

The role of cigarette smoking and statins in the development of postmenopausal osteoporosis: a pilot study utilizing the Marshfield Clinic Personalized Medicine Cohort

P. F. Giampietro · C. McCarty · B. Mukesh ·
F. McKiernan · D. Wilson · A. Shuldiner · J. Liu ·
J. LeVasseur · L. Ivacic · T. Kitchner · N. Ghebranious

Received: 3 March 2009 / Accepted: 23 April 2009

© International Osteoporosis Foundation and National Osteoporosis Foundation 2009

Abstract

Summary A cohort of postmenopausal osteoporotic females and controls with normal bone mineral density, the interleukin 6 (*IL6*) -634G>C (rs1800796) C allele of the promoter region showed association with osteoporosis.

P. F. Giampietro
Marshfield Clinic, Department of Medical Genetic Services,
1000 North Oak Avenue,
Marshfield, WI 54449, USA

C. McCarty · L. Ivacic · T. Kitchner · N. Ghebranious
Marshfield Clinic Research Foundation,
Center for Human Genetics,
Marshfield, WI, USA

B. Mukesh
Alcon Research Ltd.,
Fort Worth, TX, USA

F. McKiernan · D. Wilson
Marshfield Clinic, Department of Rheumatology,
Marshfield, WI, USA

A. Shuldiner · J. Liu
University of Maryland School of Medicine,
Baltimore, MD, USA

J. LeVasseur
University of Wisconsin-Madison,
2015 Linden Drive,
Madison, WI, USA

J. LeVasseur
Marshfield Clinic Research Foundation,
Marshfield, WI, USA

P. F. Giampietro (✉)
Department of Pediatrics, University of Wisconsin-Madison,
Waisman Center 351, 1500 Highland Avenue,
Madison, WI 53705-9345, USA
e-mail: pfgiampietro@pediatrics.wisc.edu

The lipoprotein receptor-related protein 5 (*LRP5*) gene showed association between C135242T C/T alleles and osteoporosis only in smokers, suggesting a role for environmental interaction.

Introduction A nested case-control study within a population-based cohort was undertaken to assess the relative impact of cigarette smoking, statin use, genetic polymorphisms, and one-way interaction of these factors on development of osteoporosis in postmenopausal women.

Methods Genotyping of 14 single-nucleotide polymorphisms (SNPs) corresponding to *vitamin D receptor gene*, *estrogen receptor 1*, *collagen type 1 alpha 1*, *IL6*, *transcription growth factor beta*, *apolipoprotein E*, and *LRP5* genes was performed in cases ($n=309$) with osteoporosis and controls ($n=293$) with normal bone mineral density drawn from a homogeneous Caucasian population. SNPs were chosen based on known functional consequences or prior evidence for association and genotyped using matrix-assisted laser desorption ionization time-of-flight technology.

Results Cases differed from controls relative to body mass index, age, and smoking but not statin use. After adjusting for age, the *IL6* -634G>C (rs1800796) allele showed association with osteoporosis (odds ratio (OR) for CC+CG=2.51, $p=0.0047$), independent of statin use or smoking status. On stratification for smoking, association with *LRP5* C135242T (rs545382) and osteoporosis emerged (OR 2.8 in smokers for CT alleles, $p=0.03$), suggestive of potential environmental interaction.

Conclusion Evidence suggested a role for genetic variation in *IL6* and *LRP5* in conferring risk for osteoporosis in Caucasian women, with the latter manifest only in smokers.

Keywords Cigarette · Gene-environment interaction · *IL6* · *LRP5* · Osteoporosis · Smoking

Introduction

Osteoporosis is defined as a skeletal disorder associated with compromised bone strength and increased risk of fracture (2000 NIH Consensus Development Conference). Osteoporotic bone is characterized by decreased bone mass and deterioration of its microarchitecture [1]. The clinical significance of osteoporosis is the occurrence of low-energy fractures, most notably of the hip, spine, and distal forearm. Since there is no practical *in vivo* measurement of bone strength, bone mineral density (BMD) is used as a surrogate measure for fracture risk. The most common means of assessment of BMD is dual-energy X-ray absorptiometry (DXA). This measurement is estimated to account for 70% of bone strength [2]. The remainder of bone strength is attributable to the quality of bone and includes factors such as microarchitecture, quality of organic matrix, degree of mineralization, accumulation of microdamage, state of bone turnover, bone size, and geometry [3].

The public health significance of osteoporosis is significant since approximately 20% of all postmenopausal women in Western countries meet densitometric criteria for osteoporosis and 1.5 million fractures are attributable to osteoporosis annually in the US. Given increasing longevity, annual hip fracture rates are expected to increase globally from 1.66 million in 1990 to 6.26 million by 2050 [4]. Restraining the increasing prevalence of osteoporosis will require changes in lifestyle and improvements in treatment modalities.

Osteoporosis is a multifactorial disease, which is determined by complex interactions between the genetic and environmental factors which govern peak bone mass, rate of bone loss, bone strength, and bone turnover. Genetic factors are estimated to account for 50–80% of the interindividual variation in BMD [5, 6]. Most genetic studies have focused on BMD as a phenotypic marker for osteoporosis. Association with BMD and/or osteoporosis has been reported for over 60 genes, including those related to calciotropic hormones and receptors, cytokines, growth factors and receptors, bone matrix proteins, and various other proteins [7]. These genes account for less than 5% of the variance in BMD attributable to genetic factors within the general population [8]. Multiple demographic and environmental factors have been evaluated for contribution to the development of osteoporosis and/or low BMD with inconsistent results. These include cigarette smoking [9], alcohol excess, low dietary intake of calcium, vitamin D insufficiency, low body mass index (BMI) [10], elevated parathyroid hormone, homocysteine [11], Caucasian race, female sex, and prior history of fracture [12].

Since the contribution of any individual gene to the development of osteoporosis is expected to be relatively small on a population level, we undertook a study to analyze genetic

and environmental factors in women with postmenopausal osteoporosis in the Marshfield Clinic Personalized Medicine Research Project (PMRP) Cohort. Our working hypothesis states that gene–gene and gene–environmental interaction is associated with postmenopausal osteoporosis. The clinical application of this analysis would facilitate identification of individuals with underlying genetic susceptibilities with potential to interact with defined environmental exposures in order to target avoidance of those risk factors in order to minimize risk.

Methods

A nested case–control study design was undertaken utilizing banked data and DNA from the PMRP Cohort [13]. Logistic regression analysis was undertaken to define genetic and environmental interactions with osteoporosis defined as the outcome parameter. Risk factors analyzed included cigarette smoking and statins as the environmental exposures of interest and 14 polymorphic alleles occurring within the, vitamin D receptor gene (*VDR*), estrogen receptor 1, collagen type 1 alpha 1 (*COL1A1*), interleukin 6 (*IL6*), transcription growth factor beta (*TGF- β*), apolipoprotein E, and lipoprotein receptor-related protein 5 (*LRP5*) genes.

Personalized medicine research project cohort

PMRP has established a population-based DNA and phenotypic biobank which serves as a resource for studies related to the genetic basis of health and disease [13]. Approximately 20,000 subjects age 18 years and over have been enrolled in PMRP to date. Participating subjects reside in the Marshfield Clinic Epidemiologic Study Area (MESA) in a 14-zip-code area surrounding the clinic and receive care almost exclusively from the clinic so that very complete, comprehensive, electronic medical records are available for these individuals. In addition, information relative to environmental exposures was collected and stored on individuals at the time of enrollment. Participants consented to DNA banking and access to their electronic medical record for the purpose of obtaining study-related phenotypic data.

Potential cases were initially identified electronically applying “rule of 2” in which documentation of at least two occurrences of an International Classification of Diseases 9 code is required to validate the clinical diagnoses of osteoporosis. FreePharma software was used to identify documentation of utilization of pharmaceuticals prescribed for management of osteoporosis including collection of at least two occurrences of documentation of dose and frequency of use. Established practice guidelines were

utilized to define BMD cutoff values that identified presence of osteoporosis [14]. Postmenopausal subjects aged 50 years and greater were classified as having osteoporosis if they met either of the following two criteria:

1. BMD T-score ≤ -2.5 at lumbar spine (or assessable subregion), proximal femur or nondominant forearm
2. One or more low-energy fractures of the spine, hip, radius, or pelvis

All DXA scans were performed at Marshfield Clinic or satellite centers. Scans performed at satellite centers were reviewed by one of two rheumatologists who were study coinvestigators. The practice guidelines of International Society for Clinical Densitometry (ISCD) for interpretation of BMD results were followed [14].

Potential controls were excluded if they were taking estrogen or had an existing diagnosis of osteoporosis, osteopenia, or a low-energy fracture. Controls must have had a DXA scan within 24 months of the date of the electronic interrogation of the medical record.

Cigarette-smoking history in cases and controls was abstracted from the environmental exposure questionnaires completed by subjects on enrollment into PMRP. Two smoking variables were constructed to facilitate logistic modeling: (1) ever/never binary variable and (2) current/past/never ordinal variable.

Statin use was ascertained by electronic phenotyping using FreePharma software to minimize potential errors associated with reliance on patient recall of exposure [15, 16]. Interrogation of the medical record was undertaken for the following statins: lovastatin, fluvastatin, cerivastatin, pravastatin, simvastatin, atorvastatin, and rosuvastatin. This approach for detecting exposure to statins has been piloted

previously at Marshfield Clinic and exhibited a sensitivity of 100% and specificity of 96%. Actual statin dosage was manually abstracted. Two statin variables were created for inclusion in the multivariate models: (1) ever/never user as a binary variable and (2) “statin-years” of exposure, similar to pack-years of smoking that incorporated both dose and duration of statin use.

Quality assurance (QA) was performed in two phases. Phenotypic data from a subset of 150 cases and controls (25% of the total study population) from a prior phenotyping engine pilot study were compared with the phenotypic data obtained from the current study for validation. In addition, a second QA of 19/602 (3%) cases and controls was performed using double-blind data extraction. The concordance rate for both QAs was 100%. ISCD-certified study coinvestigators (DW, FM) reviewed individual DXA scans when appropriate.

Genotyping

Genes and single-nucleotide polymorphisms (SNPs) and their corresponding minor allele frequencies listed in Table 1 were chosen for evaluation in this study because of prior demonstration of association with osteoporosis. The genotyping analysis of the SNP alleles in Table 1 was performed using the Sequenom MassARRAY SNP genotyping system (San Diego, CA, USA). The system utilizes the iPLEX assay, a primer extension reaction chemistry designed to detect sequence differences at the single-nucleotide level. Each sample was amplified in a multiplexed reaction utilizing specific primers prepared using sequence templates defined in Table 2. Matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry was used to detect mass difference that allows discrimination among the encoded SNP alleles.

Table 1 Osteoporosis candidate genes and SNPs analyzed

Gene	SNP	Minor allele frequency	Ref
<i>VDR</i>	rs1544410 (<i>BsmI</i>)	0.29	[40]
<i>VDR</i>	rs7975232 (<i>ApaI</i>)	0.47	[41]
<i>VDR</i>	rs731236 (<i>TaqI</i>)	0.30	[41]
<i>VDR</i>	rs10735810 (<i>FokI</i>)	0.31	[42]
<i>ESR1</i>	rs9340799 (<i>XbaI</i>)	0.29	[43]
<i>ESR1</i>	rs2234693 (<i>PvuII</i>)	0.46	[44]
<i>COL1A1</i>	rs1800012	0.19	[45]
<i>IL6</i>	rs1800796	0.24	[39]
<i>TGFBeta</i>	rs8179182 (713-8delC)	0.02	[46]
<i>ApoE</i>	rs429358	0.07	[47]
<i>ApoE</i>	rs7412	0.03	[47]
<i>LRP5</i>	rs545382 (C135242T)	0.15	[20]
<i>LRP5</i>	rs2306862 (C141759T)	0.16	[20]
<i>LRP5</i>	rs4988320 (G121513A)	0.20	[20]
<i>LRP5</i>	rs4988321 (G138351A)	0.13	[20]

Table 2 Primer sequences used for multiplex reactions

SNP identification	Forward PCR primer	Reverse PCR primer	Extension primer
rs1544410	ACGTTGGATGAATGTTGAGCCCAAGTTCACG	ACGTTGGATGGAGGAACCTAGATAAGCAGGG	TAGCCTGAGTATTGGGAATG
rs7975232	ACGTTGGATGTTGAGTGTCTGTGTGGGTGG	ACGTTGGATGAGAGAAGAAGGCACAGGAGC	TGGGATTGAGCAGTGAGG
rs731236	ACGTTGGATGTTGGACAGCGGCTCTGGAT	ACGTTGGATGTTCTATATCCCGTGCCCA	CGGTCTGGATGGCCTC
rs10735810	ACGTTGGATGAAGTCTCCAGGTCAGGCA	ACGTTGGATGTGGCCTGCTTGTGTTCTTA	TGGCCGCCATTGCCTCC
rs9340799	ACGTTGGATGGAACCATAGAGACCAATGC	ACGTTGGATGATGCTTGTCTCTGTTTCCC	ATGCTCATCCCAACTC
rs2234693	ACGTTGGATGACTCAGGGTCTCTGGAAAC	ACGTTGGATGTCCATCATGTTTCACTGAG	ACAGAGACAAAGCATAAAAC
rs1800012	ACGTTGGATGGCCACCCCACTGCCCCAG	ACGTTGGATGAGAGACAGGAGGAGGGCGAG	CCCACCTGCCAGGGAATG
rs1800796	ACGTTGGATGCCCTTGAAGTAACTGCACGAA	ACGTTGGATGTTCTGTGTTCTGGCTCTCCC	GCAGTTCTACAACAGCC
rs8179182	ACGTTGGATGGGTTCAATGCCATGAATGGTG	ACGTTGGATGTGTGTATGTCCCTATCC	TAGTGAACCCCTGCTTTG
rs429358	ACGTTGGATGGCTGGCGCGGACATGGAG	ACGTTGGATGTCGGTGTCTTGCCGAGCAT	GCGGACATGGAGGACGTG
rs7412	ACGTTGGATGTAAGCGGCTCTCCCGGAT	ACGTTGGATGGCCCGGCTGTACTG	CGATGACCTGCAGAAG
rs545382	ACGTTGGATGATGTGTGATGGACGAAGAGG	ACGTTGGATGCAGTCCAGTAGATGAAGTCC	AGCTCCCGCACATTTT
rs2306862	ACGTTGGATGACACGAGGACTTGCCTGGAAC	ACGTTGGATGATGGCAAGAACCTCTACTG	CCGGCCACTTCGATTTCT
rs4988320	ACGTTGGATGTGACCCCATGAGTCTGTCTG	ACGTTGGATGACATTTAGCCATGTGATGGG	AGTCTGTCTGGTCTCTG
rs4988321	ACGTTGGATGTCTCCCTCGAGACCAATAAC	ACGTTGGATGGGTTGTTGGACACATCAAAG	gTCGAGACCAATAAACACGAC

PCR reaction

Genomic DNA (30–45 ng) was amplified in a 5- μ L reaction containing 0.5 U of HotStar Taq enzyme (Qiagen, Valencia, CA, USA), 1.25 \times buffer, 1.6 mM MgCl₂, 0.5 mM each dNTP (Invitrogen, Carlsbad, CA, USA) and 100 nM of each gene-specific forward and reverse primer (Integrated DNA Technologies, Inc., Coralville, IA, USA). The reactions were run in a 384-microtiter plate format and amplified in a 9700 thermocycler (Applied Biosystems, Foster City, CA, USA). Cycling conditions were 15 min at 94°C, 45 cycles of 20 s at 94°C, 30 s at 56°C, 60 s at 72°C, and final extension time of 3 min at 72°C. After the polymerase chain reaction (PCR) amplification, Arctic shrimp alkaline phosphatase is added to the samples, which are then incubated for 40 min at 37°C to dephosphorylate any residual amplification nucleotides.

iPLEX reaction

The extension primers, iPLEX enzyme, iPLEX buffer, and the iPLEX termination mixture of mass-modified dideoxynucleotide triphosphates are added to initiate the iPLEX primer extension reaction. This reaction generates allele-specific primer extension products that are one base longer than the original MassEXTEND primer. The extend mixture (2 μ L) is added to the PCR/SAP mixture (7 μ L) for a total volume of 9 μ L containing 0.222 \times iPLEX buffer, 1 \times iPLEX Termination Mix, 0.625 to 1.25 μ M of each extension primer, and 1 \times iPLEX enzyme. The extension reaction consists of a two-step 200 short cycles program consisting of one loop of five cycles inside a loop of 40 cycles. The sample is denatured at 94°C for 30 s. Strands are then annealed at 52°C for 5 s and extended at 80°C for 5 s. The annealing and extension cycle is repeated four more times for a total of five cycles and then looped back to a 94°C denaturing step for 5 s and then enters the five-cycle annealing and extension loop again. The five annealing and extension steps with the single denaturing step are repeated an additional 39 times for a total of 40, which equate to a total number of 200 cycles. Following the extension reaction, MassEXTEND clean resin is added to the reaction to remove extraneous salts that interfere with MALDI-TOF analysis.

MALDI-TOF analysis

The sample is then transferred in 15-nL quantities from the 384-well plate and spotted onto the pad of the 384 SpectroCHIP array chip. The chip is placed into the MALDI-TOF, which detects the mass difference that differentiates between SNP alleles.

Statistical analysis

All factors (genetic and environmental) were modeled in univariate logistic models using the package SAS (Cary, NC, USA). This modeling evaluated independent associations between each independent genetic and environmental factor and osteoporosis. Models were also tested for all one-way interactions (gene–gene and gene–environment). This analysis evaluated multiple gene–gene and gene–environmental interactions and their potential contribution to the development of osteoporosis. BMI was included as a potential confounder in all models. Low BMI increases risk of osteoporosis while high BMI increases risk of elevated lipids and use of statins. A *p* value of 0.05 was considered statistically significant.

Results

Age, smoking status, BMI, T-score, and statin usage for the study population are summarized in Table 3. Significant differences were observed among cases and controls. Cases were significantly older and had a greater percentage of smoking individuals and lower T-score values than the control individuals. Statin use was equivalent among case and control groups.

Logistic regression analysis adjusted for IL6, age, BMI, and smoking status is shown in Table 4. The *IL6* –634G>C (rs1800796) allele of the promoter region exhibited association with osteoporosis (odds ratio (OR) for CC+CG=2.51 (95% confidence interval (CI)=1.33, 4.75; *p*=0.0047)), independent of statin use or smoking status.

Logistic regression analysis of *LRP5* adjusting for age, BMI, and smoking was performed with all *LRP5* alleles listed in Table 1. The ORs obtained indicate a lack of significant differences among *LRP5* genotypes between cases and controls suggesting that *LRP5* genotype alone was not associated with osteoporosis in this population (data not shown). Gene–gene and gene–environment modeling was significant for association between *LRP5* C135242T and cigarette smoking with an OR of 2.8 in smokers with the C/T alleles (95% CI=1.1, 7.0 *p*=0.03). These data are shown in Table 5.

Discussion

Since the relative contribution of individual genes to susceptibility for osteoporosis is expected to be small, we undertook an analysis to determine whether gene–environment associations for osteoporosis could be identified and to evaluate their relative contribution to risk. This study is the first to demonstrate evidence for an interaction between cigarette smoking and *LRP5* in association with osteoporosis. A significant association with an OR of 2.78 was observed between the *LRP5* C135242T SNP and BMD when cases and controls were stratified by smoking status. The *LRP5* gene encodes a transmembrane protein involved in Wnt signaling: LRP5. The Wnt pathway plays an important role in bone development and metabolism [17, 18]. Recent experimental evidence in mice indicates that LRP5 indirectly regulates bone mass by inhibiting tryptophan hydroxylase (Tph), a rate-limiting enzyme for serotonin synthesis in duodenal enterochromaffin cells

Table 3 Baseline characteristics of study population

Baseline characteristics	Controls (<i>N</i> =293)	Cases (<i>N</i> =309)	<i>p</i> value
Age (years)	61.6±8.8	70.4±9.4	<0.001
Smoke			
Never smoked	176 (60.1%)	217 (70.2%)	0.02
Past smoker	27 (9.2%)	15 (4.9%)	
Current smoker	90 (30.7%)	77 (24.9%)	
Body mass index	32.9±6.7	26.9±5.0	<0.001
BMD T-scores			
ROI spine ^a	0.5 (–1.0, +4.0)	–2.5 (–5.3, +1.2)	<0.001
Hip ^a	–0.1 (–1.0, +2.7)	–2.6 (–5.0, –0.6)	<0.001
Femoral neck ^a	–0.1 (–1.0, +2.2)	–2.5 (–3.8, –0.5)	<0.001
Trochanter ^a	0.1 (–1.0, +3.1)	–2.3 (–5.0, –0.4)	<0.001
Total hip ^a	0.4 (–0.9, +3.2)	–1.9 (–3.9, –0.1)	<0.001
Distal radius ^a	0.2 (–0.8, +2.2)	–2.5 (–5.6, –0.4)	<0.001
Ever use of statin			
Yes	115 (39.3%)	118 (38.2%)	0.79
No	178 (60.8%)	191 (61.8%)	

^aData represent median (range)

Table 4 Logistic regression analysis for IL6 adjusting for age, BMI, and smoking

Genotype rs1800796 (IL6—634G>C)	Controls (N=293)	Cases (N=309)	Odds ratio (95% CI)
GG	265 (90.4%)	266 (86.1%)	1.00
CG	24 (8.2%)	43 (13.9%)	3.35 (1.71, 6.60)*
CC	4 (1.4%)	0 (0%)	N/A
CC+CG			2.51 (1.33, 4.75)**

* $p=0.0004$; ** $p=0.0047$

[19]. This represents a significant departure from the prevailing view that *LRP5* exhibits direct effects on osteoblast function. There was no significant association between *LRP5* C135242T and BMD in osteoporotic cases and controls in the absence of stratification for smoking. Prior reported associations between *LRP5* and BMD are summarized in Table 6.

Koay et al. [20] observed a strong association ($p=0.007$) between *LRP5* and BMD in a cohort of 152 osteoporotic male and female probands and their families. This polymorphism is located at +65 bases from the start of exon 8 and represents a phe→phe same-sense mutation. Significant differences were found in the haplotype consisting of the C135242T G121513A, G138351A, and C141759T SNPs ($\chi^2=21.2$; $p=0.002$), a three-marker haplotype consisting of G121513A, C135242, and G138351A ($\chi^2=23.9$; $p=0.001$), and a three-marker haplotype consisting of C135242T, G138351A, and C141759T ($\chi^2=23.9$, $p=0.001$). It is possible that the C135242T SNP does not directly affect BMD but is in linkage disequilibrium with a SNP(s) which does affect BMD. Alternatively, this SNP may alter an mRNA splice site. Since males and females were analyzed in Koay's study and only females were analyzed in our study, a gender-specific effect of the C135242T SNP in the absence of smoking is suggested.

The mechanism(s) by which cigarette smoking contributes to osteoporosis is unclear. Inhibition of the chemotactic response of osteoprogenitor cells and osteoblast-like cells to platelet-derived growth factor-BB and fibronectin following exposure to cigarette-smoking extract (CSE) has been demonstrated [21]. These chemotactic stimuli play a role in recruitment of mesenchymal cells during bone restructuring. CSE also inhibited bone cell contraction of collagen gels by osteogenic progenitor cells and osteoblast-like cells.

Some experimental evidence for association between cigarette smoking, *LRP5* insufficiency, and bone fragility

has been reported in murine models [22]. *LRP5* knockout mice exposed to cigarette smoke exhibited a 45% decrease in trabecular bone structure and apparent density and midshaft ultimate load compared to unexposed controls.

Unpublished data collected by A Schuldiner (coauthor) were evaluated further to assess association of BMD and *LRP5* C135242T in a different population. Genotypic and phenotypic data including BMD and tobacco exposure limited to tobacco chewing collected from an old Order Amish population were analyzed. Adult males in the Old Order Amish population who chewed tobacco exhibited no significant association ($p=0.29$) between tobacco chewing and BMD. Potential explanations for this inconsistency may be that the effect of smoking on BMD is gender specific or, alternatively, that the metabolism and toxicity of cigarettes components may differ with route of exposure.

IL6 is produced by a variety of lymphoid and non-lymphoid cells including T and B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangial cells, and some tumor cells. IL6 plays an important role in hematopoiesis by acting synergistically with *IL3* and *IL6* by formation of multilineage blast cell colonies. Through its stimulatory activities on osteoclasts, IL6 plays a role in the pathogenesis of bone loss. Osteoclasts are activated through binding of the nuclear factor kappa B (NF- κ B) ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) to the RANK receptor [23]. Estrogen deficiency results in increased bone loss that may be mediated through the expression of bone cytokines which stimulate bone resorption including IL1, IL6, tumor necrosis factor alpha, and M-CSF, subsequently resulting in increased osteoclast formation and differentiation [24]. Table 7 summarizes the association studies previously performed between BMD and IL6.

The A1 genotype at the IL6 locus has 18 CA repeats and is associated with a lower radial BMD in postmenopausal

Table 5 Association of *LRP5* C135242T SNP adjusting for age and BMI stratified by smoking status

Genotype	Controls (N=293)	Cases (N=309)	Odds ratio (95% CI)
Ever smokers (N)	117	92	
CC	100 (85.5%)	75 (81.5%)	1.00
CT	17 (14.5%)	17 (18.5%)	2.78 (1.10, 7.03)*
Nonsmokers (N)	176	217	
CC	143 (81.3%)	176 (81.1%)	1.00
CT	33 (18.8%)	41 (18.9%)	0.99 (0.52, 1.88)

* $p=0.031$

Table 6 Summary of association studies between *LRP5* and BMD

Study reference	Population	Cohort size	BMD parameters measured	<i>LRP5</i> SNP or microsatellite polymorphism	Comments
Kooy et al. 2004 [20]	Caucasian (British)	152 osteoporotic male and female probands and their families (597) and 160 females with elevated BMD	LS spine Femoral Neck	C135242T Haplotypes of 2 to 3 of SNPs of G121513A, C135242T, G138351A, and C141759T	$p=0.007$ $p<0.003$
Ferrari SL 2005[48]		78 males with idiopathic osteoporosis	Total hip	C171346A in intron 21 and total BMD within families	$p<1 \times 10^{-5}$ in men only; $p=0.0019$ in men and women $p<0.003$
Van Meurs et al. 2008 [49]	Caucasian	37,534 Males and females GENOMOS consortium	Lumbar spine	C171346A in intron 21 and comparison of osteoporotic probands and unrelated elevated BMD in women c.2047A-.4037T haplotype LRP5 Met 667	$p=0.004$ Lumbar spine: $p=3.3 \times 10^{-8}$ Femoral neck: $p=3.3 \times 10^{-5}$ Lumbar spine: $p=2.6 \times 10^{-9}$ Femoral: $p=5 \times 10^{-6}$ $p=6.3 \times 10^{-12}$
Richards et al. 2008 [50]	Caucasian European	2,904 females	Femoral neck Lumbar spine (L1–L4) Femoral neck	LRP5 Val11330 rs4988321 rs736228	
Agueda et al. [51]	Spanish	Replication set of 6,463 individuals Genome-Wide Association Study 964 postmenopausal women	Lumbar spine and femoral neck	rs2508836 Lumbar spine Femoral neck	$p=0.025$ additive model $p=0.032$, recessive model $p=0.011$ recessive model $p=0.007$ additive model $p=0.019$ recessive
Current study	Caucasian	309 postmenopausal females	Lumbar spine Forearm Femur	rs312009 associated with LS BMD rs729635 and rs643892 associated with fracture C135242T	Association observed among smokers only $p=0.03$

Table 7 Summary of prior association studies of *IL6* and BMD

Study reference	Population	Cohort size	BMD parameters measured	IL6 SNP or microsatellite polymorphism	Comments
Murray et al. 1997 [33]	Caucasian (Scottish) menopausal and postmenopausal females	200	Spine, hip	AT minisatellite in 3' flanking region	Spine BMD values were significantly higher in the C/F genotype as compared to F/F genotype, $p=0.04$ (spine), $p=0.11$ (femoral neck)
Tsukamoto et al. 1999 [25]	Japanese postmenopausal females	73	Radial BMD	CA repeat polymorphism	A1 genotype (134 bp, 18 CA repeats) associated with lower BMD ($p=0.0221$)
Ota et al. 2001 [39]	Japanese postmenopausal females	470	Radial BMD	G/C substitution at position -634 in the regulatory promoter region*	BMD lowest in G/G homozygotes, intermediate in G/C heterozygotes and highest in C/C homozygotes ($p<0.05$)
Huang et al. 2003 [52]	Caucasian male and females of European origin	1,816	Hip/spine	CA repeat polymorphism	Weak association with spine BMD ($p=0.069$) Weak association with hip BMD ($p=0.06$)
Feng et al. 2003 [53]	Elderly female Japanese	47	Femoral neck	G/C polymorphisms at position -573	Negative relationship between CC genotype and femoral neck BMD and IL6 level ($p=0.0161$)
Chung et al. 2003 [54]	Postmenopausal Korean females	335	Radius	-572 C promoter SNP ^a	IL6 572 C containing genotype have increased BMD ($p=0.02$, codominant model; $p=0.007$ dominant model)
Ferrari et al. [30]	Caucasian (offspring of Framingham cohort)	1,574	Hip	IL6 -174 allele	BMD lower in elderly females (>15years postmenopause) with 174 GG without estrogen and low calcium intake (p values 0.003–0.096)
Magana et al. 2008 [55]	Osteoporotic and nonosteoporotic Mexican women	140	Lumbar BMD	-572 C>G ^a -174 G > C	No association with -572C>G allele Association with C allele of G-174C SNP ($p<0.0001$)
Yamada et al. 2003 [29]	Japanese—aged 40–79 years	1,113 women and 1,126 men	Radius, right femoral neck, right trochanter, right Ward's triangle and L2-L4 total body, lumbar spine, total hip, femoral neck	-634 C>G*	GG associated with reduced bone mass in postmenopausal women $p\leq 0.05$
Li et al. 2007 [28]	Premenarche Chinese girls 9–11 years	176	Spine, hip, femoral neck, trochanter, intertrochanter	-634 C>G ^a	-634C/C genotype associated with greater BMD and bone mineral content $p<0.05$
Lei et al. 2005 [27]	Chinese nuclear families with a daughter 25–37 years of age	1,263	Spine, hip, femoral neck, trochanter, intertrochanter	-634 C>G ^a	Trend for linkage between the IL6 gene and -634C/G marker $p<0.05$
Current study	Postmenopausal females	309	LS spine Forearm Femur	-634 G>C (rs1800796) ^a G/C substitution at position -634 in the regulatory promoter region	BMD lowest in C/C homozygotes BMD highest in G/G homozygotes $p=0.0047$

^a *IL6* -634 G>C and -572 G>C are both rs1800796.

The numbering of -634 G>C corresponds to the published sequence of Yamasaki et al. [56]

Japanese women [25]. Further, IL6 epistasis was observed between *COL1A1* and *IL6* with variation in bone density in a cohort of Japanese individuals [26]. In our study, the occurrence of a C allele in rs1800796 SNP of the *IL6* gene was associated with a significantly increased OR of 2.51 for osteoporosis, providing further evidence for an association between the *IL6* locus and osteoporosis. No gene–gene interaction was observed in our study population between *COL1A1*, *VDR*, *ESR*, *TGF- β* , and *LRP5*. Our findings replicate those of Ota et al. [17] and other investigators [27–29] who studied Asian populations, except the “C” allele was associated with osteoporosis in our study, while the “G” allele was associated with osteoporosis in the Ota study. This suggests that the causative variant may be on a different haplotype background in these two populations. One possible mode of action for the –634 G>C (rs1800796) allele could be to modulate *IL6* levels, which can then regulate osteoclast production. The function of this allele could vary across populations and especially across races or ethnicities. Functional assays utilizing both alleles could provide evidence to support this hypothesis.

The strength of our study lies in the phenotypic precision for cases and controls. Careful attention was paid to selection of controls who had no estrogen exposures for a period of at least 2 years. The large majority of DXA scans were performed at Marshfield Clinic by ISCD-certified study coinvestigators or were verified when obtained from regional clinic centers serving the MESA population. The electronic medical record facilitated capture of all DXA data for cases and controls.

Limitations of our study include limitations inherent in the use of DXA as a measure of osteoporosis. Given the difficulty of capturing fracture data and classification of low- vs. high-energy fracture, DXA analysis represents a quantitative but limited measure of skeletal fragility in humans. There were also significant differences in age between controls and cases (61.6 vs. 70.4 years) and data were adjusted for age during statistical analysis. It is intriguing to speculate that “CC” homozygotes in our population have the highest susceptibility for osteoporosis and that both our results and those of Ferrari et al. [30] indicate a possible age effect relative to *IL6* polymorphisms. Our study was performed using a single *IL6* allele. Haplotype analysis would likely provide more robust information. While not within the scope this study, further investigation will pursue validation of these postulations in the future

The potential applicability of these data and other prior associations proposed for different genes remains uncertain. In a genome-wide association study involving 5,861 Icelandic subjects and 301,019 SNPs, five genomic regions of significance were identified including receptor activator of nuclear factor kappa B ligand gene, *RANKL* (13q14),

osteoprotegerin gene (8q24), *ESR 1* (6q25), and a region on 1p36 and 6p21 [31]. Collective evidence in the literature supports the hypothesis that the development of osteoporosis is a common end point which can be mediated through a variety of genetically and/or environmentally mediated pathways. While these variants are not useful in the prediction of fracture risk in a given individual, they may provide insights into the biochemical pathways associated with osteoporosis development and consequently inform design of therapeutic strategies for affected patients.

In addition to playing a major regulatory role in immune response and hematopoiesis, the cytokine *IL6* and its receptor stimulate osteoclast development. Loss of gonadal function is associated with an increase in *IL6* as well as other cytokines and is postulated to result in osteoclast stimulation and subsequent bone loss [32]. The A1 genotype at the *IL6* locus has 18 CA repeats and is associated with a lower radial BMD in postmenopausal Japanese women [25]. Similar observations were made in a study by Murray [33]. Multiple investigations in different populations have demonstrated association between *IL6* polymorphisms and BMD (Table 7).

Statins are used to lower low-density lipoprotein concentrations and act mainly through the inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase [34]. Statins stimulated bone formation in vitro and resulted in expression of the bone morphogenic protein-2 gene in bone cells [35]. Conflicting results exist relative to the association of fracture risk with statin usage. Several studies have documented a decrease in fracture risk related to statin use [36, 37] while another study showed a similar rate of hip fracture among subjects taking statins compared to those on other lipid-lowering agents [38]. Although statin use did not differ significantly between our cases and controls, in our study, osteoporosis was defined by the BMD score ≤ 2.5 standard deviations below the young adult mean as opposed to fracture occurrence.

In conclusion, these results confirm a previously reported association between the *IL6* –634G>C (rs1800796) C in the promoter region and decline in BMD levels [39]. A previously undefined significant interaction between *LRP5* C135242T (rs4988321) and smoking was observed. Future studies will focus on functional analysis and replication of environmental effects in different populations in addition to studying TPH alleles and their association with osteoporosis. Recognition of gene–environment associations are important in the implementation of preventive strategies for osteoporosis and permit targeted education of genetically susceptible individuals in order to reduce risks through lifestyle modification.

Acknowledgements The authors thank Adeline Kaam for her assistance with phenotyping and Ingrid Glurich, Alice Stargardt, and Marie

Fleisner, Marshfield Clinic Research Foundation, Office of Scientific Writing, for their editorial assistance in the preparation of this manuscript.

Funding Marshfield Clinic Research Foundation; Donors to Personalized Medicine and Rheumatology

Conflicts of interest None.

References

- Anonymous (1993) Consensus development conference: diagnosis, prophylaxis and treatment of osteoporosis. *Am J Med* 94:646–650
- Smith J, Shoukri K (2000) Diagnosis of osteoporosis. *Clin Cornerstone* 2:22–33
- Nguyen TV, Blangero J, Eisman JA (2000) Genetic epidemiological approaches to the search for osteoporosis genes. *J Bone Miner Res* 15:392–401
- Cooper C, Campion G, Melton LJ III (1992) Hip fractures in the elderly: a worldwide projection. *Osteoporos Int* 2:285–289
- Evans RA, Marel GM, Lancaster EK et al (1988) Bone mass is low in relatives of osteoporotic patients. *Ann Intern Med* 109:870–873
- Pocock NA, Eisman JA, Hopper JL et al (1987) Genetic determinants of bone mass in adults. A twin study. *J Clin Invest* 80:706–710
- Shen H, Recker RR, Deng HW (2003) Molecular and genetic mechanisms of osteoporosis: implication for treatment. *Curr Mol Med* 3:737–757
- Kung AW, Huang QY (2007) Genetic and environmental determinants of osteoporosis. *J Musculoskelet Neuronal Interact* 7:26–32
- Ward KD, Klesges RC (2001) A meta-analysis of the effects of cigarette smoking on bone mineral density. *Calcif Tissue Int* 68:259–270
- Johnell O, Gullberg B, Kanis JA et al (1995) Risk factors for hip fracture in European women: the MEDOS Study. Mediterranean osteoporosis study. *J Bone Miner Res* 10:1802–1815
- Herrmann M, Peter Schmidt J, Umanskaya N et al (2007) The role of hyperhomocysteinemia as well as folate, vitamin B(6) and B (12) deficiencies in osteoporosis: a systematic review. *Clin Chem Lab Med* 45:1621–1632
- Waugh EJ, Lam MA, Hawker GA et al (2009) Risk factors for low bone mass in healthy 40–60 year old women: a systematic review of the literature. *Osteoporos Int* 20:1–21
- McCarty C, Wilke R, Giampietro P et al (2005) Marshfield Clinic Personalized Medicine Research Project (PMRP): design, methods and recruitment for a large population-based biobank. *Personalized Med* 2:49–79 <http://www.marshfieldclinic.org/chg/pages/Proxy.aspx?Content=MCRF-Centers-PMRP-Pubs-McCarthyMethodsCHG0522122553.1.pdf>
- Leib ES, Lewiecki EM, Binkley N et al (2004) Official positions of the international society for clinical densitometry. *J Clin Densitom* 7:1–6
- Wilke RA, Carillio MW, Ritchie MD (2005) Pacific symposium on biocomputing—computational approaches for pharmacogenetics. *Pharmacogenomics* 6:111–113
- Sirohi E, Peissig P (2005) Study of effect of drug lexicons on medication extraction from electronic medical records. In: Altman RB DA, Hunter L, Jung TA, Klein TE (eds) Pacific symposium on biocomputing. World Scientific, Singapore, pp 308–318 <http://helix-web.stanford.edu/psb05/sirohi.pdf>
- Gong Y, Slee RB, Fukai N et al (2001) LDL receptor-related protein 5 (*LRP5*) affects bone accrual and eye development. *Cell* 107:513–523
- Little RD, Carulli JP, Del Mastro RG et al (2002) A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone mass trait. *Am J Hum Genet* 70:11–19
- Yadav VK, Ryu JH, Suda N et al (2008) *LRP5* controls bone formation by inhibiting serotonin synthesis in the duodenum. *Cell* 135:825–837
- Koay MA, Woon PY, Zhang Y et al (2004) Influence of *LRP5* polymorphisms on normal variation in BMD. *J Bone Miner Res* 19:1619–1627
- Liu X, Kohyama T, Kobayashi T et al (2003) Cigarette smoke extract inhibits chemotaxis and collagen gel contraction mediated by human bone marrow osteoprogenitor cells and osteoblast-like cells. *Osteoporos Int* 14:235–242
- Akhter M, Manolides A, Cullen D, Recker RR (2008) *LRP5* G171V mutation and tobacco smoke related bone fragility. *J Bone Miner Res* 23:S454 Abstracts
- Kenny AM, Prestwood KM (2000) Osteoporosis. Pathogenesis, diagnosis and treatment in older adults. *Rheum Dis Clin North Am* 26:569–591
- Pacifici R (1996) Estrogen, cytokines and the pathogenesis of postmenopausal osteoporosis. *J Bone Miner Res* 11:1043–1051
- Tsukamoto K, Yoshida H, Watanabe S et al (1999) Association of radial bone mineral density with CA repeat polymorphism at the interleukin 6 locus in postmenopausal Japanese women. *J Hum Genet* 44:148–151
- Yang TL, Shen H, Xiong DH et al (2007) Epistatic interactions between genomic regions containing the *COL1A1* gene and genes regulating osteoclast differentiation may influence femoral neck bone mineral density. *Ann Hum Genet* 71:152–159
- Lei SF, Deng FY, Xiao SM et al (2005) Association and haplotype analyses of the *COL1A2* and ER-alpha gene polymorphisms with bone size and height in Chinese. *Bone* 36:533–541
- Li X, He GP, Zhang B et al (2008) Interactions of interleukin-6 gene polymorphisms with calcium intake and physical activity on bone mass in pre-menarche Chinese girls. *Osteoporos Int* 19:1629–1637
- Yamada Y, Ando F, Niino N, Shimokata H (2003) Association of polymorphisms of interleukin-6, osteocalcin and vitamin D receptor genes, alone or in combination with bone mineral density in community dwelling Japanese women and men. *J Clin Endocrinol Metab* 88:3372–3378
- Ferrari SL, Ahn-Luong L, Garner P et al (2003) Two promoter polymorphisms regulating interleukin-6 gene expression are associated with circulating levels of C-reactive protein and markers of bone resorption in postmenopausal women. *J Clin Endocrinol Metab* 88:255–259
- Styrkarsdottir U, Cazier JB, Kong A et al (2003) Linkage of osteoporosis to chromosome 20p12 and association to BMP2. *PLoS Biol* 1:E69
- Manolagas SC, Jilka RL (1995) Bone marrow, cytokines, and bone remodeling. Emerging insights into the pathophysiology of osteoporosis. *N Engl J Med* 332:305–311
- Murray RE, McGuigan F, Grant SF et al (1997) Polymorphisms of the interleukin-6 gene are associated with bone mineral density. *Bone* 21:89–92
- Maron DJ, Fazio S, Linton MF (2000) Current perspectives on statins. *Circulation* 101:207–213
- Mundy G, Garrett R, Harris S et al (1999) Stimulation of bone formation in vitro and in rodents by statins. *Science* 286:1946–1949
- Chan KA, Andrade SE, Boles M et al (2000) Inhibitors of hydroxymethylglutaryl-coenzyme A reductase and risk of fracture among older women. *Lancet* 355:2185–2188
- Rejnmark L, Olsen ML, Johnsen SP et al (2004) Hip fracture risk in statin users—a population-based Danish case-control study. *Osteoporos Int* 15:452–458

38. Ray WA, Daugherty JR, Griffin MM (2002) Lipid-lowering agents and the risk of hip fracture in a Medicaid population. *Inj Prev* 8:276–279
39. Ota N, Nakajima T, Nakazawa I et al (2001) A nucleotide variant in the promoter region of the interleukin-6 gene associated with decreased bone mineral density. *J Hum Genet* 46:267–272
40. Viitanen A, Kärkkäinen M, Laitinen K et al (1996) Common polymorphism of the vitamin D receptor gene is associated with variation of peak bone mass in young Finns. *Calcif Tissue Int* 59:231–234
41. Zmuda JM, Cauley JA, Ferrell RE (2000) Molecular epidemiology of vitamin D receptor gene variants. *Epidemiol Rev* 22:203–217
42. Gross C, Eccleshall TR, Malloy PJ et al (1996) The presence of a polymorphism at the translation initiation site of the vitamin D receptor gene is associated with low bone mineral density in postmenopausal Mexican–American women. *J Bone Miner Res* 11:1850–1855
43. Ioannidis JP, Ralston SH, Bennett ST et al (2004) Differential genetic effects of *ESRI* gene polymorphisms on osteoporosis outcomes. *JAMA* 292:2105–2114
44. Deng HW, Li J, Li JL et al (1998) 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* 103:576–585
45. Uitterlinden AG, Burger H, Huang Q et al (1998) Relation of alleles of the collagen type I alpha 1 gene to bone density and the risk of osteoporotic fractures in postmenopausal women. *N Engl J Med* 338:1016–1021
46. Langdahl BL, Carstens M, Stenkjaer L, Eriksen EF (2003) Polymorphisms in the transforming growth factor beta 1 gene and osteoporosis. *Bone* 32:297–310
47. Shiraki M, Shiraki Y, Aoki C et al (1997) Association of bone mineral density with apolipoprotein E phenotype. *J Bone Miner Res* 12:1438–1445
48. Ferrari SL, Deutsch S, Baudoin C et al (2005) *LRP5* gene polymorphisms and idiopathic osteoporosis in men. *Bone* 37:770–775
49. van Meurs JB, Trikalinos TA, Ralston SH et al (2008) Large-scale analysis of association between *LRP5* and *LRP6* variants and osteoporosis. *JAMA* 299:1277–1290
50. Richards JB, Rivadeneira F, Inouye M et al (2008) Bone mineral density, osteoporosis, and osteoporotic fractures: a genome-wide association study. *Lancet* 371:1505–1512
51. Agueda L, Bustamante M, Jurado S et al (2008) A haplotype-based analysis of the *LRP5* gene in relation to osteoporosis phenotypes in Spanish postmenopausal women. *J Bone Miner Res* 23:1954–1963
52. Huang QY, Shen H, Deng HY et al (2003) Linkage and association of the CA repeat polymorphism of the *IL6* gene, obesity-related phenotypes, and bone mineral density (BMD) in two independent Caucasian populations. *J Hum Genet* 48:430–437
53. Feng D, Ishibashi H, Yamamoto S et al (2003) Association between bone loss and promoter polymorphism in the *IL6* gene in elderly Japanese women with hip fracture. *J Bone Miner Metab* 21:225–228
54. Chung HW, Seo JS, Hur SE et al (2003) Association of interleukin-6 promoter variant with bone mineral density in premenopausal women. *J Hum Genet* 48:243–248
55. Magaña JJ, Gómez R, Cisneros B et al (2008) Association of interleukin-6 gene polymorphisms with bone mineral density in Mexican women. *Arch Med Res* 39:618–624
56. Yamasaki K, Taga T, Hirata Y et al (1988) Cloning and expression of the human interleukin-6 (BSF-2/IFN beta 2) receptor. *Science* 241:825–828