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Bone mineral mass is associated with interleukin 1 receptor autoantigen and TNF-α gene polymorphisms in post-menopausal Mediterranean women

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ABSTRACT. Bone mass is known to be under genetic control. Interleukin-1 (IL-1), interleukin-6 (IL-6) and TNF-α are strong inductors of bone resorption. The estrogenic deficiency that occurs during menopause leads to an increase in the production of these cytokines. We analyzed the genetic susceptibility of several polymorphisms of the interleukin-1 receptor antagonist (IL-1ra), IL-6 and TNF-α genes in lumbar spine and hip bone mass in a sample of post-menopausal Caucasian Mediterranean women with osteoporosis. 104 post-menopausal osteoporotic women (58.6±4.8 yr) and 51 post-menopausal women without osteoporosis as the control group (57.2±4.5 yr) were studied. The osteoporotic group was in turn sub-classified into severe and non-severe osteoporosis. The variable number of tandem repeats IL1-ra, IL-6 SfaNI and TNF-α NcoI genetic polymorphisms were studied. Biochemical markers of bone turnover were measured in blood and urine. Women carrying the A2 allele (A2+) of the IL-1ra gene showed greater BMD in the lumbar spine (*p*=0.02) and hip (*p*=0.006), compared to those not carrying the allele (A2-). The IL-6 polymorphism studied in its 5' flanking region did not show any association with BMD values. The TNF-α gene G allele was associated with a greater bone mass in the non-severe osteoporotic subgroup, both in the lumbar spine (*p*=0.0007) and in the hip (*p*=0.02). Likewise, genotype combination A2+GG was associated to a greater hip BMD at the femoral neck and Ward triangle levels (*p*=0.02). We conclude that both IL-1ra and TNF-α can be candidate *loci* to be studied in the susceptibility to develop post-menopausal osteoporosis.
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INTRODUCTION

Osteoporosis is a major health problem and is responsible for one third of all fractures that appear in women over 65 yr of age (1, 2). It has a multi-factorial origin, though it has been possible to establish a genetic contribution to its etiology by means of studies on family and twins (3-5). The specific gene and allelic variants that confer osteoporotic risk have remained largely undefined, but the number of candidates has increased steadily in recent years. Previous genetic studies have been aimed at analyzing candidate genes including those that encode for growth factors (6), calciotropic hormones (7, 8), and bone matrix proteins that directly participate in BMD control (9, 10). Most of these studies suggest a polygenic susceptibility with a different degree of participation of each analyzed marker.

More recently, it has been demonstrated that the maintenance of the remodeling cycle is locally determined by cytokines. In this sense interleukin-1 (IL-1) is a potent bone resorption inductor, as it is observed for TNF-α and interleukin-6 (IL-6), which are able to stimulate the osteoblastic activity and osteoclastic differentiation (11).

In vitro studies have shown that estrogens possess a regulatory action over the cytokine network at a local level (12). The lack of estrogens would cause an activation in cytokine production which, in turn, would increase bone resorption. The activity of these cytokines can be modified according to certain genetic polymorphisms (13, 14) and thus participate in the susceptibility to osteoporosis.

Key-words: IL-1ra, TNF-α, gene polymorphisms, bone markers, post-menopausal Mediterranean women.

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Polymorphisms of genes for cytokines and factors involved in regulation of bone cell function have been involved in bone phenotypic differences in human and mouse models. The studies published to date have found a weak association between both the A2 allele of the IL-1 receptor antagonist (IL-1ra) (15) and a repeat AT polymorphism in the 3' flanking region of the IL-6 (16), with lumbar spine bone mass loss in post-menopausal women. IL-6 gene has also been linked to bone density in family linkage studies (17), though other Authors have not confirmed this association in recent works (18). The narrow relationship that these 3 proinflammatory cytokines maintain, as well as their regulation and the decisive role they presumably play in bone remodeling, make them candidates to be analyzed as markers of genetic susceptibility in osteoporosis.
The present work has studied IL-1ra, TNF-α and IL-6 genetic polymorphisms in a Mediterranean post-menopausal women sample and their role as osteoporosis susceptibility markers.

Experimental subjects

A case-control study was designed starting from a cross-sectional one. Patients were recruited from the Rheumatology outpatient's unit of the Hospital Universitari Joan XXIII (Tarragona, Spain) between June to December 1999. The study was approved by the local Ethics Committee and all subjects gave their informed consent.
A total of 104 consecutive osteoporotic post-menopausal women (58.6±4.8 yr) were included in the study. An age-matched control group was composed of 51 post-menopausal women (57.2±4.5 yr), with a minimum of 2 yr progress and with normal bone absorptiometry (DEXA). Both populations were from a North-Eastern region of Spain. The absorptiometric diagnosis of either normality or osteoporosis was performed according to the WHO diagnostic categories. The severe osteoporotic subset was composed of those women having osteoporosis by absorptiometry and who had suffered from a fracture (spine, hip, distal radius or proximal humerus) as stated by WHO criteria (19).
None of the women were suffering from a chronic or acute disease at the moment of the study nor were they taking any pharmacological treatment that could modify bone metabolism. All women abstained from toxic habits such as alcohol or tobacco and none of them had undergone previous surgery on the back-lumbar spine or intra-raquideum contrast which could alter BMD levels. A lumbar spine radiograph was performed on each patient (anteroposterior and lateral) in order to discard spondylosis or aortic calcifications.

MATERIALS AND METHODS

BMD was measured in the lumbar spine (anteroposterior of L2-L4) and proximal femur, using a DPX-L Double Energy X-Ray Absorptiometer (DEXA, Lunar®). In the proximal femur 3 sites were measured: the femoral neck, the trochanteric region, and Ward's triangle within the femoral neck. For the diagnosis of post-menopausal osteoporosis, patients should present abnormal absorptiometric values in lumbar spine, independently of those found in the femoral neck.

Laboratory methods

The following biochemical markers of bone turnover were analyzed in a serum sample: total ALP and osteocalcin; and in an urine sample collected according to the Nordin method (20) were determined N-telopeptides and the ratio calcium/creatinine.
Total ALP, calcium and creatinine were analyzed by colorimetric methods. Osteocalcin was determined by means of a RIA (Diagnostic System Laboratories, U.S.A.). N-telopeptides (NTx) were studied by ELISA (Ortho-Clinical Diagnostics, Johnson & Johnson, U.S.A.). NTx values were corrected according to urine creatinine values.
A 10-ml sample of venous blood was collected in an EDTA vacutainer. Genomic DNA was isolated from the buffy coat using QiaAMPTM spin columns (Qiagen, Chatsworth, U.S.A.). 100 ng of DNA were used for the polymorphism analysis by PCR.

Variable number of tandem repeats (VNTR) IL-1ra gene analysis

The second intron of the IL-1ra gene, located in chromosome 2, region q13- 14 was analyzed using primers: 5' CTCAGCAACACTCCTAT 3' and 5' TCCTGGTCTGCAGGTAA 3'. PCR conditions comprised a denaturing step at 94 C for 30 s and then 30 cycles of 94 C for 30 s, 58 C for 1 min and 72 C for 1 min.
VNTR IL-1ra analysis was performed in 51 controls and 104 osteoporotic women. A five-allele polymorphism which produced 5 bands of different sizes was obtained. A 410 bp band corresponded to A1 allele (4 repeats), a 240 bp band corresponded to A2 allele (2 repeats), a 500 bp band corresponded to A3 allele (5 repeats), a 325 bp band corresponded to A4 allele (3 repeats), and a 595 bp band corresponded to A5 allele (6 repeats).

Restriction fragment length polymorphism (RFLP)-IL-6 gene analysis

The replacement of G by C at position 174 of the IL-6 gene was analyzed by SfaNI RFLP using primers 5' TGACTTCAGCTTTACTCTTTGT 3' and 5' CTGATTGGAAACCTT ATTAAG 3'. PCR conditions comprised a denaturing step at 95 C for 10 min and then 35 cycles of 94 C for 1 min, 58 C for 1 min, 35 s and 72 C for 1 min.
This genetic region was analyzed in 51 controls and 94 osteoporotic women. The identified genotypes were named according to the presence or absence of the enzyme restriction sites, so SfaNI (G/G), (G/C), and (C/C) are homozygotes for the presence of the site (140/58 bp), heterozygotes for the presence and absence of the site (198/140/58 bp), and homozygotes for the absence of the site (198 bp), respectively.

Table 1 - *General characteristics of all subjects.*

	Controls (no.=51)	Non-severe osteoporosis (no.=58)	Severe osteoporosis (no.=46)
Age (yr)	57.5±4.6	59.6±4.0	58.2±5.3
BMI (kg/m^2)	28.2±3.1	27.0±3.5	27.3±4.0
Dietary calcium (mg/day)	615.7±274.3	587.1±260.9	584.0±283.6
Years since menopausal age	11.2±4.5	13.6±8.3	11.9±6.4
ALP (U/l)	174.4±38.1	176.5±41.8	180.2±51.5
Log Osteocalcin (ng/ml)	1.01±0.28*	1.17±0.28	1.16±0.31
Log NTx/Cr (nmol/mmol)	1.89±0.25	1.92±0.29	1.98±0.25
Ca/Cr (mmol/mmol)	0.41±0.18	0.44±0.22	0.48±0.28
Lumbar spine BMD (g/cm^2)	1.11±0.07**	0.81±0.04	0.74±0.10
Femoral neck BMD (g/cm^2)	0.92±0.10**	0.75±0.08	0.72±0.09
Ward triangle BMD (g/cm^2)	0.83±0.11**	0.62±0.09	0.59±0.11
Trochanter BMD (g/cm^2)	0.81±0.09**	0.66±0.10	0.63±0.09
Lumbar spine BMD (T-score)	-0.73±0.64**	-3.21±0.38	-3.80±0.82
Lumbar spine BMD (Z-score)	0.49±0.73**	-1.73±0.35	-2.58±0.77

Ca: Calcium; Cr: Creatinine; NTx: N-telopeptides. Values are expressed as mean±SD. *p=0.01; **p=0.0001.

RFLP-308 TNF-α gene analysis

The replacement of of G by A at position -308 of the TNF-α gene was analyzed by NcoI RFLP using primers 5' AGGCAATAGGTT-TT-GAGGGCCAT 3' and 5' TCCTCCCTG CTCCGATTCCG 3'. PCR conditions comprised an initial cycle of 94 C 3 min, 60 C 1 min and 72 C 1 min, and then 35 cycles of 94 C 1 min, 60 C 1 min and 72 C 1 min.

This genetic region was analyzed in 51 controls and 94 osteoporotic women. The identified genotypes were named according to the presence or absence of the enzyme restriction sites, so NcoI (G/G), (G/A), and (A/A) are homozygotes for the presence of the site (87/20 bp), heterozygotes for the presence and absence of the site (107/87/20 bp), and homozygotes for the absence of the site (107 bp), respectively.

Table 2 - *Relationship between clinic and absorptiometric parameters and IL-1ra, IL-6 and TNF-α genotypes in the post-menopausal osteoporotic women group.*

	IL-1ra gene		IL-6 gene		TNF-α gene	
	Allele A2+ (no.=41)	Allele A2- (no.=63)	GG (no.=30)	CC+CG (no.=64)	(no.=78)	AA+GA (no.=16)
Age (yr)	59.3±4.6	58.8±4.8	59.4±3.9	58.8±5.1	58.7±4.4	59.8±5.4
BMI (kg/m^2)	26.9±3.6	27.3±3.8	26.5±3.9	27.1±3.9	27.2±3.8	26.7±3.8
Dietary calcium (mg/day)	593.9±293.3	583.3±257.3	690.5±218.9	541.3±253.3	600±261.7	531.2±345.8
Years since menopausal age	13.6±7.5	12.2±7.7	11.8±6.9	12.0±6.7	12.2±6.8	13.4±9.8
ALP (U/l)	175.6±40.6	180.4±49.9	174.5±45.8	182.1±49.9	180.7±49.4	165.5±33.2
Log osteocalcin (ng/ml)	1.15±0.29	1.17±0.30	1.13±0.27	1.19±0.29	1.16±0.31	1.10±0.25
Log NTx/Cr (nmol/mmol)	1.93±0.26	1.96±0.28	1.96±0.24	1.96±0.29	1.96±0.28	1.92±0.22
Ca/Cr (mmol/mmol)	0.47±0.22	0.46±0.27	0.50±0.27	0.46±0.27	0.46±0.24	0.51±0.29
Lumbar spine BMD (g/cm^2)	0.79±0.06	0.77±0.09	0.78±0.11	0.77±0.09	0.78±0.09	0.76±0.08
Femoral neck BMD (g/cm^2)	0.77±0.08**	0.72±0.09	0.73±0.09	0.74±0.08	0.74±0.09	0.72±0.09
Ward triangle BMD (g/cm^2)	0.63±0.09*	0.59±0.10	0.62±0.12	0.61±0.10	0.61±0.11	0.58±0.11
Trochanter BMD (g/cm^2)	0.66±0.10	0.63±0.10	0.66±0.12	0.64±0.09	0.65±0.11	0.63±0.08
Lumbar spine BMD (T-score)	-3.33±0.49	-3.56±0.78	-3.46±0.88	-3.59±0.75	-3.44±0.72	-3.70±0.62
Lumbar spine BMD (Z-score)	-1.91±0.69*	-2.23±0.72	-2.01±0.62	-2.23±0.83	-2.11±0.76	-2.18±0.61

Ca: Calcium; Cr: Creatinine; NTx: N-telopeptides. Values are expressed as mean±SD. *p=0.02; **p=0.006.

Statistical analysis

The SPSS/PC+ statistical program (v. 6.1.3) was used. Results are given as mean±SD. Non-parametric variable distribution was normalized by logarithmic transformation. Differences between group means were tested by *t*-test or one-way analysis of variance depending on the number of variable categories. The chi-square test was used to compare frequencies. Multiple linear regression analysis was performed to study the relationship between variables controlling for confounding factors. Statistical significance occurred if a computed two-tailed probability value was less than 5% (p=0.05).

Due to the low observed frequency of some genotypes in the studied *loci*, we gathered genotypes according to the following criteria: for the IL-1ra gene according to previous described associations (15) and for the TNF-α and IL-6 genes according to differences in transcription rates of these genes' mRNA (13, 21).

RESULTS

Clinical, metabolic and densitometric characteristics of the studied populations are shown in Table 1. The severe osteoporosis group displayed significant differences in BMD, T-score and Z-score compared to the non-severe osteoporosis and control groups.

Genotypic and allelic frequencies between the control and osteoporotic groups were similar. The genotypic frequencies for the IL-1ra gene were: A1A1 51.9% *vs* 57.7%, A1A2 33.3% *vs* 34.6%, A1A3 1.9% *vs* 2.9%, A2A2 13% *vs* 4.8%; for the IL-6 gene: A1A1 11.9% *vs* 25.4%, A2A2 42.9% *vs* 31.3%, A1A2 45.2% *vs* 43.3%; for the TNF-α: T1T1 74% *vs* 83%, T2T2: 0% *vs* 4.3%, T1T2 26% *vs* 12.8%; %; for control and osteoporotic women, respectively. The allelic frequencies for the IL-1ra gene were: A2+ allele 29.6% *vs* 22.1%, A2- allele 70.4% *vs* 77.9%; for the IL-6 gene: A1 allele 34.5% *vs* 47%, A2 allele 65.5% *vs* 53%; for the TNF-α gene: G allele 87% *vs* 89.4%, A allele 13% *vs* 10.6%; for control and osteoporotic women, respectively. Observed genotypic and allelic frequencies did not differ from that predicted from Hardy-Weinberg equilibrium.

There were no differences in the distribution of the biochemical markers of bone turnover among the studied genotypes (Table 2). Patients carrying the A2 allele (A2+) of the IL-1ra gene showed greater BMD in lumbar spine (p=0.02) and hip (p=0.006), compared to those not carrying the allele (A2-). The remaining genotypic groups performed for IL-6 and TNF-α showed no differences in bone mass (Table 2). When osteoporotic women were classified according to their severity degree, we observed that the presence of the GG –308 TNF-α genotype was associated with a greater bone mass in the non-severe osteoporotic subgroup, either in the lumbar spine (p=0.0007) and in hip (p=0.02) (Table 3).

Table 3 - *Relationship between clinic and absorptiometric parameters and TNF-α polymorphism in the non-severe and severe post-menopausal osteoporotic subgroups.*

	TNF-α gene	
	GG	AA+GA
Non-severe osteoporosis		
No.	41	9
Age (yr)	58.7±4.0	61.7±3.6
BMI (kg/m^2)	26.9±3.4	26.9±4.3
Dietary calcium (mg/day)	606.1±252.5	522.2±338.3
Menopausal age (yr)	12.2±7.9	15.6±8.6
Lumbar spine BMD (g/cm^2)	0.83±0.04	0.77±0.06
Femoral neck BMD (g/cm^2)	0.76±0.08*	0.69±0.09
Ward triangle BMD (g/cm^2)	0.64±0.09**	0.54±0.09
Trochanter BMD (g/cm^2)	0.67±0.11*	0.60±0.07
Lumbar spine BMD (T-score)	-3.11±0.32°	-3.60±0.46
Lumbar spine BMD (Z-score)	-1.72±0.34	-1.79±0.44
Severe osteoporosis		
No.	37	7
Age (yr)	58.76±4.8	57.4±6.5
BMI (kg/m^2)	27.4±4.4	26.6±2.3
Dietary calcium (mg/day)	593.2±274.9	542.8±382.3
Menopausal age (yr)	12.3±5.5	10.6±11.3
Lumbar spine BMD (g/cm^2)	0.74±0.11	0.74±0.09
Femoral neck BMD (g/cm^2)	0.71±0.09	0.75±0.10
Ward triangle BMD (g/cm^2)	0.58±0.11	0.63±0.11
Trochanter BMD (g/cm^2)	0.62±0.10	0.67±0.08
Lumbar spine BMD (T-score)	-3.81±0.86	-3.83±0.81
Lumbar spine BMD (Z-score)	-2.55±0.85	-2.68±0.41

Values are expressed as mean±SD. *p=0.02; **p=0.006; °p=0.0007.

The genotype interaction analysis showed an association between the IL1-ra A2 allele (A2+) together with the GG –308 TNF-α and a greater hip BMD at the femoral neck and Ward triangle levels (p=0.02) (Table 4).

Multiple regression analysis of the observed univariate associations between the genotypes and

Table 4 - *Relationship between absorptiometric parameters and IL1-ra and TNF-α genotypic combinations in the osteoporotic women group.*

	Genotypic combination	
	A2+GG (no.=31)	Remainder genotypes (no.=63)
Lumbar spine BMD (g/cm^2)	0.79±0.07	0.77±0.09
Femoral neck BMD (g/cm^2)	0.76±0.08*	0.72±0.09
Ward triangle BMD (g/cm^2)	0.64±0.09*	0.59±0.11
Trochanter BMD (g/cm^2)	0.66±0.11	0.64±0.10
Lumbar spine BMD (T-score)	-3.34±0.54	-3.55±0.75
Lumbar spine BMD (Z-score)	-1.96±0.73	-2.19±0.72

Values are expressed as mean±SD. *p=0.02.

Table 5 - *Multiple regression analysis R and P values of the observed univariate associations.*

	Lumbar spine BMD (g/cm^2)	Femoral neck BMD (g/cm^2)	Ward triangle BMD (g/cm^2)	Trochanter BMD (g/cm^2)	Lumbar spine BMD (T-score)	Lumbar spine BMD (Z-score)
IL-1ra						
Allele A2+	R=0.35	R=0.43	-	-	-	R=0.45
	P=0.04	P=0.002		-	-	P=0.04
TNF-α*						
GG	R=0.59	-	R=0.42	-	R=0.61	-
	P=0.009	P=0.107	P=0.01	P=0.130	P=0.003	
IL-1ra+TNF-α						
A2+GG	-	R=0.40	R=0.32	-	-	-
		P=0.01	P=0.03			

*In the non-severe osteoporosis group. All associations were controlled for age, BMI and years since menopause. P: significance level; R: regression coefficient; -: not applicable.

the densitometric measures were performed, maintaining the contribution of the genotypes to BMD parameters except for the GG-TNFα genotype and the femoral neck and trochanter BMD in the non-severe osteoporosis group (Table 5).

DISCUSSION

There have been several reports of gene variants being associated with susceptibility to osteoporosis. This is the first one to implicate a combination of cytokine gene variants in susceptibility to BMD. This data suggest that carriage of "A2" allele of IL-1ra and GG genotype of -308 TNF-α genes is significantly associated with the presence of a greater bone mass in post-menopausal women.

The distribution of the studied genotypes was similar in both the osteoporotic (severe and non severe) and control groups. However, it must be emphasized that the specific effect of the single analyzed gene for these mutations could not be completely discarded in order to confer a greater susceptibility to develop osteoporosis.

Most of the *in vitro* studies suggest that IL-1, IL-6 and TNF-α contribute to bone remodeling at a local level. Moreover, in post-menopausal women the level of cytokines secreted by non-stimulated monocytes has been found to be significantly increased for IL-1 β and TNF-α (22-24).

IL-1 is a powerful stimulant of osteoclastic bone resorption, and its activity is increased in estrogen-deficient states with increased production of IL-1 and inhibition of the IL-1ra. Treatment with IL-1ra blocks the bone loss associated with ovariectomy in animals (25). In a previous work analyzing the same VNTR IL-1ra polymorphism of the second intron of the gene, the presence of the A2 allele was associated with a low rate of bone loss in lumbar spine in early post-menopause (15). More recently, Langdahl *et al.* have reported an association between this region and osteoporotic fractures (22). In our patients the presence of the A2 allele was associated with a higher BMD in all the analyzed areas, especially in the lumbar spine and hip. As regards our findings, which partially agree with those of Keen *et al.* (15), these results suggest a protective role of this allele over bone tissue mainly in femoral neck. It is tempting to speculate that this action may represent a protective effect against hip fracture which is the main cause of the morbi-mortality that appears in late post-menopause. However, more extensive longitudinal studies are needed to confirm this hypothesis.

On the other hand, TNF-α by itself is an important contributor to the pathophysiology of bone loss in osteoporosis. A lower transcription rate of the TNF-α mRNA associated with low TNF activity in patients carrying the G/G genotype has been demonstrated *in vitro* and *in vivo* studies (26). This may be clinically translated into a high BMD as it has been observed in the group with non severe osteoporosis mainly in the lumbar spine zone (Table 3); however, the specific effect of the homozygous state for this mutation in severe osteoporosis could not be fully addressed in our study because of the small sample size. This genotype has been found to be associated with other chronic age-related diseases as obesity and insulin-resistance (27). It is interesting to note that body size, as measured by bw, lean mass or fat mass, has one of the strongest associations with bone density and bone mass in a wide range of studies (28). The same genotype involved in fat mass (27) seems also to be linked to osteoporosis. Thus, different developmental diseases (osteoporosis, obesity, atherosclerosis and insulin-resistance) share some evolutive characteristics with an increased incidence as the population grows older

and with a predominant role of the cytokine network in their pathogenesis (29).

In contrast to previous studies, we have not found any positive association between the IL-6 genotype and BMD. Still, one must take into account that in our study we have analyzed a mutation located 5' upstream due to its effect on the IL-6 mRNA transcription, unlike to what reported by other Authors where the region associated to a different BMD was located 3' downstream of this gene.

Despite the limited sample size, the finding of a genotype combination which associates to a greater BMD in osteoporotic women (A2+GG for IL-1ra and TNF-α genes, respectively) mainly at the expense of hip bone mass, suggests a protective role of these genes and is another argument in favor of the genetic participation of these cytokines in the bone remodeling process.

These results, together with those obtained by other groups on proinflammatory cytokine genetic variations, make it necessary to enlarge the study of these genetic regions in relation to the pathogenesis of osteoporosis.

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