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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

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ABSTRACT

Osteoporosis is the most common bone disorder worldwide and is associated with a reduced quality of life with important clinical and economic consequences. The most widely accepted etiopathogenic hypothesis on the origin of osteoporosis and its complications is that they are a consequence of the synergic action of environmental and genetic factors. Bone is constantly being remodelled through anabolic and catabolic pathways in which inflammation, the NF-kB pathway and the renin-angiotensin-aldosterone system (RAAS) are crucial. The aim of our study was to determine whether polymorphisms in genes implicated in inflammation, the NF-kB pathway and RAAS modified the risk of osteoporotic fracture. We analysed 221 patients with osteoporotic fracture and 354 controls without fracture from the HORTEGA sample after 12–14 years of follow up. In addition, we studied the genotypic distribution of 230 single nucleotide polymorphisms (SNPs) in genes involved in inflammation, NF-kB pathway and RAAS. Our results showed that be carrier of the C allele of the rs2228145 *IL6R* polymorphism was the principal genetic risk factor associated with osteoporotic fracture. The results also showed that variant genotypes of the rs4762 *AGT*, rs4073 *IL8*, rs2070699 *END1* and rs4291 *ACE* polymorphisms were important genetic risk factors for fracture. The study provides information about the genetic factors associated with inflammation, the NF-kB pathway and RAAS, 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 (OMIM:166710), the most common bone disorder worldwide, is characterized by low bone mineral density (BMD), reduced bone mass, alteration of bone microarchitecture and an increased risk of osteoporotic bone fracture [1,2]. Osteoporosis is a silent, progressive disease with dramatic clinical and economic consequences. It is reported that approximately one in three postmenopausal women have osteoporosis and the majority will have an osteoporotic fracture at some time. Osteoporotic fractures are associated with increased

Abbreviations: BMD, Bone mineral density; NF-kB, nuclear factor kB; RANKL, receptor activator of NF-kB ligand; RANK, receptor activator of NF-kB; RAAS, renin-angiotensin-aldosterone system; BMI, body mass index; PIXI, peripheral instantaneous X-ray imaging; SNPs, single nucleotide polymorphisms; ANOVA, one-way analysis of variance; OR, Odds ratio; CI, confidence intervals; CART, classification and regression tree; OF, osteoporotic fracture

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morbidity, disability and mortality and a worse quality of life [3,4].

The aetiology of osteoporosis and osteoporotic fracture is unclear. The most widely accepted etiopathogenic hypothesis is that they are a consequence of the synergic action of environmental and genetic factors. Osteoporosis may be secondary to the aging process in combination with reductions in sex hormones or as a consequence of metabolic alterations, nutritional deficiencies or adverse medication effects [5,6]. Twin and family studies show that genetic factors may explain 50–80% of the risk of osteoporosis [7]. It is reported that a family history of osteoporosis is a crucial risk factor for osteoporotic bone fracture [4,8]. However, the genetic risk factors are not clear.

Bone is constantly remodelled through the anabolic and catabolic pathways, which maintain tissue homeostasis and it is suggested that inflammation plays a crucial role in bone metabolism [9]. It is reported that pro-inflammatory cytokine secretion may be associated with activation of osteoclast differentiation and subsequent bone resorption through activation of the nuclear factor kB (NF-kB) pathway [10–12], which is also crucial in the modulation of inflammatory response, as it regulates the expression of many pro-inflammatory genes [13]. The renin-angiotensin-aldosterone system (RAAS) has also been associated with bone metabolism. It is reported that angiotensin II induces expression of the receptor activator of NF-kB ligand (RANKL) in osteoblasts, leading to the activation and differentiation of osteoclasts through the NF-kB pathway [14,15]. Osteoclast precursors, which express the receptor activator of NF-kB (RANK) on their surface, differentiate into mature osteoclasts, as they identify RANKL through direct cell interaction with osteoblasts [16–18]. Likewise, the crucial role of RAAS in inflammation and aging through activation of the NF-kB pathway has been shown [19–21].

The aim of this study was to determine whether polymorphisms in genes implicated in inflammation, the NF-kB pathway and RAAS modify the risk of osteoporotic fracture.

2. Subjects and methods

2.1. Subjects

The Hortega Study is a population-based survey of adult residents of University Hospital Rio Hortega (East Valladolid Health Department, 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 representative sample of 1502 subjects. The follow-up of the 1502 Hortega Study participants (baseline examination visit and collection of biological specimens in 2001–2003), added information on mortality and incident health endpoints as of 30 November 2015 [22–25]. 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 participants 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 fracture (N = 221) were considered cases and those without (N = 354) as controls.

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 were collected from each subject. BMI was calculated by dividing weight in kilograms by height in metres squared. Obesity, hypertension and type 2 diabetes mellitus were recorded using 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 [26]. Non-vertebral fractures were obtained from the medical record. Osteoporotic fractures were determined after a follow-up of 12–14 years.

Table 1
Demographic, anthropometric and clinical characteristics of study subjects.

	Control subjects	Patients with OF	Patients with clinical OF
Age, mean ± SD	61.88 ± 16.32	61.37 ± 17.88	59.89 ± 17.92
Female sex, n (%)	189 (53.4%)	107 (48.4%)	46 (50.0%)
Height, mean ± SD	164.53 ± 9.16	164.19 ± 9.50	164.73 ± 10.43
Weight, mean ± SD	71.35 ± 13.54	70.96 ± 12.78	70.04 ± 11.77
BMI, mean ± SD	26.32 ± 4.30	26.29 ± 4.12	25.82 ± 3.80
Obesity, n (%)	84 (24.9%)	59 (28.2%)	19 (21.3%)
Overweight, n (%)	123 (36.5%)	79 (37.8%)	35 (39.3%)
Central obesity, n (%)	82 (24.0%)	59 (27.4%)	21 (23.6%)
Hypertension, n (%)	143 (40.4%)	83 (37.6%)	25 (27.2%)**
Diabetes mellitus, n (%)	27 (7.6%)	14 (6.3%)	7 (7.6%)
Smoking, n (%)	81 (23.2%)	58 (26.5%)	30 (32.6%)
Alcohol, n (%)	67 (19.0%)	54 (24.4%)	24 (26.1%)
Postmenopausal, n (%)	165 (46.61%)	89 (40.27%)	38 (41.30%)
Corticosteroids, n (%)	38 (10.79%)	29 (13.1%)	16 (17.4%)
Family history of OF, n (%)	1 (0.3%)	10 (4.5%)*	5 (5.4%)**
BMD, mean ± SD	0.55 ± 0.11	0.53 ± 0.11	0.53 ± 0.13
BMD (t-score), mean ± SD	−0.17 ± 1.10	−0.19 ± 1 0.38	−0.18 ± 1.32

OF: osteoporotic fracture.
BMI: body mass index.
BMD: bone mineral density.
* : p-value < .05 between control subjects and patients with osteoporotic fracture.
** : p-value < .05 between control subjects and patients with clinical osteoporotic fracture.

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

Venous blood samples were collected in tubes containing EDTA. DNA 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. We selected 230 single nucleotide polymorphisms (SNPs) for genotyping (supplementary table 1). SNPs were selected according to the following considerations: functionality (previously described or possible effect), distribution along the gene, with preference given to those located in exons or contiguous regions. We selected polymorphisms with minor allele frequencies > 5%. Genecard (www.genecards.org) and NCBI (www.ncbi.nlm.nih.gov/snp) databases were used to determine the gene and pathway involved in each polymorphism included.

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 tests to compare qualitative variables. The control group was tested for

Table 2
Polymorphisms in genes involved in inflammation significantly associated with the risk of osteoporotic fracture (*p*-value and OR (95% CI) were calculated with reference to controls).

Gene	SNP	Genotype	Control	Patients with osteoporotic fracture			Patients with clinical osteoporotic fracture		
			n (%)	n (%)	p-value	OR (CI = 95%)	n (%)	p-value	OR (CI = 95%)
EDN1	rs2070699	GG	186 (54.7%)	90 (42.7%)	–	1.00	38 (44.2%)	0.161	
		GT	129 (37.9%)	82 (38.9%)	0.153	1.31 (0.90–1.91)	38 (44.2%)		
		TT	25 (7.4%)	39 (18.5%	< 0.001	3.22 (1.83–5.65)	10 (11.6)		
		GG	186 (54.7%)	90 (42.7%)	–	1.00	38 (44.2%)	0.081	
		GT + TT	154 (45.3%)	121 (57.3%)	0.006	1.62 (1.14–2.29)	48 (55.8%)		
	rs3087459	CC	241 (71.1%)	131 (60.6%)	–	1.00	62 (70.5%)	0.989	
		CA	91 (26.8%)	71 (32.9%)	0.060	1.43 (0.98–2.09)	24 (27.3%)		
		AA	7 (2.1%)	14 (6.5%)	0.006	3.67 (1.44–9.34)	2 (2.3%)		
		CC	241 (71.1%)	131 (60.6%)	–	1.00	62 (70.5%)	0.907	
		CA + AA	98 (28.9%)	85 (39.4%)	0.011	1.59 (1.11–2.28)	26 (29.5%)		
IL8	rs4073	AA	57 (17.4%)	54 (26.5%)	–	1.00	19 (22.4%)	0.579	
		AT	154 (47.1%)	98 (48.0%)	0.083	0.67 (0.42–1.05)	38 (44.7%)		
		TT	116 (35.5%)	52 (25.5%)	0.003	0.47 (0.28–0.77)	28 (32.9%)		
		AA	57 (17.4%)	54 (26.5%)	–	1.00	19 (22.4%)	0.297	
		AT+TT	270 (82.6%)	106 (66.3%)	< 0.001	0.41 (0.26–0.64)	66 (77.6%)		
	rs2227543	CC	134 (38.6%)	66 (30.4%)	–	1.00	35 (39.8%)	0.473	
		CT	162 (46.7%)	104 (47.9%)	0.176	1.30 (0.88–1.91)	36 (40.9%)		
		TT	51 (14.7%)	47 (21.7%)	0.013	1.87 (1.14–3.06)	17 (19.3%)		
		CC	134 (38.6%)	66 (30.4%)	0.058		35 (39.8%)	0.842	
		CT + TT	213 (61.4%)	151 (69.6%)			53 (60.2%)		
IL18RAP	rs2293224	CC	92 (27.1%)	38 (18.1%)	–	1.00	19 (21.6%)	–	1.00
		CT	170 (50.1%)	103 (49.0%)	0.095	1.46 (0.93–2.30)	37 (42.0%)	0.866	1.05 (0.57–1.93)
		TT	77 (22.7%)	69 (32.9%)	0.002	2.17 (1.31–3-57)	32 (36.4%)	0.033	2.01 (1.05–3.82)
		CC	92 (27.1%)	38 (18.1%)	–	1.00	19 (21.6%)	0.290	
		CT + TT	247 (72.9%)	172 (81.9%)	0.016	1.68 (1.10–2.57)	69 (78.4%)		
	rs2293225	CC	210 (61.8%)	108 (50.2%)	–	1.00	55 (62.5%)	0.417	
		CT	120 (35.3%)	87 (40.5%)	0.062	1.41 (0.98–2.02)	28 (31.8%)		
		TT	10 (2.9%)	20 (9.3%)	0.001	3.88 (1.75–8.60)	5 (5.7%)		
		CC	210 (61.8%)	108 (50.2%)	–	1.00	55 (62.5%)	0.899	
		CT + TT	130 (38.2%)	107 (49.8%)	0.008	1.60 (1.13–2.26)	33 (37.5%)		
IL10RB	rs2834167	AA	194 (56.4%)	106 (46.7%)	–	1.00	45 (50.6%)	–	1.00
		AG	139 (40.4%)	100 (44.1%)	0.123	1.31 (0.92–1.86)	35 (39.3%)	0.744	1.08 (0.66–1.77)
		GG	11 (3.2%)	21 (9.3%)	0.001	3.49 (1–62-7.52)	9 (10.1%)	0.008	3.52 (1.38–9.01)
		AA	194 (56.4%)	106 (46.7%)	–	1.00	45 (50.6%)	0.324	
		AG + GG	150 (43.6%)	121 (53.3%)	0.023	1.47 (1.05–2.06)	44 (49.4%)		
IL6R	rs2228145	AA	139 (41.6%)	63 (29.6%)	–	1.00	27 (31.4%)	0.102	
		AC	140 (41.9%)	93 (43.7%)	0.059	1.46 (0.98–2.17)	47 (54.7%)		
		CC	55 (16.5%)	57 (26.8%)	0.001	2.28 (1.42–3.67)	12 (14.0%)		
		AA	139 (41.6%)	63 (29.6%)	–	1.00	27 (31.4%)	0.084	
		AC + CC	195 (58.4%)	150 (70.4%)	0.005	1.69 (1.17–2.44)	59 (68.6%)		

OR: odds ratios.
CI: confident interval.

Table 3
Polymorphisms in genes involved in the NF-kB pathway that were significantly associated with the risk of osteoporotic fracture (p-value and OR (95% CI) were calculated with reference to controls).

Gene	SNP	Genotype	Control	Patients with osteoporotic fracture			Patients with clinical osteoporotic fracture		
			n (%)	n (%)	p-value	OR (CI = 95%)	n (%)	p-value	OR (CI = 95%)
SLC39A8	rs35411892	TT	283 (81.1%)	150 (69.1%)	–	1.00	76 (83.5%)	0.791	
		TC	54 (15.5%)	45 20.7%)	0.045	1.57 (1.01–2.44)	13 (14.3%)		
		CC	12 (3.4%)	22 (10.1%)	0.001	1.68 (1.23–1.99)	2 (2.2%)		
		TT	283 (81.1%)	150 (69.1%)	–	1.00	76 (83.5%)	0.595	
		TC + CC	66 (18.9%)	67 (30.9%)	0.001	1.91 (1.29–2.83)	15 (16.5%)		
BANK1	rs3733197	AA	211 (61.3%)	97 (44.9%)	–	1.00	34 (37.8%)	–	1.00
		AG	114 (33.1%)	97 (44.9%)	0.001	1.85 (1.28–2.65)	46 (51.1%)	< 0.001	2.50 (1.52–4.12)
		GG	19 (5.5%)	22 (10.2%)	0.006	2.51 (1.30–4.87)	10 (11.1%)	0.006	3.26 (1.40–7.61)
		AA	211 (61.3%)	97 (44.9%)	–	1.00	34 (37.8%)	–	1.00
		AG + GG	133 (38.7%)	119 (55.1%)	< 0.001	1.94 (1.37–2.74)	56 (62.2%)	< 0.001	2.61 (1.62–4.21)

OR: odd ratios.
CI: confident interval.

conformity to the Hardy-Weinberg equilibrium using the chi-square test for each polymorphism. The association between the polymorphisms included in our study and patients-controls were analysed by chi-square for contingency table. In the case of statistical signification, the strength of association was evaluated using odds-ratios (OR) and corresponding 95% confidence intervals (CI) adjusted by sex, age, BMI, bone mineral density (BMD), menopause, hypertension and family history of osteoporotic fracture. The Benjamini-Hochberg adjustment was applied.

Table 4
Polymorphisms in genes involved in the renin-angiotensin-aldosterone system that were significantly associated with the risk of osteoporotic fracture (p-value and OR (95% CI) were calculated with reference to controls).

Gene	SNP	Genotype	Control	Patients with osteoporotic fracture			Patients with clinical osteoporotic fracture		
			n (%)	n (%)	p-value	OR (CI = 95%)	n (%)	p-value	OR (CI = 95%)
REN	rs11571080	AA	164 (46.5%)	83 (37.7%)	–	1.00	38 (41.3%)	0.362	
		AG	159 (45.0%)	99 (45.0%)	0.265	1.23 (0.85–1.77)	42 (45.7%)		
		GG	30 (8.5%)	38 (17.3%)	0.001	2.50 (1.44–4.32)	12 (13.0%)		
		AA	164 (46.5%)	83 (37.7%)	–	1.00	38 (41.3%)	0.678	
		AG + GG	189 (46.5%)	137 (62.3%)	0.040	1.43 (1.01–2.02)	54 (58.7%)		
AGT	rs4762	CC	241 (79.0%)	126 (70.4%)	–	1.00	51 (69.9%)	0.199	
		CT	63 (20.7%)	47 (26.3%)	0.109	1.42 (0.92–2.20)	22 (30.1%)		
		TT	1 (0.3%)	6 (3.4%)	0.007	11.4 (1.36–96.3)	0 (0.0%)		
		CC	241 (79.0%)	126 (70.4%)	–	1.00	51 (69.9%)	0.094	
		CT + TT	64 (21.0%)	53 (29.6)	0.033	1.58 (1.03–2.41)	22 (30.1%)		
	rs5049	AA	298 (85.4%)	166 (76.9%)	–	1.00	75 (85.2%)	0.554	
		AG	31 (8.9%)	26 (12.0%)	0.148	1.50 (0.86–2.62)	10 (11.4%)		
		GG	20 (5.7%)	24 (11.1%)	0.016	2.15 (1.15–4.07)	3 (3.4%)		
		AA	298 (85.4%)	166 (76.9%)	–	1.00	75 (85.2%)	0.970	
		AG + GG	51 (14.6%)	50 (23.1%)	0.011	1.76 (1.14–2.71)	13 (14.8%)		
	rs2071406	CC	309 (88.3%)	187 (85.0%)	0.162		75 (81.5%)	–	1.00
		CT	39 (11.1%)	28 (12.7%)			13 (14.1%)	0.358	1.37 (0.69–2.70)
		TT	2 (0.6%)	5 (2.3%)			4 (4.3%)	0.016	8.24 (1.48–45.8)
		CC	309 (88.3%)	187 (85.0%)	0.256		75 (81.5%)	0.087	
		CT + TT	41 (11.7%)	33 (15.0%)			17 (18.5%)		
ACE	rs4291	AA	159 (58.0%)	78 (47.3%)	–	1.00	35 (53.8%)	0.234	
		AT	101 (36.9%)	66 (40.0%)	0.172	1.33 (0.88–2.01)	23 (35.5%)		
		TT	14 (5.1%)	21 (12.7%)	0.003	3.05 (1.47–6.33)	7 (10.8%)		
		AA	159 (58.0%)	78 (47.3%)	–	1.00	35 (53.8%)	0.540	
		AT + TT	115 (42.0%)	87 (52.7%)	0.029	1.54 (1.04–2.27)	30 (46.2%)		
NR3C2	rs1403142	AA	88 (25.4%)	52 (24.4%	0.067		40 (44.4%)	–	1.00
		AG	165 (47.6%)	120 (56.3%)			38 (42.2%)	0.010	0.50 (0.30–0.84)
		GG	94 (27.1%)	41 (19.2%)			12 (13.3%)	< 0.001	0.28 (0.13–0.57)
		AA	88 (25.4%)	52 (24.4%	0.802		40 (44.4%)	–	1.00
		AG + GG	259 (74.6%)	161 (75.6%)			50 (55.6%)	< 0.001	0.42 (0.26–0.68)
	rs1040288	CC	101 (29.3%)	44 (20.0%)	–	1.00	17 (18.5%)	–	1.00
		CG	173 (50.1%)	118 (53.6%)	0.038	1.56 (1.02–2.39)	40 (43.5%)	0.314	1.37 (0.74–2.54)
		GG	71 (20.6%)	58 (26.4%)	0.013	1.87 (1.14–3.07)	35 (38.0%)	0.001	2.92 (1.52–5.63)
		CC	101 (29.3%)	44 (20.0%)	–	1.00	17 (18.5%)	–	1.00
		CG + GG	244 (70.7%)	176 (80.0%)	0.014	1.65 (1.10–2.47)	75 (81.5%)	0.040	1.82 (1.02–3.24)
	rs5522	AA	286 (81.3%)	162 (73.6%)	–	1.00	61 (66.3%)	–	1.00
		AG	63 (17.9%)	49 (22.3%)	0.139	1.37 (0.90–2.09)	26 (28.3%)	0.015	1.93 (1.13–3.30)
		GG	3 (0.9%)	9 (4.1%)	0.013	5.29 (1.41–19.8)	5 (5.4%)	0.006	7.81 (1.81–33.5)
		AA	286 (81.3%)	162 (73.6%)	–	1.00	61 (66.3%)	–	1.00
		AG + GG	66 (18.8%)	58 (26.4%)	0.032	1.55 (1.03–2.31)	31 (33.7%)	0.002	2.20 (1.31–3.66)
	rs2248038	AA	210 (63.3%)	108 (53.2%)	–	1.00	39 (44.8%)	–	1.00
		AG	48 (14.5%)	29 (14.3%)	0.541	1.17 (0.70–1.96)	15 (17.2%)	0.130	1.68 (0.85–3.29)
		GG	74 (22.3%)	66 (32.5%)	0.008	1.73 (1.15–2.60)	33 (37.9%)	0.001	2.40 (1.40–4.09)
		AA	210 (63.3%)	108 (53.2%)	–	1.00	39 (44.8%)	–	1.00
		AG + GG	122 (37.7%)	95 (46.8%)	0.022	1.51 (1.06–2.15)	48 (55.2%)	0.002	2.11 (1.31–3.41)

OR: odds ratios.
CI: confident interval.

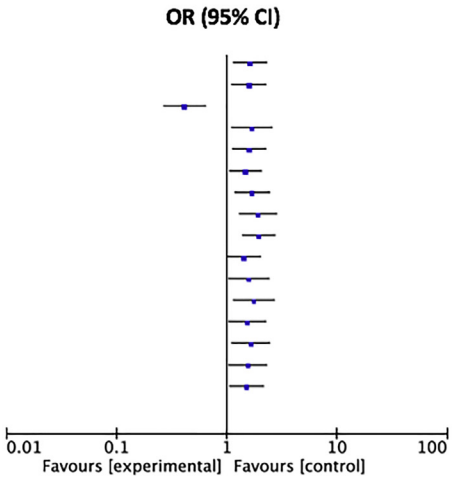
To explore potential interactions between polymorphisms significantly associated with osteoporotic fracture, demographic, anthropometric and clinical characteristics and the risk of osteoporotic fracture, we used a classification and regression tree approach (CART) [27]. CART analysis is a binary recursive partitioning method that produces a graphical structure that resembles a decision tree. In this context, we can identify subgroups for subjects with higher risk of suffering osteoporotic fracture. The approach begins with a set of patients, containing the entire sample, classified in groups by a dependent factor (in this case: patients with osteoporotic fracture 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). We did not perform CART analysis to evaluate the risk of clinical osteoporotic fracture as the sample size was too small. 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. A power = 0.99947 with degree freedom: 2; effect size small: 0.23; level of significance: 0.05 and sample size: 500 was reported. The analysis to detect the power of 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 performed with the R Stats package. Differences with a p-value < .05 were considered as statistically significant.

A)

Polymorphism	Patients Events	Patients Total	Controls Events	Controls Total	Weight	OR (95% CI)
EDN1 rs2070699	121	211	154	340	7.1%	1.62 [1.15, 2.30]
END1 rs3087459	85	216	98	339	6.5%	1.60 [1.11, 2.29]
IL8 rs4073	106	160	270	327	8.5%	0.41 [0.27, 0.64]
IL18RAP rs2293224	172	210	247	339	4.8%	1.69 [1.10, 2.58]
IL18RAP rs2293225	107	215	130	340	7.2%	1.60 [1.13, 2.26]
IL10RB rs2834167	121	227	150	344	7.9%	1.48 [1.05, 2.07]
IL6R rs2228145	150	213	195	334	6.4%	1.70 [1.18, 2.45]
SLC39A8 rs34411892	67	217	66	349	5.0%	1.92 [1.29, 2.84]
BANK1 rs3733197	119	216	133	344	6.5%	1.95 [1.38, 2.75]
REN rs11571080	137	220	189	353	7.8%	1.43 [1.02, 2.02]
AGT rs4762	53	179	64	305	4.7%	1.58 [1.04, 2.42]
AGT 5049	50	216	51	349	4.2%	1.76 [1.14, 2.72]
ACE rs4291	87	165	115	274	5.8%	1.54 [1.05, 2.27]
NR3C2 rs1040288	176	220	244	345	5.4%	1.66 [1.11, 2.48]
NR3C2 rs5522	58	220	66	352	5.3%	1.55 [1.04, 2.32]
NR3C2 rs2248038	95	203	122	332	7.0%	1.51 [1.06, 2.16]



B)

Polymorphism	Patients Events	Patients Total	Controls Events	Controls Total	Weight	OR (95% CI)
BANK1 rs3733197	56	90	133	344	20.2%	2.61 [1.62, 4.22]
NR3C2 rs1403142	50	90	259	347	20.2%	0.42 [0.26, 0.69]
NR3C2 rs1040288	75	92	244	345	19.4%	1.83 [1.03, 3.25]
NR3C2 rs5522	31	92	66	352	20.0%	2.20 [1.32, 3.66]
NR3C2 rs2248038	48	87	122	332	20.2%	2.12 [1.31, 3.42]

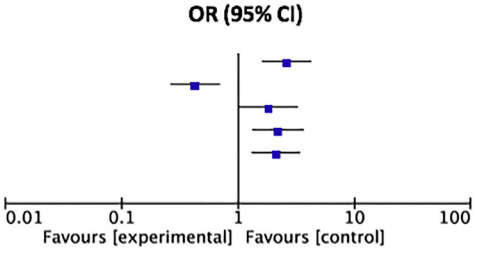


Fig. 1. Forest plot for the polymorphisms in genes involved in inflammation, in the NF-kB pathway and renin-angiotensin-aldosterone system that were significantly associated with the risk of osteoporotic fracture in patients with osteoporotic fracture (A) and in patients with clinical osteoporotic fracture (B). OR: odds ratio, CI: confident interval.

3. Results

We analysed 221 patients with osteoporotic facture, 92 of whom had a clinical fracture, and 354 controls after a 12–14 year follow up. The demographic, anthropometric and clinical characteristics of study subjects are shown in Table 1. There were significant differences only in the prevalence of hypertension and the family history of osteoporotic fractures. The prevalence of hypertension was significantly lower in patients with clinical fracture than in controls and a family history of osteoporotic fracture was higher in patients with fractures than in controls (Table 1).

Tables 2-4 show the polymorphisms that significantly associated with the risk of osteoporotic fracture. The distribution of the genotypes of these polymorphisms in the control sample was in Hardy-Weinberg equilibrium. With respect to the polymorphisms in genes involved in inflammation, the results showed that the variant genotypes of the polymorphisms in the *END1*, *IL8*, *IL18RAP*, *IL10RB* and *IL6R* genes were associated with an increased risk of osteoporotic fracture (Table 2). The TT genotype of the rs2293224 *IL18RAP* and the GG genotype of the rs2834167 *IL10RB* polymorphisms were also associated with clinical fracture (Table 2). The TT genotype of the rs4073 *IL8* polymorphism was associated with a reduced risk of osteoporotic fracture (Table 2). With respect to the polymorphisms in genes implicated in the NF-kB pathway, the CC genotype of the rs35411892 *SLC39A8* and the GG genotype of the rs3733197 *BANK1* polymorphisms were associated with an increased risk of osteoporotic fracture (Table 3). The rs3733197 *BANK1* polymorphism was also associated with an increased risk of clinical fracture (Table 3). With respect to the genes involved in RAAS, the variant genotypes of the polymorphisms in the *REN*, *AGT*, *ACE* and *NR3C2* genes were associated with an increased risk of osteoporotic fracture (Table 4). The rs1040288, rs5522 and rs2248038 *NR3C2* polymorphisms were also associated with clinical fracture (Table 4). In addition, the rs2071406 *AGT* and rs1403142 *NR3C2* polymorphisms were associated with a reduced risk of clinical fracture (Table 4). Co-dominance analysis confirmed that being a carrier of the variant allele of the polymorphisms described above was associated with osteoporotic fracture (Tables 2-4). Fig. 1 shows the forest plot for the polymorphisms in genes involved in inflammation, in the NF-kB pathway and renin-angiotensin-aldosterone system that were significantly associated with the risk of osteoporotic fracture in patients with osteoporotic fracture and in patients with clinical osteoporotic fracture. The results after Benjamini-Hochberg adjustment did not significantly differ from those reported above, although codominance analysis of the rs5522 *NR3C2* polymorphism showed no significant differences when comparing patients with osteoporotic fracture with controls and in the comparison of patients with clinical osteoporotic fracture and controls, the rs2293224 *IL18RAP*, rs2071406 *AGT* and rs1040288 *NR3C2* polymorphisms showed no significant differences (Supplementary table 2).

CART analysis showed that being a carrier of the C allele of the rs2228145 *IL6R* polymorphism was the principal genetic risk factor for osteoporotic fracture. In patients who were carriers of the C allele of the rs2228145 *IL6R* polymorphism, subjects with the T allele of the rs4762 *AGT* polymorphism showed the highest risk of osteoporotic fracture. Variant genotypes of the rs4073 *IL8*, rs2070699 *END1* and rs4291 *ACE* polymorphisms were also important genetic risk factors for fracture. CART analysis showed no associations between demographic,

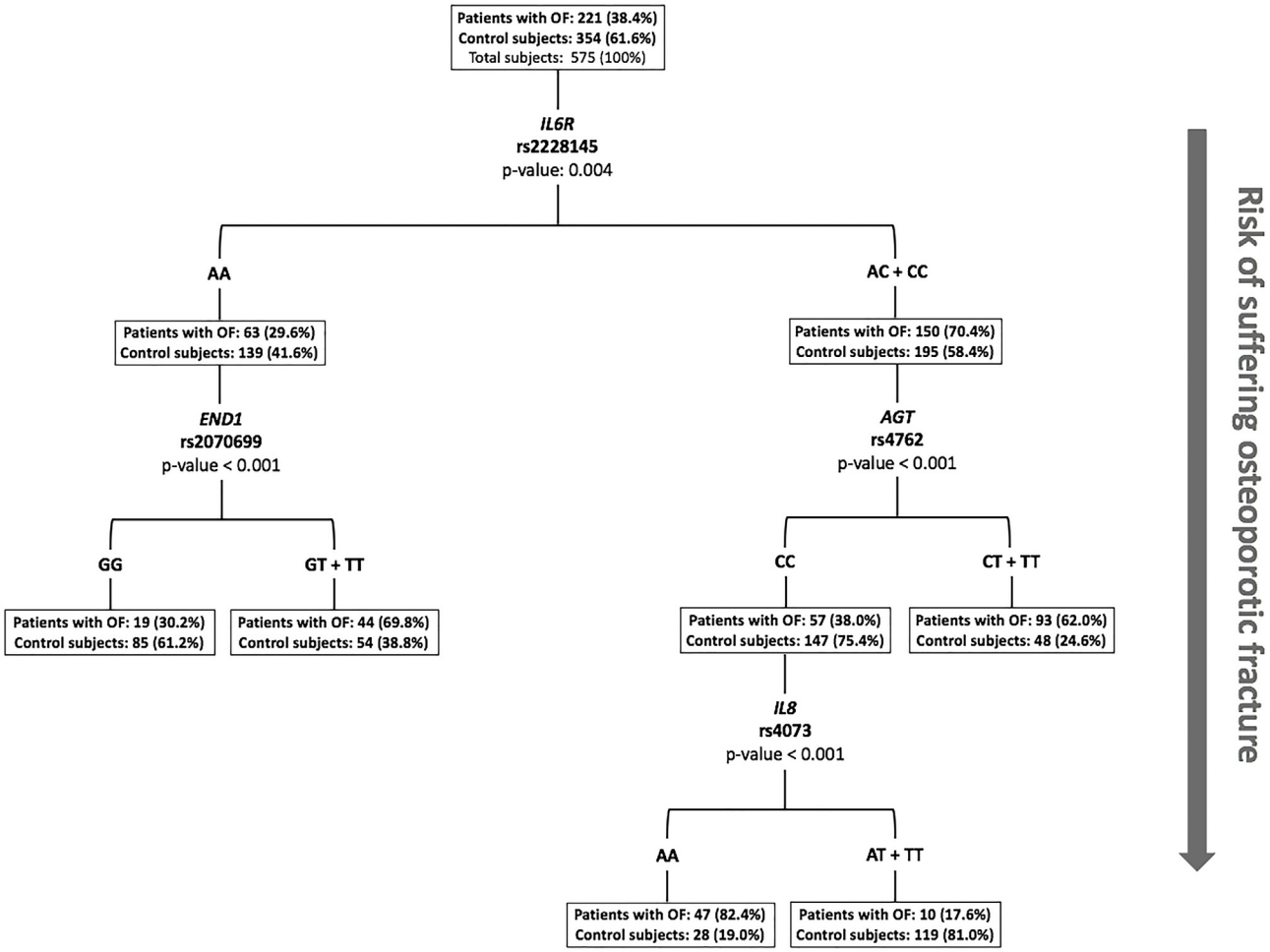


Fig. 2. Classification and regression tree (CART) analysis to explore the association between the variant allele of the polymorphisms included in the study and the risk of osteoporotic fracture. The procedure examined all possible independent variables (polymorphisms) and selected the one that was most closely associated with the risk of osteoporotic fracture, generating different groups (or nodes). OF: osteoporotic fracture.

anthropometric and clinical characteristics and the risk of osteoporotic fracture (Fig. 2).

4. Discussion

Osteoporosis and osteoporotic fractures are associated with a reduced quality of life [3,4]. Our results confirmed that genetic predisposition is crucial in the etiopathogenesis of osteoporosis [5,6], as the family history of osteoporotic fracture was significantly higher in patients with fracture than in controls.

Evidence suggests that pro-inflammatory cytokines are involved in bone metabolism, hence it had been reported that inflammation could be crucial in the etiopathogenic origin of osteoporosis. One characteristic of the aging process is increased production of pro-inflammatory cytokines may also be associated with an increased risk of osteoporosis [28–30] and an increased risk of osteoporosis in inflammatory conditions is reported (12). Genetic variants in inflammatory genes have been associated with the prevalence of osteoporosis, and polymorphisms in the *IL6* and *IL6R* genes have frequently been associated with an increased risk of bone loss [31–34]. Our results showed that the principal genetic risk factor for osteoporotic fracture is being a carrier of the C allele of the rs2228145 *IL6R* polymorphism. The rs2228145 *IL6R* polymorphism implies the Asp358Ala variation and is suggested that it could be associated with elevated IL6 pathway activity [35]. Our hypothesis is that the C allele of this polymorphism could increase inflammation, favouring the risk of osteoporotic fracture. We also found an association of the risk of fracture and gene polymorphisms in the *END1* (rs2070699 and rs3087459), *IL8* (rs4073 and rs2227543), *IL18RAP* (rs2293224 and rs2293225) and *IL10RB* (rs2834167) genes. These genetic variants have been associated with inflammatory and other diseases [36–39], but to the best of our knowledge, this is the first time that these inflammatory polymorphisms have been associated with the risk of osteoporotic fracture, as they could affect inflammation and, therefore the risk of fracture.

The NF-kB pathway is involved regulates some aspects of inflammation [13]. It is reported that the NF-kB pathway controls the expression of various pro-inflammatory genes inducing the synthesis of crucial cytokines and chemokines. NF-kB plays a a crucial role in the regulation of the activation, differentiation and survival of innate immune cells and inflammatory T cells [40–42]. Therefore, deregulation of the NF-kB pathway could contribute to the etiopathogenesis of inflammatory diseases [13]. The NF-kB pathway is also crucial in the differentiation and activation of osteoclasts and, therefore, in the activation of bone resorption [16–18]. Our results show that the CC genotype of the rs35411892 *SLC39A8* polymorphism and the GG genotype of the rs3733197 *BANK1* polymorphism were associated with an increased risk of osteoporotic fracture. The *SLC39A8* gene is a negative feedback regulator of the NF-Kb pathway [43] and the *BANK* gene is crucial in calcium mobilization [44,45]. Thus, we hypothesize that these polymorphisms could modify the NF-kB pathway and thereby modify the inflammatory state or the activation of osteoclasts, which could increase the risk of osteoporotic fracture. This is the first report to

show an association between genetic variants in the *SLC39A8* and *BANK1* genes and the risk of osteoporotic fracture. On the other hand, it has also been reported that RAAS modulates the activation-differentiation of osteoclasts and inflammation by NF-kB pathway activation [14,15,19–21] and that RAAS has been associated with alterations in bone metabolism, particularly bone loss [14,46–48]. Our results showed an association between the risk of fracture and gene polymorphisms in the *REN* (rs11571080), *AGT* (rs4762, rs5049, rs2071406), *ACE* (rs4291) and *NR3C2* (rs1403142, rs1040288, rs5522, rs2248038) genes. These genetic variants have been associated with hypertension or other cardiovascular diseases [49–51], but this is the first time they have been associated with the risk of bone fracture. These genetic variants could modify RAAS regulation and thereby alter osteoclast activation or inflammation, increasing the risk of osteoporotic fracture. According to our results the second principal genetic risk factor for bone fracture is being a carrier of the T allele of the rs4762 *AGT* polymorphism. This is a missense variant that implies the Thr207Met variation in the angiotensinogen protein, a vital module of RAAS [52]. Our hypothesis is that the rs4762 *AGT* polymorphism could modify the regulation of RASS and thereby increase the risk of osteoporotic fracture.

The study had some limitations. First, it might have been interesting to evaluate the influence of these polymorphisms on the risk of osteoporosis, but we only recorded calcaneal bone densitometry making it difficult to diagnose osteoporosis. Secondly, the size of the sample was limited. Other limitation, it was that the associations reported in our results should be taken with caution due to the high number of comparisons made, our work 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 follow up, and the similarity of study and control subjects, which is important in determining the influence of genetic factors.

In summary, we found an association between polymorphisms in genes implicated in inflammation, NF-kB pathway and RASS 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 about the genetic factors associated with inflammation, the NF-kB pathway and RAAS, which are involved in the risk of osteoporotic fracture. Future studies are required to define the role of these polymorphisms in the risk of osteoporotic fracture.

CRediT authorship contribution statement

Ricardo Usategui-Martín:Conceptualization, Formal analysis, Validation, Writing - original draft, Writing - review & editing.**Verónica Lendinez-Tortajada**:Investigation, Formal analysis, Validation, Writing - review & editing.**José Luis Pérez-Castrillón**:Conceptualization, Formal analysis, Validation, Writing - review & editing.**Laisa Briongos-Figuero**:Resources, Writing - review & editing.**Jesica Abadía-Otero**:Resources, Writing - review & editing.**Javier Martín-Vallejo**:Formal analysis.**Francisco Lara-Hernandez**:Investigation, Writing - review & editing.**Felipe J. Chaves**:Investigation, Formal analysis, Validation, Writing - review & editing.**Ana B. García-Garcia**:Investigation, Formal analysis, Validation, Writing - review & editing.**Juan Carlos Martín-Escudero**:Conceptualization, Resources, Formal analysis, Validation, Project administration, Supervision, Writing - review & editing.

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Declaration of competing interest

The authors report no potential conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bone.2020.115477.

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