In: Liver Cirrhosis Research
Editor: J. L. Burns, pp. 59-78
ISBN: 1-59454-155-8

Chapter V

GENE POLYMORPHISMS IN PRIMARY BILIARY CIRRHOSIS: ASSOCIATION WITH THE DISEASE AND HEPATIC OSTEOPATHY

Peter Laszlo Lakatos[1,*], Eva Bajnok[1], Claudia Willheim-Polli[2], Peter Ferenci[2], Istvan Takacs[1], Peter Lakatos[1] and Ferenc Szalay[1]

[1]1st Department of Medicine, Semmelweis University, Budapest, Hungary
[2]4th Department of Medicine, University of Vienna,Vienna, Austria

ABSTRACT

Genetic factors have been implicated in the pathogenesis of osteoporosis, a common disorder in primary biliary cirrhosis (PBC). Estrogen receptor-alpha gene (ER-□), vitamin-D-receptor gene (VDR) and IL-1-receptor-antagonist gene (IL-1RN) are all attractive candidates for osteoporosis susceptibility. Furthermore insulin-like growth factor-I (IGF-I) gene microsatellite repeat polymorphism was found to be associated with osteoporosis in some studies and collagen-I□1 (COLIA1) Sp1 s allele was associated with lower bone mineral density (BMD) in one study in PBC. IGF-I treatment restored osteopenia and reduced fibrogenesis in experimental cirrhosis. In this study we summarize our results on polymorphisms of the above genes and bone disease in Hungarian PBC patients.

Patients and methods: 70 female patients with PBC were enrolled (age:57.6yrs, range:37-76yrs, each AMA-M2 positive, stage II-IV). 139 age-matched female subjects served as controls (age: 55.9 yrs, range:43-72 yrs). COLIA1 Sp1 and IGF-I microsatellite polymorphisms were determined by PCR in all patients and controls. VDR BsmI, IL-1RN variable-number tandem repeat (VNTR) and ER-□ PvuII and XbaI polymorphisms

* Correspondence concerning this article should be addressed to Lakatos Péter László, MD, PhD, 1st Department of Internal Medicine, Semmelweis University, Koranyi str. 2/A, H-1083 Budapest, Hungary, e-mail: kislakpet@bel1.sote.hu.

were detected in 33 patients and controls. BMD was measured by dual energy x-ray absorptiometry (Lunar,Prodigy,USA) in lumbar spine (LS) and femoral neck (FN).

Results: There was no difference in IGF-I microsatellite repeat polymorphism (192/192=34.2%, 194/192=28.6%, other=37.2%) and COLIA1 Sp1 polymorphism (SS=72.9%, Ss=22.8% and ss=4.3%) and IL-1 VNTR polymorphism between PBC patients and controls, however, the COLIA1 Sp1 s allele was significantly less frequent in patients with PBC (p=0.038). The genotype frequency of VDR BsmI (BB=57.5%, Bb=33.3%, bb=9.1%, p=0.01) and ER-a PvuII (PP=18.2%, Pp=75.6%, pp=6.2%, p=0.03) and XbaI (XX=9.1%, Xx=90.9%, xx=0%, p=0.0003) of the patients was different from that of the control group, with higher frequency of the BB, Pp and Xx genotypes in PBC. Osteoporosis (t score<-2.5) was detected in 22 patients (31.4%). Osteoporotic patients were elder and had longer disease history (p=0.01 for both). An association was found between the IGF-I genotypes and ODM data, the 192/192 genotype was associated with higher FN Z-score compared to other genotypes (p=0.036).

Conclusions: In contrast to previous studies the COLIA1 Sp1 s allele was less frequent in patients with PBC, and its presence was not associated with BMD. We confirmed previous findings on higher frequency of VDR BsmI BB genotype in patients with PBC. The ER-α PvuII and XbaI Pp and Xx genotypes were more frequent in PBC patients, while IL-1RN VNTR and IGF-I microsatellite repeat polymorphism was not different. Since IGF-I polymorphism was associated to BMD, it may be hypothesized that not COLIA1 but IGF-I together with other genetic and environmental factors may be involved in the complex regulation of BMD in PBC.

Keywords: Primary biliary cirrhosis, hepatic osteopathy, gene polymorphisms, estrogen receptor gene, vitamin D receptor gene, IL-1 receptor antagonist gene, COLIA1, IGF-I, microsatellite repeat polymorphism.

INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic disease believed to be of autoimmune origin, which is characterized by the inflammation of the small bile ducts, affecting middle age women [1]. Osteoporosis (OP) is a common complication in primary biliary cirrhosis (PBC), even in the absence of biochemical hyperbilirubinaemia. It is a major source of morbidity and mortality [2,3 ,4].

The pathogenesis of bone disease in PBC is not fully understood. Numerous factors may be involved in the pathogenesis, such as immobility, poor nutrition, disturbances in hormonal status, immunological alterations, as well as risk factors commonly associated with primary osteoporosis (postmenopausal status, cigarette smoking, and alcohol consumption). Possible mechanisms include Ca and vitamin D malabsorption as a consequence to bile salt deficiency, vitamin K deficiency, altered parathyroid function [5,6,7]. Both low and high bone turnover states have been reported in PBC [8,9,10]. Recent study by our group showed high osteoprotegerin and low RANKL level in patients with PBC that might represent a compensatory mechanism to negative balance of bone remodeling [8].

Bone mass is partially determined by genetic factors and which may account for up to 75-80% of the variability in BMD in healthy women [11]. Twins studies and segregation analysis in normal families have suggested polygenic regulation of BMD [11,12]. Several

candidate genes involved in the control of bone formation have been identified. The best characterized genetic association is the relationship between Vitamin D receptor (VDR) allelic polymorphisms and BMD in prae- and postmenopausal women [13,14,15,16] with or without hormone replacement therapy [17]. The presence of BB genotype or B allele was associated with greater risk to develop osteoporosis [14, 15].

This association was not confirmed in some studies [18,19]. Likewise, data in patients with PBC are conflicting. VDR genotypes are associated with PBC, independent of changes of BMD [20,21]. However, they may explain the role of hormonal influence in the development of hepatic osteopathy in PBC [22]. Another polymorphisms related to osteoporosis is located in the first intron of the collagen type 1α1 (COLIA1) gene Sp1 [23,24,25,26]. The s (T) allele is associated with increased bone loss and fracture frequency and decreased yield threshold in otherwise healthy subjects. The ss genotype was associated with a 2-fold increased rate of yearly bone loss and a 5.9-fold elevated risk of bone fractures [23,24]. In a meta-analysis of Efstathiadou and colleagues [27] the total average attributable fraction of fractures due to the s allele in Europe/U.S. population was estimated as 9.4%. Finally, the Ss genotype was associated with reduced yield strength of bone samples compared to SS genotypes [28]. In the only study in PBC patients COLIA1 genotype was a genetic marker of peak bone mass [29]. Because of the impact of estrogens on bone formation the estrogen receptor alpha (ER-α) gene PvuII, XbaI- and (TA)n promoter repeat polymorphisms are also of particular interest [30,31,32,33]. In Japanese postmenopausal women a strong association between ER-α gene PvuII polymorphism and reduced bone mass in the spine was observed [30,34] but data in Caucasians are conflicting [31,32,33,35]. Experimental studies support the role of the ER-α gene in the regulation of BMD. In transgenic ER knockout mice BMD is 20-25% lower than in normal mice [36]. So far, ER-α gene polymorphisms were not studied in patients with PBC. Insulin-like growth factor-I (IGF-I) is a ubiquitous polypeptide involved in cell growth and differentiation, stimulation of linear growth and proliferation of chondrocytes. It also plays an important role in the acquisition of peak bone mineral density (BMD) [37,38,39]. Serum IGF-I levels are decreased in chronic liver diseases [40,41,42], but there was no relationship between serum IGF-I and BMD. A microsatellite repeat polymorphism located 1kb upstream from the IGF-I gene transcript start site appears to exert a genetic control of bone formation, the 192/192 genotype is associates with lower BMD [37,43]. No data are available on IGF-I microsatellite repeat polymorphisms in chronic liver diseases.

Interleukin-1 receptor antagonist (IL-1Ra) is a competitive inhibitor of IL-1α and β, which completely inhibits the stimulatory effects of interleukin-1 (IL-1) on bone resorption in vitro [44]. A trend for higher IL-1/IL-1Ra ratio found in osteoporotic women compared with healthy age-matched controls [45]. Some, but not all studies reported an association between the number of tandem repeats (VNTR) of interleukin-1 receptor antagonist gene (IL-1RN) and postmenopausal bone loss in the spine [46,47]. The A2 allele was associated with some inflammatory and autoimmune diseases [48,49,50,51]. In this study the polymorphisms of the above mentioned genes was investigated in two cohorts of Hungarian PBC patients and correlated them with clinical, laboratory and osteodensitometric findings.

PATIENTS AND METHODS

Patients

VDR BsmI, ER- α, and IL-1RN VNTR polymorphisms were studied 33 (group 1; mean age: 54.7 ± 11.7 yrs; 27 postmenopausal), IGF-I microsatellite repeat and COLIA1 Sp1 polymorphism in 70 patients (group 2; mean age: 57.6 ± 10.6 yrs; 62 postmenopausal; histologic stage: I: 11, II: 31, III: 18, and IV: 10 patients). All patients were female with biochemical evidence of cholestasis, consistent histological findings and anti-mitochondrial antibody (AMA) M2 positivity. Patients with evidence of extrahepatic cholestasis were excluded. All patients received ursodeoxycholic acid (UDCA) as well as 1000 mg Ca and individually 1000-3000 IU Vitamin D3 supplementation orally. None of the patients received other treatment for osteoporosis. The serum Ca and vitamin D3 values of the patients were in the normal range. The clinical data of the PBC patients are summarized in Table 1.

The onset of the disease was defined as the date of the first documentation of abnormal liver function test, the duration of liver disease as the time from disease onset to the date of the BMD measurement. For the study of VDR BsmI, ER- α, and IL-1RN VNTR polymorphisms 82 age matched, healthy female controls (mean age: 54.1 ± 4.7 yrs, postmenopausal: 71/82, 86 %) were selected from a panel undergoing DNA genotyping. None of the healthy controls had osteoporosis, 58.5% had osteopenia. We also selected 76 female subjects with osteoporosis as as "disease" control group (mean age: 56.8 ± 6.1 yrs, 93 %, 71/76 postmenopausal, weight: 62.1 ± 10.1 height: 160 ± 6.9, BMD LS: 0.847 ± 0.123, Z-score: -1.627 ± 1.083, T-score: -2.987 ± 0.961, BMD FN: 0.749 ± 0.105 Z-score: -0.721 ± 0.846, T-score: -1.901 ± 0.962). For the study of IGF-I microsatellite repeat and COLIA1 Sp1 polymorphism 139 age-matched, healthy females served as controls (mean age: 55.9 ± 6.8 yrs, postmenopausal: 118/139, 84.9%). 53 control subject had osteopenia (46.1%).

The studies were approved by the Semmelweis University Regional and Institutional Committee of Science and Research Ethics. After written informed consent, all patients and controls underwent DNA genotyping and BMD measurements.

Methods

Laboratory Determinations

Serum bilirubin concentration, aspartate-aminotransferase (AST), alanine-amino-transferase (ALT), alkaline phosphatase (ALP) and albumin activities were measured by Olympus AU600 (Olympus Co. Ltd, Shizuoka, Japan) autoanalyser at 37 °C. The enzyme activities are expressed in U/L, albumin in g/L and serum bilirubin concentration is given in μmol/L. AMA M2 was determined by immunofluorescent method (Binding Site kit, Birmingham, UK), vitamin D3 level by RIA (25-OH D3 vitamin kit, Med-elpho Ltd, Budapest, Hungary).

Serum osteocalcin (OC) levels were measured with the N-MID Osteocalcin Electrochemiluminescence Immunoassay (ELICA) kit (Roche Diagnostics GmbH, Mannheim, Germany). The serum levels of C-terminal cross-linking telopeptide of type I

collagen (CTX-I) was determined by the Elecsys β-Crosslaps immunoassay kit (Roche Diagnostics GmbH, Mannheim, Germany). Both measurements were performed by Elecsys 2010 immunoassay system (Hitachi, Tokyo, Japan).

Table 1. The summary of the clinical and laboratory data of PBC patients according to the presence of osteoporosis (values are mean ± SD, *p=0.01, #p=0.03, between patients with osteoporosis vs. without osteoporosis, in the brackets see normal values.)

	Study 1			Study 2		
	total	with osteoporosis	without osteoporosis	total	with osteoporosis	Without osteoporosis
Number of patients	33	14	19	70	22	48
Age (years)	54.7 ± 11.7	58 ± 10*	52 ± 12*	57.6 ± 10.6	60.8 ± 8.8	56.2 ± 11.1#
Duration of the disease (years)	7.8 ± 5.7	9.5 ± 5.1*	5.4 ± 3.3*	6.8 ± 5.5	9.0 ± 6.8	55.8 ± 4.#
Serum bilirubin (0-20.5 μmol/l)	31.4 ± 75.6	49.1 ± 112.8	18.5 ± 23.7	29.1 ± 58.2	35.1 ± 93.1	26.2 ± 30.5
ASAT (1-46 U/l)	41.2 ± 23.9	44.9 ± 27.0	38.5 ± 21.7	42.4 ± 21.2	44.6 ± 21.9	41.4 ± 21.1
ALAT (1-49 U/l)	42.3 ± 26.6	45.6 ± 33.6	39.9 ± 20.5	44.9 ± 25.5	48.1 ± 30.2	43.3 ± 23.1
GGT (9-52 U/l)	166.6 ± 146.6	162.7 ± 181.8	169.6± 117.9	205.5 ± 211.4	169.4 ± 173.9	223.1 ± 227.3
ALP (98-290 U/l)	496.2 ± 227.3	517.7 ± 214.8	480.4 ± 240.6	551.8 ± 326.2	480.2 ± 231.1	586.8 ± 361.1
Albumin (35-50 g/L)	43.4 ± 4.2	42.8 ± 4.5	44.0 ± 4.2	41.7 ± 5.0	42.7 ± 3.9	41.1 ± 5.5
Ca (2.25-2.61 mmol/l)	2.44 ± 0.14	2.45 ± 0.14	2.43 ± 0.14	2.37 ± 0.16	2.43 ± 0.13	2.35 ± 0.17
P (0.85-1.45 mmol/l)	1.11 ± 0.14	1.12 ± 0.14	1.11 ± 0.14	1.09 ± 0.13	1.10 ± 0.14	1.18 ± 0.14
Vitamin D (60-180 nmol/l)	151.5 ± 61.6	131.5 ± 40.2	183.6 ± 72.5	146.5 ± 47.4	134.4 ± 30.1	172.3 ± 56.7
OC (20-48 ng/ml)	-	-	-	22.9 ± 13.5	25.9 ± 17.9	21.1 ± 9.8
CTX-I (0-320 pg/ml)	-	-	-	329.0 ± 278.6	401.1 ± 414.1	283.4 ± 128.3

Genomic DNA Analysis

Genomic DNA was extracted from peripheral mononuclear cells using DNA isolation-kit (NukleoSpin Blood Quickpure, Macherey-Nagel, Düren, Germany) according to the description of the manufacturer and amplified by polymerase chain reaction (PCR) with sequence-specific primers using a Hybaid Express thermocycler (Teddington, Middlesex, UK). PCR products were separated by electrophoresis on 2% agarose gel at 120V for 30 min and visualized by ethidium bromide staining if not stated otherwise.

Vitamin D Receptor Genotyping

A fragment of the vitamin D receptor was amplified by PCR with primers as described previously [13]. The amplified DNA was subjected to digestion by BsmI resriction enzyme

(65^{o}C, 90 min) with enzyme Bsm1, Hybaid, Teddington, UK) and the fragments were separated by gel electrophoresis. The absence or presence of the BsmI restriction site is indicated by B (825 bp) or b (650 + 175 bp) respectively. All samples with BB genotype were repeatedly digested and analyzed to avoid possibility of enzyme inhibition and the false assignment of bb or Bb genotype to truly BB individuals.

Estrogen Receptor Alpha Genotyping

ER-α genotyping was performed by PCR using previously described primers and amplification conditions [30]. For the digestion of ER PCR product enzymes PvuII, XbaI (Hybaid, Teddington, UK) were used **(**37^{o}C 90 min). The genotype assignments were PP, Pp, pp for PvuII and XX, Xx, xx for XbaI, with uppercase letters signifying the absence of and lower case letters the presence of the restriction site.

IL-1-RN genotyping

DNA was subjected to PCR amplification for the 86 bp tandem repeat polymorphism of the IL-1RN gene using primers as described [52]. Allele assignments were made in accordance with previous reports: allele 1 (A1), 410 bp-four repeats; allele 2 (A2), 240 bp-two repeats; allele 3 (A3), 500 bp-five repeats; allele 4 (A4),325 bp-three repeats; allele 5 (A5), 595 bp-six repeats.

IGF-I Genotyping

The PCR was performed with primers and amplification conditions as described previously [53], to amplify a polymorphic microsatellite composed of variable cytosine-adenine (CA) repeats, situated 1kb upstream from the transcription start site of the IGF-I gene. The forward primer was radiolabeled with ^{32}P using T4 polynucleotid kinase (Invitrogen Corp., Carlsbad, CA, USA). Radiolabeled PCR products were screened for length variation by a 6.5% polyacrilamide gel electrophoresis at 70 watts for 165 min. Autoradiographs were exposed for 4-12 h in cassettes without intensifying screens. Genotypes were scored by two independent investigators. Allele assignments were made according to the size of the CA repeat.

COLIA1 genotyping

COLIA1 Sp1 genotypes were determined by PCR using previously described primers and amplification conditions using [54]. The amplified DNA was subjected to digestion by BSA restriction enzyme (37^{o}C for 30 h, New England BioLabs GmbH, Frankfurt Germany) and the fragments were separated by gel electrophoresis (2% agarose at 120V for 30 min) and visualized by ethidium bromide staining. The alleles were assigned as “S” (G) or “s” (T) [54].

BMD Assessment

Bone density was measured at the lumbar spine (L2-4) and at the femoral neck (FN) by dual-energy X-ray absorptiometry (Lunar Prodigy, Lunar corp., Madison, WI, USA). BMD was expressed as areal density m grams per square centimeter, as well as in t-scores (difference from the mean BMD value of healthy young people divided by its standard

deviation) and z-scores (difference from the mean BMD value of age-matched people divided by its standard deviation). Established osteoporosis was diagnosed if the BMD t-score value was below –2.5 at either position [55].

Statistical Analysis

Shapiro Wilk's W test was used to test normality. To test the differences between genotype / allele frequencies of the cohort and controls and between subgroups of the patients Chi square analysis or Fischer exact with Yates correction test was computed. To test the differences in clinical data between osteoporotic and non-porotic patients as well as between patients and controls t-test with separate variance estimates and in study 2 Mann-Whitney U test was used. The correlation between laboratory findings and BMD was tested by using Spearman Rank Order Correlation. The association between genotype or allele frequencies and BMD was tested by using ANOVA and post hoc Scheffe analysis. For the purposes of comparison the patients were considered osteoporotic if they fulfilled t-score criteria at either lumbar spine or femoral neck. For the statistical analysis Statistica for Windows 6.0 (StatSoft Inc., OK, USA) was used. A p value <0.05 was considered significant.

RESULTS

Osteoporosis (OP) was detected in 14 (42.4%) and 22 patients (31.4%) in groups 1 and 2, respectively. Each or them was in the postmenopause and they were approximately 5-years older and had a longer disease history compared to patients without OP ($p=0.01$ and $p=0.03$, for both). In group 1 OP was associated with increased fracture frequency (at least one fracture in 6/14 OP patients vs. 1/19 non-OP, $p<0.01$). The BMD data of patients and controls is summarized in Table 2. The intraindividual correlation between BMD measurements at spine and femur of the patients showed a strong correlation in both groups ($r=0.68$-88, $p<0.0001$). In group 2 an inverse correlation was found between the age and LS BMD and t-score values ($p=0.011$-0.023), and between disease duration and LS BMD, z- and t-score values ($p=0.015$-0.03). No correlation was found between the laboratory markers of the liver disease (bilirubin, ASAT, ALAT, GGT, ALP) and BMD values at either position.

The genotype frequency of VDR Bsm I, ER-α PvuII and XbaI is shown in table 3, the genotype and allele frequency of IL-1RN in patients and controls in table 4. The VDR genotype frequency in controls was consistent with other studies performed in healthy populations of Caucasian origin [13,14,15]. In osteoporotic controls the genotype frequency was similar to that of the healthy controls. In contrast, in PBC patients the BB genotype (n= 19, 57.5 %, $p=0.01$) and B allele (n= 49, 74.2 %) was overrepresented compared to the control groups ($p<0.01$ for both).

The IGF-I genotype and allele frequencies of PBC patients were not different from that of the control group (table 5). The association of BMD data and IGF-I microsatellite repeat polymorphism are presented in Figures 1 and 2. Patients with the IGF-I 192/192 genotype had higher FN z-score values than patients with other genotypes ($p=0.036$) despite almost

identical disease duration (192/192: 7.2 yrs vs. other genotypes: 6.4 yrs) and matching body mass index (192/192: 25.6 kg/m^2, vs. other genotypes 24.9 kg/m^2). The same tendency was observed between this genotype and LS BMD and z-score (p=0.07 for both) or FN BMD and t-score (p=0.058 and p=0.06) values. No difference was found in IGF-I genotype or allele frequencies between patients with OP (192/192=27.3%, 194/192=22.7%, other=50.0%) or without OP (192/192=37.5%, 194/192=31.

Table 2. Bone mineral density values in lumbar spine (LS) and femoral neck (FN) of controls and PBC patients (BMD g/cm^2, values are mean ± SD, *p≤0.01, #p=0.04 PBC patients vs. controls)

		LS			FN		
		BMD	Z-score	T-score	BMD	Z-score	T-score
Study 1	PBC (n=33)	0.937 ± 0.261	-0.68 ± 1.67	-2.01 ± 2.39	0.789± 0.227	-0.53 ± 1.68	-1.72 ± 2.20
	Controls (n=82)	1.085± 0.129*	-0.16 ± 0.13	-0.95 ± 1.07*	0.901 ± 0.134*	0.05 ± 1.08#	-0.66 ± 0.13*
Study2	PBC (n=70)	0.975± 0.216	-0.34 ± 1.70	-1.63± 1.98	0.831± 0.188	-0.30 ± 1.54	-1.43 ± 1.86
	Controls (n=139)	1.086± 0.142*	-0.05 ± 1.19	-0.89± 1.15*	0.898 ± 0.126*	0.04 ± 1.03	- 0.69± 1.06*

Table 3. Genotype frequency of vitamin D-receptor BsmI, estrogen receptor alpha PvuII and XbaI, IGF-I microsatellite repeat and COLIA1 Sp1 polymorphism in controls and patients with PBC (p calculated by Chi-square analysis, # p=0.026 for SS vs. Ss/ss by Fischer exact test).

	PBC n (%)	Control n (%)	p
VDR Bsm I			
BB	19 (57.5%)	23 (28.1%)	p=0.01
Bb	11 (33.3%)	41 (50.0%)	
Bb	3 (9.1 %)	18 (21.9%)	
ER-α PvuII			
PP	6 (18.2 %)	26 (31.7%)	p=0.03
Pp	25 (75.6 %)	40 (48.8%)	
pp	2 (6.2 %)	16 (19.5 %)	
ER- α XbaI			
XX	3 (9.1 %)	15 (18.3%)	p=0.003
Xx	30 (90.9%)	49 (59.8%)	
Xx	0	18 (21.9%)	
IGF I			
192/192	53 (38.2%)	24 (34.2%)	NS
194/192	43 (30.9%)	20 (28.6%)	
other	43 (30.9%)	26 (37.2%)	
COLIA1			
SS	81 (58.4%)	51 (72.9 %)	NS
Ss	50 (35.9%)	16 (22.8%)	
ss	8 (5.7%)	3 (4.3%)	

Table 4. Genotype and allele frequency of interleukin-1 receptor antagonist gene *VNTR* in controls and patients with PBC (NS: not significant by Chi square). Allele 4 was not found in either patients or controls.

A	IL-1RN VNTR, Genotype n (%)						Allele frequency n (%)		
	A1A1	A1A2	A2A2	A1A3	A2A3	A3A3	A1	A2	A3
PBC (n=33)	20 (60.6%)	10 (30.2%)	2 (6.1%)	1 (3.1%)	0	0	52 (78.8%)	13 (19.7%)	1 (1.5%)
Controls (n=82)	41 (50.0%)	28 (34.1%)	5 (6%)	7 (8.5%)	1 (1.4%)	0	117 (71.3%)	39 (23.8%)	8 (4.9%)
p value	NS						NS		

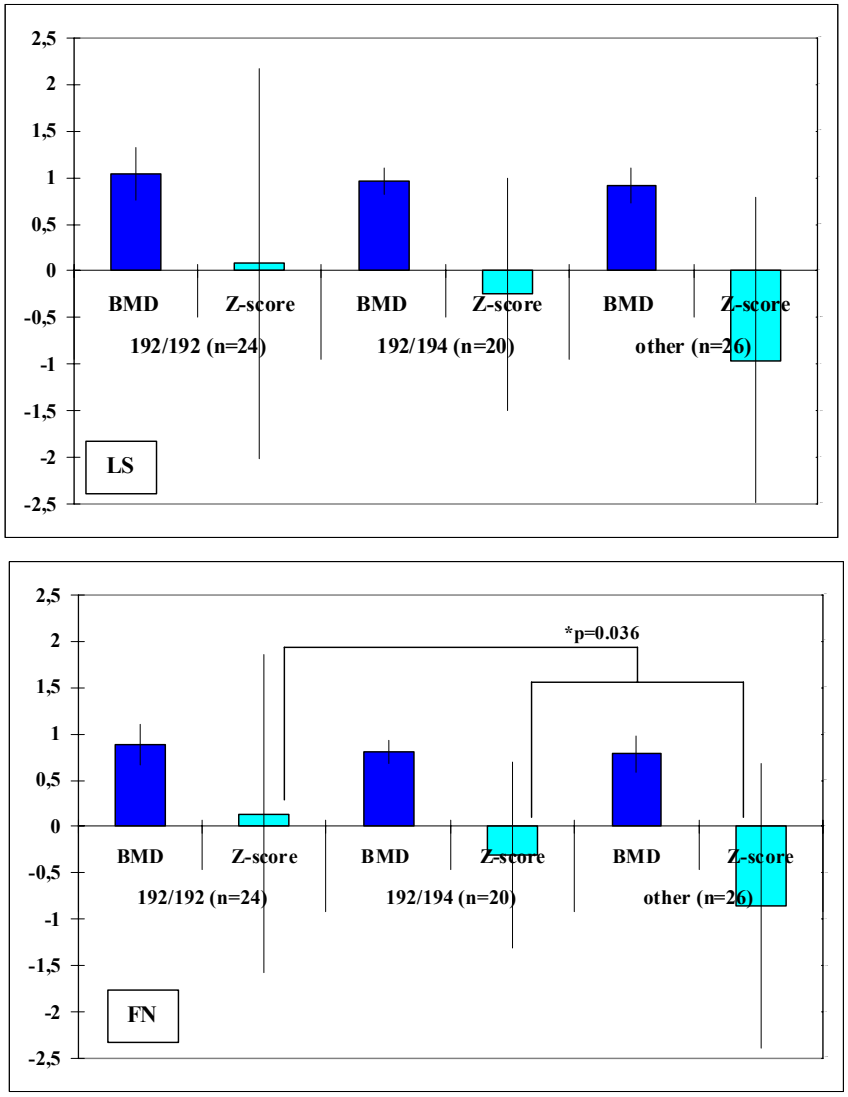

Figure 1. IGF-I microsatellite repeat genotypes, lumbar spine (LS) and left femur neck (FN) BMD and z-scores in patients with PBC (BMD=g/cm^2, values are mean ± SD, *p=0.036 for Z-score values 192/192 vs. others by Mann Whitney U test).

The IL-1RN genotype frequency of the osteoporotic controls was similar to the healthy subjects and it was not different from that of the PBC patients. 25%, other=31.25%). The COLIA1 Sp1 genotype frequency of PBC patients was also not different from that of the controls (Hardy-Weinberg disequlibrium test). The s allele was less frequent in PBC patients without OP than in controls (12.5% vs. 24.5%, p=0.01 by Fischer exact). The same tendency was observed between patients with and without OP (22.7% vs. 12.5%, p=0.08) (see table 6 and Figure 2).

Table 5.: Allele frequency of IGF-I microsatellite repeat polymorphisms in controls and patients with PBC (NS: not significant by Chi square).

B	IGF-I n (%)								
	174	176	188	190	192	194	196	198	200
Controls (n=278)	2 (0.7%)	1 (0.3%)	2 (0.7%)	15 (5.4%)	185 (66.5%)	51 (18.3%)	17 (6.2%)	4 (1.6%)	1 (0.3%)
PBC (n=140)	0	1 (0.7%)	3 (2.1%)	12 (8.6%)	84 (60.0%)	26 (18.6%)	13 (9.3%)	0	1 (0.7%)
P value	NS								

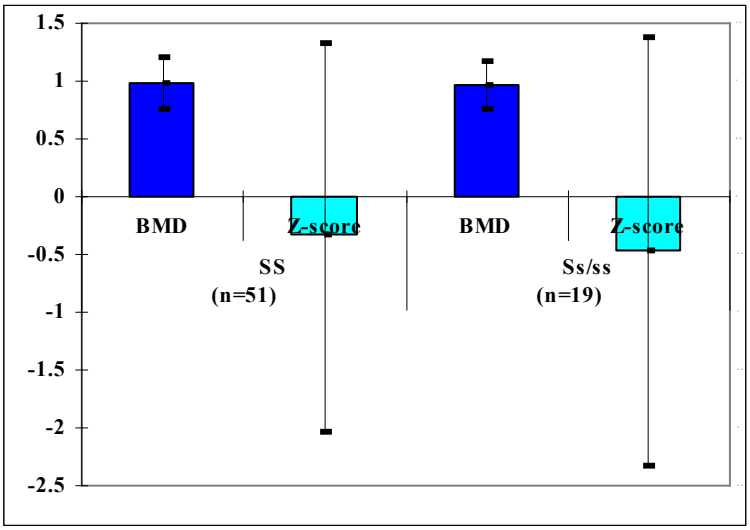

Figure 2. COLIA1 Sp1 genotypes, lumbar spine BMD and z-scores in patients with PBC (BMD=g/cm^2, values are mean ± SD. No significant difference between SS and Ss/ss by Mann-Whitney U test).

No association was found between BMD (t and z scores) and the other polymorphisms determined (VDR, ER-alpha PvuII or XbaI, IL-1RN or COLIA1 Sp1). There was a tendency of VDR BsmI bb genotype to be associated with higher BMD values however, but only 3 patients had this genotype.

Table 6. Genotype frequency the vitamin D-receptor (VDR) BsmI, estrogen receptor alpha (ER-α) PvuII and XbaI and IGF-I microsatellite repeat polymorphism and COLIA1 Sp1 polymorphism in PBC patients with and without osteoporosis.

	with osteoporosis n (%)	without osteoporosis n (%)	p
VDR Bsm I			
BB	9 (64%)	10 (53%)	NS
Bb	5 (36%)	6 (32%)	
Bb	0	3 (15%)	
ER-α PvuII			
PP	1 (8%)	4 (21%)	NS
Pp	11 (78%)	15 (79%)	
pp	2 (14%)	0	
ER-α XbaI			
XX	2 (14%)	1 (6%)	NS
Xx	12 (85%)	18 (94%)	
Xx	0	0	
IGF I			
192/192	6 (27.3%)	18 (37.5%)	NS
194/192	5 (22.7%)	15 (31.2%)	
other	11 (50.0%)	15 (31.2%)	
COLIA1			
SS	14 (63.6%)	37 (77.1%)	NS
Ss	6 (27.3%)	10 (20.8%)	
ss	2 (9.1%)	1 (2.1%)	

DISCUSSION

This study attempted to identify genetic markers for PBC and for PBC-associated hepatic osteopathy. Among many markers investigated only the BB genotype of the VDR and the XbaI Xx genotype of the ER-α ware overrepresented in middle-aged Hungarian PBC patients as compared with both healthy subjects and disease controls with non-liver disease-related osteoporosis. However, we did not find a direct association between VDR genotypes and BMD [56].

Osteoporosis is a common disorder in PBC [2,3]. Its prevalence in this study (31-42%) is in concordance with previously reported values of 24-43 % [2,7,57]. The cause of bone abnormalities in patients with cholestatic liver disease is not fully understood. Disease duration may reflect severity of the disease; however the rate of progression is variable. As expected [1-2,21] patients with osteoporosis were older and had a longer disease duration than those without osteoporosis. Both high and low bone turnover has been reported [8-10]. High turnover osteoporosis is further supported by the elevated CTX-I level found in group 2, particularly in advanced stages. In these patients OC levels were also elevated indicating increased osteoblastic activity representing a possible compensatory mechanism. An

association between the rate of bone loss and the severity of cholestasis has been reported in PBC patients [22,58], but was not confirmed in this study. This discrepancy may be explained by the fact that all of our patients were treated with UDCA [59], which improved cholestasis.

Many studies have addressed the genetic background of osteopenia and osteoporosis, several candidate genes have been investigated (VDR, ER-α, IL-6, collagen 1A1) [13-54]. VDR has been of special interest [13-17].

Similarly, a possible genetic background of PBC is under intense investigation. Associations were found at chromosomes 6p21.3 and 2q including HLA DRBI*08, CTLA4*G, IL1RN-IL1B haplotypes, CASP8 and APO-E epsilon4 [60,61]. Recently the HLA-DRB1*11 has been shown to be associated with reduced risk of developing PBC, while the presence of B*15, B*41, B*55 and B*58 was found to be more frequent in PBC [62].

The study of the role of genetic polymorphisms of bone formation in patients with PBC is complicated because they may be independently associated with PBC like the BsmI polymorphism of VDR or the Sp1 polymorphism in COLIA1 gene [20,22,63]. Furthermore, the possible genetic factor may render subjects more susceptible to the effects of pathophysiologic mechanisms which by themselves are involved in the pathogenesis of bone disease.

This problem is further complicated by the fact, that established risk factors for osteoporosis are also associated with the development of autoimmune diseases such as PBC. 1,25-(OH)2-D3 was shown to inhibit T-cell activation in vitro and in vivo, and to suppress IL-1, IL-2, IL-6, TNF and INF-γ production [64]. These cytokines are important in the development of T helper 1 cells, which are believed to be involved in the pathogenesis of autoimmune diseases, such as PBC. It has also been shown that vitamin D is able to regulate the expression of costimulatory molecules B7.1 and B7.2 [65]. Interestingly VDR genotypes have been found to be associated with susceptibility to various diseases, as Graves' disease [66,67] and hepatitis B [68]. The association between the presence of B allele or BB genotype and the susceptibility to other autoimmune diseases (like Graves' disease) suggests that VDR-polymorphisms may not only be associated with osteoporosis, but may also play a role in the development of autoimmune diseases. Not surprisingly, the reported data in PBC patients are conflicting [20,21,22]. A similar association may apply to ER-α and IL-1ra. ER-α is a strong candidate for the regulation of BMD [17, 30-33], but ER-α and β expression was also reported in peripheral and thymic lymphocytes, macrophages and endothelial cells, suggesting that estrogen might affect immune cells during development and mature function [69]. Furthermore in T cells from female patients with systemic lupus erythematosus an altered ER function was identified [70]. In autoimmune conditions and in multiple sclerosis ER-α P allele and Xx was associated with disease susceptibility and onset of the disease [71]. We also found an overrepresentation of the Pp and Xx genotypes in PBC patients.

IL-1 is a potent stimulator of the bone resorption, and it has been implicated in the pathogenesis of postmenopausal osteoporosis [45,46]. Interleukin-1 antagonist protein (IL-1ra) is a natural anti-inflammatory molecule, a competitive inhibitor of IL-1α and β. In Graves disease, polymyositis, systemic lupus erythematosus and rheumatoid arthritis elevated levels of IL-1ra have been reported [48]. Increased frequency of A2 allele was found in inflammatory and autoimmune diseases (Graves's disease, inflammatory bowel disease,

multiple sclerosis, Henoch-Schönlein nephritis, etc.) [48-51]. In vitro in the presence of A2 allele a reduced IL-1/IL-1ra ratio with increased IL-1ra production was reported [72]. Donaldson et al. found IL-1RN VNTR A1A1 genotype (60% vs. 40%, OR: 2.28) and the IL-1B 1,1 genotype (65% vs. 43%, OR: 2.37) to be more frequent in PBC compared to controls [73]. We observed a similar but not significant tendency in our PBC patients. The A1A1 genotype is not associated with reduced IL-1/IL-1Ra ratio, in concordance with the observed proinflammatory T-cell (T helper 1) profile in PBC [74]. In our study we did not find any significant difference in BMD in patients having at least one A2 allele compared to non-carriers. The presence of A2 allele was not more frequent in the non-osteoporotic patients nor we did find any correlation between IL-1RN genotype and BMD. Our results suggest that IL-1RN VNTR polymorphism may not play an essential role in the development of osteopenia in PBC, at least not in this set of patients.

Because of its important role in the acquisition of peak BMD, modeling of bone and stimulation of periosteal bone growth [39,75] several studies addressed the association between IGF-I microsatellite polymorphism and OP [39,53]. Liver derived IGF-I [75] exert a small, but significant effect on cortical periosteal bone growth and on adult axial skeletal growth. This may be affected by chronic liver diseases. Low serum IGF-I has been reported in chronic liver diseases [41,42], but no relationship was found between serum IGF-I and BMD [42]. In contrast, IGF-I treatment restored osteopenia in experimental cirrhosis [76]. In our study the 192/192 genotype was associated with higher femoral neck z-scores compared to other genotypes, which raises the possibility that IGF-I microsatellite repeat polymorphism together with genetic and/or environmental factors is involved in the complex regulation of BMD in PBC. To our knowledge this is the first report on IGF-I polymorphism in chronic liver diseases including PBC. However, in the absence in differences in genotype frequencies between patients and controls, it is unlikely that IGF-I microsatellite polymorphism plays an essential role in the pathogenesis and/or progression of PBC.

Even more complex is the relation of genetic marker with the pathogenesis and the progression of PBC. The pathogenesis of PBC is unknown, but because of the presence of typical autoantibodies it is generally considered as an autoimmune disease. Thus, the relation of some of the genetic polymorphisms to autoimmunity is of interest. Based on our data a direct relation of the investigated polymorphisms and the pathogenesis of PBC seems unlikely. However, it may be assumed, that genetic factors influence the rapidity of progression to cirrhosis.

Insulin-like growth factor-I (IGF-I) is involved in the regulation of cell growth and differentiation; it stimulates linear growth [38]. An important issue is that IGF-I treatment has been found to exert an antifibrogenic effect in experimental setting [77]. The treatment of cirrhotic rats was accompanied by the reduction hydroxyproline content, prolyl hydroxylase activity, collagen 1alphaI and 1alpha (III) m RNA expression in the liver. Furthermore IGF-I treatment partially normalized the gene expression profile and restored the expression of growth hormone receptor and the levels of global DNA methylation in experimental cirrhosis, and also enhanced the regenerative activity [78]. Low serum IGF-I has been reported in chronic liver diseases [40,41,42], however, the role of IGF-I protein in fibrogenesis is complex.

Collagen type I plays an important role in liver fibrogenesis. Early fibrotic events lead to conversion of the basement membrane-like matrix to one rich in fibrillar matrix, with dramatic increase in the expression of type I collagen [79]. Mutations in collagen type I that confers resistance to the action collagenases critically impair hepatic stellate cell apoptosis and may prevent restoration of hepatocyte mass in liver fibrosis [80], furthermore halofuginone, a collagen type I inhibitor was found to improve liver regeneration in experimental cirrhosis [81]. Since collagen type I expression is influenced by Sp1 polymorphism and lower frequency of the s allele was found in PBC, it might be hypothesized that COLIA1 may influence disease progression and fibrogenesis in patients with PBC.

It has also been suggested that IL-1RN polymorphism might influence the risk of hepatic fibrosis. In Japanese alcoholics heterozygotes for A1 allele rather than A1 homozygotes tended to have greater risk to develop fibrosis or cirrhosis [82]. Although numbers are small, in our study 7 out of 11 (63.3 %) patients heterozygous for A1 had advanced stage disease (stage III-IV), while 8 out of 22 (36.4%) of the A1 or A2 homozygotes, however, they did not differ in age nor had a longer disease history. Further studies are needed to determine, whether IL-1RN VNTR polymorphism could be associated with a more rapid disease progression.

In summary, in our studies we confirmed previous findings on higher frequency of the VDR BsmI BB genotype and B allele in patients with PBC. Our study was the first study to our knowledge to report on the ER-α XbaI and PvuII polymorphisms in PBC patients. An overrepresentation of the ER-α Pp and Xx genotypes was found in Hungarian PBC patients, while there was no difference in the IL-1RN VNTR and IGF I microsatellite repeat polymorphism between PBC patients and controls. Since IGF-I polymorphism was associated to BMD in PBC, it may be hypothesized that not COLIA1 but IGF-I together with other genetic and environmental factors may be involved in the complex regulation of BMD in PBC. In contrast to previous studies the s allele was less frequent in patients with PBC, and its presence was not associated with BMD.

REFERENCES

[1] Talwalkar JA, Lindor KD. Primary biliary cirrhosis. *Lancet* 2003;362:53-61.

[2] Guanabens N, Pares A, Marinoso L, Brancós MA, Pierra C, Serrano S, et al. Factors influencing the development of metabolic bone disease in primary biliary cirrhosis. *Am J Gastroenterol* 1990;85:1356-62.

[3] Vleggar FP, van Buuren HR, Wolfhagen FH, Schalm SW, Pols HA. Prevention and treatment of osteoporosis in primary biliary cirrhosis. *Eur J Gastroenterol Hepatol* 1999;11:617-21.

[4] Monegal A, Navasa M, Guanabens N, Peris P, Pons F, Martinez de Osaba MJ, et al. Osteoporosis and bone mineral metabolism disorders in cirrhotic patients referred for orthotopic liver transplantation. *Calcif Tissue Int* 1997;60:148-54.

[5] Long RG, Meinhard E, Skinner RK, Varghese Z, Wills MR, Sherlock S. Clinical, biochemical, and histological studies of osteomalacia, osteoporosis, and parathyroid function in chronic liver disease. *Gut* 1978;19:85-90.

[6] Arnaud SB. 25-hydroxyvitamin D3 treatment of bone disease in primary biliary cirrhosis. *Gastroenterology* 1982;83:137-49.

[7] Lakatos PL, Firneisz G, Lakatos P, Horvath C, Szalay F. Follow-up study of bone mineral density in postmenopausal patients with primary biliary cirrhosis. *Orv Hetil* 2001;142;503-8.

[8] Szalay F, Hegedus D, Lakatos PL, Bajnok E, Tornai I, Lakatos P. High serum osteoprotegerin and low RANKL levels in patients with primary biliary cirrhosis. *J Hepatol* 2003;38:395-400.

[9] Stellon AJ, Webb A, Compston J, Williams R. Low bone turnover state in primary biliary cirrhosis. *Hepatology* 1987;7:137-42.

[10] Hodgson SF, Dickson ER, Eastell R, Eriksen EF, Bryant SC, Riggs BL. Rates of cancellous bone remodelling and turnover in osteopenia associated with primary biliary cirrhosis. *Bone* 1993;14:819-27.

[11] Pocock NA, Eisman JA, Hopper JL, Yeates MG, Sambrook PN, Ebert S. Genetic determinants of bone mass in adults: A twin study. *J Clin Invest* 1987;80:700-710.

[12] Gueguen R, Jouanny P, Guillemin F, Kuntz C, Pourel J, Siest G. Segregation analysis and variance components analysis of bone mineral density in healthy families. *J Bone Miner Res* 1995;12:2017-20.

[13] Morrison NA, Qi JC, Tokita A, Kelly P, Crofts L, Nguyen TV, et al. Prediction of bone density from vitamin D receptor alleles. *Nature* 1994;367:706-710.

[14] Cooper GS, Umbach DM. Are vitamin D receptor polymorphisms associated with bone mineral density? A meta-analysis. *J Bone Miner Res* 1996;12:1841-49.

[15] Fleet JC, Harris SS, Wood RJ, Dawson-Hughes B. The BsmI vitamin D receptor restriction fragment lenght polymorphism (BB) predicts low bone density in premenopausal black and white women. *J Bone Miner Res* 1995;10:985-90.

[16] Willing M, Sowers M, Aron D, Clarck MK, Burns T, Bunten C, et al. Bone mineral density and its change in white women: estrogen and vitamin D receptor genotypes and their interaction. *J Bone Miner Res* 1998;13:695-705.

[17] Deng HW, Li J, Li JL, Johnson M, Gong G, Davis KM, et al. Change of bone mass in postmenopausal Caucasian women with and without hormone replacement therapy is associated with vitamin D receptor and estrogen receptor genotypes. *Hum Genet* 1998;103:576-85.

[18] Garnero P, Borel O, Sornay-Rendu E, Arlot ME, Delmas PD. Vitamin D receptor gene polymorphisms are not related to bone turnover, rate of bone loss, and bone mass in postmenopausal women: the OFELY study. *J Bone Miner Res* 1996;11:827-34.

[19] Tsai KS, Hsu SH, Cheng WC, Chen CK, Chieng PU, Pan WH. Bone mineral density and bone markers in relation to vitamin D receptor gene polymorphism in Chinese men and women. *Bone* 1996;19:513-8.

[20] Halmos B, Szalay F, Cserniczky T, Nemesanszky E, Lakatos P, Barlage S, et al. Association of primary biliary cirrhosis with vitamin D receptor BsmI genotype in a Hungarian population. *Dig Dis Sci* 2000;45:1091-95.

[21] Springer JE, Cole DEC, Rubin LA, Cauch-Dudek K, Harewood L, Evrovski J, et al. Vitamin D-receptor genotypes as independent genetic predictors of decreased bone mineral density in primary biliary cirrhosis. *Gastroenterology* 2000:118:145-51.

[22] Pares A, Guanabens N, Alvarez L, De Osaba MJ, Oriola J, Pons F, et al. Collagen type Ialpha1 and vitamin D receptor gene polymorphisms and bone mass in primary biliary cirrhosis. *Hepatology* 2001;33:554-60.

[23] MacDonald HM, McGuigan FA, New SA, Campbell MK, Golden MH, Ralston SH, et al. COLIA1 Sp1 polymorphism predicts premenopausal and elderly postmenopausal spinal bone loss. *J Bone Miner Res* 2001;16:34-41.

[24] Bernard M, Martinez ME, Escalona M, Gonzalez ML, Gonzalez C, Garces MV et al. Polymorphism in the type I collagen (COLIA1) gene and risk of fractures in posmenopausal women. *Bone* 2002;30:223-8.

[25] Sluis IM, Muinck Keizer-Schrama SM, Pols HA, Lequin MH, Krenning EP, Uitterlinden AG. Collagen Ia1 Polymorphism is Associated with Bone Characteristics in Caucasian Children and Young Adults. *Calcif Tissue Int* 2002;71:393-9.

[26] Kann P, Bergink AP, Fang Y, Van Daele PL, Hofman A, Van Leeuwen JP et al. The collagen Ia1 Sp1 polymorphism is associated with differences in ultrasound transmission velocity in the calcaneus in postmenopausal women. *Calcif Tissue Int* 2002;70:450-6.

[27] Efstathiadou Z, Tsatsoulis A, Ioannidis JPA. Association of collagen I□1 Sp1 polymorphism with the risk of prevalent fractures: a meta-analysis. *J Bone Miner Res* 2001;16:1586-92.

[28] Mann V, Hobson EE, Li B, Stewart TL, Grant SFA, Robins SP et al. A COLIA1 Sp1 binding site polymorphism predisposes to osteoporotic fracture by affecting bone density and quality. *J Clin Invest* 2001;107:899-907.

[29] Pares A, Guanabens N, Alvarez L, De Osoba MJ, Oriola J, Pons F et al. Collagen type I alpha1 and vitamin D receptor gene polymorphisms and bone mass in primary biliary cirrhosis. *Hepatology* 2001;33:554-60.

[30] Kobayashi S, Inoue S, Hosoi T, Ouchi Y, Shiraki M, Orimo H. Association of bone mineral density with polymophism of the estrogen receptor gene. *J Bone Miner Res* 1996;11:306-11.

[31] Vandevyver C, Vanhoof J, Declerck K, Stinissen P, Vandervorst C, Michiels L, et al. Lack of association between estrogen receptor genotypes and bone mineral density, fracture history, or muscle strength in elderly women. *J Bone Miner Res* 1999;14:1576-82.

[32] Albagha OME, McGuigan FEA, Reid DM, Ralston ST. Estrogen receptor a gene polymorphisms and bone mineral density: haplotype analysis in women from United Kingdom. *J Bone Miner Res* 2001;16:128-134.

[33] Salmén T, Heikkinen AM, Mahonen A, Kröger H, Komulainen M, Saarikoski S, et al. Early postmenopausal bone loss is associated with PvuII estrogen receptor gene polymorphism in Finnish women: effect of hormone replacement therapy. *J Bone Miner Res* 2000;15:15:315-321.

[34] Ho AZ, Yeung SS, Kung AW. PvuII polymorphisms of the estrogen receptor alpha and bone mineral density in healthy southern Chinese women. *Calcif Tissue Int* 2000;66:405-8.

[35] Bagger YZ, Jorgensen HL, Heegaard AM, Bayer L, Hansen L, Hassager C. No major effect of estrogen gene polymorphisms on bone mineral density or bone loss in postmenopausal Danish women. *Bone* 2000;26:111-16.

[36] Korach KS. Insights from the study of animals lacking functional estrogen receptor. *Science* 1994;266:1524-27.

[37] Rosen CJ, Kurland ES, Vereault D, Adler RA, Rackoff PJ, Craig WY et al. Association between serum insulin growth factor-I (IGF-I) and simple sequence repeat in IGF-I gene: implications for genetic studies of bone mineral density. *J Clin Endocrinol Metab* 1998;83:2286-90.

[38] Rosen CJ. Serum insulin-like growth factors and insulin-like growth factor proteins:clinical implications. *Clin Chem* 1999;45:1384-90.

[39] Yakar S, Rosen CJ, Beamer WG, Ackert-Bicknell CL, Wu Y, Liu JL et al. Circulating levels of IGF-1 directly regulate bone growth and density. *J Clin Invest* 2002;110:771-81.

[40] Ormarsdottir S, Ljunggren Ö, Mallmin H, Olofsson H, Blum WF, Lööf L. Circulating levels of insulin-like growth factors and their binding proteins in patients with chronic liver disease: lack of correlation with bone mineral density. *Liver* 2001;21:123-8.

[41] Yoshida S, Nio M, Hayashi Y, Ohi R, Kawamura I, Goto T. Serum insulin like growth factor-I in biliary atresia. *J Pediatr Surg* 2003;38:211-5.

[42] Okan A, Comlekci A, Akpinar H, Okan I, Yesil S, Tankurt E, Simsek I. Serum concentrations of insulin-like growth factor-I and insulin-like growth factor binding protein 3 in patients with chronic hepatitis. *Scand J Gastroenterol* 2000;35:1212-5.

[43] Rosen CJ. IGF-I and osteoporosis. *Clin Lab Med*. 2000;20:591-602.

[44] Guise TA, Garrett R, Bonewald LF, Mundy GR. Interleukin-1 receptor antagonist inhibits the hypercalcaemia mediated by interleukin-1. *J Bone Miner Res* 1993;8:583-7.

[45] Khosla S, Peterson JM, Egan K, Jones JD, Riggs BL. Circulating cytokine level in osteoporotic and normal women. *J Clin Endocrinol Metab* 1994;79:707-711.

[46] Keen RW, Woodford-Richens KL, Lanchbury JS, Spector TD. Allelic variation at the interleukin-1 receptor antagonist gene is associated with early postmenopausal bone loss in the spine. *Bone* 1998;23:367-71.

[47] Bajnok É, Takács I, Vargha P, Speer G, Nagy Z, Lakatos P. Lack of association beween interleukin-1 receptor antagonist protein gene polymorphism and bone mineral density in Hungarian Postmenopausal women. *Bone* 2000;27:559-62.

[48] Arend WP, Malyak M, Guthridge CJ, Gabay C. Interleukin-1 receptor antagonist: role in biology. *Annu Rev Immunol* 1998;16:27-35.

[49] De la Concha EG, Arroyo R, Crusius JBA, Campillo JA, Martin C, De Seijas EV, et al. Combined effect of HLA-DRB1*1501 and interleukin-1 receptor antagonist gene allele 2 in susceptibility to relapsing/remitting multiple sclerosis. *J Neuroimmunol* 1997;80:172-78.

[50] Blackmore AI, Watson PF, Weetman AP, Duff GW. Association of Graves' disease with an allele of interleukin-1 receptor antagonist gene. *J Clin Endocrinol Metab* 1995;80:111-5.

[51] Nemetz A, Kope A, Molnar T, Kovacs A, Feher J, Tulassay Z, et al. Significant differences in interleukin-1beta and interleukin-1 receptor antagonist gene polymorphisms in a Hungarian population with inflammatory bowel disease. *Scand J Gastroenterol* 1999;34:175-9.

[52] Tarlow JK, Blakemore AIF, Lennard A, Solari R, Hughes HN, Steinkasserer A, Duff GW. Polymorphism in human IL-1 receptor antagonist gene intron 2 is caused by variable numbers of an 86-by tandem repeat. *Hum Genet* 1993;91:403-404.

[53] Takacs I, Koller DL, Peacock M, Christian JC, Hui SL, Coneally PM, et al. Sibling pair linkage and association studies between bone mineral density and the insulin-like growth factor I gene locus. *J Clin Endocrinol Metab* 1999;84:4467-71.

[54] Grant SFA, Reid DM, Blake G, Herd R, Fogelman I, Ralston SH. Reduced bone density and osteoporosis associated with a polymorphic Sp1 site in the collagen type I alpha 1 gene. *Nat Genet* 1996; 14: 203-5.

[55] Kanis JA, Melton LJ, Christiansen C, Johnston CC, Khaltaes N. The diagnosis of osteoporosis. *J Bone Miner Res* 1994;9:1137-41.

[56] Lakatos LP, Bajnok E, Hegedus D, Toth T, Lakatos P, Szalay F. Vitamin D receptor Bsm I, estrogen receptor alpha gene Pvu II and Xba I and IL-1 receptor-antagonist gene VNTR polymorphism and bone mineral density in Hungarian patients with primary biliary cirrhosis *Eur J Gastroenterol Hepatol* 2002;14:733-40.

[57] Crippin JS, Jorgensen RA, Dickson ER, Lindor KD. Hepatic osteodystrophy in primary biliary cirrhosis: effects of medical treatment. *Am J Gastroenterol* 1994;89:47-51.

[58] Pereira SP, Bray GP, Pitt PI, Li F, Moniz C, Williams R. Non-invasive assessment of bone mineral density in primary biliary cirrhosis. *Eur J Gastroenterol Hepatol* 1999;11:323-8.

[59] Lindor KD, Janes CH, Crippin JS, Jorgensen RA, Dickson ER. Bone disease in primary biliary cirrhosis: does ursodeoxycholic acid make a difference? *Hepatology* 1995;21:389-392.

[60] Jones DE, Donaldson PT. Genetic factors in the pathogenesis of primary biliary cirrhosis. *Clin Liver Dis* 2003;7:841-64.

[61] Corpechot C, Benlian P, Barbu V et al.. Apolipoprotein E polymorphism, a marker of disease severity in primary biliary cirrhosis. *J Hepatol* 2001;35:324-8.

[62] Invernizzi P, Battezzati PM, Crosignani A et al. Peculiar HLA polymorphisms in Italian patietns with primary biliary cirrhosis. *J Hepatol* 2003;38:401-6.

[63] Vogel A, Strassburg CP, Manns MP. Genetic association of vitamin D receptor polymorphisms with primary biliary cirrhosis and autoimmune hepatitis. *Hepatology*. 2002;35:126-31.

[64] Lemire JM. Immunmodulatory actions of 1,25 dihydroxyvitamin D3. *J Steroid Biochem Mol Biol* 1995;53:599-602.

[65] Clavreul A, D'Hellencourt CL, Montero-Menei C, Potron G, Couez D. Vitamin D differentially regulates B7.1 and B7.2 expression on human peripherial blood monocytes. *Immunology* 1998;95:272-77.

[66] Ban Y, Taniyama M, Ban Y. Vitamin D receptor gene polymorphism is associated with Graves' disease in the Japanese population. *J Clin Endocrinol Metab* 2000;85:4639-4643.

[67] Obermayer-Pietsch BM, Frühauf GE, Chararas K, Mikhail-Reinisch S, Renner W, Berghold A, et al. Association of the vitamin D receptor genotype BB with low bone density in hyperthyroidism. J *Bone Miner Res* 2000;15:1950-55.

[68] Bellamy R, Ruwende C, Corrah T et al: Tuberculosis and chronic hepatitis B virus infection in Africans and variation in the Vitamin D receptor gene. *J Infect Dis* 1999;179:721-4.

[69] Tornwall J, Carrey AB, Fox RI, Fox HS. Estrogen in autoimmunity: expression of estrogen receptors in thymic and autoimmune T cells. *J Gend Specif Med* 1999;2:33-40.

[70] Rider V, Jones SR, Evans M, Abdou NI. Molecular mechenisms involved in the estrogen-dependent regulation of calcineurin in systemic lupus erythematosus T cells. *Clin Immunol* 2000;95:124-34.

[71] Niino M, Kikuchi S, Fuazawa T, Yabe I, Tashiro K. Estrogen receptor gene polymorphism in Japanese patients with multiple sclerosis. *J Neurol Sci* 2000;179:70-5.

[72] Danis VA, Millington M, Hyland VJ, Grennan D. Cytokine production by normal human monocytes: inter-subject variation and relationship to an IL-1 receptor antagonist (IL-1Ra) gene polymorphism. *Clin Exp Immunol* 1995;99:303-10.

[73] Donaldson P, Agarwal K, Craggs A et al. HLA and interleukin 1 gene polymorphisms in primary biliary cirrhosis: association with disease progression and disease susceptibility. *Gut* 2001;48:397-402.

[74] Harada K, Van de Water J, Leung PSC et al. In situ nuclein acid hybridization of cytokines in primary biliary cirrhosis: Predominance of the Th1 subset. *Hepatology* 1997;25:791-6.

[75] Sjögren K, Sheng M, Moverare S, Liu JL, Wallenius K, Törnell J et al. Effects of liver-derived insulin-like growth factor I on bone metabolism in mice. *J Bone Miner Res* 2002;17:1977-87.

[76] Cemborain A, Catilla-Cotazar I, Garcia M, Muguerza B, Delgado G, Diaz-Sanchez M, et al. Effects of IGF-I treatment on osteopenia in rats with advanced liver cirrhosis. *J Physiol Biochem* 2000;56:91-9.

[77] Muguerza B, Castilla-Cortazar I, Garcia M, Quiroga J, Santidrian S, Pitero J. Antifibrogenic effect in vivo of low doses of insulin-like growth factor-I in cirrhotic rats. *Biochim Biophys Acta* 2001;1536:185-95.

[78] Mirpuri E, Garcija-Trevijano ER, Castilla-Cortazar I, Berasain C, Quiroga J, Rodrigez-Ortigosa C et al. Altered gene expression in CCL4-cirrhotic rats is partially normalized by insulin-like growth factor-I. *Int J Biochem Cell Biol* 2002;34:242-52.

[79] Yang C, Zeisberg M, Mosterman B, Sudhakar A, Yerramalla U, Holthaus K et al. Liver fibrosis: insights into migration of hepatic stellate cells in response to extracellular matrix and growth factors. *Gastroenterology* 2003;114:147-59.

[80] Issa R, Zhou X, Trim N, Millward-Sadler H, Krane S, Benyon C, et al. Mutation in collagen-1 that confers resistance to the action of collagenase results in failure of

recovery from CCl4-induced liver fibrosis, persistence of activated hepatic stellate cells and diminished hepatocyte regeneration. *FASEB J* 2003;17:47-9.

[81] Spira G, Mawasi N, Paizi M, Anbinder N, Genina O, Alexiev R, et al. Halofuginone, a collagen type I inhibitor improves liver regeneration in cirrhotic rats. *J Hepatol* 2002;37:331-9.

[82] Takamatsu M, Yamauchi M, Maezawa Y, Ohata M, Saitoh S, Toda G. Correlation of a polymorphism in the interleukin-1 receptor antagonist gene with hepatic fibrosis in Japanese alcoholics. *Alcohol Clin Exp Res* 1998;22(suppl3):s141-s144.