήρης απασχόληση – Δημόσιος Υπάλληλος ☐

37.1.2 Πλήρης απασχόληση – Ιδιωτικός Υπάλληλος ☐

37.1.3 Μερική απασχόληση – Δημόσιος Υπάλληλος ☐

37.1.4 Μερική απασχόληση – Ιδιωτικός Υπάλληλος ☐

37.1.5 Ελεύθερος Επαγγελματίας ☐

37.1.6 Άνεργος ☐

37.1.7 Συνταξιούχος ☐

37.1.8 Οικιακά ☐

37.2.Περιγράψτε το επάγγελμά σας…….

38. ΦΥΣΙΚΗ ΔΡΑΣΤΗΡΙΟΤΗΤΑ

Παρακαλούμε σκεφτείτε **<u>τις τελευταίες 7 μέρες (εβδομάδα)</u>**.

Θα θέλαμε να μας δώσετε κάποιες πληροφορίες για την φυσική σας δραστηριότητα.

38.1. ΦΥΣΙΚΗ ΔΡΑΣΤΗΡΙΟΤΗΤΑ ΣΤΗΝ ΕΡΓΑΣΙΑ

- Ποια είναι η βασική σας απασχόληση; _____
- Εργαστήκατε τις τελευταίες 7 μέρες;

Όχι ☐ **προχωρήστε στην ενότητα 2**

Ναι ☐ Πόσες μέρες; _____ (*1*)

- Πόσες ώρες τη μέρα κατά μέσο όρο; _____ώρες/ ημέρα εργασίας(*2*)
- Εκ των οποίων πόσο χρόνο κατά μέσο όρο καταναλώσατε:

	Ώρες/ ημέρα εργασίας	
καθιστή/ος		(*3*)
όρθια/ος		(*4*)
σε κίνηση		(*5*)
μεταφέροντας βάρος		(*6*)
Συνολικός χρόνος εργασίας		

- Πόσος χρόνος χρειάστηκε για τη μετακίνηση σας **από και προς** τη δουλειά σας αυτές τις μέρες;

_____ λεπτά/ ημέρα (7)

- **Εκ του οποίου χρόνου** πόση ώρα α)περπατήσατε; _____ λεπτά/ ημέρα που πήγα στη δουλειά(*8*)

β)οδηγήσατε; _____ λεπτά/ ημέρα που πήγα στη δουλειά(*9)*

38.2: ΦΥΣΙΚΗ ΔΡΑΣΤΗΡΙΟΤΗΤΑ ΣΤΟ ΣΠΙΤΙ

- Κατά τη διάρκεια των τελευταίων 7 ημερών πόσες ώρες (κατά μέσο όρο) **την <u>ημέρα</u>:**
  - κοιμηθήκατε (συμπεριλαμβανομένου και τυχόν μεσημεριανού ύπνου); _____ώρες/ ημέρα *(10)*
  - είδατε τηλεόραση-βίντεο; _____ ώρες/ ημέρα (*11*)

- Κατά τη διάρκεια των τελευταίων 7 ημερών πόσες ώρες **<u>συνολικά</u>** καταναλώσατε:
  - για ελαφριές δουλειές σπιτιού (π.χ. μαγείρεμα, πλύσιμο πιάτων κλπ); _____ώρες/ εβδομάδα (*12*)
  - για βαριές δουλειές σπιτιού(π.χ. πλύσιμο στο χέρι, σφουγγάρισμα κλπ); _____ώρες/ εβδομάδα(*13*)
  - για διάβασμα και στον υπολογιστή (εκτός ωρών εργασίας); _____ ώρες/ εβδομάδα (*14*)

8.3: ΦΥΣΙΚΗ ΔΡΑΣΤΗΡΙΟΤΗΤΑ ΓΙΑ ΨΥΧΑΓΩΓΙΑ

- Τις τελευταίες 7 μέρες πόσες ώρες **συνολικά**:

	Ώρες/ εβδομάδα	
χορέψατε σε club ή/και bar:		(*15*)
ήσασταν καθιστός/η ή στεκόσασταν όρθιος/α με φίλους σε καφετέρια – μπαρ – ταβέρνα – εστιατόριο- θέατρο-σινεμά;		(*16*)
περπατήσατε για ψυχαγωγία (βόλτα στα μαγαζιά, στο πάρκο κλπ) και για μετακίνηση (εκτός μετακίνησης προς και από τη δουλειά):		(*17*)

- Τις τελευταίες 7 μέρες γυμναστήκατε;

Ναι ☐ Όχι ☐

- Αν ναι τι ακριβώς κάνετε και πόσες ώρες **συνολικά** τις τελευταίες 7 μέρες:

	Ώρες/ εβδομάδα	
		(*18*)
		(*19*)
		(*20*)

- Με τι μέσο μετακινηθήκατε κυρίως την τελευταία εβδομάδα (σημειώστε **μόνο ένα**);

Μοτοσικλέτα ☐ Ι.Χ. ☐ Περπατώντας ☐ Ποδήλατο ☐

Μέσα Μαζικής Μεταφοράς (πχ. λεωφορείο, μετρό κλπ) ☐ Ταξί ☐

39. ΚΑΠΝΙΣΤΙΚΕΣ ΣΥΝΗΘΕΙΕΣ

39.1.Ποια από τις παρακάτω προτάσεις σας περιγράφει καλύτερα; (Παρακαλούμε σημειώστε μόνο ένα κουτάκι)

39.1.1 Καπνίζω καθημερινά ☐

39.1.2 Καπνίζω περιστασιακά, αλλά όχι καθημερινά ☐

39.1.3 Συνήθιζα να καπνίζω καθημερινά, αλλά τώρα δεν καπνίζω καθόλου ☐

39.1.4 Συνήθιζα να καπνίζω περιστασιακά, αλλά τώρα δεν καπνίζω καθόλου ☐

39.1.5 Δεν έχω καπνίσει ποτέ ☐

39.2. Πόσο χρονών ήσασταν όταν αρχίσατε το κάπνισμα;……..

39.3. Πόσο χρονών ήσασταν όταν σταματήσατε το κάπνισμα;……..

39.4. Πόσα χρόνια καπνίζατε συνολικά;……....

Εάν καπνίζετε παρακαλούμε απαντήστε στις επόμενες ερωτήσεις.

39.5. Τι συνηθίζετε να καπνίζετε; (Παρακαλούμε σημειώστε μόνο ένα κουτάκι)

39.5.1. Τσιγάρα με φίλτρο ☐

39.5.2 Τσιγάρα χωρίς φίλτρο ☐

39.5.3 Στριφτά τσιγάρα ☐

39.5.4 Πούρα ☐

39.5.5 Πίπα ☐

39.5.6 Άλλο ☐ **Διευκρινίστε: ……**

39.6. Μπορείτε να προσδιορίσετε την ποσότητα που καπνίζετε σε ημερίσια βάση;

	<2	**3-5**	**6-10**	**11-20**	**21-30**	**31-40**	**>40**
39.6.1 Τσιγάρα με φίλτρο	☐	☐	☐	☐	☐	☐	☐
39.6.2 Τσιγάρα χωρίς φίλτρο	☐	☐	☐	☐	☐	☐	
39.6.3 Στριφτά τσιγάρα	☐	☐	☐	☐	☐	☐	☐
39.6.4 Πούρα	☐	☐	☐	☐	☐	☐	
39.6.5 Πίπα	☐	☐	☐	☐	☐	☐	
39.6.6 Άλλο	☐	☐	☐	☐	☐	☐	

39.7. Είστε Παθητικός/ή Καπνιστής / τρια;

39.7.1 Στο σπίτι ☐ **Ναι** ☐ **Όχι**

39.7.2 Στη δουλειά ☐ **Ναι** ☐ **Όχι**

39.7.3 Στο καφενείο ☐ **Ναι** ☐ **Όχι**

39.7.4 Αλλού ☐ **Ναι** ☐ **Όχι** **Διευκρινίστε: ……**

40. ΔΙΑΤΡΟΦΙΚΕΣ ΣΥΝΗΘΕΙΕΣ

	Πάντα	**Συχνά**	**Μερικές Φορές**	**Περιστασιακά**	**Ποτέ**
40.1. Προετοιμάζετε εσείς τα γεύματά σας;	☐	☐	☐	☐	☐
40.2. Τρώτε μόνος/ μόνη;	☐	☐	☐	☐	☐

40.3. Πόσο συχνά τρώτε μαγειρεμένο φαγητό;

(Παρακαλούμε σημειώστε μόνο ένα κουτάκι).

40.3.1. Κάθε μέρα ☐

40.3.2. Τακτικά (μία ή περισσότερες φορές την εβδομάδα) ☐

40.3.3. Περιστασιακά (λιγότερο από μία φορά την εβδομάδα) ☐

40.3.4. Ποτέ (λιγότερο από μία φορά το μήνα) ☐

40.4. Έχετε προβλήματα στη μάσηση; ☐ **ΝΑΙ** ☐ **ΟΧΙ**

40.5. Έχετε τεχνητές οδοντοστοιχίες; ☐ **ΝΑΙ** ☐ **ΟΧΙ**

40.6. Αγοράζετε προ - μαγειρεμένα γεύματα; ☐ **ΝΑΙ** ☐ **ΟΧΙ**

Αν Ναι, αυτά είναι:

40.6.1. Συντήρησης ☐

40.6.2. Κατεψυγμένα ☐

40.6.3. Κονσέρβες ☐

40.6.4. Άλλα ☐ **Διευκρινίστε:……**

40.7. Ακολουθείτε ειδική διατροφή; ☐ **ΝΑΙ** ☐ **ΟΧΙ**

Αν Ναι, τι είδους διατροφή είναι αυτή;

40.7.1. Χαμηλή σε αλάτι ☐

40.7.2. Χαμηλή σε λίπος ☐

40.7.3. Χαμηλή σε θερμίδες ☐

40.7.4. Για μείωση βάρους ☐

40.7.5. Άλλη ☐ **Διευκρινίστε:……**

41. ΔΙΑΤΡΟΦΙΚΗ ΑΝΑΚΛΗΣΗ ΕΙΚΟΣΙΤΕΤΡΑΩΡΟΥ

ΩΡΑ	ΦΑΓΗΤΟ / ΠΟΤΟ	ΠΕΡΙΓΡΑΦΗ	ΠΟΣΟΤΗΤΑ

41.1.Ήταν μια τυπική μέρα; ☐ ΝΑΙ ☐ ΟΧΙ

41.2. Ποια μέρα ήταν αυτή; ……

41.3. Λαμβάνετε διατροφικά συμπληρώματα; ☐ ΝΑΙ ☐ ΟΧΙ

Αν ΝΑΙ, προσδιορίστε: **Τύπο Διατροφικού Συμπληρώματος** **Ποσότητα**

1.

2.

41.4. Χρησιμοποιείτε αλάτι; ☐ ΝΑΙ ☐ ΟΧΙ

41.5. Αλατίζετε το φαγητό στο πιάτο πριν το δοκιμάσετε; ☐ ΝΑΙ ☐ ΟΧΙ

41.6. Προσθέτετε αλάτι στο μαγείρεμα; ☐ ΝΑΙ ☐ ΟΧΙ

41.7. Υπάρχουν τρόφιμα / ποτά που δεν τρώτε για λόγους θρησκείας; ☐ ΝΑΙ ☐ ΟΧΙ

Αν ΝΑΙ, προσδιορίστε: **Τρόφιμο / Ποτό** **Διάρκεια αποφυγής**

1.

2.

42. ΕΡΩΤΗΜΑΤΟΛΟΓΙΟ ΣΥΧΝΟΤΗΤΑΣ ΚΑΤΑΝΑΛΩΣΗΣ ΤΡΟΦΙΜΩΝ

Σημειώστε ΠΟΣΟ ΣΥΧΝΑ καταναλώσατε τα παρακάτω τρόφιμα το <u>τελευταίο μήνα</u> Θα πρέπει να απαντήσετε έχοντας ως **<u>μερίδα αναφοράς την ποσότητα που αναγράφεται</u>** στις παρενθέσεις. (Συντμήσεις: *φ* = φορές, *γρ*. = γραμμάρια, *τμχ*. = τεμάχιο, *φλ*. = φλιτζάνι τσαγιού = 240 ml)						
	Ποτέ Σπάνια	**1-3 φορές /μήνα**	**1-2 φορές /εβδομάδα**	**3-6 φορές /εβδομάδα**	**1 φορά /ημέρα**	**≥ 2 φορά /ημέρα**
42.1. Γάλα / Γιαούρτι πλήρες (1 ποτήρι / 1 κεσεδάκι)	α	β	γ	δ	ε	στ
42.2. Γάλα / Γιαούρτι χαμηλό σε λιπαρά (1 ποτήρι / 1 κεσεδάκι)	α	β	γ	δ	ε	στ
42.3. Τυρί κίτρινο, τυρί σε κρέμα (30 γρ)	α	β	γ	δ	ε	στ
42.4.Τυρί φέτα, ανθότυρο (30 γρ)	α	β	γ	δ	ε	στ
42.5. Τυρί άπαχο ή χαμηλό σε λιπαρά (light, cottage) (30 γρ)	α	β	γ	δ	ε	στ
42.6. Αυγό (βραστό, τηγανιτό, ομελέτα) (1 τμχ)	α	β	γ	δ	ε	στ
42.7. Ψωμί άσπρο (1 φέτα 30γρ ή φέτα τοστ) Φρυγανιά (2 τμχ)	α	β	γ	δ	ε	στ
42.8. Ψωμί ολικής αλέσεως (1 φέτα 30γρ ή φέτα τοστ)Φρυγανιά (2 τμχ)	α	β	γ	δ	ε	στ
42.9. Κουλούρι Θες/κης, πίτα (σουβλάκι) Ψωμάκια μπέργκερ (1 τμχ)	α	β	γ	δ	ε	στ
42.10. Κριτσίνια (2 λεπτά), παξιμάδια (1 μέτριο) Κουλούρια (2 μέτρια)	α	β	γ	δ	ε	στ
42.11. Δημητριακά πρωινού (½ φλ) Μπάρες δημητριακών (1 τμχ)	α	β	γ	δ	ε	στ
42.12. Ρύζι λευκό (1 φλ)	α	β	γ	δ	ε	στ
42.13. Ρύζι καστανό (1 φλ)	α	β	γ	δ	ε	στ
42.14. Μακαρόνια, κριθαράκι, χυλοπίτες, άλλα ζυμαρικά (1 φλ)	α	β	γ	δ	ε	στ
42.15. Ζυμαρικά ολικής αλέσεως (1 φλ)	α	β	γ	δ	ε	στ
42.16. Πατάτες βραστές, φούρνου, πουρές (1 μέτρια / ½ φλ)	α	β	γ	δ	ε	στ
45.17. Πατάτες τηγανιτές (½ μερίδα εστιατορίου)	α	β	γ	δ	ε	στ
42.18. Μοσχάρι (μπριζόλα, κομμάτι) (150 γρ)	α	β	γ	δ	ε	στ
42.19. Μπιφτέκι (2 τμχ), κεφτεδάκια (4 τμχ), κιμάς (1 κουτάλα)	α	β	γ	δ	ε	στ

42.20. Κοτόπουλο/ γαλοπούλα (όλα τα είδη) (150 γρ)	α	β	γ	δ	ε	στ
42.21. Χοιρινό (μπριζόλα, κομμάτι, σουβλάκι) (150 γρ)	α	β	γ	δ	ε	στ
42.22. Αρνί, κατσίκι, κυνήγι, παϊδάκια (150 γρ)	α	β	γ	δ	ε	στ
42.23. Αλλαντικά (1 φέτα)	α	β	γ	δ	ε	στ
42.24. Λουκάνικα (1 μέτριο), μπέικον (2 φέτες)	α	β	γ	δ	ε	στ
42.25. Αλλαντικά/ κρεατοσκευάσματα άπαχα ή light (όπως παραπάνω)	α	β	γ	δ	ε	στ
42.26. Ψάρια μικρά (150 γρ)	α	β	γ	δ	ε	στ
42.27. Ψάρια μεγάλα (150 γρ)	α	β	γ	δ	ε	στ
42.28. Θαλασσινά (χταπόδι, καλαμάρι, γαρίδες) (150 γρ)	α	β	γ	δ	ε	στ
42.29. Όσπρια (π.χ. φακές, φασόλια, ρεβίθια) (1 πιάτο)	α	β	γ	δ	ε	στ
42.30. Σπανακόρυζο / λαχανόρυζο (1 πιάτο), γεμιστά (2 μέτρια)	α	β	γ	δ	ε	στ
42.31. Παστίτσιο, μουσακάς, παπουτσάκια (1 μερίδα = 150 γρ)	α	β	γ	δ	ε	στ
42.32. Αρακάς, φασολάκια, μπάμιες, αγκινάρες (1 πιάτο)	α	β	γ	δ	ε	στ
42.33. Τομάτα, αγγούρι, καρότο, πιπεριά (1 φλ. ωμά)	α	β	γ	δ	ε	στ
42.34. Μαρούλι, λάχανο, σπανάκι, ρόκα (1 φλ. ωμά)	α	β	γ	δ	ε	στ
42.35. Μπρόκολο, κουνουπίδι, κολοκυθάκια, (½ φλ. βραστά)	α	β	γ	δ	ε	στ
42.36. Χόρτα, πράσο, σπανάκι, σέλινο (½ φλ. βραστά)	α	β	γ	δ	ε	στ
42.37. Πορτοκάλι (1 μέτριο)	α	β	γ	δ	ε	στ
42.38. Μήλο, αχλάδι (1 μέτριο)	α	β	γ	δ	ε	στ
42.39. Άλλα χειμερινά φρούτα (1 ολόκληρο ή ½ φλ)	α	β	γ	δ	ε	στ
42.40. Μπανάνα (1 μέτρια)	α	β	γ	δ	ε	στ
42.41. Άλλα καλοκαιρινά φρούτα (1 ολόκληρο ή ½ φλ)	α	β	γ	δ	ε	στ
42.42. Χυμός φρούτων (1 ποτήρι)	α	β	γ	δ	ε	στ
42.43.. Αποξηραμένα φρούτα (¼ φλ.)	α	β	γ	δ	ε	στ
42.44. Ξηροί καρποί, σπόροι (1 φλιτζανάκι καφέ)	α	β	γ	δ	ε	στ
42.45. Πίτες σπιτικές (π.χ. τυρόπιτα, σπανακόπιτα) (1 κομμάτι)	α	β	γ	δ	ε	στ
42.46. Πίτες έτοιμες (1 κομμάτι)	α	β	γ	δ	ε	στ

42.47. Τοστ, σάντουιτς (1 ολόκληρο)	α	β	γ	δ	ε	στ
42.48. Γλυκά ταψιού (1 τμχ)	α	β	γ	δ	ε	στ
42.49. Γλυκά κουταλιού, κομπόστα, ζελέ (1 μερίδα)	α	β	γ	δ	ε	στ
42.50. Πάστες, τάρτα (1 τμχ)	α	β	γ	δ	ε	στ
42.51. Κρουασάν (1), γκοφρέτες (1 μέτρια), κέικ (1 φέτα) Μπισκότα (3-4)	α	β	γ	δ	ε	στ
42.52. Σοκολάτα (όλα τα είδη) (1 μέτρια ~ 60 γρ)	α	β	γ	δ	ε	στ
42.53. Παγωτό, μιλκ σέικ, κρέμα, ρυζόγαλο (1 τμχ)	α	β	γ	δ	ε	στ
42.54. Πατατάκια, γαριδάκια, ποπ κορν (1 σακουλάκι ~70 γρ)	α	β	γ	δ	ε	στ
42.55. Μέλι, μαρμελάδα, ζάχαρη (π.χ. σε ψωμί, καφέ) (1 κουτ. γλυκού)	α	β	γ	δ	ε	στ
42.56. Ελιές (10 μικρές/ 5 μεγάλες)	α	β	γ	δ	ε	στ
42.57. Κρασί (1 ποτήρι = 125 ml)	α	β	γ	δ	ε	στ
42.58. Μπύρα (1 ποτήρι = 240 ml)	α	β	γ	δ	ε	στ
42.59. Άλλο είδος αλκοόλ (1 ποτό)	α	β	γ	δ	ε	στ
42.60. Αναψυκτικά (1 κουτί ~ 330 ml)	α	β	γ	δ	ε	στ
42.61. Αναψυκτικά light (1 κουτί ~ 330 ml)	α	β	γ	δ	ε	στ
42.62. Καφές (1 φλ. ή ποτήρι)	α	β	γ	δ	ε	στ
42.63. Τσάι, άλλα αφεψήματα (1 φλ)	α	β	γ	δ	ε	στ
42.64. Μαγιόνεζα, σως (1 κουτ. σούπας)	α	β	γ	δ	ε	στ
42.65. Μαγιονέζα/ σως λάιτ (1 κουτ. σούπας)	α	β	γ	δ	ε	στ
42.66. Πόσες φορές χρησιμοποιείς ελαιόλαδο (οπουδήποτε);	α	β	γ	δ	ε	στ
42.67. Πόσες φορές χρησιμοποιείς σπορέλαιο (οπουδήποτε);	α	β	γ	δ	ε	στ
42.68. Πόσες φορές χρησιμοποιείς μαργαρίνη (οπουδήποτε);	α	β	γ	δ	ε	στ
43.69. Πόσες φορές χρησιμοποιείς βούτυρο (οπουδήποτε);	α	β	γ	δ	ε	στ
42.70. Πόσο τρως από το ορατό λίπος και την πέτσαστο κρέας;	Όλο		Περισσότερο	Μέρος		Καθόλου
42.71. Πόσο συχνά παραγγέλνεις από έξω ή τρως εκτός σπιτιού;	α	β	γ	δ	ε	στ
42.72. Πόσο συχνά καταναλώνεις πρωινό;	α	β	γ	δ	ε	--
42.73. Πόσα γεύματα έχεις συνολικά την ημέρα μαζί με τα σνακ;	1-3		4-5		>6	
42.74. Πόσα από αυτά είναι κυρίως γεύματα(πρωινό, μεσ/νό, βρ/νό);	1		2		3	
42.75. Καταναλώνεις βιολογικά προϊόντα ή	ΝΑΙ			ΟΧΙ		

προϊόντα σόγιας;		
42.76. Παίρνεις συμπληρώματα διατροφής (π.χ. βιταμίνες);	ΝΑΙ	ΟΧΙ

43. Ερωτηματολόγιο έκθεσης στον ήλιο

1. Πόση ώρα κάθεστε στον ήλιο μεταξύ 9.00-18.00 κατά μέσο όρο ανά εβδομάδα (σημειώστε τον αριθμό των ωρών)

   …….ώρες τον χειμώνα

   …….ώρες το καλοκαίρι

2. Χρησιμοποιείτε αντηλιακό

   - **Πάντα**
   - **Μερικές φορές**
   - **Ποτέ**

3. Τι τύπο δέρματος έχετε;

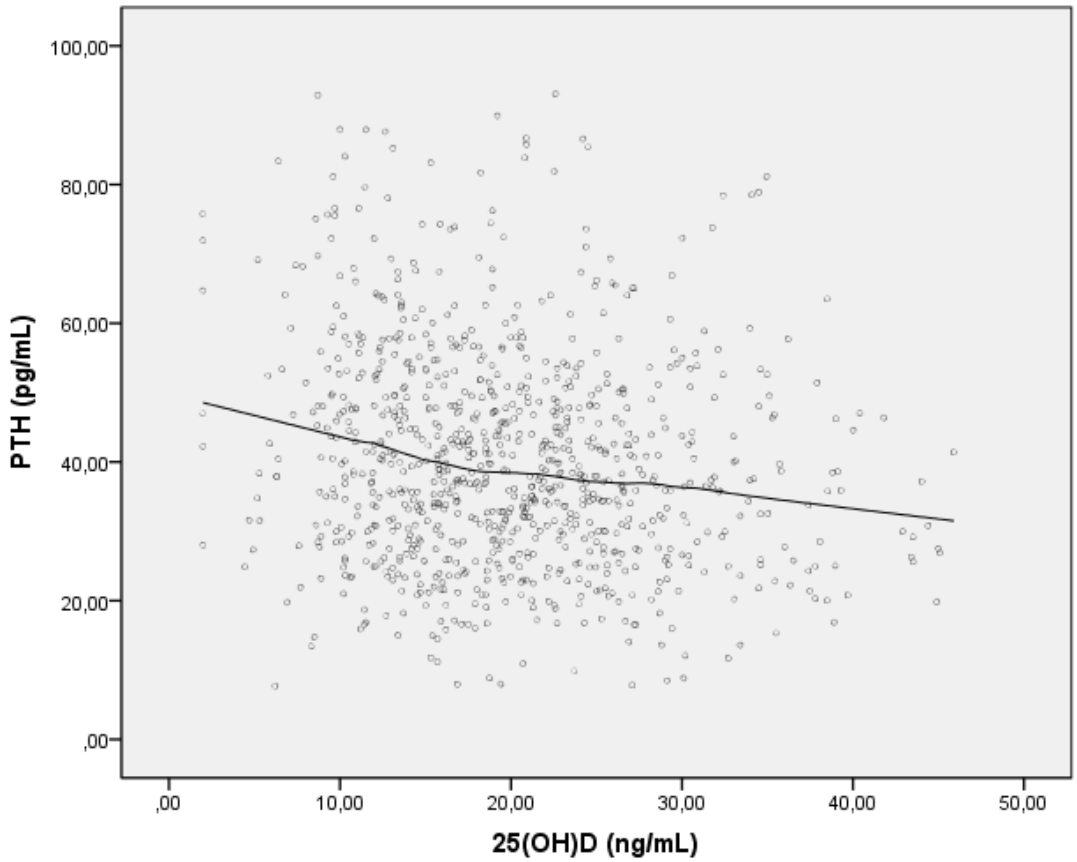
   - **Τύπος Ι**: πάντα καίγεται και ποτέ δεν μαυρίζει
   - **Τύπος ΙΙ**: συχνά καίγεται και μαυρίζει δύσκολα
   - **Τύπος ΙΙΙ**: καμιά φορά καίγεται μέτρια και σταδιακά μαυρίζει
   - **Τύπος ΙV**: σπάνια καίγεται και μαυρίζει εύκολα

4. Εργάζεστε:

   - **Σε εσωτερικό χώρο**
   - **Σε εξωτερικό χώρο**
   - **Και τα δύο**

Appredix 2

Figure A2: A negative correlation between serum 25(OH)D and PTH concentration was observed in all subjects (r = -0.162, P=0.00), as it sawn in table 3 of the manuscript 1. Relationship between serum 25-hydroxyvitamin D [25(OH)D] and parathyroid hormone (PTH) (P =0·000). LOESS plot with 95% CI.



Appredix 3

Table A3. Mean dietary pattern score among sex, age groups, serum Vitamin D status, season of blood sampling and area of residence.

	1- vegetables-fruit	2- fast food	3-western	4- healthy	5-sweets	6- traditional
Sex (%population)						
male (11.5%)	-0.0827±1.01	0.0929±1.03	0.2652±1.17*	-0.0971±1.02	-0.1147±1.00	0.2304±1.21*
female (88.5%)	0.0406±1.00	-0.0211±0.97	-0.0197±1.00*	0.0287±1.00	0.0025±1.00	-0.0314±0.04*
Age group (years) (%population)						
18-50 (50.8%)	-0.1878±1.01*	0.2412±1.09*	0.0985±1.00*	-0.0054±0.98	0.0332±1.02	-0.0246±1.01
51-65 (37.0%)	0.1698±0.93*	-0.2130±0.87*	-0.0347±0.97	0.0300±0.98	-0.0547±0.9	0.0156±1.02
>65 (12.2%)	0.4820±1.00*	-0.3892±0.7*	-0.2131±1.02*	0.0893±1.12	-0.0397±1.07	0.0105±1.01
Vitamin D status						
25(OH)D <20 ng/mL (52.9%)	0.0867±1.00	-0.0455±0.96	0.0326±1.03	0.0662±0.93*	0.0826±1.07*	-0.0166±0.98
25(OH)D ≥20 ng/mL (47.1%)	-0.0390±1.01	0.0363±1.03	-0.0203±0.93	0.1020±1.06*	-0.1143±0.86*	0.0161±1.05
Season of blood sampling						
Winter-Spring (34.5%)	0.0585±1.06	0.0043±0.97	0.0291±1.036	-0.0625±0.98	0.0269±0.92	0.0725±0.97
Summer-Autumn (65.5%)	0.0113±0.97	-0.0166±1.01	-0.0033±0.98	0.0543±1.006	-0.0293±1.02	-0.0412±1.03
Area of residence						
Urban (71.9%)	0.0005±0.97	0.0251±1.02	-0.0741±0.96*	0.1650±1.03*	-0.0051±1.05	-0.0121±0.024
Rural (28.1%)	0.0973±1.07	-0.0976±0.94	0.2180±1.06*	-0.3732±0.8*	-0.0223±0.81	0.0240±0.92

Appredix 4

Table A4. Biochemical, anthropometric and QUS parameters according to genotype of 8 polymorphisms of OSTEOS study

polymorphisms Genotype (frequency %)	**25(OH)D** mean ± SD (*p-Value*)	**PTH** mean ± SD (*p-Value*)	**BMI** mean ± SD (*p-Value*)	**BUA** mean ± SD (*p-Value*)	**SOS** mean ± SD (*p-Value*)	**SI** mean ± SD (*p-Value*)
rs11520772						
AA (59.7)	20.33±7,99	**40.36±15.51***	27.68±5.37	113.53±17.17	**1558.56±36.49***	90.52±19.00
TA (31.8)	20.46±7.69	**43.85±15.05***	27.91±5.49	113.52±15.11	1546.18±125.36	91.08±19.28
TT (8.6)	18.10±7.21	39.73±2.46	27.63±5.64	111.52±18.6	**1511.07±239.14***	86.52±21.88
rs2908007						
AA (32.7%)	20.5±7.98	43.76±15.96	27.4±5.14	111.9±15.09	1539.78±153.33	88.32±18.58
GA (48.7)	19.87±8.22	43.96±15.25	28.09±5.35	112.13±18.13	1548.12±116.86	88.79±20.34
GG (18.6)	20.27±7.87	41.02±15.51	27.64±5.44	111.85±14.3	1553.37±34.51	90.74±16.99
rs2982552						
AA (37.6)	20.27±7.7	43.45±15	27.8±5.29	113.3±15.54	1558.56±35.71	90.54±19.2
GA (2.7)	20.38±8.23	42.86±15.5	27.5±5.12	111.74±16.4	1545.67±129.69	89.63±19.03
GG (19.7)	19.49±8.43	44.16±15.9	28.35v5.73	110.24±18.75	1526.15±182.55	84.34±19.28
rs3000634						
AA (73.5)	20.22±8.16	43.65±15.8	27.92±5.4	112.21±16.7	1540.71±138.81	88.05±18.78
GA (24.2)	20.08±7.72	42.8±14.16	27.54±5.15	112.33±16.5	1564.66±35.64	92.2±20.23
GG (2.3)	19.46±9.14	36.09±10.1	26.10±4.36	102.13±13.8	1538.76±32.44	83.78±19.64
rs3020331						
CC (35.2)	19.78±8.9	44.13±15.26	27.99±5.4	110.55±33.98	1550.91±33.98	86.66±18.0
TC (46.7)	20.47±7.67	42.03±15.72	27.42±5.25	113.34±17.42	1540.29±172.6	91.47±20.0
TT (18.1)	20.11±7.38	44.81±14.31	28.34±5.31	111.56±16.37	1554.14±39.18	87.02±19.06
rs597319						
AA (42.3)	20.66±8.0	43.58±15.62	27.86±5.15	114.2±17.2	1558.64±38.03	**92.2±16.67***
GA (44.8)	19.6±8.23	43.13±14.73	27.86±5.37	110.8±16.24	1534.72±174.73	87.74±18.67
GG (13.0)	20.89±7.53	42.31±16.73	26.94±5.29	109.06±14.63	1548.01±28.9	**82.09±16.57***
rs7741021						
AA (35.1)	20.24±8.4	44.03±15.0	27.57±5.24	109.82±14.99	1541.22±138.17	86.41±19.65
CA (46.8)	20.12±7.83	41.78±15.38	27.87±5.41	114.37±17.77	1557.71±37.18	91.25±19.51
CC (18.1)	20.48±7.94	45.16±16.18	27.69±5.13	110.64±15.83	1529.36±196.51	87.35±16.48
rs9292469						
CC (36.9)	19.67±8.53	41.45±14.74	27.63±5.17	110.69±20.33	1525.89±20.33	86.03±15.22
TC (48.2)	20.78±8.1	44.37±16.33	27.7±5.46	112.65±17.43	1555.1±35.89	89.72±20.39
TT (14.9)	19.60±6.9	42.74±13.18	27.93±4.86	114.0±16.77	1563.64±41.31	91.34±22.0

Appredix 5

Poster presentations

SERUM 25-HYDROXYVITAMIN D LEVELS OF HEALTHY MEN IN GREECE

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Abstract

Objective: The objective of this observational cross-sectional study is to identify the prevalence of vitamin D deficiency in healthy adult men in Greece, as reflected by the levels of 25-hydroxyvitamin D (25(OH)D), since recent data indicate that vitamin D deficiency can be common in countries previously considered as low risk (e.g Mediterranean countries).

Material and Methods: A population of 134 community dwelling men was recruited at the health promotion events carried out by the Hellenic Society for the Support of Patients with Osteoporosis in rural and urban areas throughout Greece. Serum total calcium (Ca), phosphorus (P), creatinine, parathyroid hormone (PTH) and 25(OH)D were measured. The study was approved by the Ethics Committee of Harokopio University.

Results: The mean age of the population was 44.34 years, (range, 18-78 years) while 88.7% were18-65 years old. Mean serum 25(OH)D was 21.94 ng/mL, mean PTH was 40.01 pg/mL and mean Ca, P and creatinine were 9.21, 3.22 and 0.88 mg/dL respectively. Concerning the vitamin D levels, 42.5% of the subjects had deficient (0-19.9 ng/mL), 35.8% had insufficient (20-29.9 ng/mL) and only 21.6% had adequate (30-150 ng/mL) levels, while 8.2% had vitamin D levels ≤10ng/mL. PTH was at normal range (15-65 pg/mL) for 89.3% of the population and 8.4% had high PTH (>65 pg/mL). The levels of serum Ca, P, and creatinine, were within the normal range.

Conclusions: Approximately the half of Greek adult men (45.2%) in this study had vitamin D levels below 20ng/mL. Given that low levels of 25(OH)D are associated with increased risk for fractures and exacerbate bone loss, this study highlights the emerging issue of 25(OH)D insufficiency in Greek men population and the need for targeted interventions even in age groups not previously considered as at risk.

Introduction

Osteoporosis is a major public health concern. Women have a higher risk of osteoporotic fracture compared with men (1 in 3 women and 1 in 5 men over 50 years). Vitamin D deficiency is among the most important clinical risk factors. Although recent data indicate that prevalence of vitamin D deficiency can be common in countries previously considered as low risk (e.g Mediterranean countries) [1] ,few data are available for Greek population. Low levels occur in elderly[2], mothers after delivery[3], children and adolescents[4] and there are few data concerning adults[5]. Therefore, the evaluation of vitamin D levels is of immense significance[6]. is an observational cross-sectional study that aims to identify the prevalence of vitamin D deficiency of healthy adults in Greece.

Methods

A population of 134 community dwelling men was recruited at the health promotion events carried out by the Hellenic Society for the Support of Patients with Osteoporosis in rural and urban areas throughout

Greece. Following a 12 hour fast, all subjects will have a blood drawn of venous blood used for serum isolation. Total calcium (Ca), phosphorus (P), creatinine, parathyroid hormone (PTH) and 25-hydroxyvitamin D (25(OH)D) were measured. The SPSS statistical package used for the statistical analysis. The study was approved by the Ethics Committee of Harokopio University.

Results

The mean age of the population was 44.34 years, (range, 18-86 years) while 88.7% were18-65 years old. Mean serum 25(OH)D, PTH, Ca, P and creatinine are presented in Table 1. Vitamin D levels, are presented in Table 2. PTH was at normal range (15-65 pg/mL) for 89.3% of the population and 8.4% had high PTH (>65 pg/mL). The levels of serum Ca, P, and creatinine, were within the normal range

Table 1: Descriptive characteristics

	Mean ± SD
Age (years)	44.34 ± 15.52
25(OH)D (ng/mL)	21.94 ± 9.10
PTH (pg/mL)	40.01 ± 169.88
Ca (mg/dL)	9.21 ± 0.57
P (mg/dL)	3.22 ± 0.59
Creatinine (mg/dL)	0.88 ± 0.018

Table 2: 25(OH)D levels

25(OH)D status (ng/mL)	Percentage of population
Severe Deficiency (<10)	8.2%
Deficiency (10-19.9)	34.3 %
Insufficiency (20-29.9)	35.8 %
Adequacy (≥30)	21.6%

Conclusions

A high proportion of Greek adult men (42.5%) in this study had vitamin D deficiency (<20ng/mL) and only 21.6% had levels above the adequacy (30ng/mL). Low levels of 25(OH)D are associated with increased risk for fractures and exacerbate bone loss. Beyond the skeletal consequences, low serum vitamin D, has numerous nonkeletal effects[7]. This study highlights the emerging issue of 25(OH)D insufficiency in Greek men population, that should get priority in public health strategies and the need for targeted interventions even in age groups and gender, not previously considered as at risk, like adult men.

Acknowledgements

The study was supported by the Hellenic Society for the Study of Bone Metabolism

References

1. Holick MF. Vitamin D Deficiency. N Engl J Med 2007;357:266-281. 2. Papapetrou PD, et al. Vitamin D deficiency in the elderly in Athens, Greece. Bone Miner Metab 2007;25:198–203. 3. Nicolaidou P, et al. Low Vitamin D Status in Mother-Newborn Pairs in Greece. Calcif Tissue Int 2006;78:337-342.. 4. Lapatsanis D, et al. Vitamin D: A Necessity for Children and Adolescents in Greece. Calcif Tissue Int 2005;77:348-355. 5. Pazaitou-Panayiotou K, et al. Height, whole Body Surface Area, gender, working outdoors, and sunbathing in previous summer are important determinants of serum 25-hydroxyvitamin D levels. Exp Clin Endocrinol Diabetes. 2012;120(1):14-22. 6. Dusso AS, et al. Vitamin D. Am J Physiol Renal Physiol 2005;289:F8–F28. 7. Grober U, et al. Vitamin D: Update 2013 From rickets prophylaxis to general preventive healthcare. Dermatoendocrinol. 2013;5(3):331-347

ECTS – BUDAPEST 2019

HEEL QUANTITATIVE ULTRASOUND PARAMETERS, SERUM 25-HYDROXYVITAMIN D AND PARATHYROID HORMONE LEVELS OF HEALTHY ADULT WOMEN IN GREECE

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Abstract

Objective: The objective of this observational cross-sectional study is to identify heel bone properties, specifically broadband ultrasound attenuation (BUA), speed of sound (SOS) and stiffness index (SI), in healthy Greek women, as well the relation of these parameters with age, serum 25-hydroxyvitamin D (25(OH)D) and parathyroid hormone (PTH).

Material and Methods: A population of 738 community dwelling women was recruited at the health promotion events carried out by the Hellenic Society for the Support of Patients with Osteoporosis in rural and urban areas throughout Greece. PTH and 25(OH)D were measured. Heel bone properties measured using quantitative ultrasound (QUS) device. The study was approved by the Ethics Committee of Harokopio University.

Results: The descriptive data of population are shown in the table 1 as mean ± standard deviation. There is a significant difference of BUA (114,05±15,7 ≠ 107,66±15,7, p=0,039) and SOS (1555,67±34,26 ≠ 1506,0±18, p=0,00) between normal(15-65pg/mL) and high(>65pg/mL) PTH level respectively. SI and BUA are significantly different between all age groups as shown in table1 with p=0,00 for both parameters. SI is higher in 18-50 age group in relation to 51-65 and >65 age group (p=0,00 and p=0,03 respectively).

Table 1: Descriptive characteristics

	25(OH)D (ng/mL)	PTH (pg/mL)	SI	BUA	SOS
Total sample	19.87±7.57	39.67±14.75	89.95±18.38	113.78±15.67	1551.92±70.14
18-50 years	20.14±7.78	36.05±14.15	96.75±17.00	117.89±15.01	1560.13±88.95
51-65 years	19.36±7.66	43.29±14.85	84.51±16.7	110.12±14.6	1544.71±31.76
>65 years	17.64±6.52	47.29±16.03	75.5±16.19	104.37±16	1528.91±29.00

Conclusions: The mean vitamin D levels of older Greek women (>50years) is below 20ng/mL, as well as BUA, SOS and SI are lower in older age groups (51-65 and >65). Given that low levels are associated with increased risk for fractures, this study highlights the emerging issue of 25(OH)D insufficiency in Greek women and the need for targeted interventions.

All authors state that they have no conflicts of interest.

Introduction

Osteoporosis is a major public health concern. Women have a higher risk of osteoporotic fracture compared with men (1 in 3 women and 1 in 5 men over 50 years). [1] Quantitative bone ultrasound (QUS) is emerging as a low-cost , ionizing radiation–free, simple, and portable screening technique that is able to identify women at risk for the osteoporosis and that may be used by general practitioners in primary care. Lower values of the QUS parameters were associated with a significant increase of any subsequent fracture at any site.[2] Vitamin D deficiency is also among the most important clinical risk factors. Although recent data indicate that prevalence of vitamin D deficiency can be common in countries previously considered as low risk (e.g Mediterranean countries) [1] ,few data are available for Greek population. Low levels occur in elderly[3], mothers after delivery[4], children and adolescents[5] and there are few data concerning adults[6]. Therefore, the evaluation of QUS parameters and vitamin D levels is of immense significance[7]. is an observational cross-sectional study that aims to identify the prevalence of vitamin D deficiency of healthy adults in Greece.

Methods

A population of 738 community dwelling women was recruited at the health promotion events carried out by the Hellenic Society for the Support of Patients with Osteoporosis in rural and urban areas throughout Greece. Following a 12 hour fast, all subjects will have a blood drawn of venous blood used for serum isolation. Total calcium (Ca), phosphorus (P), parathyroid hormone (PTH) and 25-hydroxyvitamin D (25(OH)D) were measured. Heel bone properties measured using the Achilles quantitative ultrasound (QUS) device, a water-bath ultrasound system into which the subject places his heel. Achilles generates a band of frequencies from 200 to 600 kHz. It measures the broadband ultrasound attenuation (BUA), expressed in dB/MHz, and the speed of sound (SOS), expressed in m/s. The third variable, stiffness index (SI), is automatically calculated by Achilles from the BUA and the SOS, using the equation SI=(0.67*BUA)+(0.28*SOS)-420. The SPSS statistical package used for the statistical analysis. The study was approved by the Ethics Committee of Harokopio University.

Results

The mean age of the population was 50.40±12.94 years, (range, 20-86 years. The descriptive data of population are shown in the table 1 as mean ± standard deviation. There is a significant difference of BUA (114.05±15.7 ≠ 107.66±15.7, p=0.04) and SOS (1555.67±34.26 ≠ 1506.0±18.00 p=0.00) between normal (15-65pg/mL) and high (>65pg/mL) PTH level respectively. SI and BUA are significantly different between all age groups as shown in table1 with p=0.00 for both parameters. SI is higher in 18-50 age group in relation to 51-65 and >65 age group (p=0.00 and p=0.03 respectively).

Table 1: Descriptive characteristics

	25(OH)D (ng/mL)	PTH (pg/mL)	SI	BUA	SOS
Total sample	19.87±7.57	39.67±14.75	89.95±18.38	113.78±15.67	1551.92±70.14
18-50 years	20.14±7.78	36.05±14.15	96.75±17	117,89±15,01	1560.13±88.95
51-65 years	19.36±7.66	43.29±14.85	84.51±16.7	110,12±14,6	1544.71±31.76
>65 years	17.64±6.52	47.29±16.03	75.5±16.19	104,37±16	1528.91±29

Conclusions

The mean serum 25(OH)D touch thresholds for the age group 18-50 years and is below the reference values (20ng/mL) for ages over 50years. Additionally, as older age groups, the lower the levels of QUS parameters (BUA, SOS and SI) are lower in older age groups (51-65 and >65). BUA and SOS values are significant higher in subjects with normal PTH (15-65pg/mL), than those with high levels (>65pg/mL). Low levels of 25(OH)D and QUS parameters, are associated with increased risk for fractures and exacerbate bone loss.[1,2] Beyond the skeletal consequences, low serum vitamin D, has numerous nonkeletal effects[8]. This study highlights the emerging issue of 25(OH)D insufficiency and the decrease of BUA, SOS and SI at older ages, in Greek population, that should get priority in public health strategies and the need for targeted interventions.

Acknowledgements

The study was supported by the Hellenic Society for the Study of Bone Metabolism

References

1. Holick MF. Vitamin D Deficiency. N Engl J Med 2007;357:266-281. 2. Marin F, et al. Relationship Between Bone Quantitative Ultrasound and Fractures: A Meta-Analysis. J Bone Miner Res 2006;21:1457–1463 3. Papapetrou PD, et al. Vitamin D deficiency in the elderly in Athens, Greece. Bone Miner Metab 2007;25:198–203. 4. Nicolaidou P, et al. Low Vitamin D Status in Mother-Newborn Pairs in Greece. Calcif Tissue Int 2006;78:337-342., 5. Lapatsanis D, et al. Vitamin D: A Necessity for Children and Adolescents in Greece. Calcif Tissue Int 2005;77:348-355. 6. Pazaitou-Panayiotou K, et al. Height, whole Body Surface Area, gender, working outdoors, and sunbathing in previous summer are important determinants of serum 25-hydroxyvitamin D levels. Exp Clin Endocrinol Diabetes. 2012;120(1):14-22. 7. Dusso AS, et al. Vitamin D. Am J Physiol Renal Physiol 2005;289:F8–F28. 8. Grober U, et al. Vitamin D: Update 2013 From rickets prophylaxis to general preventive healthcare. Dermatoendocrinol. 2013;5(3):331-347

ECTS-ROME 2016

SERUM 25-HYDROXYVITAMIN D LEVELS OF HEALTHY ADULT WOMEN IN GREECE

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Abstract

Objective: The objective of this observational cross-sectional study is to identify the prevalence of vitamin D deficiency in healthy women in Greece, as reflected by the levels of 25-hydroxyvitamin D (25(OH)D), since recent data indicate that vitamin D deficiency can be common in countries previously considered as low risk (e.g. Mediterranean countries).
Material and Methods: A population of 840 community dwelling women was recruited at the health promotion events carried out by the Hellenic Society for the Support of Patients with Osteoporosis in rural and urban areas throughout Greece. Serum total calcium (Ca), phosphorus (P), creatinine, parathyroid hormone (PTH) and 25(OH)D were measured. The study was approved by the Ethics Committee of Harokopio University.
Results: The mean age of the population was 50.33 years, (range, 20-86 years) while 86.9% were 20-65 years old. Mean serum 25(OH)D was 19.95 ng/mL, mean PTH was 41.16 pg/mL and mean Ca, P and creatinine were 9.50, 3.63 and 0.77 mg/dL respectively. Concerning the vitamin D levels, 55.3% of the subjects had deficient (0-19.9 ng/mL), 34.2% had insufficient (20-29.9 ng/mL) and only 10.5% had adequate (30-150 ng/mL) levels, while 8.0% had vitamin D levels ≤10ng/mL. PTH was at normal range (15-65 pg/mL) for 89.7% of the population and 8.2% had high PTH (>65 pg/mL). The levels of serum Ca, P, and creatinine, were within the normal range.
Conclusions: The majority of Greek women (88%) in this study had vitamin D levels below 30ng/mL. Given that low levels of 25(OH)D are associated with increased risk for fractures and exacerbate bone loss, this study highlights the emerging issue of 25(OH)D insufficiency in Greek women and the need for targeted interventions even in age groups not previously considered as at risk.

Introduction

Osteoporosis is a major public health concern. Women have a higher risk of osteoporotic fracture compared with men (1 in 3 women and 1 in 5 men over 50 years). Vitamin D deficiency is among the most important clinical risk factors. Although recent data indicate that prevalence of vitamin D deficiency can be common in countries previously considered as low risk (e.g Mediterranean countries) [1] ,few data are available for Greek population. Low levels occur in elderly[2], mothers after delivery[3], children and adolescents[4] and there are few data concerning adults[5]. Therefore, the evaluation of vitamin D levels is of immense significance[6]. This is an observational cross-sectional study that aims to identify the prevalence of vitamin D deficiency of healthy women in Greece.

Methods

A population of 840 community dwelling women was recruited at the health promotion events carried out by the Hellenic Society for the Support of Patients with Osteoporosis in rural and urban areas throughout Greece. Following a 12 hour fast, all subjects will have a blood drawn of venous blood used for serum isolation. Total calcium (Ca), phosphorus (P), creatinine, parathyroid hormone (PTH) and 25-hydroxyvitamin D (25(OH)D) were measured. The SPSS statistical package used for the statistical analysis. The study was approved by the Ethics Committee of Harokopio University.

Results

The mean age of the population was 50.33 years, (range, 20-86 years) while 86.9% were 20-65 years old. Mean serum 25(OH)D, PTH, Ca, P and creatinine are presented in Table 1. Vitamin D levels, are presented in Table 2. PTH was at normal range (15-65 pg/mL) for 89.7% of the population and 8.2% had high PTH (>65 pg/mL). The levels of serum Ca, P, and creatinine, were within the normal range.

Table 1: Descriptive characteristics

	Mean ± SD
Age (years)	50.33 ± 13.01
25(OH)D (ng/mL)	19.95 ± 8.51
PTH (pg/mL)	41.16 ± 18.06
Ca (mg/dL)	9.50 ± 0.65
P (mg/dL)	3.63 ± 0.74
Creatinine (mg/dL)	0.77 ± 0.16

Table 2: 25(OH)D levels

25(OH)D status (ng/mL)	Percentage of population
Deficiency (<10)	8%
Deficiency (10-19.9)	47.3 %
Insufficiency (20-29.9)	34.2 %
Adequacy (≥30)	10.5%

Conclusions

The majority of Greek women (55.3%) in this study had vitamin D deficiency (<20ng/mL) and only 10.5% had levels above the adequacy (30ng/mL). Low levels of 25(OH)D are associated with increased risk for fractures and exacerbate bone loss. Beyond the skeletal consequences, low serum vitamin D, has numerous nonkeletal effects[7]. This study highlights the emerging issue of 25(OH)D insufficiency in Greek women, that should get priority in public health strategies and the need for targeted interventions even in age groups not previously considered as at risk, like adults.

Acknowledgements

The study was supported by the Hellenic Society for the Study of Bone Metabolism

References

1. Holick MF. Vitamin D Deficiency. N Engl J Med 2007;357:266-281. 2. Papapetrou PD, et al. Vitamin D deficiency in the elderly in Athens, Greece. Bone Miner Metab 2007;25:198–203. 3. Nicolaidou P, et al. Low Vitamin D Status in Mother-Newborn Pairs in Greece. Calcif Tissue Int 2006;78:337-342. 4. Lapatsanis D, et al. Vitamin D: A Necessity for Children and Adolescents in Greece. Calcif Tissue Int 2005;77:348-355. 5. Pazaitou-Panayiotou K, et al. Height, whole Body Surface Area, gender, working outdoors, and sunbathing in previous summer are important determinants of serum 25-hydroxyvitamin D levels. Exp Clin Endocrinol Diabetes. 2012;120(1):14-22. 6. Dusso AS, et al. Vitamin D. Am J Physiol Renal Physiol 2005;289:F8–F28. 7. Grober U, et al. Vitamin D: Update 2013 From rickets prophylaxis to general preventive healthcare. Dermatoendocrinol. 2013;5(3):331-347

ECTS 2014 PRAGUE

SERUM 25-HYDROXYVITAMIN D LEVELS OF HEALTHY ADULTS IN GREECE

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Abstract

Objective: The objective of this observational cross-sectional study is to identify the prevalence of vitamin D deficiency in healthy adults in Greece, as reflected by the levels of 25-hydroxyvitamin D (25(OH)D), since recent data indicate that vitamin D deficiency can be common in countries previously considered as low risk (e.g Mediterranean countries).

Material and Methods: A population of 974 community dwelling adults (134 males, 840 females) was recruited at the health promotion events carried out by the Hellenic Society for the Support of Patients with Osteoporosis in rural and urban areas throughout Greece. Serum total calcium (Ca), phosphorus (P), creatinine, parathyroid hormone (PTH) and 25(OH)D were measured. The study was approved by the Ethics Committee of Harokopio University.

Results: The mean age of the population was 49.58 years, (range, 18-86 years) while 87.2% were18-65 years old. Mean serum 25(OH)D was 20.38 ng/mL, mean PTH was 41.24 pg/mL and mean Ca, P and creatinine were 9.97, 3.57 and 0.79 mg/dL respectively. Concerning the vitamin D levels, 53.6% of the subjects had deficient (0-19.9 ng/mL), 34.4% had insufficient (20-29.9 ng/mL) and only 12.0% had adequate (30-150 ng/mL) levels, while 8.0% had vitamin D levels ≤10ng/mL. PTH was at normal range (15-65 pg/mL) for 89.7% of the population and 8.2% had high PTH (>65 pg/mL). The levels of serum Ca, P, and creatinine, were within the normal range.

Conclusions: The majority of Greek adults (88%) in this study had vitamin D levels below 30ng/mL. Given that low levels of 25(OH)D are associated with increased risk for fractures and exacerbate bone loss, this study highlights the emerging issue of 25(OH)D insufficiency in Greek population and the need for targeted interventions even in age groups not previously considered as at risk.

Introduction

Osteoporosis is a major public health concern. Women have a higher risk of osteoporotic fracture compared with men (1 in 3 women and 1 in 5 men over 50 years). Vitamin D deficiency is among the most important clinical risk factors. Although recent data indicate that prevalence of vitamin D deficiency can be common in countries previously considered as low risk (e.g Mediterranean countries) [1] ,few data are available for Greek population. Low levels occur in elderly[2], mothers after delivery[3], children and adolescents[4] and there are few data concerning adults[5]. Therefore, the evaluation of vitamin D levels is of immense significance[6]. is an observational cross-sectional study that aims to identify the prevalence of vitamin D deficiency of healthy adults in Greece.

Methods

A population of 974 community dwelling adults (134 males, 840 females) was recruited at the health promotion events carried out by the Hellenic Society for the Support of Patients with Osteoporosis in rural and urban areas throughout Greece. Following a 12 hour fast, all subjects will have a blood drawn of venous blood used for serum isolation. Total calcium (Ca), phosphorus (P), creatinine, parathyroid hormone (PTH) and 25-hydroxyvitamin D (25(OH)D) were measured. The SPSS statistical package used for the statistical analysis. The study was approved by the Ethics Committee of Harokopio University.

Results

The mean age of the population was 49.58 years, (range, 18-86 years) while 87.2% were18-65 years old. Mean serum 25(OH)D, PTH, Ca, P and creatinine are presented in Table 1. Vitamin D levels, are presented in Table 2. PTH was at normal range (15-65 pg/mL) for 89.7% of the population and 8.2% had high PTH (>65 pg/mL). The levels of serum Ca, P, and creatinine, were within the normal range

Conclusions

The majority of Greek adults (53.6%) in this study had vitamin D deficiency (<20ng/mL) and only 12% had levels above the adequacy (30ng/mL). Low levels of 25(OH)D are associated with increased risk for fractures and exacerbate bone loss. Beyond the skeletal consequences, low serum vitamin D, has numerous nonskeletal effects[7]. This study highlights the emerging issue of 25(OH)D insufficiency in Greek population, that should get priority in public health strategies and the need for targeted interventions even in age groups not previously considered as at risk, like adults.

Acknowledgements

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Table 1: Descriptive characteristics of population

	Mean ± SD
Age (years)	49.58 ± 13.5
25(OH)D (ng/mL)	20.38 ± 8.95
PTH (pg/mL)	41.24 ± 17.99
Ca (mg/dL)	9.97 ± 0.93
P (mg/dL)	3.57 ± 0.73
Creatinine (mg/dL)	0.79 ± 1.16

Table 2: 25(OH)D levels of population

25(OH)D status (ng/mL)	Percentage of population
deficiency (<10)	8%
deficiency (10-19.9)	45.6 %
insufficiency (20-29.9)	34.4 %
adequacy (≥30)	12%

References

1. Holick MF. Vitamin D Deficiency. N Engl J Med 2007;357:266-281.
2. Papapetrou PD, et al. Vitamin D deficiency in the elderly in Athens, Greece. Bone Miner Metab 2007;25:198–203.
3. Nicolaidou P, et al. Low Vitamin D Status in Mother-Newborn Pairs in Greece. Calcif Tissue Int 2006;78:337-342.
4. Lapatsanis D, et al. Vitamin D: A Necessity for Children and Adolescents in Greece. Calcif Tissue Int 2005;77:348-355.
5. Pazaitou-Panayiotou K, et al. Height, whole Body Surface Area, gender, working outdoors, and sunbathing in previous summer are important determinants of serum 25-hydroxyvitamin D levels. Exp Clin Endocrinol Diabetes. 2012;120(1):14-22
6. Dusso AS, et al. Vitamin D. Am J Physiol Renal Physiol 2005;289:F8–F28.
7. Grober U, et al. Vitamin D: Update 2013 From rickets prophylaxis to general preventive healthcare. Dermatoendocrinol. 2013;5(3):331-347