Gene 809 (2022) 146036

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Association between genetic variants in oxidative stress-related genes and osteoporotic bone fracture. The Hortega follow-up study

Ricardo Usategui-Martín [a,b,1,*], José Luis Pérez-Castrillón [c,d,1,*], María L. Mansego [e,f], Francisco Lara-Hernández [e], Iris Manzano [e], Laisa Briongos [c,d], Jesica Abadía-Otero [c], Javier Martín-Vallejo [g], Ana B. García-García [e,h], Juan Carlos Martín-Escudero [c,d], Felipe J. Chaves [e,h]

[a] IOBA, University of Valladolid, Valladolid. Spain
[b] Cooperative Health Network for Research (RETICS), Oftared, National Institute of Health Carlos III, ISCIII, Madrid. Spain
[c] Department of Internal Medicine, Rio Hortega Universitary Hospital, Valladolid, Spain
[d] Department of Medicine. Faculty of Medicine. University of Valladolid, Valladolid, Spain
[e] Genomic and Genetic Diagnosis Unit, INCLIVA Biomedical Research Institute, Valencia, Spain
[f] Department of Bioinformatics. Making Genetics S.L. Pamplona. Spain
[g] Department of Statistics. University of Salamanca. Salamanca Biomedical Research Institute (IBSAL), Salamanca. Spain
[h] CIBER of Diabetes and Associated Metabolic Diseases (CIBERDEM), Madrid. Spain

ARTICLE INFO

Keywords:
Osteoporosis
Fracture
Bone metabolism
Aging
Oxidative stress
Reactive oxygen species

ABSTRACT

The most widely accepted etiopathogenesis hypothesis of the origin of osteoporosis and its complications is that they are a consequence of bone aging and other environmental factors, together with a genetic predisposition. Evidence suggests that oxidative stress is crucial in bone pathologies associated with aging. The aim of this study was to determine whether genetic variants in oxidative stress-related genes modified the risk of osteoporotic fracture. We analysed 221 patients and 354 controls from the HORTEGA sample after 12–14 years of follow up. We studied the genotypic and allelic distribution of 53 SNPs in 24 genes involved in oxidative stress. The results showed that being a carrier of the variant allele of the SNP rs4077561 within *TXNRD1* was the principal genetic risk factor associated with osteoporotic fracture and that variant allele of the rs1805754 *M6PR*, rs4964779 *TXNRD1*, rs406113 *GPX6*, rs2281082 *TXN2* and rs974334 *GPX6* polymorphisms are important genetic risk factors for fracture. This study provides information on the genetic factors associated with oxidative stress which are involved in the risk of osteoporotic fracture and reinforces the hypothesis that genetic factors are crucial in the etiopathogenesis of osteoporosis and its complications.

1. Introduction

Osteoporosis, the most common bone disorder worldwide (OMIM: 166710), is characterized by low bone mineral density (BMD), reduced bone mass and alteration of the bone microarchitecture, leading to enhanced bone fragility and an increased risk of bone fracture (Kanis 1997; Yang et al. 2020). Osteoporosis is a silent, progressive systemic bone disease with dramatic clinical, social, and economic consequences. The principal clinical consequence of osteoporosis is bone fracture. Age is an independent risk factor for bone fracture, with older subjects having a fracture risk up to 10 times higher than younger subjects. Bone fractures are associated with a worse quality of life and increased disability, morbidity and mortality (Adachi et al. 2010; Kanis 2002; Sheer et al. 2020).

Abbreviations: BMD, Bone mineral density; GWAS, Genome-wide association studies; ROS, Reactive oxygen species; FoxO, Forkhead box, class O; WHO, World Health Organization; PIXI, Peripheral instantaneous X-ray imaging; SNPs, Single nucleotide polymorphisms; ANOVA, One-way analysis of variance; ORs, Odd ratios; 95% CI, 95% confidence intervals; CART, Classification and regression tree approach; Txn, Thioredoxin; TxnR1, Txn reductase 1; Txn2, Thioredoxin 2; GPxs, Glutathione peroxidases; M6pr, Mannose-6-phosphate receptor.

* Corresponding authors.
E-mail addresses: ricardo.usategui@uva.es (R. Usategui-Martín), joseluis.perez@uva.es (J.L. Pérez-Castrillón).

[1] Ricardo Usategui-Martín and José Luis Pérez-Castrillón contributed equally.

https://doi.org/10.1016/j.gene.2021.146036
Received 26 August 2021; Received in revised form 15 October 2021; Accepted 19 October 2021
Available online 21 October 2021

The most widely accepted etiopathogenic hypothesis of the aetiology of osteoporosis and osteoporotic bone fracture is the synergic action of environmental and genetic factors. Twin and family studies have shown that genetic variants could explain 50–80 % of the risk of osteoporosis. In addition, a family history of osteoporosis had been associated with an increased risk of bone fracture. In this scenario, Genome-wide association studies (GWAS) have been crucial providing information about the genetic architecture of osteoporosis and bone fracture predisposition (Trajanoska and Rivadeneira 2019; Stewart and Ralston 2000; Rivadeneira and Mäkitie 2016). With respect to environmental factors, osteoporosis and fracture may be secondary to the bone aging process in combination with reductions in sex hormone levels, other metabolic alterations, nutritional deficiencies and adverse medication effects (Kanis 1997; Yang et al. 2020; Kanis 2002).

The increase in fracture risk associated with bone aging is a very complex process which involves systemic, local and genetic factors (Corrado et al. 2020; Khosla et al., 2018; Feehan et al., 2019). An increase in reactive oxygen species (ROS) is an important factor in the aging of tissues, including bone (Corrado et al. 2020). Several reports have suggested that ROS and oxidative stress could play a crucial role in bone alterations associated with senescence (Corrado et al. 2020; Almeida et al. 2007; Essers et al. 2005). The relationship between high levels of ROS and low BMD has been reported in various studies (Sharma et al. 2015; Zhou et al. 2016; Bonaccorsi et al. 2018; Domazetovic et al. 2017). High levels of ROS promote the activation of Forkhead box, class O (FoxO) transcription factor, which is involved in the activation of the cell defence mechanisms against oxidative stress (Essers et al. 2005), but it has also been observed that the increase in FoxO was also associated with a concomitant reduced β-catenin expression (Manolagas and Almeida 2007). β-catenin plays a crucial role in the Wnt pathway and, therefore, in osteoblast differentiation (Bennett et al. 2005; Riancho et al. 2011). Bone loss and the increased risk of fracture associated with high levels of ROS and oxidative stress have been attributed to the fact that high levels of ROS cause a reduction in the activation of the β-catenin-Wnt pathway (Corrado et al. 2020; Manolagas and Almeida 2007). Therefore, the aim of this study was to determine whether polymorphisms in genes implicated in oxidative stress modify the risk of osteoporotic fracture.

2. Subjects and methods

2.1. Subjects

The Hortega Study is a population-based survey of adult residents from the East Valladolid Health Department (Rio Hortega University Hospital, Spain) that investigated cross-sectional and prospective associations between genetic, metabolomic and environmental risk factors and chronic diseases. The multi-stage complex sampling yielded a

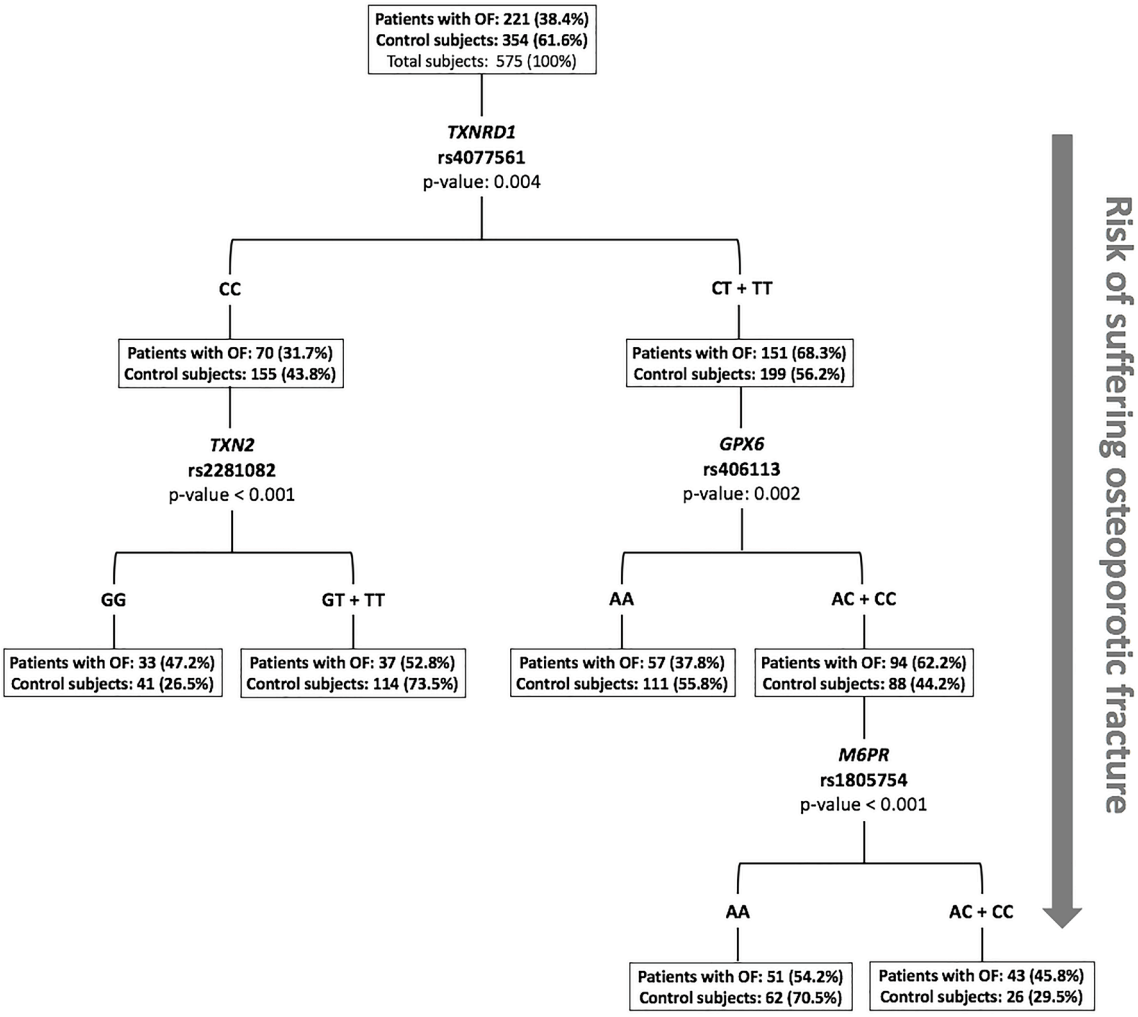

Fig. 1. Classification and regression tree (CART) analysis to assess the association between the variant allele of the polymorphisms included in the study and the risk of osteoporotic fracture. The procedure examined all possible independent variables (polymorphisms) and selected the one most closely associated with the risk of osteoporotic fracture, generating different groups (or nodes). OF: osteoporotic fracture.

representative sample of 1502 subjects. The follow up of the Hortega Study participants started in 2001–2003 (baseline examination visit and collection of biological specimens) and added information on mortality and incident health endpoints in November 2015 (Tellez-Plaza et al. 2019; Mansego et al. 2008; Morales-Suárez-Varela et al. 2011; de Marco et al. 2019; Usategui-Martín et al. 2020).

To analyse the influence of genetic factors on the risk of osteoporotic fracture we selected subjects from the Hortega Study, as previously described (Usategui-Martín et al. 2020). In brief, of the 1502 baseline participants, we excluded 137 subjects due to lack of demographic, anthropometric or clinical data, resulting in a study population of 1365. After analysis of subjects aged ≥ 50 years (N = 702), we excluded 49 participants missing follow-up information, 15 with a personal history of bone fractures at baseline and 63 participants without bone X-ray, resulting in a study population of 575 subjects. Subjects with bone fracture (N = 221) were considered cases and those without (N = 354) controls (Supplementary Fig. 1).

From each subject we collected demographic, anthropometric, and clinical characteristics including age, sex, height, weight, body mass index (BMI), smoking, alcohol consumption, menopause, corticosteroid use, family history of osteoporotic fracture and calcaneal bone densitometry. BMI was calculated by dividing weight in kilograms by height in metres squared. Obesity, hypertension, and type 2 diabetes mellitus were diagnosed according to World Health Organization (WHO) criteria (http://www.who.int). Calcaneal bone densitometry was performed on the right calcaneus using the peripheral instantaneous X-ray imaging (PIXI) DXA system (General Electric Lunar Pixi, Boston, MA, USA). Vertebral fractures were determined according to the Genant classification (Genant et al. 1993). Non-vertebral fractures were obtained from the medical record. Osteoporotic fractures were determined after a follow up of 12–14 years.

The experimental protocol was in accordance with the Declaration of Helsinki (2008) of the World Medical Association, and was approved by the University Hospital of Valladolid Ethics Committee and was in compliance with Spanish data protection laws (LO 15/1999) and specifications (RD 1720/2007). All subjects who agreed to participate gave written informed consent.

2.2. DNA isolation and polymorphism genotyping

Genomic DNA from peripheral blood leukocytes was isolated by standard commercial procedures (Chemagic Magnetic Separator, Chemagen, Baesweiler, Germany). DNA was quantified and diluted to a final concentration of 100 ng/μL. Genotyping was performed using the SNPlex oligonucleotide ligation assay (Applied Biosystems, Foster City, CA, USA) following the manufacturer's recommendations. The selection of single nucleotide polymorphisms (SNPs) was conducted by SYSNP (Hirschhorn and Daly 2005; Lorente-Galdos et al. 2012) and computer-based searches of the following databases: PubMed, Web of Science, Scopus, and Embase electronic databases using the terms such as "bone metabolism", "reactive oxygen species", "bone", "SNPs", "polymorphisms", "genetic variants". SNPs were selected according to the following considerations: functional known or potentially functional effect, location in promoter regions, MAF > 0.1 in Caucasian subjects, localization and distribution along the gene (including upstream and downstream regions) and low described linkage disequilibrium between candidate SNPs. NCBI and HapMap databases were used to collet information about SNPs and determine the gene and pathway involved in each polymorphism included. We selected 53 polymorphisms for genotyping in 24 genes involved in oxidative stress pathway (Table 1). 15 genes were classified as antioxidant genes (*CAT, GCLC, GCLM, GPX6, GSR, GSS, M6PR, MSRB2, OGG1, SOD1, SOD2, SOD3, TXN, TXN2* and *TXNRD1*) and 9 genes as reactive species generators (*CYBB, NCF2, NCF4, NOS2A, NOX1, NOX3, NOX4, NOX5* and *XDH*). We included genetic variants with potential influence in the gene expression and function, we also included the most relevant polymorphisms described in the literature associated to oxidative stress genes (Forsberg et al., 2001; Rodrigues et al. 2014).

Table 1
Summary of the 53 selected polymorphisms in 24 genes.

Gene	Chr	SNP id	Alleles	Feature
CAT	11	rs1049982	C/T	5́UTR
		rs511895	A/G	Intronic
		rs7104301	A/G	Downstream
CYBB	X	rs5964125	A/G	Intronic
		rs5964151	G/T	3́UTR
GCLC	6	rs1014852	A/T	Intronic
		rs11415624	T/A	3́UTR
		rs3736729	A/C	Intronic
GCLM	1	rs7515191	A/G	Intronic
GPX6	6	rs406113	A/C	Missense
		rs974334	C/G	Intronic
GSR	8	rs1002149	G/T	Promoter
		rs2911678	A/T	Intronic
GSS	20	rs2273684	G/T	Intronic
M6PR	12	rs1805754	A/C	Promoter
MSRB2	10	rs11013291	C/T	Intronic
NCF2	1	rs2274064	C/T	Missense
		rs2296164	C/T	Intronic
NCF4	22	rs2072712	C/T	Synonymous
NOS2A	17	rs2779248	C/T	Upstream
NOX1	X	rs4827881	A/C	Upstream
		rs5921682	A/G	Upstream
NOX3	6	rs3749930	G/T	Missense
NOX4	11	rs490934	C/G	Intronic
NOX5	15	rs34990910	A/G	Intronic
		rs2036343	A/C	Promoter
OGG1	3	rs1052133	C/G	Missense
SOD1	21	rs17881274	C/T	Upstream
SOD2	6	rs2842980	A/T	Downstream
		rs2855116	G/T	Intronic
SOD3	4	rs2284659	G/T	Promoter
TXN	22	rs4135168	A/G	Intronic
		rs4135179	A/G	Intronic
		rs4135225	C/T	Intronic
TXN2	22	rs2281082	G/T	Intronic
TXNRD1	12	rs4964778	C/G	Intronic
		rs4964779	C/T	Intronic
		rs5018287	A/G	Intronic
		rs10861201	A/C	Intronic
		rs4077561	C/T	Promoter
		rs4964287	C/T	Synonymous
XDH	2	rs17011368	C/T	Missense
		rs206801	C/T	Intronic
		rs206812	A/G	Promoter
		rs207454	A/C	Intronic
		rs10175754	C/T	Intronic
		rs1346644	C/G	Intronic
		rs1429374	A/G	Intronic
		rs17011353	C/T	Intronic
		rs17323225	C/T	Missense
		rs1884725	A/G	Synonymous
		rs2073316	A/G	Intronic
		rs761926	C/G	Intronic

2.3. Statistical analysis

Quantitative variables were expressed as mean ± standard deviation (SD) and qualitative variables as absolute (n) and relative (%) frequencies. One-way analysis of variance (ANOVA) was used to determine differences between quantitative variables, and the chi-square test to compare qualitative variables. The healthy subject group was tested for conformity to the Hardy-Weinberg equilibrium using the chi-square test for each polymorphism. Odd ratios (ORs) and 95 % confidence intervals (95 % CI) were estimated for each polymorphic variant using unconditional logistic regression models to evaluate the association with osteoporotic fracture risk: p-values were adjusted by sex, age, BMI, BMD, menopause, hypertension and family history of osteoporotic fracture. Given the high number of significance test to detect association between

polymorphic variant and experimental groups, Benjamini-Hochberg corrections was carried out.

The classification and regression tree approach (CART) was used to assess potential interactions between polymorphisms significantly associated with osteoporotic fracture, demographic, anthropometric and clinical characteristics and the risk of osteoporotic fracture (Breiman et al., 1984). CART analysis is a binary recursive partitioning method which produces a graphical structure that resembles a decision tree. This enables identification of subgroups of subjects with a higher risk of osteoporotic fracture. A set of patients containing the entire sample is classified into groups by a dependent factor (in this case: patients and control subjects). The procedure examines all possible independent factors (or variables) and selects the one that is most closely associated with respect to the dependent variable and creates two new groups (nodes). The partition process is repeated in each node and stops when there is no association between the dependent variable and independent variables, or the sample size of the group is small (N ⟨100). Bonferroni adjustment was applied in the CART analysis.

We made a power analysis related to the chi-square test (contingency table) and OR to study the association between polymorphisms and bone fracture (yes/no). The results showed a power = 1 with degree freedom: 2; effect size moderate: 0.5; level of significance: 0.05 and sample size: 500. The results also showed a power = 0.99947 with degree freedom: 2; effect size small: 0.23; level of significance: 0.05 and sample size: 500. The analysis to detect the power of the statistical significance of OR with level of significance: 0.05, event probability: 0.40, genotypic basal probability: 0.20 and sample size: 500 showed OR = 1.88 (power = 0.80), OR = 2.43 (power = 0.97) and OR = 3.1 (power = 0.99).

The statistical analyses were performed using SPSS software. The Benjamini-Hochberg adjustment was carried out with the R-stats package. P values < 0.05 were considered as statistically significant.

3. Results

A total of 221 patients with osteoporotic fracture and 354 control subjects were analysed after a 12–14 year follow up. Table 2 summarizes the demographic, anthropometric and clinical characteristics of study subjects. The only significant difference was that the family history of osteoporotic fracture was higher in patients with fractures than in controls (Table 2).

Table 3 shows the genotypic frequencies of polymorphisms significantly associated with the risk of osteoporotic fracture. The genotype distribution of these polymorphisms in the control sample were in Hardy-Weinberg equilibrium. The variant genotypes of the rs1805754 *M6PR*, rs4964779 *TXNRD1,* rs4077561 *TXNRD1,* rs406113 *GPX6* and rs974334 *GPX6* polymorphisms were associated with an increased risk of osteoporotic fracture (Table 3). In addition, the variant genotype of the rs2281082 *TXN2* polymorphism was associated with a reduced risk of osteoporotic fracture (Table 3). Codominance analysis confirmed that being a carrier of the variant genotype of the polymorphisms described above was associated with the risk of osteoporotic fracture (Table 3). Table 4 shows the allelic frequencies of the polymorphisms in genes involved in oxidative stress that were significantly associated with the risk of osteoporotic fracture. The C allele of rs1805754 *M6PR*, the C allele of rs4964779 *TXNRD1*, the T allele of rs4077561 *TXNRD1*, the C allele of rs406113 *GPX6* and the G allele of rs974334 *GPX6* were associated with an increased risk of osteoporotic fracture (Table 4). In addition, the T allele of the rs2281082 *TXN2* polymorphism was associated with a reduced risk of osteoporotic fracture (Table 4). The results after Benjamini-Hochberg adjustment did not significantly differ from those reported above (Supplementary Table 1). The frequencies of polymorphisms that were not associated with the risk of osteoporotic fracture are summarized in Supplementary Table 2.

CART analysis showed that being a carrier of the variant genotype (CT + TT) of the rs4077561 *TXNRD1* polymorphism was the principal genetic risk factor for osteoporotic fracture. In carriers of the variant genotype of the rs4077561 *TXNRD1* polymorphism, subjects with the variant genotype (AC + CC) of the rs406113 *GPX6* polymorphism showed the highest risk of osteoporotic fracture. Variant genotypes of the rs2281082 *TXN2* and rs1805754 *M6PR* polymorphisms were also important genetic risk factors for fracture. Thus, the CT + TT of the rs4077561 *TXNRD1,* AC + CC of the rs406113 *GPX6* and the AC + CC of the rs1805754 *M6PR* polymorphisms are the combination of genotypes with an increased risk of suffering osteoporotic fracture. CART analysis showed no associations between demographic, anthropometric and clinical characteristics, and the risk of osteoporotic fracture (Fig. 1).

Table 2
Demographic, anthropometric and clinical characteristics of study subjects.

	Control subjects	Patients with OF
Age (years old), mean ± SD	61.88 ± 16.32	61.37 ± 17.88
Female sex, n (%)	189 (53.4 %)	107 (48.4 %)
Height (cm), mean ± SD	164.53 ± 9.16	164.19 ± 9.50
Weight (kg), mean ± SD	71.35 ± 13.54	70.96 ± 12.78
BMI (kg/m^2), mean ± SD	26.32 ± 4.30	26.29 ± 4.12
Obesity, n (%)	84 (24.9 %)	59 (28.2 %)
Overweight, n (%)	123 (36.5 %)	79 (37.8 %)
Central obesity, n (%)	82 (24.0 %)	59 (27.4 %)
Hypertension, n (%)	143 (40.4 %)	83 (37.6 %)
Diabetes mellitus, n (%)	27 (7.6 %)	14 (6.3 %)
Smoking, n (%)	81 (23.2 %)	58 (26.5 %)
Alcohol, n (%)	67 (19.0 %)	54 (24.4 %)
Postmenopausal, n (%)	165 (46.61 %)	89 (40.27 %)
Corticosteroids, n (%)	38 (10.79 %)	29 (13.1 %)
Family history of OF, n (%)	1 (0.3 %)	10 (4.5 %) *
BMD (g/cm^2), mean ± SD	0.55 ± 0.11	0.53 ± 0.11
BMD (*t*-score), mean ± SD	−0.17 ± 1.10	−0.19 ± 1 0.38

*: p-value < 0.05 between control subjects and patients with osteoporotic fracture.
OF: osteoporotic fracture.
BMI: body mass index.
BMD: bone mineral density.

4. Discussion

The most widely accepted hypothesis on the origin of osteoporosis and its complications is that they are a consequence of aging and other environmental factors together with a genetic predisposition (Yang et al. 2020; Kanis 2002; Mitek et al. 2019). Our results confirm the crucial role of genetic factors, as the family history of osteoporotic fracture was significantly higher in patients than in control subjects. Evidence suggests that ROS and oxidative stress may be crucial in bone alterations associated with aging, such as osteoporosis and bone fracture (Sharma et al. 2015; Zhou et al. 2016; Bonaccorsi et al. 2018; Domazetovic et al. 2017) and it has also been reported that genetic variability plays an important role in the oxidative stress response to aging (Dato et al. 2013). Therefore, we studied whether genetic variants in genes involved in oxidative stress modify the risk of bone fracture. Our results showed that the variant allele of the rs1805754 *M6PR*, rs4964779 *TXNRD1,* rs4077561 *TXNRD1,* rs406113 *GPX6,* rs2281082 *TXN2* and rs974334 *GPX6* polymorphisms were associated with osteoporotic fracture. The results are in line with our previous report which suggested that the polymorphisms in genes involved in pathways associated with aging may be a crucial factor in the risk of osteoporotic fracture (Usategui-Martín et al. 2020).

The thioredoxin (Txn) pathway is the most important cell antioxidant system and regulates the cells redox status, which plays a crucial role in antioxidant defence (Lu and Holmgren 2014; Pannala and Dash 2015). The Txn endogenous regulator is Txn reductase 1 (TxnR1), a selenoprotein that reduces Txn and other compounds, thereby detoxifying cells from oxidative injures (Arnér 2009; Turanov et al. 2010). It has been reported that Txn and TxnR1 are crucial to neuroprotection, inflammation regulation, antiapoptosis processes and the immune function (Holmgren and Jun, 2010). In has been suggested that *TXNRD1*

Table 3
Genotypic frequencies of polymorphisms in genes involved in oxidative stress significantly associated with the risk of osteoporotic fracture.

Gene	SNP	Genotype	Control subjects (n (%))	Patients with OF (n (%))	p-value	OR (95 % CI)
M6PR	rs1805754	AA	254 (71.8 %)	117 (52.9 %)	–	1.00
		AC	87 (24.6 %)	80 (36.2 %)	<0.001	1.99 (1.37–2.90)
		CC	13 (3.7 %)	24 (10.9 %)	<0.001	3.99 (1.97–8.14)
		AA	254 (71.8 %)	117 (52.9 %)	–	1.00
		AC + CC	100 (28.2 %	104 (47.1 %)	<0.001	2.25 (1.58–3.20)
		AA + AC	341 (96.3 %)	197 (89.1 %)	–	1.00
		CC	13 (3.7 %)	24 (10.9 %)	0.001	3.19 (1.59–6.41)
TXNRD1	rs4964779	TT	285 (81.4 %)	148 (69.8 %)	–	1.00
		TC	45 (12.9 %)	39 (18.4 %)	0.034	1.66 (1.04–2.67)
		CC	20 (5.7 %)	25 (11.8 %)	0.006	2.40 (1.29–4.47)
		TT	285 (81.4 %)	148 (69.8 %)	–	1.00
		TC + CC	65 (18.6 %)	64 (30.2 %)	0.002	1.89 (1.27–2.82)
		TT + TC	330 (94.3 %)	187 (88.2 %)	–	1.00
		CC	20 (5.7 %)	25 (11.8 %)	0.12	2.20 (1.19–4.07)
	rs4077561	CC	155 (43.8 %)	70 (31.7 %)	–	1.00
		CT	158 (44.6 %)	111(50.2 %)	0.020	1.55 (1.07–2.25)
		TT	41 (11.6 %)	40 (18.1 %)	0.004	2.16 (1.28–3.63)
		CC	155 (43.8 %)	70 (31.7 %)	–	1.00
		CT + TT	199 (56.2 %)	151 (68.3 %)	0.004	1.68 (1.18–2.39)
		CC + CT	313 (88.4 %)	181 (81.9 %)	–	1.00
		TT	41 (11.6 %)	40 (18.1 %)	0.030	1.68 (1.05–2.70)
TXN2	rs2281082	GG	104 (29.4 %)	101 (45.9 %)	–	1.00
		GT	157 (44.4 %)	92 (41.8 %)	0.008	0.60 (0.41–0.87)
		TT	93 (26.3 %)	27 (12.3 %)	<0.001	0.29 (0.18–0.49)
		GG	104 (29.4 %)	101 (45.9 %)	–	1.00
		GT + TT	250 (70.6 %)	119 (54.1 %)	<0.001	0.49 (0.34–0.69)
		GG + GT	261 (73,7%)	193 (87.7 %)	–	1.00
		TT	93 (26.3 %)	27 (12.3 %)	<0.001	0.39 (0.24–0.62)
GPX6	rs406113	AA	191 (54.0 %)	88 (39.8 %)	–	1.00
		AC	129 (36.4 %)	92 (41.6 %)	0.020	1.54 (1.07–2.23)
		CC	34 (9.6 %)	41 (18.6 %)	<0.001	2.61 (1.55–4.40)
		AA	191 (54.0 %)	88 (39.8 %)	–	1.00
		AC + CC	163 (46.0 %)	133 (60.2 %)	0.001	1.77 (1.25–2.49)
		AA + AC	320 (90.4 %)	180 (81.4 %)	–	1.00
		CC	34 (9.6 %)	41 (18.6 %)	0.002	2.14 (1.31–3.49)
	rs974334	CC	271 (77.0 %)	139 (64.7 %)	–	1.00
		CG	75 (21.3 %)	61 (29.0 %)	0.022	1.58 (1.06–2.35)
		GG	6 (1.7 %)	10 (4.8 %)	0.025	3.24 (1.15–9.12)
		CC	271 (77.0 %)	139 (64.7 %)	–	1.00
		CG + GG	81 (23.0 %)	71 (33.8 %)	0.006	1.70 (1.17–2.49)
		CC + CG	346 (98.3 %)	200 (95.2 %)	–	1.00
		GG	6 (1.7 %)	10 (4.8 %)	0.043	2.88 (1.13–8.05)

OF: osteoporotic fracture.
OR: odds ratios.
CI: confident interval.

gene variability could modify the antioxidants associated with aging (Soerensen et al. 2012; Dato et al. 2014; Dato et al. 2015). We found that the principal genetic risk factor for osteoporotic fracture is being a carrier of the variant genotype of the rs4077561 polymorphism, which is a genetic variant located in the promoter region of the *TXNRD1* gene. Our hypothesis is that the variant genotype of this gene promoter polymorphism could modify *TXNRD1* expression, altering the antioxidant response associated with bone aging and favouring the risk of osteoporotic fracture. In addition, the results showed the rs4964779 *TXNRD1* polymorphism was associated with bone fracture, reinforcing the hypothesis that *TXNRD1* gene variability may be crucial in the predisposition to osteoporotic fracture. Genetic variants in *TXNRD1* have been associated with cardiovascular disease, heart failure, stroke, Alzheimer disease, arthritis, cancer and other diseases associated with aging (Soerensen et al. 2012; Dato et al. 2014; Dato et al. 2015), but this is the first time they have been associated with the risk of bone fracture. Thioredoxin 2 (Txn2) is another redox protein of the Txn pathway that is essential for the control of ROS homeostasis, apoptosis and cell viability (Cunningham et al. 2015; Pérez et al. 2008; Holzerova et al. 2016). The Txn2 protein is located in the matrix mitochondria and is encoded by the *TXN2* nuclear gene (Spyrou et al. 1997). It has been reported that genetic alterations in *TXN2* are associated with impaired mitochondrial function and increased oxidative stress (Pérez et al. 2008; Holzerova et al. 2016). We found an association between the variant genotype of

Table 4
Allelic frequencies of polymorphisms in genes involved in oxidative stress significantly associated with the risk of osteoporotic fracture.

Gene	SNP	Allele	Control subjects	Patients with OF	p-value	OR (95 % CI)
			n (%)	n (%)		
M6PR	rs1805754	A	595 (84.0 %)	314 (71.0 %)	–	1.00
		C	113 (16.0 %)	128 (29.0 %)	<0.001	2.14 (1.61–2.86)
TXNRD1	rs4964779	T	615 (87.9 %)	335 (79.0 %)	–	1.00
		C	85 (12.1 %)	89 (21.0 %)	<0.001	1.92 (1.38–2.66)
	rs4077561	C	468 (66.1 %)	251 (56.8 %)	–	1.00
		T	240 (33.9 %)	191 (43.2 %)	0.002	1.48 (1.16–1.89)
TXN2	rs2281082	G	365 (51.6 %)	294 (66.2 %)	–	1.00
		T	343 (48.4 %)	146 (33.2 %)	<0.001	0.49 (0.41–0.67)
GPX6	rs406113	A	511 (72.2 %)	268 (60.6 %)	–	1.00
		C	197 (27.8 %)	174 (39.4 %)	<0.001	1.68 (1.30–2.16)
	rs974334	C	617 (87.6 %)	339 (80.7 %)	–	1.00
		G	87 (12.4 %)	81 (19.3 %)	0.002	1.69 (1.21–2.35)

OF: osteoporotic fracture.
OR: odds ratios.
CI: confident interval.

the rs2281082 *TXN2* polymorphism and a reduced risk of osteoporotic fracture, suggesting the G allele resulted in increased risk of bone fracture. The rs2281082 *TXN2* polymorphism has also been studied in other conditions associated with aging (Rodrigues et al. 2014; Harris et al. 2007; Seibold et al. 2011) but this is the first time a significant association has been found. Our results confirm that genetic variants in genes involved in the Txn pathway could be crucial to the risk of osteoporotic fracture.

Glutathione peroxidases (GPxs) are crucial in protecting against oxidative stress and eight GPxs family members with antioxidant capacity in different physiological and pathological situations have been identified (McLean et al. 2005; Kryukov et al. 2003; Ramming et al. 2014; Mehmeti et al. 2017; Chen et al. 2020). In addition, it has been suggested that the inactivation of GPxs is associated with the induction of oxidative stress (Miyamoto et al. 2003). We found that the second principal genetic risk factor for bone fracture is being a carrier of the C allele of the rs406113 *GPX6* polymorphism. This is a missense variant in the first exon of the *GPX6* gene that involves the Phe13Leu variation of the signal peptide region of the GPx6 protein. The rs406113 *GPX6* polymorphism could be located in an exon splicing site, this fact has only predicted in silico, therefore the real effect of the SNP should be assessed in specific experiments. Even so, the polymorphism may have regulatory gene expression implications (Xu and Taylor 2009; Rupérez et al. 2014). We hypothesize that it could modify GPx6 protein synthesis and thereby modify the response to oxidative stress, increasing the risk of osteoporotic fracture. We also found that the rs974334 *GPX6* gene variant was associated with the risk of bone fracture, reinforcing the idea that *GPX6* polymorphisms could play an important role in the predisposition to osteoporotic fracture. Polymorphisms in the *GPX6* gene have been studied in congenital heart diseases, obesity and cancer (Chowdhury et al. 2012; Rodrigues et al. 2014; Rupérez et al. 2014; Kuchenbaecker et al. 2015; Costa-Urrutia et al. 2020) but, to the best of our knowledge, this is the first time that *GPX6* genetic variants have been associated with the risk of bone fracture. Oxidative stress and ROS damage play an important role in cell death induced by lysosome dysfunction and may be associated with alterations in the autophagy degradation pathway, inhibition of the lysosome enzyme function and lysosome membrane damage. Oxidative stress may cause lysosomal damage directly or by causing secondary damage though the increase in damaged macromolecules and organelles (Pivtoraiko et al. 2009; Antunes et al., 2001). Mannose-6-phosphate receptor (M6pr) is crucial in lysosome function as it is involved in the transport of lysosomal hydrolases from the Golgi apparatus to the lysosomes. M6pr is a transmembrane glycoprotein which is encoded by the *M6PR* gene (Porter et al. 2013). We analysed whether genetic variants in the *M6PR* gene modified the risk of osteoporotic fracture and found that the C allele of the rs1805754 polymorphism increased the risk. The rs1805754 genetic variant is located in the promoter region of the *M6PR* gene, and therefore could modify the gene expression. We found that the C allele of the rs1805754 polymorphism could alter *M6PR* gene transcription and therefore modify the lysosomal function in conditions of oxidative stress, increasing the risk of fracture.

The study had some limitations. First, it might have been interesting to analyze the influence of these genetic variants on central bone mineral density (vertebral and hip) but we only recorded calcaneal bone densitometry. Secondly, the size of the sample was limited, although the statistical power analysis showed that the sample size was sufficient for our objective. Thirdly, the associations reported in our results should be taken with caution due to the high number of comparisons made: our study lays the foundation for future research to define the role of these polymorphisms. The main strengths of the study are the cohort of subjects drawn from a study with 12–14 years of follow up, and the similarity of patients and control subjects, which is important in determining the influence of genetic factors.

In summary, we found an association between polymorphisms in genes involved in oxidative stress and the risk of bone fracture, reinforcing the hypothesis that genetic factors are crucial in the etiopathogenesis of osteoporosis and its complications. We also provide information, for the first time, on the importance that genetic variants in genes encoding antioxidant proteins have in the risk of osteoporotic fracture. Future studies are required to define the role of these polymorphisms in the risk of osteoporotic fracture including studies in other series of patients will be necessary to validate our findings.

Author contribution

RUM and JLPC performed the statistical analysis, analysed and interpreted results and wrote the manuscript. JMV performed the statistical analysis. FLH, ABGG and FJC obtained genetic data, contributed to analysis and interpretation and reviewed the manuscript. LBF, JAO, JCME obtained population data, made critical revisions of the manuscript and contributed to the discussion. RUM, JLPC, FJC and JCME designed the study

Funding

This study was supported by grants from Instituto de Salud Carlos III (Ministry of Economy and Competitiveness, Spanish Government) (ISC IIII-FEDER: PI14/00874 and PI17/00544).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

the work reported in this paper.

Acknowledgements

We thank patients for their participation and the members of the Hortega Study who collected clinical data.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gene.2021.146036.

References

Adachi, J.D., Adami, S., Gehlbach, S., et al., 2010. Impact of Prevalent Fractures on Quality of Life: Baseline Results from the Global Longitudinal Study of Osteoporosis in Women. Mayo Clin. Proc. 85 (9), 806–813.

Almeida, M., Han, L.i., Martin-Millan, M., et al., 2007. Skeletal Involution by Age-Associated Oxidative Stress and Its Acceleration by Loss of Sex Steroids. J. Biol. Chem. 282 (37), 27285–27297.

Antunes, F., Cadenas, E., Brunk, U.T., 2001. Apoptosis Induced by Exposure to a Low Steady-State Concentration of H2O2 Is a Consequence of Lysosomal Rupture. Biochem. J. 356 (Pt 2), 549–555.

Arnér, E.S.J., 2009. Focus on Mammalian Thioredoxin Reductases-Important Selenoproteins with Versatile Functions. BBA 1790 (6), 495–526.

Bennett, C.N., Longo, K.A., Wright, W.S., et al., 2005. Regulation of Osteoblastogenesis and Bone Mass by Wnt10b. PNAS 102 (9), 3324–3329.

Bonaccorsi, G., Piva, I., Greco, P., Cervellati, C., 2018. Oxidative Stress as a Possible Pathogenic Cofactor of Post-Menopausal Osteoporosis: Existing Evidence in Support of the Axis Oestrogen Deficiency-Redox Imbalance-Bone Loss. Ind. J. Med. Res. 147 (4), 341–351.

Breiman, L., Friedman, J., Olshen, R., Stone, C., 1984. Classification and Regression Trees, 1st ed. Wadsworth International Group, Belmont, California.

Chen, Y., Wang, K., Zhang, D., et al., 2020. GPx6 Is Involved in the in Vitro Induced Capacitation and Acrosome Reaction in Porcine Sperm. Theriogenology 156, 107–115.

Chowdhury, S., Hobbs, C.A., MacLeod, S.L., et al., 2012. Associations between Maternal Genotypes and Metabolites Implicated in Congenital Heart Defects. Mol. Genet. Metab. 107 (3), 596–604.

Corrado, Addolorata, Daniela Cici, Cinzia Rotondo, Nicola Maruotti, and Francesco Paolo Cantatore 2020 Molecular Basis of Bone Aging. International Journal of Molecular Sciences 21(10).

Costa-Urrutia, Paula, Aline Mariana Flores-Buendía, Iván Ascencio-Montiel, et al. 2020 Antioxidant Enzymes Haplotypes and Polymorphisms Associated with Obesity in Mexican Children. Antioxidants (Basel, Switzerland) 9(8).

Cunningham, G.M., Roman, M.G., Flores, L.C., et al., 2015. The Paradoxical Role of Thioredoxin on Oxidative Stress and Aging. Arch. Biochem. Biophys. 576, 32–38.

Dato, S., Soerensen, M., Lagani, V., et al., 2014. Contribution of Genetic Polymorphisms on Functional Status at Very Old Age: A Gene-Based Analysis of 38 Genes (311 SNPs) in the Oxidative Stress Pathway. Exp. Gerontol. 52, 23–29.

Dato, S., Crocco, P., D'Aquila, P., et al., 2013. Exploring the Role of Genetic Variability and Lifestyle in Oxidative Stress Response for Healthy Aging and Longevity. Int. J. Mol. Sci. 14 (8), 16443–16472.

Dato, Serena, Francesco De Rango, Paolina Crocco, Giuseppe Passarino, and Giuseppina Rose 2015 Antioxidants and Quality of Aging: Further Evidences for a Major Role of TXNRD1 Gene Variability on Physical Performance at Old Age. Oxidative Medicine and Cellular Longevity 2015. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4429211/, accessed October 2, 2020.

Domazetovic, Vladana, Gemma Marcucci, Teresa Iantomasi, Maria Luisa Brandi, and Maria Teresa Vincenzini 2017 Oxidative Stress in Bone Remodeling: Role of Antioxidants. Clinical Cases in Mineral and Bone Metabolism: The Official Journal of the Italian Society of Osteoporosis, Mineral Metabolism, and Skeletal Diseases 14(2): 209–216.

Essers, Marieke A. G., Lydia M. M. de Vries-Smits, Nick Barker, et al. 2005 Functional Interaction between Beta-Catenin and FOXO in Oxidative Stress Signaling. Science (New York, N.Y.) 308(5725): 1181–1184.

Feehan, J., Saedi, A.A., Duque, G., 2019. Targeting Fundamental Aging Mechanisms to Treat Osteoporosis. Exp. Opin. Therap. Targ. 23 (12), 1031–1039.

Forsberg, L., de Faire, U., Morgenstern, R., 2001. Oxidative Stress, Human Genetic Variation, and Disease. Arch. Biochem. Biophys. 389 (1), 84–93.

Genant, H.K., Wu, C.Y., van Kuijk, C., Nevitt, M.C., 1993. Vertebral Fracture Assessment Using a Semiquantitative Technique. J. Bone Min. Res Off. J. Am. Soc. Bone Miner. Res. 8 (9), 1137–1148.

Harris, S.E., Fox, H., Wright, A.F., et al., 2007. A Genetic Association Analysis of Cognitive Ability and Cognitive Ageing Using 325 Markers for 109 Genes Associated with Oxidative Stress or Cognition. BMC Genet. 8, 43.

Hirschhorn, J.N., Daly, M.J., 2005. Genome-Wide Association Studies for Common Diseases and Complex Traits. Nat. Rev. Genet. 6 (2), 95–108.

Holmgren, A., Jun, L.u., 2010. Thioredoxin and Thioredoxin Reductase: Current Research with Special Reference to Human Disease. Biochem. Biophys. Res. Commun. 396 (1), 120–124.

Holzerova, Eliska, Katharina Danhauser, Tobias B. Haack, et al. 2016 Human Thioredoxin 2 Deficiency Impairs Mitochondrial Redox Homeostasis and Causes Early-Onset Neurodegeneration. Brain: A Journal of Neurology 139(Pt 2): 346–354.

Kanis, J. A. 1997 Diagnosis of Osteoporosis. Osteoporosis International: A Journal Established as Result of Cooperation between the European Foundation for Osteoporosis and the National Osteoporosis Foundation of the USA 7 Suppl 3: S108-116.

Kanis, J.A., 2002. Diagnosis of Osteoporosis and Assessment of Fracture Risk. Lancet (London, England) 359 (9321), 1929–1936.

Khosla, S., Farr, J.N., Kirkland, J.L., 2018. Inhibiting Cellular Senescence: A New Therapeutic Paradigm for Age-Related Osteoporosis. J. Clinic. Endocrinol. Metabol. 103 (4), 1282–1290.

Kryukov, Gregory V., Sergi Castellano, Sergey V. Novoselov, et al. 2003 Characterization of Mammalian Selenoproteomes. Science (New York, N.Y.) 300(5624): 1439–1443.

Kuchenbaecker, K.B., Ramus, S.J., Tyrer, J., et al., 2015. Identification of Six New Susceptibility Loci for Invasive Epithelial Ovarian Cancer. Nat. Genet. 47 (2), 164–171.

Lorente-Galdos, B., Medina, I., Morcillo-Suarez, C., et al., 2012. Select Your SNPs (SYSNPs): A Web Tool for Automatic and Massive Selection of SNPs. Int. J. Data Min. Bioinform. 6 (3), 324–334.

Lu, J., Holmgren, A., 2014. The Thioredoxin Antioxidant System. Free Radical Biol. Med. 66, 75–87.

Manolagas, S.C., Almeida, M., 2007. Gone with the Wnts: Beta-Catenin, T-Cell Factor, Forkhead Box O, and Oxidative Stress in Age-Dependent Diseases of Bone, Lipid, and Glucose Metabolism. Mol. Endocrinol. (Baltimore, Md.) 21 (11), 2605–2614.

Mansego, M.L., Redon, J., Marin, R., et al., 2008. Renin Polymorphisms and Haplotypes Are Associated with Blood Pressure evels and Hypertension Risk in Postmenopausal Women. J. Hypertens. 26 (2), 230–237.

de Marco, G., Garcia-Garcia, A.B., Real, J.T., et al., 2019. Respiratory Chain Polymorphisms and Obesity in the Spanish Population, a Cross-Sectional Study. BMJ Open 9 (2), e027004.

McLean, C.W., Mirochnitchenko, O., Claus, C.P., Noble-Haeusslein, L.J., Ferriero, D.M., 2005. Overexpression of Glutathione Peroxidase Protects Immature Murine Neurons from Oxidative Stress. Dev. Neurosci. 27 (2–4), 169–175.

Mehmeti, I., Lortz, S., Avezov, E., Jörns, A., Lenzen, S., 2017. ER-Resident Antioxidative GPx7 and GPx8 Enzyme Isoforms Protect Insulin-Secreting INS-1E β-Cells against Lipotoxicity by Improving the ER Antioxidative Capacity. Free Radical Biol. Med. 112, 121–130.

Mitek, T., Nagraba, Ł., Deszczyński, J., et al., 2019. Genetic Predisposition for Osteoporosis and Fractures in Postmenopausal Women. Adv. Exp. Med. Biol. 1211, 17–24.

Miyamoto, Y., Koh, Y.H., Park, Y.S., et al., 2003. Oxidative Stress Caused by Inactivation of Glutathione Peroxidase and Adaptive Responses. Biol. Chem. 384 (4), 567–574.

Morales-Suárez-Varela, M.M., Mansego, M.L., Vicedo-Cabrera, A.M., et al., 2011. Inefficient Arterial Hypertension Control in Patients with Metabolic Syndrome and Its Link to Renin-Angiotensin-Aldosterone System Polymorphisms. Hypertens. Res. Off. J. Japan. Soc. Hypert. 34 (6), 758–766.

Pannala, V.R., Dash, R.K., 2015. Mechanistic Characterization of the Thioredoxin System in the Removal of Hydrogen Peroxide. Free Radical Biol. Med. 78, 42–55.

Pérez, V.I., Lew, C.M., Cortez, L.A., et al., 2008. Thioredoxin 2 Haploinsufficiency in Mice Results in Impaired Mitochondrial Function and Increased Oxidative Stress. Free Radical Biol. Med. 44 (5), 882–892.

Pivtoraiko, V.N., Stone, S.L., Roth, K.A., Shacka, J.J., 2009. Oxidative Stress and Autophagy in the Regulation of Lysosome-Dependent Neuron Death. Antioxid. Redox Signal. 11 (3), 481–496.

Porter, K., Nallathambi, J., Lin, Y., Liton, P.B., 2013. Lysosomal Basification and Decreased Autophagic Flux in Oxidatively Stressed Trabecular Meshwork Cells. Autophagy 9 (4), 581–594.

Ramming, T., Hansen, H.G., Nagata, K., Ellgaard, L., Appenzeller-Herzog, C., 2014. GPx8 Peroxidase Prevents Leakage of H2O2 from the Endoplasmic Reticulum. Free Radical Biol. Med. 70, 106–116.

Riancho, J.A., Olmos, J.M., Pineda, B., et al., 2011. Wnt Receptors, Bone Mass, and Fractures: Gene-Wide Association Analysis of LRP5 and LRP6 Polymorphisms with Replication. Eur. J. Endocrinol. 164 (1), 123–131.

Rivadeneira, F., Mäkitie, O., 2016. Osteoporosis and Bone Mass Disorders: From Gene Pathways to Treatments. Trend. Endocrinol. Metabol. TEM 27 (5), 262–281.

Rodrigues, P., de Marco, G., Furriol, J., et al., 2014. Oxidative Stress in Susceptibility to Breast Cancer: Study in Spanish Population. BMC Cancer 14, 861.

Rupérez, A.I., Olza, J., Gil-Campos, M., et al., 2014. Association of Genetic Polymorphisms for Glutathione Peroxidase Genes with Obesity in Spanish Children. J. Nutrigenet. Nutrig. 7 (3), 130–142.

Seibold, P., Hein, R., Schmezer, P., et al., 2011. Polymorphisms in Oxidative Stress-Related Genes and Postmenopausal Breast Cancer Risk. Int. J. Cancer 129 (6), 1467–1476.

Sharma, T., Islam, N., Ahmad, J., Akhtar, N., Beg, M., 2015. Correlation between Bone Mineral Density and Oxidative Stress in Postmenopausal Women. Indian J. Endocrinol. Metabol. 19 (4), 491–497.

Sheer, R.L., Barron, R.L., Sudharshan, L., Pasquale, M.K., 2020. Validated Prediction of Imminent Risk of Fracture for Older Adults. Amer. J. Manag. Care 26 (3), e91–e97.

Soerensen, M., Dato, S., Tan, Q., et al., 2012. Human Longevity and Variation in GH/IGF-1/Insulin Signaling, DNA Damage Signaling and Repair and pro/Antioxidant Pathway Genes: Cross Sectional and Longitudinal Studies. Exp. Gerontol. 47 (5), 379–387.

Spyrou, G., Enmark, E., Miranda-Vizuete, A., Gustafsson, J., 1997. Cloning and Expression of a Novel Mammalian Thioredoxin. J. Biolog. Chem. 272 (5), 2936–2941.

Stewart, T.L., Ralston, S.H., 2000. Role of Genetic Factors in the Pathogenesis of Osteoporosis. J. Endocrinol. 166 (2), 235–245.
Tellez-Plaza, M., Briongos-Figuero, L., Pichler, G., et al., 2019. Cohort Profile: The Hortega Study for the Evaluation of Non-Traditional Risk Factors of Cardiometabolic and Other Chronic Diseases in a General Population from Spain. BMJ Open 9 (6), e024073.
Trajanoska, K., Rivadeneira, F., 2019. The Genetic Architecture of Osteoporosis and Fracture Risk. Bone 126, 2–10.
Turanov, A.A., Kehr, S., Marino, S.M., et al., 2010. Mammalian Thioredoxin Reductase 1: Roles in Redox Homoeostasis and Characterization of Cellular Targets. Biochem. J. 430 (2), 285–293.
Usategui-Martín, R., Lendinez-Tortajada, V., Pérez-Castrillón, J.L., et al., 2020. Polymorphisms in Genes Involved in Inflammation, the NF-KB Pathway and the Renin-Angiotensin-Aldosterone System Are Associated with the Risk of Osteoporotic Fracture. The Hortega Follow-up Study. Bone 138, 115477.
Xu, Zongli, and Jack A. Taylor 2009 SNPinfo: Integrating GWAS and Candidate Gene Information into Functional SNP Selection for Genetic Association Studies. Nucleic Acids Research 37(Web Server issue): W600-605.
Yang, T.-L., Shen, H., Liu, A., et al., 2020. A Road Map for Understanding Molecular and Genetic Determinants of Osteoporosis. Nat. Rev. Endocrinol 16 (2), 91–103.
Zhou, Q., Zhu, L.i., Zhang, D., et al., 2016. Oxidative Stress-Related Biomarkers in Postmenopausal Osteoporosis: A Systematic Review and Meta-Analyses. Dis. Markers 2016, 7067984.