ONE mineral density (BMD) is a quantitative trait determined by the interaction of genetic, metabolic, and environmental factors, and its diminution is deemed a major risk factor for fractures. To date, several candidate genes have received great attention, among them the collagen IA1 (COLIA1) and estrogen receptor α (ERα) genes. The COLIA1 gene encodes the most abundant bone matrix protein, collagen type I α1. Estrogen receptor genes mediate in women the effects of estrogen, probably the major systemic hormone for maintaining normal bone turnover, and its deficiency leads to bone mass loss. While there is some evidence that the ERα gene may be involved in regulation of BMD in men, the main evidence for its regulatory role of BMD in women derives from studies performed in postmenopausal women of different ethnic origins.

We recently conducted haplotype-based association analysis between combined PvuII and XbaI restriction fragment-length polymorphisms (RFLPs) in intron 1 of ERα gene and phalan-geal hand BMD, on pedigree data collected from a population of European origin, residing in Russia. We found that the Px haplotype of the ERα gene was associated with low cancellous and cortical BMD in a maternal subgroup, actually comprised of elderly women (62.7 ± 6.5 years of age). The transmission disequilibrium test (TDT) revealed evidence suggestive of a linkage disequilibrium between the Px haplotype of the ERα gene and the BMD putative gene in female (but not in male) offspring. Additionally in our elderly women sample, the “s” allele of COLIA1 gene substantially enhanced the influence of the Px haplotype of the ERα gene by further reducing BMD in subjects carrying the combined genotypes (Px haplotype and “s” allele).

It has been shown that increased/decreased levels of calcitropic hormones (parathyroid hormone [PTH] and 25 hydroxyvitamin D [25(OH)D]), and biochemical indices of bone formation like the carboxyterminal propeptide of type I procollagen (PICP), or osteocalcin, may be associated with increased risk for osteoporosis fracture in postmenopausal women, independently of each other or of BMD. The present study was therefore undertaken to evaluate whether the association of the Px haplotype of the intronic ERα gene and allele “s” of Sp1 locus in the COLIA1 gene with phalangeal hand BMD could be related to calcitropic hormones and/or biochemical indices of bone formation. Specifically we addressed the following 4 questions: (1) Is there an association between high or low levels of each of the above biochemical indices and low BMD? (2) Is there an association between high or low levels of each of the above biochemical indices and either or both the Px haplotype of the ERα gene and the “s” allele of the COLIA1 gene? (3) Is there a specific genotype whose carriers are characterized by a lowest mean BMD and highest (or lowest) circulating levels of...
the biochemical indices? (4) What is the quantitative contribution of the Px haplotype and other studied variables to the variance of BMD in elderly women?

**MATERIALS AND METHODS**

**Subjects and BMD Measurements**

The Chuvashaiian population chosen for our study is ethnically a Caucasian population living in numerous small villages in the forested or hilly portions of the Volga riverside in Russia. Their ancestors were most likely Bulgars from the Volga and Kama riverside who intermarried with the local Finno-Ugric tribes. The individuals assessed in the present research were part of a pedigree sample that resided in the area for at least 3 generations. Sharing a similar environment, as well as similar socioeconomic conditions, and also a minimal genetic flow, is what characterizes this rural population, which has been described by us in greater detail elsewhere.\(^1\)\(^7\)\(^13\) The population does not have access to modern medical services, nor to the use of hormone replacement therapy or calcium supplements. Our study did not include individuals with known bone diseases or with risk factors for increased BMD loss, such as steroid hormone therapy, diabetes, or hyperparathyroidism. Our research team randomly selected families for study after direct contact with all the households in the small villages. The subjects recruited for study were all members of nuclear families who agreed to participate and signed on informed consent. The project was approved by the Tel-Aviv University ethics committee and encompassed 122 elderly women from the total pedigree-based sample (463 individuals). The mean age (± SD) of these women, representing the maternal moiety of the original pedigrees, was 62.7 years (±6.5) and mean of years since menopause (± SD) was 14.6 years (±7.4). Plain radiographs of both hands were obtained from each individual with an x-ray source. BMD of cancellous and cortical bone separately, at the distal and middle phalanges of the third finger on both hands, was evaluated by digital microdensitometer, using a standard methodology.\(^1\)\(^6\)\(^2\)\(^0\) We used average BMD measure of the third finger bones of both hands, and calculated separately the Z score values for cancellous bone and cortical bone, as previously described.\(^1\)\(^7\)\(^1\)\(^8\) We should note that the Z-transformation was based on the data of the total pedigrees sample (>700 individuals), and took into account size and structure of the studied families.

**Determination of ERα and COL1A1 Genotypes**

Genomic DNA, extracted from peripheral leukocytes by either standard phenol-chloroform procedure or via a Nucleon kit (Amersham Life Science, London, UK), was used for specific polymerase chain reaction (PCR) amplification. The PCR product of the ERα gene (~1.3-kb fragment), containing a part of intron 1 and exon 2, was amplified according to the Kobayashi et al protocol.\(^3\) To analyze the PvuII and XbaI RFLPs in this fragment, we used direct haplotyping procedure. Haplotypes of ERα gene represented the combination of both polymorphic sites (P/p and X/x) on each of the sixth pair chromosomes in each individual. Direct haplotype analysis was enabled by simultaneously digesting the PCR product with the 2 restriction enzymes, PvuII and XbaI, as per our recent report.\(^1\) Absence or presence of the sites for the restriction enzymes PvuII and XbaI was recorded as P or p and X or x respectively. Genotypes by haplotypes were determined for each subject as PXP, Pxp, pxP, pxp, etc. The PCR product of the COL1A1 gene (~260-bp fragment) was amplified by using mismatched primer as previously described,\(^1\) taking into account that the fragment contains an introduced restriction site for Mrcl enzyme, at the SpI binding site in the first intron of the gene. Absence or presence of the Mrcl restriction site was recorded as S or s, respectively.

In the sample of unrelated parents, both “mothers” and “fathers,” the genotype frequency distribution at both loci, i.e., PvuII and XbaI RFLPs in the ERα gene and Mrcl RFLP in the COL1A1 gene, was in conformity with the Hardy-Weinberg equilibrium.\(^1\)\(^3\)

**Determination of Plasma Levels of Bone Turnover Markers, Calcitropic Hormones, and Steroid Sex Hormones**

Plasma was separated from whole blood samples (collected in the morning after a 12-hour fast, during the autumn months) and stored at −80°C until assay. Plasma-intact osteocalcin was measured by immunoassay using ELISA-OSTEO kit (CIS Bio International, ORIS Group, France). Carboxyterminal PICP was measured by radioimmunoassay, using the \(^12\)I-RI A kit (DiaSorin, Stillwater, MN). Intact (I)-PTH was measured by immunoradiometric assay, using N-tact PTH SP kit (Inctar, Stillwater, MN). 25(OH)D was assayed by radioimmunoassay using TESTO-CT2 and ESTR-US-CT kits (CIS Bio International). The intra-assay and interassay coefficients of variation (CVs) for the above kits varied from 3.2% to 12.5% and from 4.9% to 17.6%, respectively. The kit detection limit for estradiol had been assessed as 5 pmol/L. All assays were performed in duplicate and the results were reported in detail.\(^2\)\(^1\)

**Statistical Analysis**

Plasma levels of each of the biochemical indices were age adjusted and standardized within the “mothers” subgroup of the pedigree sample in order to achieve a mean equaling 0 and a SD of 1.0 (Z values). To test for any association between high or low levels of BMD and each of the potential predictive variables, we first divided the BMD Z scores (ranging under normal distribution from −2.5 to 2.3 Z scores) into 3 subgroups as follows: A = Z < −1, B = −1 ≤ Z ≤ 1, and C = Z > 1, for each cancellous and cortical bone tissue. The mean values of all the hormones and biochemical markers in these 3 BMD subgroups were then compared by 1-way analysis of variance (ANOVA). Student’s t test was used to test the hypothesis that a candidate gene polymorphism exerts an influence on calcitropic hormones or bone turnover markers. Additionally, when combined di-loci genotypes were used for similar comparisons, ANOVA was implemented. Forward stepwise multiple regression analysis was performed to evaluate the independent contribution of the potential predictor variables for Z scores (continuous variable) of each of the 2 BMD types. The calcitropic hormones, sex steroid hormones and bone turnover markers, were introduced as quantitative variables (age-adjusted standardized residuals). Genotypes represented by haplotypes as categorical variables were entered as ‘dummy’ variables by coding them numerically: “1” for subjects carrying 1 or 2 copies of the Px haplotype (genotypes PxPx, PXPX, Ppxx) and “2” for subjects lacking this haplotype (genotypes PXXP, PXXP, pxpx). For the sake of convenience some additional explanations will be given in the relevant text of the Results section. All the above analyses were performed using the STATISTICA package for Windows (version 5.0, StatSoft, 2000).

**RESULTS**

The plasma levels of PTH, 25(OH)D, PICP, osteocalcin, estradiol and testosterone are shown in Table 1. Mean age-adjusted PTH levels differed significantly between the subgroups of categorical BMD Z scores of both types of bone tissue, with the highest values observed in the subgroup of Z < −1, and the lowest in subgroup Z > 1 (Fig 1). Similar examination of all the other biochemical variables vis-a-vis cancellous BMD evinced that between subgroups, only the testosterone concentrations showed marginally significant differences
(P = .059), whereas the other indices were unremarkable. The same examination for cortical bone showed that 25(OH)D levels correlated negatively with BMD, while circulating testosterone decreased in parallel with cortical BMD decrease (Fig 2).

Figure 3 clearly demonstrates negative correlation between BMD (both types) and PTH, in line with the Px haplotype status. In other words, subjects who carried 1 or 2 copies of the Px haplotype (combined group of PxPX, Pxpx, pxpx genotypes) had significantly higher levels of PTH and lower values of BMD (as a continuous variable), than subjects lacking this haplotype (PXpx, PXpx, pxpx genotypes). No significant differences were noted for 25(OH)D, estradiol, and testosterone. Levels of the bone formation markers PICP and osteocalcin displayed the same trend as PTH (as expected), but the differences here did not reach statistical significance. We also assessed the predictive power of the Px haplotype and each of the age-adjusted levels of hormones [PTH, 25(OH)D, estradiol, and testosterone] and biochemical markers (PICP or osteocalcin) on age-adjusted BMD, using the multiple regression analysis (Table 2). Table 2 provides a simultaneous F test of the hypothesis that all the regression coefficients are equal to zero, and individual t tests for each coefficient separately. As can be seen in Table 2, the overall multivariate F ratio was highly significant (P < .001) in both the undertaken analyses. However, different arrays of predictor variables were retained for cancellous and cortical BMD. The regression model explained 12.9% of the total observed variance of cancellous BMD (Table 2). PTH and the carrying of Px haplotype were the most significant independent predictors for low BMD values, with some 7.6% (P = .004) and 4.1% (P = .028) of the BMD variance attributable to PTH levels and genotype. The contributions of testosterone and osteocalcin to the predictive power of the model were only marginally significant (.05 < P < .11). Multiple regression analysis of cortical BMD revealed that about 18% of the total variance was explained by the selected set of variables. The results of the analysis suggested that 25(OH)D and possession of the Px haplotype exerted a most substantial effect (7.5% and 6.2%, respectively). The rest was explained by the estradiol and PTH effects. Osteocalcin made no significant (P = .228) contribution to the BMD variation, yet its exclusion from the analysis significantly diminished the overall likelihood of the model fit.

Next we attempted to assess possible associations between each of the hormones and biochemical markers and the com-

<table>
<thead>
<tr>
<th>Table 1. Descriptive Statistics for Studied Sample</th>
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<tr>
<td>Variable</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Age (yr)</td>
</tr>
<tr>
<td>Cancellous BMD (Z scores)</td>
</tr>
<tr>
<td>Cortical BMD (Z scores)</td>
</tr>
<tr>
<td>PTH (pg/mL)</td>
</tr>
<tr>
<td>25 (OH)D (ng/mL)</td>
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<tr>
<td>PICP (ng/mL)</td>
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<tr>
<td>Osteocalcin (ng/mL)</td>
</tr>
<tr>
<td>Estradiol (pg/mL)</td>
</tr>
<tr>
<td>Testosterone (ng/mL)</td>
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</table>

Fig 1. Z means (SEM) of PTH, according to 3 categorized Z scores of cancellous or cortical bone; comparisons are between group Z < -1 v Z > 1 (Duncan test): *P = .017; **P = .031.

Fig 2. Z means (SEM) of 25(OH)D, testosterone according to 3 categorized Z scores of cortical bone; comparisons are between group Z < -1 v Z > 1 (Duncan test): *P = .003; **P = .017.
combination of genotypes at both polymorphic ERα and COLIA1 genes. To this end, analyses of variance for the alternative combinations of these di-loci genotypes were conducted. We designated as “1” the genotypes of ERα carrying 1 or 2 copies of the Px haplotype, and as “2” the genotypes lacking the Px haplotype. The genotypes of COLIA1 carrying one or two copies of “s” allele (ie, Ss and ss) were defined as “A” and the SS genotype as “B.” Altogether the combinations formed 4 genotypic groups as follows: “1A,” “2A,” “1B,” “2B.” 

In an earlier phase of our ongoing pedigree-based study of the radiographic phalanges BMD in the Chuvasha population we observed a strong influence of the putative genetic factors on the interindividual variation of the BMD. 18 In subsequent research on this population sample we found that the Px haplotype of the ERα gene was associated with low phalanges BMD in elderly women, and that the combination of the Px

**DISCUSSION**

4 shows that women of group 1A had the lowest BMD Z scores for both cancellous and cortical bone, the lowest mean 25(OH)D, and the highest means for PTH, PICP, and osteocalcin. In this group, PTH was inversely correlated with both types of BMD and with 25(OH)D. Women with the alternative combination of genotypes (group 2B), had BMD (both cancellous and cortical bone), 25(OH)D, PTH, PICP, and osteocalcin values close to Z = 0, which actually is the overall sample mean of all the 4 subgroups of women. The differences between these 2 groups reached statistical significance for cancellous BMD and PTH (P < .03 and P < .001, respectively), but not for the other variables, namely, cortical BMD (P = .087), 25(OH)D (P = .077), PICP (P = .119), or osteocalcin (P = .548). The sex hormones (data not shown) had the lowest values within group 1A, and values around the median (mean Z = 0) within group 2B. The later differences, however, were not significant. It can also be seen in Fig 4 that women lacking Px haplotype and carrying “s” allele (group 2A) differed significantly insofar as the values of their PTH, 25(OH)D, PICP and cancellous BMD from women carrying both Px haplotype and “s” allele (group 1A). The difference for osteocalcin did not attain significance (P = .156). In regard to the mentioned variables, we observed that the mean level of each in group 1A was opposite to the mean in group 2A (higher or lower than the median value). This is best demonstrated for PICP where the difference was 1.2 Z (P = .012) between the 2 groups.

**NOTE.** BMD Z scores were modeled as a continuous variable. Age-adjusted plasma concentrations of PTH, 25(OH)D$_3$, estradiol, testosterone, and osteocalcin were used as independent variables in the regression model. $\beta$ and $\text{SE}_\beta$ is regression coefficient and its standard error; $R^2$, multiple $R^2$; $\Delta R^2$, change of multiple $R^2$ at each step of regression.

<table>
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<th>Predictor</th>
<th>$\beta$</th>
<th>$\text{SE}_\beta$</th>
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<th>$\Delta R^2$</th>
<th>$P$ Value</th>
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<td>Cancellous bone</td>
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<tr>
<td>Intercept</td>
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<td>Final adjusted $R^2 = 0.179$</td>
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*The Px haplotype was grouped according to its presence: subjects with 1 or 2 copies of the haplotype were coded as group “1”; subjects who are lacking the haplotype were coded as group “2”.

Fig 3. Z means (SEM) of PTH, and cancellous and cortical BMD, according to Px haplotype status; t test: *$P = .010$; **$P = .003$; ***$P = .025$. 

Table 2. Multiple Linear Regression Analysis of BMD for Cancellous and Cortical Bone in the Group of Elderly Women

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haplotype and “s” allele of COLIA1 gene further decreased the BMD. In the present study, we have added biochemical predictors to the model, and used multiple regression analysis of BMD (dependent variable) adjusted for age, body height, and weight. As gleaned from Table 2, the model explained 12.9% and 17.9% of the remaining variance in the 2 types of BMD; furthermore, PTH and Px haplotype proved to be independent predictors of both types of BMD variation, while 25(OH)D and estradiol contributed additionally only to cortical bone variation.

Hormones and genes that are involved in bone metabolism constitute part of a very complex matrix of metabolic pathways and consequently can influence BMD in more than 1 pathway. As the hormones PTH, 25(OH)D, and estradiol, as well as the Px haplotype of the ERα gene, were all found to be independent predictors of BMD in our elderly women sample, we deemed it worthwhile to further investigate their interrelationships. Possession of the Px haplotype was associated both with higher PTH levels and lower BMD for the 2 bone tissue types. Moreover, this tendency became even better expressed when polymorphism at the COLIA1 gene was taken into account, for then the highest mean value of PTH and the lowest mean BMD were observed in subjects carrying both Px haplotype and “s” allele (Fig 4). These findings suggest the possible interaction between ERα and COLIA1 genes on the one hand, and on the other—that the effects of the ERα gene on BMD can be partially mediated by the PTH hormone [as well as by 25(OH)D]. Multiple regression analysis showed (Table 2) that both the ERα haplotype and PTH exerted an independent effect on BMD variation, assumedly through other actions on extraskeletal calcium homeostasis, as previously demonstrated.

This assumption is supported by the findings of Khosla et al. who showed that Letrozole treatment in late postmenopausal women reduced serum estrone and estradiol to near undetectable levels, and decreased serum PTH by 22%. Since we could not detect correlation between circulating levels of estradiol and PTH, we tend to assume that the gamut of low levels of estradiol presented in our elderly women is exerting at least some of its actions through the transcription factor ERα, depending on the allele of ERα gene encoding it. While in subjects not carrying the Px haplotype, estrogen exerts its influence by at least maintaining BMD, subjects that do carry this haplotype, have an augmented response of PTH to estrogen (probably through the already demonstrated presence of estrogen receptors in the parathyroid glands), which results in lower BMD. This explanation is receiving credence from emerging data that indicate that allelic variants in the ERα gene may modulate estrogen’s effects, especially in regard to BMD and lipid metabolism.

Elevated levels of serum PTH have been considered to be associated with high bone turnover, which can be distinguished by markers of bone formation, PICP, and intact osteocalcin. Our data show that women carrying the Px haplotype have significantly higher mean PTH, and also tend to have higher levels of PICP and osteocalcin (albeit not significant). Women carrying both the Px haplotype and “s” allele have significantly the highest mean PTH (P < .001) and PICP (P < .05) levels, and a similar but nonsignificant trend for osteocalcin. In our sample, the women lacking the Px haplotype and carrying the “s” allele had a low mean PTH and the lowest mean PICP and osteocalcin levels (Fig 4). It would seem that allele “s” (at the Sp1 recognition locus), when present in women lacking the Px haplotype, impairs the transcription of the COLIA1 gene, which results in lower levels of PICP. The opposite obtains in women carrying both the “s” allele and the Px haplotype. It has already been suggested that the COLIA1
Sp1 polymorphism is a functional genetic variant, and that the “s” allele increases binding affinity for the transcription factor Sp1. This in turn leads to increased ratio of α1(1) protein relative to α2(1), as well as to reduced bone strength in “Ss” individuals as compared to “SS” ones. It has also been shown that in osteoblasts, estrogen regulates collagen type I levels mainly by the ERα isofrom. Our own data affirm the possible protein-protein interaction between ERα and Sp1, such as may regulate a gene transcription in line with that proposed in Klinge’s review article, and as has already been demonstrated for several genes.

Possibly the above-suggested interaction might further involve the receptor pathways of any of the calcitropic hormones [PTH and 1,25(OH)2 D], because the levels of PTH and 25(OH)D observed by us appeared to be unique for subjects with the di-loci genotype of Px haplotype and “s” allele, all of which resulted in altered expression of the protein product (the collagen type I α1 polypeptide chain) and higher levels of PICP. However, the impact of these polymorphisms on the transcription and clinical end points has yet to be assessed.

If to recap, the current study indicates that elderly women carrying the Px haplotype in combination with the “s” allele are at greater risk of low BMD, which might partially be the result of high circulating levels of PTH. Admittedly, there are several potential limitations with this study. To begin with, it was performed on a modest sample size of 122 elderly women. Second, as the study population does not have access to modern medical services, nor to treatments such as hormone replacement therapy or calcium supplements, it is not possible to evaluate the contribution of potential covariates or to extrapolate the present results to the general modern population. Furthermore, the data from this study are useful in generating hypotheses, and these need to be tested experimentally. Finally, the fact that our group of women carrying both the Px haplotype and “s” allele was comprised of only 4 individuals implies that any results obtained by analyses performed with this minute group need to be considered with due caution. Yet, we should bear in mind that the group represented a combination of 2 relatively rare genotypes and consistently produced extreme mean values of PTH, PICP, osteocalcin, 25(OH)D, and BMD. We believe and hope that future studies would resort to combination of genotypes as a rule rather than an exception, and to be sure, further investigation is needed to ascertain whether our findings regarding this interlocus interaction effects on hormones, bone turnover markers and bone density in elderly women are reproducible also in other populations.

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