CLINICAL STUDY

Variable number of tandem repeats polymorphism in parathyroid hormone-related protein as predictor of peak bone mass in young healthy Finnish males

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Abstract

Objective: Mice with osteoblast-specific deletion of parathyroid hormone-related protein (PTHrP) exhibit impaired recruitment and increased apoptosis of osteogenic cells resulting in decreased bone formation and premature osteoporosis. The PTHrP levels within the bone microenvironment are therefore critical in influencing bone mass acquisition. Whether this is applicable in humans has not been established. Here, we studied the association of a variable number of tandem repeats (VNTR) polymorphism in *PTHrP* with peak bone mass.

Methods: Enrolled in the study were 234 young Finnish males, with median age of 19.6 years (range 18.3–20.6 years). Lifestyle factors, serum bone markers, osteodensitometric measurements (lumbar spine and hip) and calcaneal quantitative ultrasound readings were obtained. The *PTHrP* VNTR length was determined by the PCR amplification of genomic DNA extracted from peripheral blood and correlated to bone parameters by the multiple regression models.

Results: The presence of at least one 252 bp allele was associated with increased lumbar spine bone mineral density (BMD; $P < 0.0034$), broadband ultrasound attenuation (BUA; $P < 0.0012$) and speed-of-sound (SOS; $P < 0.0023$) measurements. The correlation with increased lumbar spine BMD ($P = 0.0008$), BUA ($P = 0.005$) and SOS ($P = 0.001$) was further strengthened by the pairing of the 252 bp allele with a 460 bp allele in comparison with those without any 252 bp allele. Electrophoretic mobility shift assays were used to illustrate the potential transcriptional functionality of the VNTR sequence.

Conclusion: The results indicate that the *PTHrP* VNTR sequence likely modulates local *PTHrP* expression within the skeletal microenvironment and could serve as a diagnostic predictor of peak bone mass acquisition.

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Introduction

Osteoporosis is a highly prevalent skeletal disorder characterized by compromised bone strength that predisposes to an increased risk for fracture at the hip, spine and other skeletal sites with significant associated morbidity and mortality (1, 2). Osteoporosis and related fractures are common in ageing individuals and contribute substantially to poor quality of life for those affected as well as increased healthcare costs and burden of illness to society at large. Although a number of factors such as rate of bone loss, bone size and structure, and propensity to fall contribute to the risk of osteoporosis fractures, the most important parameter in

determining the risk for developing osteoporosis and ensuing fractures is the peak bone mass attained during young adulthood (3). Skeletal bone mass reaches over 90% of its maximum by age 18 (earlier in females than males). Peak bone mass is influenced by a number of environmental factors such as lifestyle, concomitant diseases and diet. In addition, twin studies have suggested that up to 85% of the variance in bone mineral density (BMD) is genetically determined depending on the site examined (3). Hence, genetically determined factors cause hereditary low bone mass that reduces peak bone mass acquisition (4). Consequently, bone mass is lower than the reference range at skeletal maturity (age 25–30 years). Patients with hereditary

low bone mass, therefore, have less bone to lose in later life before fracture risk further increases and have low BMD early in adult life. Genetic factors may also increase bone loss in later life. It would therefore be desirable to identify those individuals who are at high risk for osteoporosis prior to the onset of the disease, so that they can benefit from prophylactic interventions and earlier treatment.

Parathyroid hormone-related peptide (PTHrP) is expressed in the bone-forming cells of the skeleton (osteoblasts) and is critical for their proper proliferation, differentiation and function, processes that are pivotal for attaining and maintaining appropriate bone mass thereby preventing the development of osteoporosis. Mice homozygous for *PTHrP* inactivation exhibit chondrodysplastic abnormalities and altered endochondral bone formation that culminate in their death in the immediate peripartum period (5, 6). On the other hand, mice heterozygous for the inactivated *PTHrP* allele are phenotypically normal at birth but develop by 3 months of age premature and advanced osteoporosis (7). Moreover, mice generated with osteoblast-specific deletion of *PTHrP* demonstrate impaired recruitment of osteoprogenitor cells and premature programmed cell death of osteogenic cells, resulting in decreased bone formation and thereby, premature osteoporosis (8).

The human *PTHrP* (on chromosome region 12p12.1–p11.2) has also been reported to play an important role in the development and specifically in skeletogenesis (9–11). Whether PTHrP expression within the human skeletal microenvironment is critical for attainment of peak bone mass during adolescence and maintenance of skeletal homeostasis later in adult life as in mice remains to be determined. Polymorphisms in *PTHrP* have not been reported in association with either peak bone mass attained or propensity for osteoporosis and associated fracture risk. A variable number of tandem repeats (VNTR) polymorphism in *PTHrP* located in intron V, 100 bp downstream of exon VI (12) has been described (13). In the general population, it is characterized by eight different alleles ranging in length from 252 to 460 bp containing the repeat unit (G(TA)*n*C)N, where *n* = 4–11 and *n* = 3–17 (Fig. 1).

In this study, we examined the *PTHrP* VNTR polymorphism in young Finnish males and evaluated its potential usefulness as a diagnostic predictor of peak bone mass attained by the age of 20 years (14). Moreover, the potential role of the VNTR sequence in the regulation of *PTHrP* gene expression was assessed using electrophoretic mobility shift assays. Our findings indicate that particular alleles and combinations of specific *PTHrP* VNTR alleles correlate with variability in peak bone mass

Figure 1 The *PTHrP* VNTR. (A) Schematic of the genomic organization of the human *PTHrP* gene (not drawn to scale). Boxes with corresponding Roman numerals on top represent exons: light grey boxes, 5' non-coding; black boxes, coding; and dark grey boxes, 3' non-coding. Arrows indicate transcription initiation sites. The vertical arrow indicates the VNTR that starts 100 bp downstream from the end of exon VI. (B) Nucleotide sequence of the 252 bp *PTHrP* VNTR allele. The PCR primers are shown in bold italic type, while the proposed repeats are in bold type. These repeats correspond to the consensus sequence for binding of CF2-II as determined by TFSEARCH (arrows). In the 378 bp VNTR allele, the other most common *PTHrP* VNTR allele, there are 13 such repeats (not shown).

in this population, raising the possibility that this genotypic approach may serve as an important tool in identifying individuals with a genetic predisposition to low bone mass acquisition at a relatively young age and hence at a higher risk for developing osteopenia and osteoporosis in later life. This may arise from the observed capacity of the VNTR sequence to specifically bind to nuclear extracts from osteoblast-like cells, implicating its potential regulatory role in *PTHrP* expression within the local bone microenvironment.

Experimental subjects

The study population comprised 234 young men, aged 18.3–20.6 years. They were participants in a study aimed at elucidating the role of genes, hormones and lifestyle factors as determinants of peak bone mass, and studying exercise-induced changes in bone mass during military service. The 184 men were recruits of the Finnish Army. Fifty men of similar age, who had postponed their military service for reasons unrelated to health, formed a control group in the exercise part of the study, in which the effect on bone parameters of physical loading associated with military study was examined. Both groups were examined at the same time at the very beginning of the military service of the recruits, when bone mass and ultrasound measurements were obtained. For the purpose of the present study, the two groups were combined. The study was approved by the Ethical Review Committee of Department of Medicine, Helsinki University Central Hospital, and a written consent was obtained from the participants.

Materials and methods

Study design

Both groups were examined at the same time from the very beginning of the military service of the recruits. BMD was measured and quantitative ultrasonography of the heel was performed.

Lifestyle factors

Current exercise, smoking, calcium intake and alcohol consumption were recorded using a structured questionnaire. The participation in various physical activities over the last year was recorded by questions addressing 30 different types of physical activity and training, which were weighted according to their bone-loading effect. Light or non-weight-bearing activities such as walking, cycling and swimming were scored as 1, activities producing repetitive weight-bearing impact (e.g. endurance running and skiing) were scored as 1.5, and high-impact or high-magnitude loading (e.g. jumping, sprint running and weightlifting) were scored as 2. For each

type of exercise practised, the subjects were asked to indicate the average number of occasions per month and the duration (minutes) and intensity (1, light; 2, moderate and 3, heavy) of each occasion. An exercise index was then calculated by summing up the products of frequency, duration, intensity and bone-loading effect of different activities during summer- and wintertime. Calcium intake was calculated on the basis of the supply from dairy products only by using the following estimations: 1 dl milk, sour milk and yogurt contain 120 mg calcium, and one slice of cheese contains 100 mg. In the calculations, 1.8 dl was used as the volume of one glass.

Biochemical measurements

Urinary type 1 collagen amino-terminal telopeptide (NTX) was measured with NTX Reagent kits (Amersham) on the VITROS ECI immunodiagnostics systems analyzer (Ortho-Clinical Diagnostics Inc., Rochester, NY, USA). The intra-assay CV was 8% at the level of 55 nM bone collagen equivalents (BCE)/l and 3% at the level of 187 nM BCE/l. The inter-assay CV was 9.8% at the level of 60 nM BCE/l and 5.3% at the level of 403 nM BCE/l. The NTX values were corrected for creatinine excretion measured by a standard laboratory method. Serum intact type 1 procollagen amino-terminal propeptide (P1NP) was determined by a competitive RIA with a commercial kit (Intact P1NP RIA) from Orion Diagnostica, Oulunsalo, Finland. Analytical sensitivity of this assay was 2 µg/l, and the intra- and inter-assay CVs ranged from 2 to 6%. Serum total osteocalcin and carboxylated osteocalcin were measured with in-house immunoassays. The intra-assay CV was < 5% and the inter-assay CV was < 8% for both assays. Serum tartrate-resistant acid phosphatase 5b (TRACP5b) activity was measured by an immunoextraction method with BoneTRAP reagents from Suomen Bioanalytiikka Oy, Turku, Finland. The analytical sensitivity of this assay was 0.1 µg/l, and the intra- and inter-assay CVs were 6% or less at relevant concentrations. Serum 25-hydroxyvitamin D (25-OHD) was measured by RIA kits from Diasorin, Stillwater, USA. At vitamin D levels of 30 and 100 nmol/l, the intra-assay CVs were 8.9 and 5.9%, and the inter-assay CVs were 12.8 and 8.8% respectively. Serum PTH was assayed using intact PTH kits from Diagnostics Product Corporation, LA, CA, USA and the IMMULITE 2000 immunoassay analyzer. At levels of 22 and 38 ng/l, the intra-assay CVs were 4.3 and 4.2%, and the inter-assay CVs were 5.3 and 3.4% at the level of 68 and 366 ng/l respectively.

Bone mass measurements

Bone mineral content (BMC) and BMD of the lumbar spine and three femoral sites (femoral neck, trochanter and total hip) were measured by dual-energy X-ray absorptiometry (DXA) using a Lunar Prodigy densitometer (Wisconsin, MA, USA).

Heel ultrasound

Broadband ultrasound attenuation (BUA) and speed-of-sound (SOS) were measured at a fixed region in the mid-calcaneus using Sahara Clinical Bone Sonometer (Hologic, Bedford, MA, USA). The sonometer was calibrated daily with a standardized phantom supplied by the manufacturer. The coefficient of variation for daily calibration measurements was 5.4% for BUA and 0.3% for SOS.

Genotyping

Genomic DNA was isolated from peripheral leucocytes by standard procedures, as described (13). The PCR amplification of the *PTHrP* VNTR was performed using the following primer sequence:

forward 5'-GACCTAGTTCTGATTGTATCCTCTACC-3'
reverse 5'-GTTCCAGGCGTAAGAATTGACGAGTG-3'

The amplifications were carried out in a 25 µl volume using a negative control where no DNA was included in the reaction. A positive control consisting of a DNA sample from the experiment was also used as an internal standard.

Genotyping and isolation of individual bands for sequencing was performed by separating the amplified *PTHrP* VNTR according to molecular size on a 1.5% agarose electrophoresis gel that was stained with ethidium bromide and visualized under u.v. light. An aliquot of 3 µl PCR product and 1 µl loading buffer was loaded onto the agarose gel. A set of six DNA samples was amplified in five independent PCR to verify the consistency of the PCR amplification.

Electrophoretic mobility shift assay

Forward and reverse oligonucleotides corresponding to the VNTR sequence 5'-TATATAC GTATATATATATAC GTATATATATAC GTA-3' containing two repeats and the corresponding oligonucleotide mutated at the repeats (indicated positions in bold type) 5'-TATATAC GTAT**TTGTCT**TATAC GGATCTACATAC GTA-3' were synthesized and purified using HPLC (Sheldon Biotechnology Centre, McGill University). Double-stranded DNA was generated by allowing 10 µg complementary oligonucleotides to anneal in 1 ml buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris, pH 8.0) placed in boiling water for 10 min, and allowed to cool to room temperature. 5'-End labelling of DNA was performed using [γ - 32 P]ATP and T4 polynucleotide kinase in an exchange reaction and probes were purified on a G-50 sephadex column.

Nuclear extracts were prepared from cultured, CV-1(simian) in origin and carrying the SV40 large T antigen (COS-1) and rat osteosarcoma (ROS) 17/2.8 cells maintained in complete Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine

serum (FBS), and aliquots were stored at -80°C . Binding reactions were performed at room temperature for 25 min in a 15 µl total volume containing 20 mM HEPES-KOH (pH 7.9), 60 mM KCl, 6 mM MgCl₂, 1 mM DTT, 10% glycerol, 1 µg poly(dI·dC), 5–10 µg nuclear extract and 20–60 K cpm γ - 32 P-ATP labelled probe. Reactions were electrophoresed on a native 5% polyacrylamide gel that was then dried and exposed to Kodak BioMax film at -80°C .

Statistical analysis

In statistical analyses, the effect of the presence versus absence of at least one copy of the individual alleles (252, 333, 378 and 460 bp) as well as the frequency-dependent relationship of the 252 bp and 378 alleles were first tested. Thereafter, the individuals were categorized into five classes: those with (1) both alleles being 252 bp; (2) one allele being 252 bp and other 333 bp; (3) one allele being 252 bp and other 378 bp; (4) one allele being 252 bp and other 460 bp and (5) other allele combinations.

The relationships between BMC, BMD, BUA and SOS and individual *PTHrP* VNTR alleles and their combinations were analysed by the multiple regression models. In addition to an individual allele or allele combination, age, height, weight, smoking, exercise, alcohol and calcium intake were put into the model. The results of these analyses are presented as least square means and their standard errors. If significance was found across the allele combinations, linear contrasts between the allele classes were programmed.

No multiple corrections for *P* values of linear contrasts were made. Also unadjusted analyses were performed, using the one-way ANOVA. All tests were performed as two sided. Due to multiple testing a significance level of 0.005 was employed. All analyses were done with SAS System (Version 8.0.2 for Windows; Cary, NC 27513 2414, US).

Results

Baseline characteristics and frequency of *PTHrP* VNTR alleles in the study population

The baseline characteristics of the study population are shown in Table 1. A total of 234 DNA samples were genotyped and the data were used for evaluating potential correlation between polymorphisms in the *PTHrP* VNTR on osteodensitometry of the lumbar spine and hip and on calcaneal quantitative ultrasound measurements. The frequency of VNTR alleles in the study population of young Finnish males was as follows: 252 (42.3%), 288 (2.2%), 333 (13%), 356 (1.2%), 378 (29%), 393 (3%), 414 (3.6%) and 460 (5.7%). Correlations with the 252, 333, 378 and 460 alleles were further explored, in as much as these were the most frequent alleles expressed.

Table 1 Baseline characteristics of the study population ($n=234$).

Characteristics	Median	Range
Age (years)	19.6	(18.3–20.6)
Height (cm)	180	(163–198)
Weight (kg)	74	(53–133)
Exercise index	10 643	(40–59 845)
Smoking (package years)	0	(0–10.5)
Calcium intake (mg/day) ^a	1264	(0–2288)
Alcohol intake (times/week)	2.0	(0.0–5.0)
Urinary NTX (nmol BCE/mmol creatinine)	86	(29–363)
Serum TRACP 5b (µg/l)	5.2	(2.4–14.0)
Serum P1NP (µg/l)	107	(41–295)
Serum TOC (µg/l)	11.5	(3.5–27.5)
Serum COC (µg/l)	11.3	(4.2–24.0)
BUA (dB/MHz)	77.6	(42.4–117.3)
SOS (m/s)	1555	(1499–1644)
Lumbar spine BMC (g)	59.3	(37.2–92.6)
Lumbar spine BMD (g/cm ²)	1.216	(0.875–1.640)
Femoral neck BMC (g)	6.3	(4.1–9.4)
Femoral neck BMD (g/cm ²)	1.167	(0.759–1.692)
Trochanter BMC (g)	14.1	(6.5–25.1)
Trochanter BMD (g/cm ²)	0.956	(0.604–1.342)
Total hip BMC (g)	41.5	(25.9–63.3)
Total hip BMD (g/cm ²)	1.163	(0.740–1.509)

^aFrom dairy products, BCE, bone collagen equivalents; BMD, bone mineral density; BMC, bone mineral content.

Bone turnover markers and serum 25-OHD and PTH

The *PTHrP* VNTR alleles did not have any effect on bone turnover markers or serum 25-OHD and PTH levels in the study population (data not shown).

Correlation of the *PTHrP* 252 bp allele with imaging parameters

Subjects with at least one copy of the 252 bp *PTHrP* allele demonstrated increased lumbar spine BMD ($P=0.0093$) as well as increased ultrasound BUA ($P=0.0016$) and SOS ($P=0.0082$). After adjusting for covariates including age, height, weight, exercise, smoking, alcohol and calcium intake, the statistically

significant associations of at least one copy of the 252 bp to lumbar spine BMD ($P=0.0034$) and to ultrasound BUA ($P=0.0012$) and SOS ($P=0.0023$) were further strengthened and there tended to be associations also to BMC of the lumbar spine, total hip and trochanteric region (P values between 0.026 and 0.046) (Table 2). Subjects expressing two 252 alleles showed increased lumbar spine BMD and ultrasound parameters but these increases were also seen with expression of only one 252 allele before and after adjustment (data not shown). Consequently, there appeared to be no frequency-dependent relationship.

Presence of the *PTHrP* 378 bp allele and imaging parameters

Subjects expressing at least one 378 bp allele showed no correlation with unadjusted parameters (data not shown). However, after adjustment for covariates, there tended to be significant reductions in lumbar spine BMC ($P=0.014$) and BMD ($P=0.034$) and trochanteric BMC ($P=0.014$; Table 2). No frequency-dependent effect of the *PTHrP* 378 bp was observed on imaging parameters, however, when subjects with zero, one or two alleles were analysed separately and adjusted for covariates (data not shown).

PTHrP 333 and 460 alleles and imaging parameters

No statistically significant correlations were found in unadjusted or adjusted BMC, BMD and ultrasound BUA and SOS values for individuals without or with at least one 333 or 460 bp allele (data not shown).

Allele combinations

In unadjusted analyses no significant differences were found between the allele combinations (data not shown). After adjustment for covariates, subjects with 252/460 combinations showed the highest mean

Table 2 Bone imaging parameters adjusted for age, height, weight, exercise, smoking, alcohol and calcium intake (least square means (s.e.m.)) in subjects without or with at least one 252 allele or one 378 allele.

Parameter	252 Allele			378 Allele		
	Without ($n=81$)	With ($n=153$)	<i>P</i>	Without ($n=116$)	With ($n=118$)	<i>P</i>
Lumbar spine BMC (g)	59.3 (1.0)	61.7 (0.7)	0.044	62.2 (0.8)	59.5 (0.8)	0.014
Lumbar spine BMD (g/cm ²)	1.202 (0.014)	1.252 (0.010)	0.0034	1.252 (0.011)	1.218 (0.011)	0.034
Femoral neck BMC (g)	6.26 (0.10)	6.50 (0.07)	0.052	6.49 (0.08)	6.36 (0.08)	0.26
Femoral neck BMD (g/cm ²)	1.155 (0.016)	1.192 (0.011)	0.064	1.192 (0.013)	1.167 (0.013)	0.18
Trochanter BMC (g)	14.1 (0.3)	15.0 (0.2)	0.026	15.1 (0.3)	14.2 (0.2)	0.014
Trochanter BMD (g/cm ²)	0.961 (0.016)	0.982 (0.011)	0.28	0.982 (0.012)	0.968 (0.013)	0.42
Total hip BMC (g)	41.4 (0.7)	43.1 (0.5)	0.046	43.1 (0.5)	41.9 (0.5)	0.13
Total hip BMD (g/cm ²)	1.160 (0.016)	1.186 (0.011)	0.18	1.184 (0.013)	1.170 (0.013)	0.42
BUA (dB/MHz)	74.4 (1.7)	81.2 (1.2)	0.0012	80.4 (1.4)	77.3 (1.4)	0.11
SOS (m/s)	1550 (3)	1562 (2)	0.0023	1561 (3)	1556 (2)	0.14

Table 3 Bone imaging parameters (least square means (S.E.M.)) adjusted for age, height, weight, exercise, smoking, alcohol and calcium intake for different allele pairs.

	252/252 (n=44)	252/333 (n=25)	252/378 (n=56)	252/460 (n=11)	Others (n=98)	Overall P
Lumbar spine BMC (g)	62.0 (1.2)	62.9 (1.6)	59.4 (1.1)	68.3 (2.7)* [†]	60.0 (0.80)	0.013
Lumbar spine BMD (g/cm ²)	1.253 (0.018)	1.283 (0.023)	1.222 (0.016)	1.348 (0.038)* [‡]	1.211 (0.012)	0.0011
Femoral neck BMC (g)	6.47 (0.13)	6.58 (0.17)	6.39 (0.11)	6.89 (0.28)	6.33 (0.09)	0.28
Femoral neck BMD (g/cm ²)	1.201 (0.021)	1.211 (0.028)	1.164 (0.019)	1.266 (0.046)	1.162 (0.014)	0.096
Trochanter BMC (g)	15.0 (0.4)	15.8 (0.6)	14.1 (0.4)	15.9 (0.9)	14.4 (0.3)	0.042
Trochanter BMD (g/cm ²)	0.977 (0.020)	1.012 (0.025)	0.961 (0.017)	1.032 (0.042)	0.967 (0.014)	0.30
Total hip BMC (g)	42.9 (0.8)	44.1 (1.1)	41.9 (0.8)	46.1 (1.8)	41.9 (0.6)	0.11
Total hip BMD (g/cm ²)	1.190 (0.020)	1.206 (0.027)	1.162 (0.018)	1.261 (0.045)	1.163 (0.014)	0.15
BUA (dB/MHz)	81.3 (2.2)	84.2 (2.8)	78.8 (1.9)	90.2 (5.0)	75.4 (1.5)	0.0046
SOS (m/s)	1565 (4)	1565 (5)	1558 (4)	1584 (9) [¶]	1551 (3)	0.0017

* $P=0.0026$ for difference between 252/378 and 252/460; [†] $P=0.0037$ for difference between 252/460 and others; [‡] $P=0.0026$ for difference between 252/378 and 252/460; [§] $P=0.0008$ for difference between 252/460 and others; ^{||} $P=0.005$ for difference between 252/460 and others; [¶] $P=0.001$ for difference between 252/460 and others. In multiple group comparisons, a significance level of 0.005 has been used.

values for all imaging parameters and they differed statistically significantly from subjects with the allele combinations not including the 252 bp with respect to lumbar spine BMD ($P=0.0008$) as well as BUA ($P=0.005$) and SOS ($P=0.001$; Table 3).

VNTR sequences bind to nuclear extracts from osteoblast-like cells

Potential functionality of VNTR sequences in the transcriptional regulation of *PTHrP* was investigated using electrophoretic mobility shift assays. Here, nuclear extracts prepared from COS-1 monkey kidney cells (unrelated control) or ROS 17/2.8 rat osteosarcoma cells (osteoblast-like cells) were incubated with labelled linear VNTR DNA sequences (encompassing two repeats) in the absence or presence of either 200-fold molar excess of unlabelled target sequence or unlabelled competitor sequence (mutated repeats) and DNA-protein interactions were assessed. The COS-1 nuclear extracts do not contain proteins that interact specifically with VNTR sequences (Fig. 2). On the other hand, nuclear extracts from ROS 17/2.8 osteoblast-like cells demonstrated specific nuclear protein binding to the VNTR region as co-incubation with competitor oligonucleotide in which the repeats were mutated failed to decrease the binding interaction. On the other hand, unlabelled target sequences were very effective in competing with the labelled oligonucleotide as shown by the failure to preserve the intensity of the shifted band.

Discussion

Adolescence is a period of rapid skeletal growth during which nearly half of the adult skeletal mass is accrued. This life stage is a window of opportunity for influencing peak bone mass and reducing the risk of osteoporosis later in life. Endocrine factors that may influence peak bone mass include insulin-like growth factor and thyroid hormones that regulate skeletal growth, and sex

hormones that stimulate epiphyseal maturation. In addition, appropriate mineralization of the skeleton requires adequate dietary intakes of minerals involved in the formation of hydroxyapatite; the most likely to be deficient is calcium. Vitamin D deficiency can contribute to calcium deficiency but also has an independent role to play in normal skeletal accrual (15, 16). Additionally weight-bearing exercise can increase bone mass. Patients with low bone mass early in adult life, therefore, have less bone to lose in later life before fracture risk further increases. Interestingly, nearly 15% of premenopausal women have BMD that is more than 1 s.d. less than the young-adult mean, and ~0.6% are more than 2.5 s.d. below young-adult mean bone density (17).

Acquisition of normal peak bone mass is generally considered to be a polygenic process arising from the interaction of common polymorphic alleles at quantitative trait loci. Over the last decade, a large number of candidate genes have been identified such as the vitamin D receptor (18), type I collagen genes (COL1A1 and COL1A2), sclerostin (SOST) (19, 20) and the low-density lipoprotein receptor-related protein 5 (LRP5), which has recently been shown to affect bone mass accrual during growth and to be involved in osteoporosis-pseudoglioma syndrome (21), a high bone mass phenotype (22, 23), and osteoporosis in both children and adults (24). Interestingly, linkage between genetic polymorphisms as determinants of BMD variations in the general population has also been reported for each of these genes (25–29). In this same cohort, we recently pointed out that the polymorphic valine variant at position 1330 of LRP5 was significantly associated with reduced BMC and BMD at several skeletal sites (29). In contrast, the association studies of the XbaI and PvuII polymorphisms of the oestrogen receptor gene and the CAG repeat polymorphism of the androgen receptor gene gave negative results (30), as did the studies of SNP polymorphisms of the lactase gene (31) and the leucine7 to proline7 polymorphism of the prepro-NPY gene (unpublished observation). Styrkarsdottir *et al.* (32) have recently shown linkage of osteoporosis to

Figure 2 Potential transcriptional role of *PTHrP* VNTR.

(A) Electrophoretic mobility gel shift assay. Nuclear extracts prepared from cultured COS-1 monkey kidney cells (control) or ROS 17/2.8 rat osteosarcoma cells (osteoblast-like cells) were incubated with labelled linear VNTR oligonucleotide (probe), in the absence or presence of either 200-fold molar excess of unlabelled (cold) target sequence or unlabelled competitor sequence in which the repeats were mutated (mutant oligonucleotide). Arrowhead indicates a band shift with ROS 17/2.8 nuclear extracts that is unaffected by co-incubation with the mutant oligonucleotide. Unlabelled target oligonucleotide was effective in competing and thereby decreasing the intensity of the shifted band suggesting a specific DNA–protein interaction. (B) Consensus sequence (inside box) for CF2-II-binding site as determined by EMSA in which bacterially produced full-length CF2-II protein was mixed with random sequence oligonucleotides (33).

chromosome 20p12 and association to variants in the bone morphogenetic protein-2 gene (*BMP2*) in several osteoporotic families in Iceland and postmenopausal women in Denmark. However, it remains to be seen if the association of osteoporosis to polymorphisms in *BMP2* gene will hold in multi-ethnic and multi-race populations.

By linkage studies using candidate genes in families of probands with osteoporosis, suggestive evidence of linkage has been reported between BMD and *PTHrP* (maximum LOD score obtained 2.7–3.5) but not for *PTH* or *PTHrP* (34). Reduced height, however, has been linked to *PTHrP* in Japanese adolescents (35). On the other hand, Hosoi and colleagues studied the association between a polymorphism of *PTH* recognized by the enzyme BSTB1 in a Japanese population and found evidence of reduced BMD in *Bb* heterozygotes when compared with *BB* homozygotes (36). The mechanisms responsible remain to be defined but we have previously reported the important anabolic role that PTH plays in stimulating foetal and neonatal growth in the animal models (37, 38).

While the existence of the *PTHrP* VNTR has been reported, no function has been ascribed to it prior to the studies described herein. Potential correlations were evaluated between specific allelic variations in the VNTR region of *PTHrP* and bone densitometric indices including bone mineral content and density values at the lumbar spine, femoral neck, trochanter and total hip, as well as with ultrasound variables of the heel. These correlations were evaluated by examining the relative frequencies of the most common alleles in this population i.e. 252, 333, 378 and 460 bp alleles or the frequency of the allele pairs 252/252; 252/333; 252/378 and 252/460 with or without adjusting for age, height, weight, exercise, smoking, calcium and alcohol intake as covariates.

The presence of a 252 bp allele in young Finnish males in this study was found to be a predictor of higher values of lumbar spine BMD, and BUA and SOS providing evidence for a relationship of specific alleles of the *PTHrP* VNTR region to the various bone parameters. Consequently, at least a single 252 allele appeared to be of benefit vis-à-vis the bone imaging parameters. However, no statistically significant frequency-dependent effect of the 252 allele was observed.

The presence or absence of at least one copy of either 333 or 460 bp allele was not found to be a predictor of any bone parameters at any site. However, in conjunction with a 252 allele the 460 allele appeared to increase bone density, particularly at the lumbar region, and the ultrasonographic parameters of the heel, in relation to the allele combinations not including the 252 bp. This occurred despite the fact that individuals with the 252/460 genotype tended to have the lowest calcium intake and exercise indices (data not shown). It is important nevertheless to note the small sample size expressing this particular genotype. Consequently, although the 460 allele alone appeared functionally silent, in combination with the 252 allele, its positive effect on bone parameters was observed.

The mechanisms that may underlie the alterations in bone mass attainment based on the specific VNTR allele combinations remains to be investigated. Potentially, sequences in the VNTR may serve as binding sites for

transcription factors and/or associated transcriptional cofactors, as shown here using nuclear extracts from osteoblast-like cells but not from kidney cells, and thereby influence the transcriptional activity of the *PTHrP* locus. For example, a VNTR polymorphic allele in intron 4 of cold-induced autoinflammatory syndrome 1 gene was shown to associate with essential hypertension and increase luciferase expression in a reporter gene assay (39). Similarly, polymorphic regions consisting of a VNTR within intron 2 of the gene coding for the serotonin transporter protein 5-HTT act as transcriptional regulators and have allele-dependent differential enhancer-like properties (40). Such enhancer properties may be dependent on steric or other properties of the VNTR and may either be positively or negatively affected by allelic variation in the VNTR. Furthermore, the possibly differential effects of individual alleles such as 252 bp and 378 bp and allele combinations within the VNTR such as the 252/460 pairing may indicate that transcriptional alterations reflect heterodimerization in this region of *PTHrP*. Irrespective of the underlying mechanism, such fine modulation in the transcriptional activity of the gene is likely to result in small alterations in the local PTHrP concentration within the skeletal microenvironment that may be responsible for differential bone-forming capacity and hence the level of peak bone mass attained during early adulthood. Interestingly, *in silico* analysis of the VNTR sequence has identified the AT-rich repeats as potential binding sites for the *Drosophila* transcription factor CF2-II that bears homology to a number of human zinc-finger proteins including the krueppel C2H2-type zinc-finger protein ZNF771 (DSC43), a transcription factor expressed in human mesenchymal cells, while undergoing osteogenic differentiation (AAH11870). Although increased local bone formation following increased PTHrP expression may arise via a number of mechanisms, inhibition of SOST (41, 42) and DKK1 (43) expression resulting in increased LRP5/wnt/ Frizzled signalling and hence stimulated osteogenic cell potential are most likely.

The strengths of our study reside in the genetic homogeneity of the Finnish population, the uniform age of men studied and the careful characterization of possible confounding factors. Although the number of young men was not high, it is unlikely that our findings were by chance and multiple testing only. First, the 252 allele was the only one that significantly associated with bone parameters after raising the significance level up to 0.005. Secondly, men with the 252 allele had higher BMD values in the lumbar spine measured by DXA as well as higher BUA and SOS values measured by a totally different technique, ultrasound. These differences did not disappear after adjusting for demographic and lifestyle factors, but even improved. On the other hand, our findings concern only the Finnish Caucasian male population and are not necessarily applicable to females and other populations and races. The findings of the present study will need to be verified in larger and more heterogeneous populations

and in both sexes. Discrepancy in association between phenotype and allelic polymorphisms amongst genders have been reported for the *INS VNTR* (44), the dopamine transporter gene (*DAT1*10*) (45) and *LRP5* where genetic variation may exert a stronger influence on BMD in males than females (28). This intriguing observation therefore raises the possibility that epigenetic or environmental factors could influence the phenotypic expression(s) of such genetic variants (reviewed in (46)). Longitudinal studies will also be required to examine whether these individuals with decreased peak bone mass do exhibit a higher incidence of osteoporosis and associated fragility fractures. Alternatively, such studies will also address whether individuals shown to have increased peak bone mass based on the length of the *PTHrP* VNTR alleles are protected from the disease.

In summary, the present findings provide first evidence for the predictive value of the length of the *PTHrP* VNTR polymorphism in the attainment of peak bone mass in young Finnish males. Clearly, this polymorphism is likely to represent one such potential marker since segregation studies in families with osteoporosis support a pattern of inheritance that is most consistent with the effects of several genes, each with modest effects, rather than a few genes with large effects. It is conceivable that a multi-gene diagnostic sensor chip based on a number of such genetic markers would predictably identify those subjects at risk of developing low bone mass and osteoporosis so that preventive interventions and/or prophylactic therapeutic measures could be instituted early and effectively.

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References

- 1 Ross PD. Osteoporosis. Frequency, consequences, and risk factors. *Archives of Internal Medicine* 1996 **156** 1399–1411.
- 2 Cauley JA, Thompson DE, Ensrud KC, Scott JC & Black D. Risk of mortality following clinical fractures. *Osteoporosis International* 2000 **11** 556–561.
- 3 Pocock NA, Eisman JA, Hopper JL, Yeates MG, Sambrook PN & Eberl S. Genetic determinants of bone mass in adults. A twin study. *Journal of Clinical Investigation* 1987 **80** 706–710.
- 4 Ralston SH. Genetics of osteoporosis. *Reviews in Endocrine and Metabolic Disorders* 2001 **2** 13–21.
- 5 Karaplis AC, Luz A, Glowacki J, Bronson RT, Tybulewicz VL, Kronenberg HM & Mulligan RC. Lethal skeletal dysplasia from targeted disruption of the parathyroid hormone-related peptide gene. *Genes and Development* 1994 **8** 277–289.

- 6 Amizuka N, Warshawsky H, Henderson JE, Goltzman D & Karaplis AC. Parathyroid hormone-related peptide-depleted mice show abnormal epiphyseal cartilage development and altered endochondral bone formation. *Journal of Cell Biology* 1994 **126** 1611–1623.
- 7 Amizuka N, Karaplis AC, Henderson JE, Warshawsky H, Lipman ML, Matsuki Y, Ejiri S, Tanaka M, Izumi N, Ozawa H & Goltzman D. Haploinsufficiency of parathyroid hormone-related peptide (PTHrP) results in abnormal postnatal bone development. *Developmental Biology* 1996 **175** 166–176.
- 8 Miao D, He B, Jiang Y, Kobayashi T, Soroceanu MA, Zhao J, Su H, Tong X, Amizuka N, Gupta A, Genant HK, Kronenberg HM, Goltzman D, Karaplis AC & Osteoblast-derived PTHrP is a potent endogenous bone anabolic agent that modifies the therapeutic efficacy of administered PTH 1–34. *Journal of Clinical Investigation* 2005 **115** 2402–2411.
- 9 Schipani E, Kruse K & Juppner H. A constitutively active mutant PTH-PTHrP receptor in Jansen-type metaphyseal chondrodysplasia. *Science* 1995 **268** 98–100.
- 10 Jobert AS, Zhang P, Couvineau A, Bonaventure J, Roume J, Le Merrer M & Silve C. Absence of functional receptors for parathyroid hormone and parathyroid hormone-related peptide in Blomstrand chondrodysplasia. *Journal of Clinical Investigation* 1998 **102** 34–40.
- 11 Karaplis AC, He B, Nguyen MT, Young ID, Semeraro D, Ozawa H & Amizuka N. Inactivating mutation in the human parathyroid hormone receptor type 1 gene in Blomstrand chondrodysplasia. *Endocrinology* 1998 **139** 5255–5258.
- 12 Yasuda T, Banville D, Hendy GN & Goltzman D. Characterization of the human parathyroid hormone-like peptide gene. Functional and evolutionary aspects. *Journal of Biological Chemistry* 1989 **264** 7720–7725.
- 13 Pausova Z, Morgan K, Fujiwara TM, Bourdon J, Goltzman D & Hendy GN. Molecular characterization of an intragenic minisatellite (VNTR) polymorphism in the human parathyroid hormone-related peptide gene in chromosome region 12p12.1-p11.2. *Genomics* 1993 **17** 243–244.
- 14 Valimäki MJ, Karkkainen M, Lamberg-Allardt C, Laitinen K, Alhava E, Heikkinen J, Impivaara O, Makela P, Palmgren J & Seppanen R. Exercise, smoking, and calcium intake during adolescence and early adulthood as determinants of peak bone mass. Cardiovascular Risk in Young Finns Study Group. *BMJ* 1994 **309** 230–235.
- 15 Panda DK, Miao D, Bolivar I, Li J, Huo R, Hendy GN & Goltzman D. Inactivation of the 25-hydroxyvitamin D 1 α -hydroxylase and vitamin D receptor demonstrates independent and interdependent effects of calcium and vitamin D on skeletal and mineral homeostasis. *Journal of Biological Chemistry* 2004 **279** 16754–16766.
- 16 Valimäki VV, Alftan H, Lehmuskallio E, Löytyniemi E, Sahi T, Stenman UH, Suominen H & Valimäki MJ. Vitamin D status as a determinant of peak bone mass in young Finnish men. *Journal of Clinical Endocrinology and Metabolism* 2004 **89** 76–80.
- 17 Lewiecki EM. Premenopausal bone health assessment. *Current Rheumatology Reports* 2005 **7** 46–52.
- 18 Morrison NA, Qi JC, Tokita A, Kelly PJ, Crofts L, Nguyen TV, Sambrook PN & Eisman JA. Prediction of bone density from vitamin D receptor alleles. *Nature* 1994 **367** 284–287.
- 19 Brunkow ME, Gardner JC, Van Ness J, Paeper BW, Kovacevich BR, Proll S, Skonier JE, Zhao L, Sabo PJ, Fu Y, Alisch RS, Gillett L, Colbert T, Tacconi P, Galas D, Hamersma H, Beighton P & Mulligan J. Bone dysplasia sclerosteosis results from loss of the SOST gene product, a novel cystine knot-containing protein. *American Journal of Human Genetics* 2001 **68** 577–589.
- 20 Balemans W, Ebeling M, Patel N, Van Hul E, Olson P, Dioszegi M, Lacza C, Wuyts W, Van Den Ende J, Willems P, Paes-Alves AF, Hill S, Bueno M, Ramos FJ, Tacconi P, Dikkers FG, Stratakis C, Lindpaintner K, Vickery B, Foerzler D & Van Hul W. Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). *Human Molecular Genetics* 2001 **10** 537–543.
- 21 Gong Y, Slee RB, Fukai N, Rawadi G, Roman-Roman S, Reginato AM, Wang H, Cundy T, Glorieux FH, Lev D, Zacharin M, Oexle K, Marcelino J, Suwairi W, Heeger S, Sabatakis G, Apte S, Adkins WN, Allgrove J, Arslan-Kirchner M, Batch JA, Beighton P, Black GC, Boles RG, Boon LM, Borrone C, Brunner HG, Carle GF, Dallapiccola B, De Paepe A, Floege B, Halfhide ML, Hall B, Hennekam RC, Hirose T, Jans A, Juppner H, Kim CA, Keppeler-Noreuil K, Kohlschuetter A, Lacombe D, Lambert M, Lemyre E, Letteboer T, Peltonen L, Ramesar RS, Romanengo M, Somer H, Steichen-Gersdorf E, Steinmann B, Sullivan B, Superti-Furga A, Swoboda W, Van Den Boogaard MJ, Van Hul W, Vikkula M, Votruba M, Zabel B, Garcia T, Baron R, Olsen BR & Warman ML. LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell* 2001 **107** 513–523.
- 22 Little RD, Carulli JP, Del Mastro RG, Dupuis J, Osborne M, Folz C, Manning SP, Swain PM, Zhao SC, Eustace B, Lappe MM, Spitzer L, Zweier S, Braunschweiler K, Benchekroun Y, Hu X, Adair R, Chee L, Fitzgerald MG, Tulig C, Caruso A, Tzellas N, Bawa A, Franklin B, McGuire S, Nogues X, Gong G, Allen KM, Anisowicz A, Morales AJ, Lomedico PT, Recker SM, Van Eerdewegh P, Recker RR & Johnson ML. A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. *American Journal of Human Genetics* 2002 **70** 11–19.
- 23 Boyden LM, Mao J, Belsky J, Mitzner L, Farhi A, Mitnick MA, Wu D, Insogna K & Lifton RP. High bone density due to a mutation in LDL-receptor-related protein 5. *New England Journal of Medicine* 2002 **346** 1513–1521.
- 24 Hartikka H, Makitie O, Mannikko M, Doria AS, Daneman A, Cole WG, Ala-Kokko L & Sochett EB. Heterozygous mutations in the LDL receptor-related protein 5 (LRP5) gene are associated with primary osteoporosis in children. *Journal of Bone and Mineral Research* 2005 **20** 783–789.
- 25 Grant SF, Reid DM, Blake G, Herd R, Fogelman I & Ralston SH. Reduced bone density and osteoporosis associated with a polymorphic Sp1 binding site in the collagen type I alpha 1 gene. *Nature Genetics* 1996 **14** 203–205.
- 26 Uitterlinden AG, Arp PP, Paeper BW, Charmley P, Proll S, Rivadeneira F, Fang Y, Van Meurs JB, Britschgi TB, Latham JA, Schatzman RC, Pols HA & Brunkow ME. Polymorphisms in the sclerosteosis/van Buchem disease gene (SOST) region are associated with bone-mineral density in elderly whites. *American Journal of Human Genetics* 2004 **75** 1032–1045.
- 27 Koay MA, Woon PY, Zhang Y, Miles LJ, Duncan EL, Ralston SH, Compston JE, Cooper C, Keen R & Langdahl Et A. Influence of LRP5 polymorphisms on normal variation in BMD. *Journal of Bone and Mineral Research* 2004 **19** 1619–1627.
- 28 Ferrari SL, Deutsch S, Choudhury U, Chevalley T, Bonjour J-P, Dermizakis ET, Rizzoli R & Antonarakis SE. Polymorphisms in the low-density lipoprotein receptor-related protein 5 (LRP5) gene are associated with variation in vertebral bone mass, vertebral bone size, and stature in whites. *American Journal of Human Genetics* 2004 **74** 866–875.
- 29 Saarinen A, Valimäki VV, Valimäki MJ, Löytyniemi E, Auro K, Uusen P, Kuris M, Lehesjoki AE & Makitie O. The A1330V polymorphism of the low-density lipoprotein receptor-related protein 5 gene (LRP5) associates with low peak bone mass in young healthy men. *Bone* 2007 **40** 1006–1012.
- 30 Välimäki VV, Piippo K, Välimäki S, Löytyniemi E, Kontula K & Välimäki MJ. The relation of the XbaI and PvuII polymorphisms of the estrogen receptor gene and the CAG repeat polymorphism of the androgen receptor gene to peak bone mass and bone turnover rate among young healthy men. *Osteoporosis International* 2005 **16** 1633–1640.
- 31 Enattah N, Välimäki VV, Välimäki MJ, Löytyniemi E, Sahi T & Järvelä I. Molecularly defined lactose malabsorption, peak bone mass and bone turnover rate in young Finnish men. *Calcified Tissue International* 2004 **75** 488–493.
- 32 Styrkarsdóttir U, Cazier JB, Kong A, Rólfsson O, Larsen H, Bjarnadóttir E, Johannsdóttir VD, Sigurdardóttir MS, Bagger Y, Christiansen C, Reynisdóttir I, Grant SF, Jonasson K, Frigge ML,

- Gulcher JR, Sigurdsson G & Stefansson K. Linkage of osteoporosis to chromosome 20p12 and association to BMP2. *PLoS Biology* 2003 **1** E69.
- 33 Gogos JA, Hsu T, Bolton J & Kafatos FC. Sequence discrimination by alternatively spliced isoforms of a DNA binding zinc finger domain. *Science* 1992 **257** 1951–1955.
 - 34 Duncan EL, Brown MA, Sinsheimer J, Bell J, Carr AJ, Wordworth BP & Wass JA. Suggestive linkage of the parathyroid receptor type 1 to osteoporosis. *Journal of Bone and Mineral Research* 1999 **14** 1993–1999.
 - 35 Minagawa M, Yasuda T, Watanabe T, Minamitani K, Takahashi Y, Goltzman D, White JH, Hendy GN & Kohno Y. Association between AAAG repeat polymorphism in the P3 promoter of the human parathyroid hormone (PTH)/PTH-related peptide receptor gene and adult height, urinary pyridinoline excretion, and promoter activity. *Journal of Clinical Endocrinology and Metabolism* 2002 **87** 1791–1796.
 - 36 Hosoi T, Miyao M, Inoue S, Hoshino S, Shiraki M, Orimo H & Ouchi Y. Association study of parathyroid hormone gene polymorphism and bone mineral density in Japanese postmenopausal women. *Calcified Tissue International* 1999 **64** 205–208.
 - 37 Xue Y, Karaplis AC, Hendy GN, Goltzman D & Miao D. Genetic models show that parathyroid hormone and 1,25-dihydroxyvitamin D3 play distinct and synergistic roles in postnatal mineral ion homeostasis and skeletal development. *Human Molecular Genetics* 2005 **14** 1515–1528.
 - 38 Miao D, He B, Karaplis AC & Goltzman D. Parathyroid hormone is essential for normal fetal bone formation. *Journal of Clinical Investigation* 2002 **109** 1173–1182.
 - 39 Omi T, Kumada M, Kamesaki T, Okuda H, Munkhtulga L, Yanagisawa Y, Utsumi N, Gotoh T, Hata A, Soma M, Umemura S, Ogihara T, Takahashi N, Tabara Y, Shimada K, Mano H, Kajii E, Miki T & Iwamoto S. An intronic variable number of tandem repeat polymorphisms of the cold-induced autoinflammatory syndrome 1 (CIAS1) gene modifies gene expression and is associated with essential hypertension. *European Journal of Human Genetics* 2006 **14** 1295–1305.
 - 40 Mackenzie A & Quinn J. A serotonin transporter gene intron 2 polymorphic region, correlated with affective disorders, has allele-dependent differential enhancer-like properties in the mouse embryo. *PNAS* 1999 **96** 15251–15255.
 - 41 Keller H & Kneissel M. SOST is a target gene for PTH in bone. *Bone* 2005 **37** 148–158.
 - 42 Bellido T, Ali AA, Gubrij I, Plotkin LI, Fu Q, O'Brien CA, Manolagas SC & Jilka RL. Chronic elevation of parathyroid hormone in mice reduces expression of sclerostin by osteocytes: a novel mechanism for hormonal control of osteoblastogenesis. *Endocrinology* 2005 **146** 4577–4583.
 - 43 Glass DA, II, Bialek P, Ahn JD, Starbuck M, Patel MS, Clevers H, Taketo MM, Long F, McMahon AP, Lang RA & Karsenty G. Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Developmental Cell* 2005 **8** 751–764.
 - 44 Thorsby PM, Berg JP & Birkeland KI. Insulin gene variable number of tandem repeats is associated with increased fat mass during adolescence in non-obese girls. *Scandinavian Journal of Clinical and Laboratory Investigation* 2005 **65** 163–168.
 - 45 Simsek M, Al-Sharbaty M, Al-Adawi S, Ganguly SS & Lawatia K. Association of the risk allele of dopamine transporter gene (DAT1×10) in Omani male children with attention-deficit hyperactivity disorder. *Clinical Biochemistry* 2005 **38** 739–742.
 - 46 Ferrari SL, Deutsch S & Antonarakis SE. Pathogenic mutations and polymorphisms in the lipoprotein receptor-related protein 5 reveal a new biological pathway for the control of bone mass. *Current Opinion in Lipidology* 2005 **16** 207–214.

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