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ORIGINAL ARTICLE

Interleukin-6 promoter polymorphism is associated with bone quality assessed by calcaneus ultrasound and previous fractures in a cohort of 75-year-old women

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Abstract Interleukin 6 (IL-6) is a multifunctional cytokine and a potent stimulator of bone resorption and has been implicated in the pathogenesis of osteoporosis in postmenopausal women. The aim of this study was to investigate if a functional IL-6 promoter polymorphism (−174) was related to bone mass and fractures in a cohort consisting of 964 postmenopausal Caucasian women aged 75 years. Bone mineral density (BMD; g/cm^2) of the femoral neck, lumbar spine and total body was measured using dual energy X-ray absorptiometry (DXA). Quantitative ultrasound (QUS) was also measured in the calcaneus and quantified as speed of sound (SOS; m/s), broadband ultrasound attenuation (BUA; dB/MHz), and stiffness index (SI). IL-6 genotypes was determined by restriction fragment length polymorphism (RFLP) using the restriction enzyme NlaIII. The frequencies of the different IL-6 genotypes were 27.5% (GG), 47.9% (GC), 24.6% (CC). The IL-6 polymorphism (presence of G) was independently related to a lower stiffness ($\beta = -0.07$; $P = 0.03$) and BUA ($\beta = -0.08$; $P = 0.02$), but not to BMD at any site measured by DXA. In the cohort, 420 subjects (44%) reported at least one fracture during their lifetime, and 349 (36%) reported at least one fracture after the age of 50. Using binary logistic regression, the IL-6 polymorphism (presence of G) was significantly related to an increased risk of a previous fracture during life (odds ratio 1.46, 95% CI 1.08–1.97) and to an increased risk of a fracture occurring after 50 years of age (odds ratio 1.37, 95% CI 1.004–1.88). The risk was further increased for fractures grouped as osteoporotic fractures (odds ratio 1.67, 95% CI 1.14–2.45), including forearm fractures (odds ratio 1.59, 95% CI 1.05–2.40). In conclusion, presence of G allele in the IL-6 promoter polymorphism at position –174 is independently related to previous fractures in postmenopausal women. This association may be related primarily to an altered bone quality identified by QUS and not a lower bone mass. This is also the first demonstration of association of IL-6 gene polymorphism to calcaneal QUS.

Keywords Fractures · IL-6 polymorphism · Postmenopausal women · QUS

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Introduction

Osteoporosis is a global health care problem, increasing largely due to improved health in the elderly, preventive health measures and subsequent delay in mortality [1]. It is characterized by reduced bone mass, micro structural deterioration with advancing age and an increase in fracture rate. Fractures as a result of a weak and osteoporotic bone are an increasing cause of mortality and painful physical impairment of the elderly, particularly in the western world [2,3,4].

Several studies have reported a plenitude of risk factors for the osteoporotic fractures including: advanced age, previous fractures, low bone mineral density (BMD), low body mass index (BMI), muscle weakness, impaired vision, cognitive impairment, corticosteroid therapy and use of sedatives [5]. However, relatively few efforts have been made to investigate whether genetic factors contribute directly to the fracture itself [6,7].

A possible candidate gene for low bone density as well as fractures is interleukin 6 (IL-6), a multifunctional cytokine [8,9,10] that stimulates osteoclast formation and bone resorption both in the absence [11,12] and presence of the IL-6 soluble receptor [13,14]. Stimulation of bone resorption by IL-6 (in the presence of the soluble receptor) has been associated with the signal transducing protein gp 130 as well as to increased mRNA and protein expression of receptor activator of NF-kB ligand [14]. It has been demonstrated that the levels of IL-6 increase during menopause in response to decreasing estradiol levels [15,16,17,18] and osteoporosis in mice due to ovariectomy has been causally linked to IL-6 [15]. Clinical studies have suggested that IL-6 mRNA expression is enhanced in bone explants derived from patients with osteoporotic vertebral fractures [19]. Furthermore, IL-6 has also been implicated in the pathogenesis of bone loss in conditions characterized by excessive osteoclast development and focal osteolytic lesions such as rheumatoid arthritis, periodontal disease, Paget's disease, multiple myeloma and hyperparathyroidism [20,21,22,23,24].

A functional polymorphism in the IL-6 promoter that is associated with an increased IL-6 promoter activity and increased plasma levels of IL-6 [25] was recently discovered to be related to low BMD in young men [26].

In the present study, we have investigated whether the previously demonstrated polymorphism in the IL-6 promoter is related to bone mineral density, quantitative ultrasound in the calcaneus and fractures in a large and well characterized cohort of Swedish women, all aged 75 years, approaching an age with increasing fracture incidence.

Materials and methods

Subjects

The Malmö Osteoporosis Prospective Risk Assessment (OPRA) study consists of 1044 women, all 75 years of age, randomly selected from the population files of the city of Malmö, Sweden. Inclusions were made during 1995–1999 by sending out letters to 1604 women 1 week after their 75th birthday. Five hundred and sixty women did not participate; 13 died shortly after the invitation, 139 could not come due to illness, 376 women were not interested or could not attend due to reasons other than illness, and 32 women were not reached despite repeated letters and phone calls. The results from this study are based on the 964 women that were possible to genotype. The primary assessments included bone density measurements, a comprehensive questionnaire and blood samples. Informed consent was obtained for all subjects in the study, which also was approved by the local ethics committee.

Bone mass

Bone mineral density (BMD) was assessed in at least one site in 995 women with a Lunar DPX-L-equipment (Lunar, Madison, Wisc., USA). Assessment of total body, lumbar spine (L2–L4) and hip (femoral neck and trochanter) BMD was performed in 931, 974 and 951 women, respectively. The technical quality was continuously checked according to standard procedure and with phantom measurements. The precision of our equipment has previously been determined to 0.5% (lumbar spine), 1.6% (femoral neck), and 2.2% (trochanter) [27].

Quantitative ultrasound of the calcaneus (QUS) was performed with a Lunar Achilles ultrasound equipment in 854 women. The precision of the ultrasound equipment as assessed accordingly in our laboratory has also earlier been determined to be 1.5% in healthy volunteers [27]. The stability of the apparatus was controlled for by daily calibration with two phantoms provided by the manufacturer. The speed of sound, broadband ultrasound attenuation and stiffness index was calculated.

Both dual-energy X-ray absorptiometry (DXA) and ultrasound measurements were performed on the same pieces of equipment and by the same two technicians during the entire study period. The main reason for not being examined with DXA was high body weight, disability not allowing supine position for the time required for measuring, or prior surgery interfering with the measurement. For QUS the main reason for not being examined was calcaneus instrument failure and water leakage, which occurred 6 times according to records.

Balance

A modified Romberg balance test was performed and consisted of five parts, (1) standing on both feet with eyes closed, (2 + 3) standing on one foot at a time with eyes open, and (4 + 5) standing on one foot at a time with eyes closed. The time until balance was lost was recorded (or maximum 60, 30, 30, 30, 30 s, respectively). A result less than 5 s was recorded as 0. Three trials per test were allowed, the best was registered and all results were added to produce a score (minimum 0, maximum 180 s).

Questionnaire

Fractures and age at fracture event were recorded through a self-assessment questionnaire. All women were, based on the questionnaire, for the purpose of comparing fracture occurrence between allele groups, divided into those without any fractures and those with at least one fracture during lifetime. In an effort to exclude fractures occurring at younger ages that are not so often related to low bone mass, we also divided the women into those without any fractures after the age of 50 and those with at least one fracture after the age of 50. Fractures of the hip, forearm, vertebra and proximal humerus were classified as osteoporotic fractures. Reported fractures were compared against radiographic and orthopedic files of the Malmö University hospital and verified at 91% and 94% for hip and forearm fractures, respectively. Subject misinterpretation of, for example, hip fracture (5 of 53) included fractures of the femur or pelvis. In addition, the present activity level was graded by each participant and reported recently (1–8; 1 = bed rest only, cannot walk, 8 = still working, no limitation of mobility) [28]. Current drug intake, smoking status, and menopausal age were also registered.

Anthropometrics

Body weight and height were measured in a standardised way.

Genomic DNA analysis

Whole blood was obtained from 969 women and genotyping for the NlaIII polymorphism was possible in 964 women. Genomic DNA was isolated from EDTA stabilised blood, using the Qia Amp Blood kit (Qiagen GmbH; Promega, Madison, Wisc., USA). Determination of the IL-6 genotypes was performed using restriction fragment length polymorphism as previously described [26] and was possible in 964 women. Genotypes for the NlaIII

polymorphism were termed GG, GC and CC, where C represents presence of the restriction site at –174. To validate the accuracy of the genotyping, 90 random patients of the total 964 patients were re-analysed for the polymorphic site and no discrepancies compared to the initial analyses were found.

Statistical methods

Differences between the genotype groups were investigated using analysis of variance with Bonferoni's post-hoc test for multiple comparisons. Independent relationships were investigated using multiple linear regression and binary logistic regression when appropriate. The SPSS package for PC (version 9.0) was used for statistical evaluation and a *P*-value of <0.05 was considered significant.

Results

The 964 postmenopausal women studied were 75.2 years old (range 75.01–75.99). The frequencies of the different IL-6 genotype were 27.5% (GG), 47.9% (GC) and 24.6% (CC).

Physical characteristics, bone density, and ultrasound measurements for all subjects and according to the different genotypes are presented in Table 1.

No significant differences were observed for weight, height, menopausal age, drug intake, smoking status or activity level in relation to the IL-6 genotypes.

The IL-6 genotypes were not associated with bone mineral density (g/cm^2) at lumbar spine, femoral neck or total body.

Ultrasound was used to estimate stiffness index (SI), broadband ultrasound attenuation (BUA), and speed of sound (SOS) of the calcaneus. Using analysis of variance, there were no significant differences when comparing the genotypes (Table 1). However, the presence of the G allele (GG, GC) was independently related to a lower stiffness ($\beta=-0.07$, $P=0.03$) and BUA ($\beta=-0.08$, $P=0.02$) of the calcaneus after adjusting for the influence of weight and height using linear regression (Table 2).

In this study, 420 of 964 women (44%) reported at least one previous fracture during their lifetime, and 349 of 964 women (36%) reported having at least one fracture after the age of 50 years. Using binary logistic regression, the presence of the G allele (GG, GC) was significantly related to an increased risk of a previous fracture during life (odds ratio 1.46, $P=0.01$), and also to an increased risk of a fracture occurring after 50 years of age (odds ratio 1.37,$P=0.047$; Table 3). In a further sub-classification of fracture types, 232 women had

Table 2 The independent relationship between the different ultrasound measurements of the calcaneus (stiffness, speed of sound *SoS*; m/s, broadband ultrasound attenuation *BUA*; dB/MHz), weight (kg), height (cm), and the IL-6 genotypes ($n=803$). The IL-6 genotypes were analyzed as presence of G. Beta values are presented, and *P*-values are presented within parentheses

Ultrasound measurements	Stiffness	BUA	SoS
Independent variables			
IL-6 (presence of G)	−0.07 ($P=0.03$)	−0.08 ($P=0.02$)	−0.06 ($P=0.08$)
Body height	−0.07 ($P=0.05$)	0.00 ($P=0.97$)	−0.12 ($P=0.001$)
Body weight	0.29 ($P<0.001$)	0.33 ($P<0.001$)	0.21 ($P<0.001$)

Table 1 IL-6 polymorphism in relation to, age, anthropometric characteristics, balance according to Romberg, ultrasound measurements, and bone density in 964 postmenopausal Caucasian women. Mean values, standard deviations, and *P*-values are presented

N1aIII allelic variants Number of subjects ($n=964$)	GG 265	GC 462	CC 237	*P*-values –[a]
Physical characteristics (mean ± SD)				
Age (years)	75 ± 0	75 ± 0	75 ± 0	–
Body weight (kg)	68 ± 11	68 ± 12	67 ± 11	0.47
Height (cm)	161 ± 5	161 ± 6	160 ± 6	0.46
Activity level (1–8)	6 ± 1	6 ± 1	6 ± 1	0.29
Balance (s)	92 ± 27	91 ± 29	92 ± 27	0.98
Menopausal age	49 + 5	49 + 5	49 + 5	0.51
Ultrasound measurements				
Stiffness	67 ± 13	66 ± 13	68 ± 13	0.13
BUA (dB/MHz)	99 ± 11	98 ± 10	100 ± 10	0.11
SOS (m/s)	1503 ± 29	1501 ± 27	1505 ± 28	0.25
Bone mineral density (g/cm^2)				
Total body	1.01 ± 0.09	1.00 ± 0.10	1.01 ± 0.10	0.41
Lumbar spine	1.01 ± 0.20	0.98 ± 0.19	0.99 ± 0.19	0.16
Femoral neck	0.76 ± 0.13	0.74 ± 0.13	0.75 ± 0.12	0.48
Number of				
Estrogen users	6	8	3	0.70[b]
Cortisone users	12	21	5	0.25
Bisphosphonate users	9	17	15	0.53
Smokers	37	68	26	0.38

[a] *P*-values for ANOVA
[b] *P*-values for Chi^2-test

Table 3 Relationship between interleukin-6 polymorphism and previous fractures. The polymorphism is investigated as presence or absence of the G allele. Fractures are investigated as percent of the 964 subjects suffering a previous fracture during their whole previous life (75 years), and subjects suffering a previous fractures after 50 years of age. Odds ratios and 95% confidence intervals (CI 95%) are presented

	At least one fracture ($n=420$) %	Never fracture during lifetime ($n=544$) %	Total cohort ($n=964$)	Odds ratio (presence of G)	CI (95%)
Presence of G	79.3	72.4	75.4	1.46	1.08–1.97
Absence of G	20.7	27.6	24.6	–	–
–	Fractures after age 50 years %	No fracture after age 50 years %	Total cohort	Odds ratio	CI (95%)
Presence of G	79.1	73.3	75.4	1.37	1.004–1.88
Absence of G	20.9	26.7	24.6	–	–

Table 4 Distribution of the common types of fractures according to IL-6 genotype, including distribution of women who has never fractured at the age of 75. Osteoporotic fractures is a sub-classification of women with at least one osteoporotic fracture (hip, forearm, vertebral or proximal humerus fractures). Odds ratios for fracture according to presence of G versus CC is calculated for fractures of sufficient number

	Total	GG	GC	CC	OR	CI (95%)
Never fracture	544	167	246	131	–	–
Types of fracture						
Hip	48	15	25	8	1.90	(0.87–4.17)
Forearm	181	53	93	35	1.59	(1.05–2.40)
Vertebral	25	8	13	4	NA	–
Proximal humerus	8	3	4	1	NA	–
Osteoporotic	232	68	121	43	1.67	(1.14–2.45)

suffered at least one osteoporotic fracture (hip, forearm, vertebra or proximal humerus) and these are reported according to genotype in Table 4. Presence of G conferred an increased risk of forearm fracture (odds ratio 1.59, 95% CI 1.05–2.40) and for the entire group of osteoporotic fractures (odds ratio 1.67, 95% CI 1.14–2.45), but not for hip fracture, a finding that may be caused by low numbers of fractures (Table 4).

After adjusting for activity level, balance, and body weight, presence of the G allele (GG, GC) was still independently related to an increased risk of a previous fracture (Table 5). This risk was significant when investigating fractures during total lifetime (odds ratio 1.45,$P=0.02$), and showed a strong tendency towards significance for fractures sustained after 50 years of age (odds ratio 1.38, $P=0.05$).

Discussion

The purpose of the present study was to investigate if a functional IL-6 polymorphism in the promotor region might be associated with postmenopausal osteoporosis, assessed not only as bone quality in terms of elasticity and microarchitecture by QUS, but also at the endpoint of interest, skeletal fractures. We found that presence of the G allele (GG, GC) was significantly independently related to an increased likelihood of previous fracture in 75-year-old women during lifetime and to fractures related to osteoporosis. To our knowledge, this is the first study to examine the IL-6 polymorphism −174 for a possible association with fractures. Only a limited number of genetic polymorphisms have previously been investigated for their possible relationships with fractures and with inconsistent findings. An association between vitamin D receptor polymorphisms and bone mineral density was demonstrated early on in certain populations [29]. In a recent prospective study, the vitamin D receptor polymorphisms Taq1 and ApaI, were not found to be associated with neither vertebral nor non-vertebral fractures [30]. Johnston et al. found no significant influence of ApoE 4 polymorphism on the

Table 5 The independent relationship between the Il-6 genotypes (presence of G), balance, physical activity, body weight, and fractures during lifetime, and after fractures after 50 years of age, in 934 postmenopausal women

Variables	Odds ratio	*P*-value	Confidence interval
Fractures during lifetime			
Il-6 genotypes (presence of G)	1.45	0.02	1.06–1.98
Balance (s)	1.01	0.047	1.00–1.01
Activity (level)	1.11	0.15	0.96–1.28
Body weight (kg)	1.01	0.02	1.00–1.03
Fractures after 50 years			
Il-6 genotypes (presence of G)	1.38	0.05	1.00–1.91
Balance (s)	1.01	0.02	1.00–1.01
Activity (level)	1.11	0.16	0.96–1.29
Body weight (kg)	1.01	0.06	1.00–1.02

risk of a previous fracture in a cohort of 899 older subjects [31]. However, the study may have suffered from lack of statistical power. In another cohort consisting of 93 patients with vertebral fractures and 88 age-matched controls, McGuigan et al. showed that the Sp1 binding site in the first intron of the collagen type 1 alpha gene (COLIA1) polymorphism was associated with fractures independently of BMD [32].

The risk of a fracture depends on bone strength, which comprises not only bone mineral density but also the bone structural composition. Individuals with similar bone mass, with and without vertebral fractures, have been compared [33,34] and those with fractures were found to have more trabecular abnormalities than those without fractures. They were also more likely to sustain further fractures, suggesting that there is a quality trait that is not accounted for when measuring BMD. Quantitative ultrasound measurements have been suggested to measure not only bone mineral density but also the micro architecture and elasticity of bone [35,36,37,38], while DXA determines only bone mass explaining 60–90% of bone strength [33,39,40]. Also, there is only a moderate correlation ($r = 0.33$–0.44) between ultrasound and BMD of the different sites as earlier has been described in this population of women [41]. Studies have shown that QUS may have an even greater predictive power for fracture risk than DXA [42] and has been rated as a better measurement for bone quality than BMD [42,43]. We therefore also related the IL-6 genotypes to quantitative ultrasound measurements.

We found a significant, although rather weak relationship between BUA and the presence of the G allele (GG, GC), suggesting that the IL-6 polymorphism may contribute to the genetic influence on bone quality. At present no other studies have reported on the possible relationship between QUS and the IL-6 –174 polymorphism. Since presence of the G allele (GG, GC) was related to both lower QUS and increased risk of previous fractures, this relationship seems causal.

There are several reports demonstrating BUA's ability to predict fractures independently of BMD [42,44]. In a study by Zmuda et al., the polymorphism in the α_2HS-glycoprotein (AHSG) was shown to be significantly related to BUA, but not to BMD, in a group of older women [45]. In addition, Arden et al. found stronger associations in BUA in monozygotic than in dizygotic twins, suggesting that BUA is under genetic influence [46].

Several lines of evidence support our suggestion that the presence of the G allele (GG, GC) located at position −174 in the IL-6 promoter may influence osteoporotic fractures in postmenopausal women. The G allele is associated with increased promoter activity and increased IL-6 levels [25]. IL-6 is a potent stimulator of bone resorption in vitro and ex vivo in murine cultures, and increased levels of IL-6 has been implicated as a stimulator of bone resorption in several different pathological conditions such as rheumatoid arthritis, periodontal disease, Paget's disease, multiple myeloma and hyperparathyroidism [20,21,22,23,24]. Of particular interest for the present study is that increased levels of IL-6 is suggested to be linked to low levels of 17β-estradiol and bone loss associated with loss of ovarian function [15,16,17].

In an earlier study, we found that the GG genotype was associated with low BMD in young males [26]. In that study, however, we did not measure QUS. The fact that the IL-6 polymorphism was not related to BMD in the present study, but to fracture rate, might suggest that IL-6 in postmenopausal women is more important for the architecture and elasticity of the remaining skeleton (revealed by QUS), than to the content of minerals (as assessed by DXA). It might also be that DXA measurement is more sensitive to the phenotype of the IL-6 polymorphism in a skeleton of a young male with high BMD. The gender difference may also be an explanation for the lack of correlation. The higher age in these women, could also make the environmental influence larger and diminish the genetic effect of this polymorphism on bone mass as measured by DXA.

Fracture risk contains multiple aspects that are mediated both dependently and independently of bone mass and bone quality. In order to further evaluate if the association of the IL-6 polymorphism and fracture was unrelated to extrinsic factors, objectively assessed balance was included in the analysis as an independent non-skeletal risk factor for fracture. Physical activity, which may be regarded as both independent of bone mass or indirectly related to bone mass, was also included. Neither of these potential confounders was affecting the results, supporting the indicated association.

The strength of the present study includes the population-based design with random sampling and high participation rate, the homogeneity of the cohort and the virtually identical age of the women, all were exactly 75 years old, which make corrections for age and gender unnecessary. As a point of limitation, when interpreting the data one has to be aware of the fact that the fractures were self-assessed with a possible risk of over- and under-reporting fractures. On the other hand, most previous published studies have also presented self-assessed fractures [31]. Underestimation, in this study as well as in other studies, is most likely affecting the evaluation of vertebral fracture risk, since a large proportion of vertebral fractures remain undiagnosed in the population and since this study did not include spinal radiograms.

In conclusion, in the present study the IL-6 promoter polymorphism at –174 was related to previous fractures, particularly osteoporotic fractures, in elderly postmenopausal women. This association seems to be related to bone quality quantified by QUS and not the bone mass. Our findings demonstrating an association between the IL-6 polymorphism and BUA and those associations found between other polymorphisms and BUA reported by others points at a genetic component also in bone quality independent of bone density.

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