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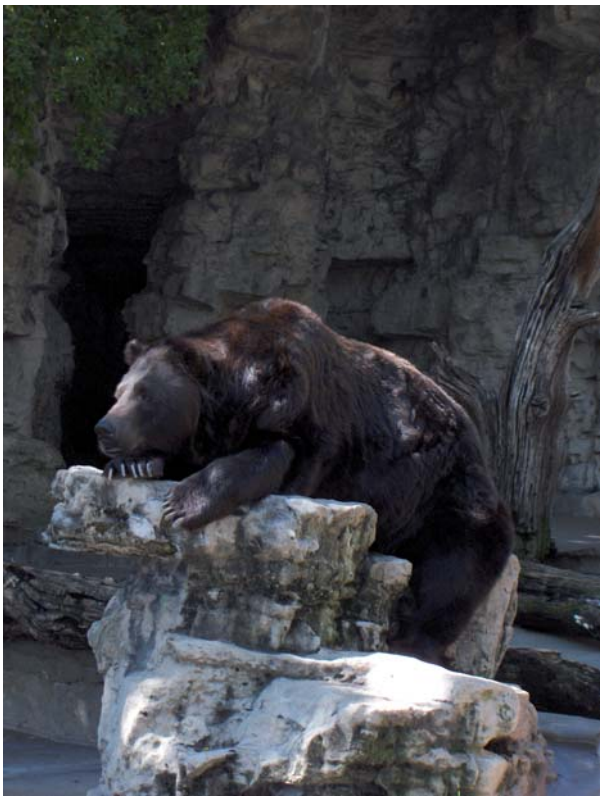
160

Genetics of Children's Bone Growth



JYVÄSKYLÄN YLIOPISTO

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Miia Suuriniemi

Genetics of Children•s Bone Growth

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UNIVERSITY OF JYVÄSKYLÄ

JYVÄSKYLÄ 2006

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Editors
Varpu Marjomäki
Department of Biological and Environmental Science
Pekka Olsbo, Marja-Leena Tynkkynen
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"No matter how much bone I lost during writing this thesis, I still have my genes I can always put my faith in"

– Miia Suuriniemi, 2005 –

ABSTRACT

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Genetics of children's bone growth
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Given the current trends of aging population and the growing adoption of unhealthy and sedentary life styles among children and adolescents, bone fragility with associated fractures will truly become a global problem in years to come. Even though the foundation for skeletal health is established during young ages and is strongly governed by the heredity, our current understanding of the genetic basis of paediatric bone mass and growth process is limited. Therefore, the purpose of this work was to elucidate this issue by investigating four important bone-related polymorphic genes in relation to several bone phenotypes and risk of fragility fractures in healthy teenage schoolgirls. Concerning the genes related to the organic matrix of bone, polymorphism of the type I collagen α1 gene (*COL1A1*) was found to associate with overall poor quantity as well as quality of bone, while that of the type I collagen α2 gene (*COL1A2*) contributed to increased risk of fractures independently of bone density. Regarding the genes related to the action and metabolism of estrogen, polymorphism of the estrogen receptor α gene (*ER-α*) was found to modulate the effect of exercise on bone density, and that of the catechol-*O*-methyltransferase gene (*COMT)* associated with an early sexual and musculoskeletal development, via elevated concentration of estrogen. Together, these findings provide new information on the genetic basis of children's bone growth, which will be useful considering the future challenge of early identification of children at risk for bone fragility and prevention of osteoporosis before a fracture occurs.

Key words: Bone; catechol-*O*-methyltransferase; estrogen receptor α; fracture; genetic polymorphism; puberty; type I collagen.

M. Suuriniemi, University of Jyväskylä, Department of Biological and Environmental Science, P.O. Box 35, FI-40014 University of Jyväskylä, Finland

Author's address

Miia Suuriniemi
University of Jyväskylä
Department of Biological and Environmental Science
P.O. Box 35
FI-40014 University of Jyväskylä, Finland
E-mail: miisu@sport.jyu.fi

Supervisors

Professor Sulin Cheng
University of Jyväskylä
Department of Health Sciences
P.O. Box 35
FI-40014 University of Jyväskylä, Finland
E-mail: Sulin.Cheng@sport.jyu.fi

Docent Vuokko Kovanen
University of Jyväskylä
Department of Health Sciences
P.O. Box 35
FI-40014 University of Jyväskylä, Finland
E-mail: Vuokko.Kovanen@sport.jyu.fi

Docent Anitta Mahonen
University of Kuopio
Department of Medical Biochemistry
P.O. Box 1627
FI-70211 Kuopio, Finland
E-mail: anitta.mahonen@uku.fi

Reviewers

Professor Emeritus Pekka Mäenpää
Nujulanlenkki 28 A
FI-41500 Hankasalmi as., Finland
E-mail: pekka.maenpaa@uku.fi

Professor Nicholas Bishop
Sheffield Children's Hospital
Stephenson Wing, Western Bank
Sheffield S10 2TH, U.K.
E-mail: n.j.bishop@sheffield.ac.uk

Opponent

Professor Kalervo Väänänen
University of Turku
Department of Anatomy/Institute of Biomedicine
Kiinamyllynkatu 10
FI-20520 Turku, Finland
E-mail: kalervo.vaananen@utu.fi

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by their Roman numerals. The work was carried out within a larger research group, where I participated in the data collection, analysis, and writing of reports and articles. Specifically, I performed a part of the genetic analyses (*COL1A1* and *COL1A2*), ultrasound measurements, a part of the anthropometric assessments (sitting height and arm and leg lengths), collection and evaluation of the fracture data, database management for the biochemical and genetic data, and most of the statistical analyses reported in the articles below. I am responsible for writing the articles I-IV and also gave my suggestions on writing the article V.

I Suuriniemi, M., Kovanen, V., Mahonen, A., Alén, M., Wang, Q., Lyytikäinen, A. & Cheng, S. *COL1A1* Sp1 polymorphism associates with bone density in early puberty. (submitted)

II Suuriniemi, M., Mahonen, A., Kovanen, V., Alén, M. & Cheng, S. 2003. Relation of *PvuII* site polymorphism in the *COL1A2* gene to the risk of fractures in prepubertal Finnish girls. Physiol. Genomics 14: 217-224.

III Suuriniemi, M., Mahonen, A., Kovanen, V., Alén, M., Lyytikäinen, A., Wang, Q., Kröger, H. & Cheng, S. 2004. The association between exercise and pubertal bone density is modulated by estrogen receptor α genotype. J. Bone Miner. Res. 19: 1758-1765.

IV Suuriniemi, M., Suominen, H., Mahonen, A., Alén, M. & Cheng, S. The effect of estrogen receptor α polymorphism on the skeletal responsiveness to exercise during puberty. (submitted)

V Eriksson, A. L., Suuriniemi, M., Mahonen, A., Cheng, S. & Ohlsson, C. 2005. The *COMT* val158met polymorphism is associated with early pubertal development, height, and cortical bone mass in girls. Pediatr. Res. 58: 71-77.

ABBREVIATIONS

aBMD	areal bone mineral density
ANCOVA	one-way analysis of covariance
ANOVA	one-way analysis of variance
AP-1	activator protein 1
BA	bone area
B-ALP	bone-specific alkaline phosphatase
BMC	bone mineral content
BMI	body mass index
bp	base pairs
BSI	body size index
BUA	broadband ultrasound attenuation
CI	confidence interval
COL1A1	type I collagen α1
COL1A2	type I collagen α2
COMT	catechol-*O*-methyltransferase
CSA	cross-sectional area
CTh	cortical thickness
CV	coefficients of variation
DASH	dynamic allele-specific hybridization
DELFIA	dissociation-enhanced lanthanide fluorescent immunoassay
DNA	deoxyribonucleic acid
DXA	dual-energy X-ray absorptiometry
E2	serum 17β-estradiol
ELISA	enzyme-linked immunosorbent assay
ER-α	estrogen receptor α
ERE	estrogen response element
ERK	extracellular-regulated kinase
FE2	free 17β-estradiol
FM	fat mass
FT	free testosterone
GH	growth hormone
HHPA	consistently high physical activity
IGF-1	insulin-like growth factor-1
IGFBP-3	insulin-like growth factor binding protein-3
IGF1-R	insulin-like growth factor-1 receptor
IJO	idiopathic juvenile osteoporosis
Ipolar	moment of inertia
K	equilibrium constant
LLPA	consistently low physical activity
LRP5	low density lipoprotein receptor-related protein 5
LSD	least significant difference
LTM	lean tissue mass

MAPK	mitogen-activated protein kinase
OC	osteocalcin
OI	osteogenesis imperfecta
OIM	murine model of osteogenesis imperfecta
OR	odds ratio
P1NP	amino-terminal propeptide of type I procollagen
PA	physical activity
PCR	polymerase chain reaction
pQCT	peripheral quantitative computed tomography
QUS	quantitative ultrasonometry
RFLP	restriction fragment length polymorphism
RIA	radioimmunoassay
SHBG	sex hormone binding globulin
SNP	single nucleotide polymorphism
SOS	speed of sound
T	testosterone
TRACP 5b	bone-specific tartrate-resistant acid phosphatase isoform 5b
UV	ultraviolet
vBMD	volumetric bone mineral density
VDR	vitamin D receptor

1 INTRODUCTION

Osteoporosis is a skeletal disorder characterized by low bone mass and micro architectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture. It is a major cause of morbidity and mortality among elderly people but can also affect children. During infancy osteoporosis may be either primary or a complication of chronic diseases or their treatments. Anyhow, as the majority of the final bone mass and structure is established by the end of longitudinal growth, the skeletal growth during childhood and adolescence is an important determinant of the life-time risk of osteoporosis. Therefore, osteoporosis should be considered as a paediatric disease with geriatric consequences. As there is no "cure" for osteoporosis in the elderly, more attention should be paid to the early preventive strategies.

The first step towards a healthy and strong skeleton is to understand the biological process of bone growth and mineral accrual. At present, bone phenotype has been generally recognized as a multi-factorial trait with a strong genetic background. However, the genes responsible for the variability in paediatric bone mass, density, and geometry are far from established. The search is challenging because multiple genes are likely to interact with each other and with environmental factors to govern the skeletal growth.

The purpose of this study was to increase our knowledge on the genetic basis of skeletal growth during puberty. The aim was approached from the standpoint of the developmental biology of the skeleton by focusing on four important bone-related polymorphic genes and their effects on several bone phenotypes and risk of fragility fractures, in healthy pubertal schoolgirls. As the organic matrix of bone is primarily composed of type I collagen, the genes coding for the α1 and α2 chains of type I collagen (*COL1A1* and *COL1A2*, respectively) were selected to represent the material component of bone. More specifically, a well-known Sp1 binding site polymorphism of the *COL1A1* gene, which has previously been found to associate with decreased bone mass and osteoporotic fractures in elderly women (Mann et al. 2001), was investigated in relation to bone (I). A *PvuII* polymorphism of the *COL1A2* gene, which has been observed in a patient with osteogenesis imperfecta (OI) and several patients

with osteoporosis (Constantinou et al. 1990), but has not been investigated thereafter, was studied as well (II). In addition, two genes related to the estrogen action and catabolism, estrogen receptor α (*ER-α*) and catechol-*O*-methyltransferase (*COMT*), were selected as representatives of the regulators of bone metabolism. An extensively studied *PvuII* polymorphism of the *ER-α* gene among elderly women (III, IV), which impact on bone mineral density (BMD) still remains debatable due to conflicting findings (Ioannidis et al. 2002), as well as a functional polymorphism of the *COMT* gene (V), which results in a 3-4 fold decreased methylation activity of the COMT enzyme (Lachman et al. 1996), were chosen as the nucleotide variants.

These studies are of importance when elucidating the genetic basis of normal vs. impaired skeletal growth during puberty. Understanding the genetic mechanisms and their interactions with environmental factors may also facilitate the prediction of the individual response to treatment, such as hormone replacement therapy or exercise. The obvious application of these studies is the discovery of genetic markers that may predict future osteoporotic fractures and allows the identification of children at risk. Such knowledge will provide a more rational way to diagnose, prevent, and treat this widespread disease with devastating health consequences.

2 REVIEW OF THE LITERATURE

2.1 Bone composition

Bone is a dynamic and highly specialized form of connective tissue whose primary functions are to provide a mechanical support system for muscular activity and physical protection for internal organs and tissues, as well as to act as a storage facility for systemic mineral homeostasis. It is a complex living tissue, where the extracellular matrix produced by the bone cells is mineralized, conferring marked rigidity and strength to the skeleton while still maintaining some degree of elasticity.

Morphologically, there are two forms of bone: cortical (compact) and trabecular (cancellous) bone. Cortical bone represents nearly 80 % of the skeletal mass and is found in the shaft regions of long bones. Therefore, it predominates in the appendicular skeleton, such as femur in the leg and radius in the arm. It is characterized by lamellae, formed by densely packed collagen fibrils, which are concentrically arranged in cylindrical structures called Haversian systems, surrounding a central canal for blood and lymph vessels as well as for nerves (Ng et al. 1997). Trabecular bone is found inside the bone cortex and predominates in the axial skeleton, such as rib cage and spine. It also occupies the ends of long bones, where the cortical bone becomes thinner. Trabecular bone is made up of a loosely organized porous network of tiny strands called trabeculae, whose position are determined by the pressure placed on the bones during development. Differences in the structural arrangement of these two bone types are related to their primary functions: cortical bone provides most of the mechanical and protective functions, while trabecular bone provides mainly metabolic functions. The three major components of bone are the bone cells, organic matrix, and mineral. Two-thirds of the dry weight of bone is mineral, and the rest is organic matrix, from which about 2 % is occupied by different bone cells (Ng et al. 1997).

2.1.1 Bone cells

Four major cell types can be found within the bone. Osteoblasts, osteoclasts, and bone lining cells are present on the bone surfaces, while osteocytes are found imbedded in the mineralized interior. Osteoblasts originate from multi-potent mesenchymal stem cells, which also give rise to adipocytes, myocytes, fibroblasts, and cartilage cells, whereas osteoclasts arise from the fusion of mononuclear precursor cells of haematopoietic origin (Ng et al. 1997). Bone lining cells and osteocytes form from the mature osteoblasts (Buckwalter et al. 1996).

Osteoblasts are responsible for the production of the bone extracellular matrix, and contain therefore well-developed endoplasmic reticulum and prominent Golgi apparatus. In addition, they regulate the differentiation and activity of the bone resorbing osteoclasts, as well as the mineralization of the newly formed bone matrix (Martin & Ng 1994). In woven bone, which is laid down rapidly during growth or repair, mineralization is initiated in specific matrix vesicles that are bud from the plasma membrane of osteoblasts, whereas in much stronger lamellar bone, which often replaces woven bone as growth continues, mineralization begins in the hole region between overlapped collagen molecules (Hohling et al. 1978, Landis et al. 1993). The mechanisms of mineralization are not completely understood, but they comprise at least collagen as a template for the initiation and propagation, as well as a variety of small proteins acting as inhibitors and regulators of the mineralization.

Osteoclasts are highly migratory, large, and multi-nucleated cells equipped with a set of proteolytic enzymes. They are responsible for the degradation and turnover of bone, as they are able to dissolve both the bone mineral and organic matrix (Harry 1998). This bone resorption allows stored calcium to be released into the systemic circulation and is an important process in regulating calcium balance. It is also essential in order to repair fatigue damage and reshape the structure to minimize stress in response to loading, as the skeleton is constantly being removed and replaced (Burr 1993). Active osteoclasts have a special ruffled border, a highly infolded area of plasma membrane where the bone resorption takes place, as well as a clear zone, which is a microfilament-rich, organelle-free area of plasma membrane that surrounds the ruffled border and serves as the anchor for the osteoclast to the bone surface undergoing resorption (Horne 1995). After resorption of bone, osteoclasts remove the degradation products via endocytosis at the ruffled border, transfer them to the basolateral membrane, and finally release them into the extracellular space.

Osteocytes are the most abundant cell types in the bone, even though their function and significance is just being unravelled. They form an interconnecting network through the bone matrix and communicate with adjacent cells by means of gap junctions (Doty 1981). This enables a large bone unit to act in concert, as individual cells are able to sense and react very fast and synchronously. Therefore, osteocytes suits well for sensing and transducing mechanical stress applied onto the bone tissue (Lanyon 1993). Furthermore,

these cells have the capacity to synthesize as well as to resorb the bone matrix to a limited extent. Even though most of the osteocytes have lost many of their cytoplasmic organelles, those being able to produce matrix have the cellular organelles characteristic of osteoblasts, while those capable for resorption contain lysosomal vacuoles and other features typical of phagocytic cells (Baud 1968).

Bone lining cells are flat and elongated cells with few cytoplasmic organelles. They are considered as inactive cells, though they might be able to differentiate back into osteoblasts if needed. As they line the bone tissue, they function as a barrier for certain ions (Rubinacci et al. 1998).

2.1.2 Bone organic matrix

The major constituent of the organic matrix of bone is type I collagen, comprising about 90 % of it. The characteristic feature of type I collagen, along with other fibrillar collagens, is a long continuous triple helix, composed of three polypeptide chains with Gly-X-Y repeats, which self-assembles into highly organized fibrils (Fig. 1). These fibrils have a very high tensile strength and play a key role in providing a structural framework, but also elasticity and flexibility for the skeleton. Each molecule of type I collagen is typically composed of two α1 chains and one α2 chain coiled around each other in a triple helix. The synthesis of collagen molecules is a multi-step process that includes transcription, intracellular modifications, extracellular processing, and fibrillogenesis (Fig. 2). Some of the posttranslational modifications, such as the nature of glycosylation and cross-linking are specific for the bone tissue, and they are crucial for to the biomechanical properties of bone. In addition to type I collagen, bone contains small amounts of type V and III collagen. Type V collagen interacts with type I fibrils and may have some regulatory role on fibril diameter and orientation. Type III collagen in bone is generally limited to anatomically distinct regions, such as sites for tendon insertion (Keene et al. 1991).

In addition to collagens, bone matrix contains a wide variety of proteoglycans and non-collagenous proteins. While most of these proteins are synthesized by the osteoblasts, some are plasma proteins prefentially absorbed by the bone matrix, and produced for example in the liver (Yang et al. 1992). Proteoglycans, such as decorin and biglycan, with protein cores composed of the leucine-rich repeat sequences are found in the mineralized matrix, whereas hyaluronan-binding forms, such as versican, are present during the early stages of osteogenesis. Proteoglycans participate in the organization of the bone matrix and regulation of the activity of growth factors (Yamaguchi et al. 1990). Glycoproteins, such as osteonectin, fibronectin, osteopontin, and bone sialoprotein, are produced at different stages of osteoblast maturation. They exhibit a broad array of functions ranging from the control of cell proliferation, cell-matrix interactions, and mediation of hydroxyapatite deposition (Arambawatta et al. 2005).

2.1.3 Bone mineral

Skeleton provides the major store for many minerals in the body, from which they are released via bone resorption when needed. The bone mineral is primarily composed of calcium and phosphate crystals in the form of hydroxyapatite, while smaller amounts of impurities, such as magnesium, carbonate, and sodium can be found absorbed on the crystals (Pellegrino et al. 1977). The mineral crystals provide mechanical rigidity and load-bearing strength for the bones. However, the size and distribution of mineral crystals in the bone matrix influences bone mechanical properties (Rho et al. 1998). For example, if there are too few crystals or crystals are too small, the mechanical strength will be compromised (Jaovisidha et al. 1999). Similarly, if there are too many crystals, bones may become brittle and thus unable to bear load (Bollerslev 1987). Therefore, there is an optimal crystal size distribution as well as an optimal amount of mineral.

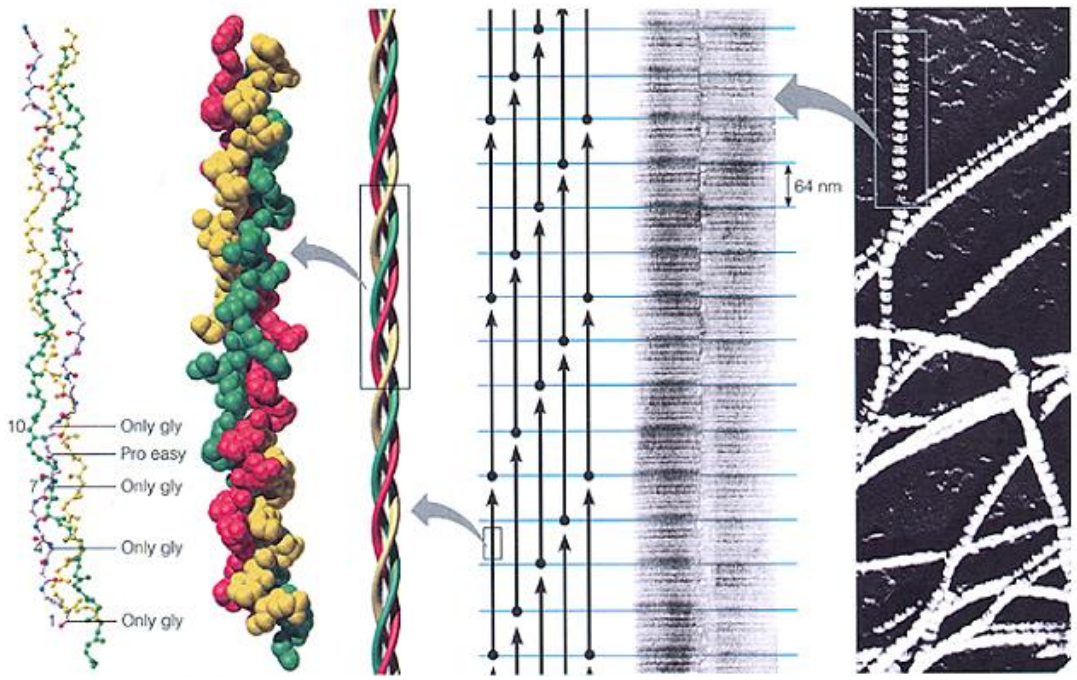

FIGURE 1 Collagen structure. Reproduced with permission from Pearson Education: Author, A. 1990. In: A. B. Mathews & C. van Holde (eds.), Biochemistry: 183. Redwoodcity, the Benjamin/Cummings Publishing Company.

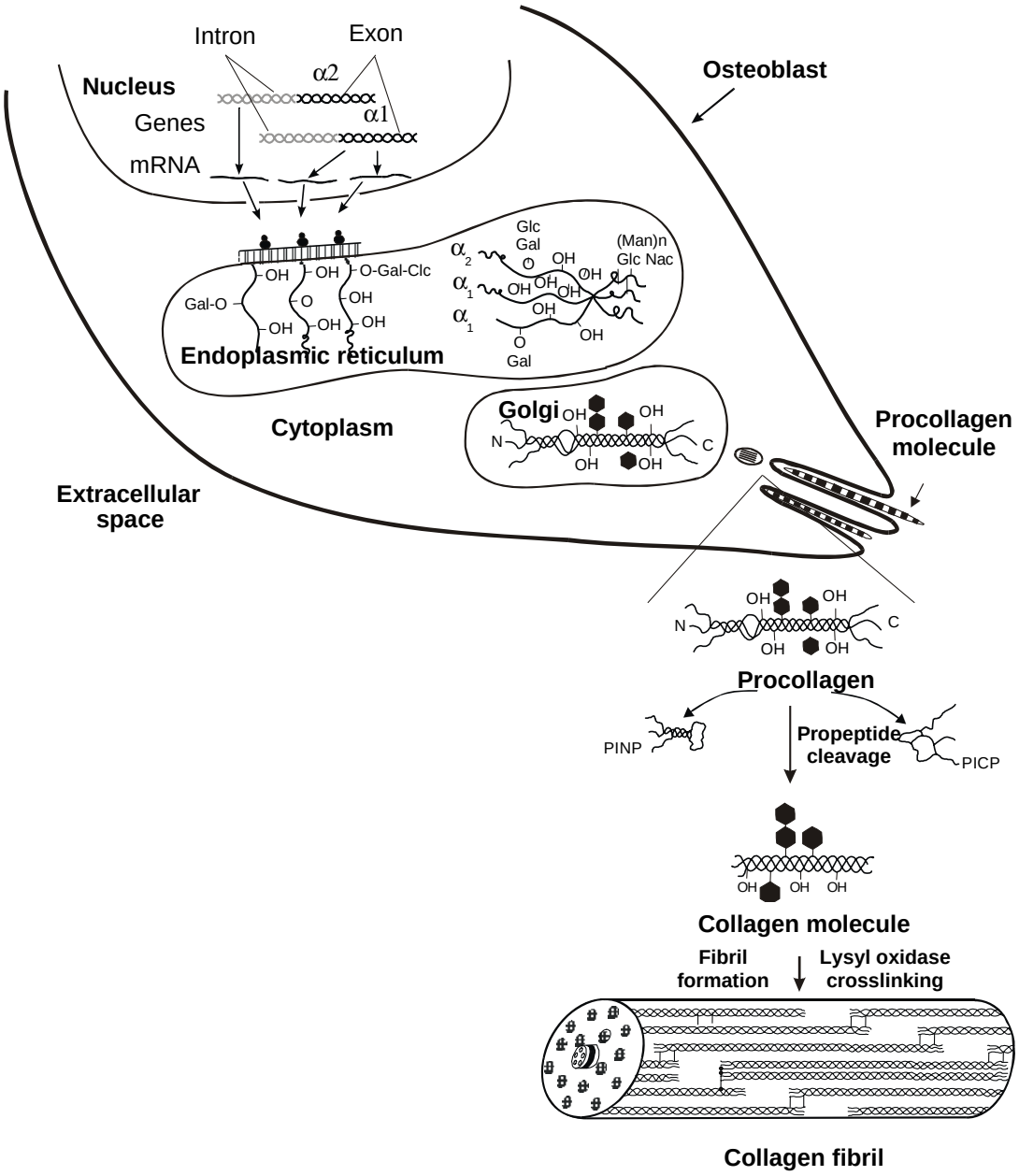

FIGURE 2 Collagen synthesis. Modified with permission from Human Kinetics: Kovanen, V. 2005. Extracellular matrix and exercise. In: F. C. Mooren & K. Völker (eds.), Molecular and Cellular Exercise Physiology: 71-93. Champaign: Human Kinetics.

2.2 Juvenile bone growth

Childhood and adolescence are characterized by longitudinal growth as well as by changes in skeletal size and shape. Growth during childhood is a relatively stable process. Until about age of 4 years girls grow slightly faster than boys,

after which both genders average a rate of 5 to 6 cm and 2.5 kg per year until the onset of puberty (Tanner 1989). From infancy through late adolescence the activity of bone formation predominates over the bone resorption, resulting in a steady accumulation of skeletal mass, which increases from approximately 70-95 g at birth to 2400-3300 g in young women and men, respectively (Trotter et al. 1974). These processes are influenced by a complex sequence of hormonal changes that interact with nutritional and environmental factors, to modify individual's full genetic potential. Completion of normal skeletal growth requires adequate production of thyroid hormone, growth hormone, insulin-like growth factors, and sex steroids. Before puberty, bone growth is largely dependent on growth hormone, but sex steroids are essential for the completion of epiphyseal maturation and mineral accrual in the teenage years.

2.2.1 Puberty and peak bone mass

Puberty is characterized by the greatest sexual differentiation since fetal life and the most rapid rate of longitudinal growth since infancy. The onset of puberty corresponds to an age of approximately 11 years in girls and 13 years in boys (Tanner et al. 1975). The widely used method for the evaluation of pubertal development, described by Tanner, classifies children into different stages and is based on visual inspection of external primary and secondary sexual characteristics, such as the size of breasts, genitalia, and development of pubic hair (Marshall & Tanner 1969). On average, girls enter as well as complete each stage of puberty earlier than boys; however, there is significant individual variation in the timing and tempo of puberty, even among children of the same gender. Along with its impact on the growth as a whole, puberty has a fundamental role in the acquisition of bone mass. Indeed, between the onset of puberty and young adulthood skeletal mass approximately doubles (Katzman et al. 1991). However, this mineral accrual occurs at different rates through the skeleton. For example, gains in the appendicular skeleton predominate before puberty, after which the spinal growth increases under the influence of sex steroids (Bass et al. 1999). Furthermore, limb growth is completed before growth of the axial skeleton. However, maximal rates of bone mineral accrual lag behind the peak velocity of body height by 6-12 months, resulting in a relatively undermineralized bone and thereby an increased fracture risk during the peripubertal years (Suuriniemi et al. 2005) (Fig. 3). Fortunately, this "fragile period" is normally only transient, as the accrual of bone mass continues after the completion of longitudinal growth. The exact age at which values for bone mass reach their peak at various skeletal sites has not yet been determined with certainty, but estimations vary from 16-18 years (spine and femoral neck) to as late as 35 years (radius, skull, and whole body) (Bonjour et al. 1991, Recker et al. 1992, Matkovic et al. 1994). A widely accepted belief is that the higher the peak bone mass accrued in young adulthood, the more an individual can afford to lose in older age. Therefore, optimal acquisition of bone mass is an important determinant of the future risk of osteoporosis and fractures.

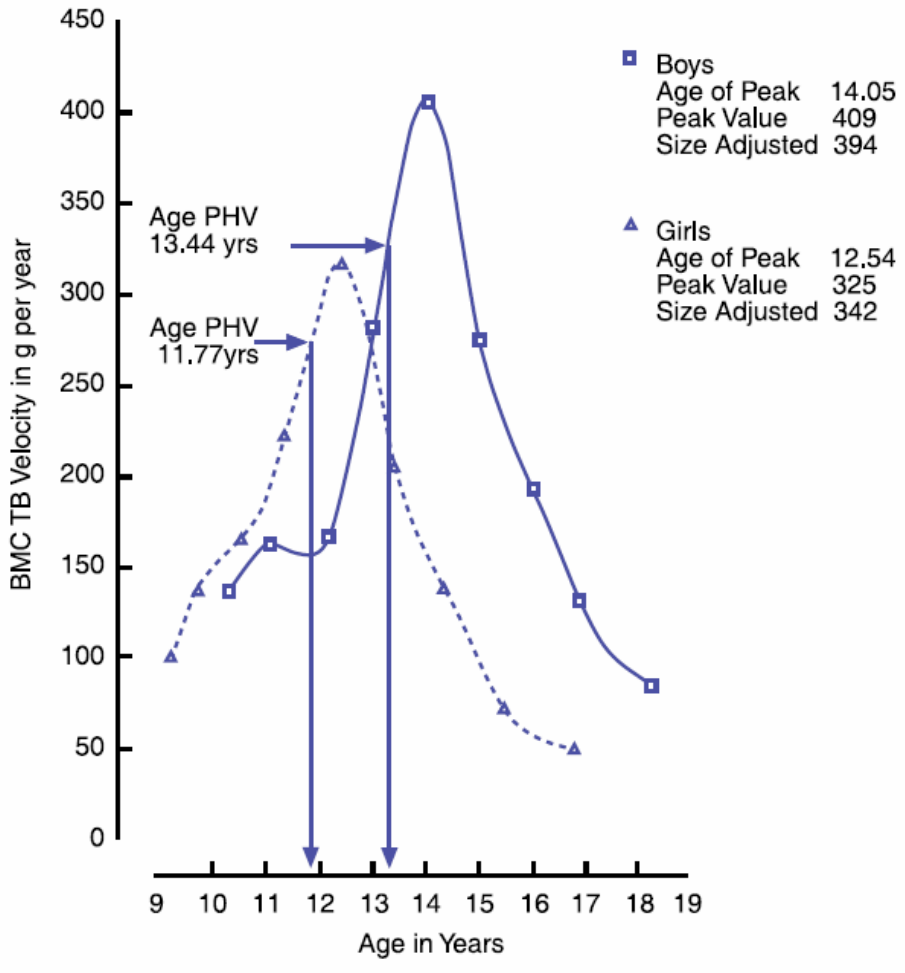

FIGURE 3 Bone mineral content (BMC) of total body and peak height velocity (PHV). Reproduced with permission from Elsevier: Javaid, M. K. & Cooper, C. 2002. Prenatal and childhood influences on osteoporosis. Best Pract. Res. Clin. Endocrinol. Metab. 16: 349-367.

2.2.2 Idiopathic juvenile osteoporosis

Idiopathic juvenile osteoporosis (IJO) is a condition applied to a primary osteoporosis of unclear aetiology present in otherwise healthy children (Lorenc 2002). Recent findings suggest that genetic factors may have a role in the disease onset at least in a subset of patients (Hartikka et al. 2005). It typically occurs before the onset of puberty, but may also be seen in younger children, especially when they are growing rapidly. The disease shows no sex predilection. Symptoms begin with an insidious onset of pain in the lower back, hips, and feet, as well as with difficulty in walking. Knee and ankle pain and fractures of the lower extremities may be present, as well as diffuse muscle weakness. The pathophysiology of IJO remains unclear, but available evidence points toward disturbed bone remodelling, which predominantly affects surfaces that are in contact with the marrow cavity and results in a very low bone formation rate and decreased trabecular bone volume (Rauch et al. 2000a).

The diagnosis is based on the exclusion of other causes of osteoporosis, including both congenital conditions, such as osteogenesis imperfecta (OI), homocysteinuria, and Turner's syndrome, and acquired disorders, such as celiac disease, endocrine disturbances, immobilization, malignancy, and the use of steroids. With the exception of a few patients who develop progressive lower extremity, spine, and chest wall deformities and require confinement to wheelchairs or bed, children with IJO usually experience complete recovery within 3-4 years, if received appropriate medical therapy and supportive care. Bone pain typically disappears within 2 years of the onset of the disease and is followed by a normalization of muscular strength (Olszaniecka et al. 1993). During the acute phase of IJO growth may be impaired, however, normal and even catch up growth may resume later on (Lorenc 2002). Even so, IJO and related symptoms should be taken seriously and investigated more to clarify their aetiology, as they debilitate the quality of life of considerable number of normal children.

2.2.3 Heritability of bone accumulation

Heritability of bone phenotype has been originally investigated using the twin model by studying the relative degree of the difference between monozygotic (identical) and same-sex dizygotic (non-identical) twins (Slemenda et al. 1991). These analyses make the assumption that twin pairs of the same age and sex share their environments and other lifestyle factors to a similar extent whether they are mono- or dizygotic. Furthermore, monozygotic twins can be used to investigate the impact of various environmental and lifestyle factors on bone characteristics, since the twin pairs are of the same age, sex, and genetic make-up. Studies over the last 30 years in healthy twins have consistently demonstrated a large genetic contribution to bone mass (Smith et al. 1973, Moller et al. 1978, Pocock et al. 1987, Slemenda et al. 1991). A number of family studies using either healthy parent-children pairs (Tylavsky et al. 1989), healthy sister pairs (Koller et al. 2000), or osteoporotic parent-children pairs (Seeman et al. 1989), have further corroborated the major role of genetics in determining bone mass. Moreover, heritability of bone mass can be detected during childhood even though the skeleton is undergoing major changes in both skeletal size and mass (Ferrari et al. 1998).

Two approaches are usually used to determine the genetic contribution to complex phenotypes: linkage and association studies. Linkage studies search for any genomic region contributing a relatively large variation in quantitative traits by using dense genetic markers covering the whole human genome (Dawn Teare & Barrett 2005). However, these studies are expensive, time consuming, and require sophisticated technology. Moreover, linkage analysis must be carried out in pedigrees (families and sibling pairs), while association can be performed at the population level. The major strength of linkage analysis is, however, that it allows screening of the entire genome with a few hundred DNA markers, which is currently not feasible with allelic association. Instead of whole genome scanning, association studies investigate specific genomic

regions at or near candidate genes, and are facilitated by our knowledge of the factors that regulate bone metabolism and the proteins that make up the bone matrix. Given the wide range of factors involved in bone metabolism, there is a seemingly unlimited supply of candidate genes for bone growth, although relatively few have been studied so far (Table 1). A problem with the candidate gene approach is that it may still miss some essential genes by looking for candidates among the pathways that are already known. The advantage over linkage analysis is, however, that association studies can detect genetic regions with only small effects on the particular trait.

Polymorphisms in the vitamin D receptor (*VDR*), *ER-α*, and *COL1A1* genes are among the most intensively studied in relation to bone phenotypes (Liu et al. 2003). However, far more studies have been conducted in older adults than in young children despite the likelihood that genetic influences have a greater impact on bone mineral acquisition than on loss (Eisman 1999). Three different polymorphisms in the *VDR* gene have been investigated in relation to children's bone properties, with partly inconsistent findings. In two independent studies an association was found between the *BsmI* polymorphism in the *VDR* gene and spine BMD in prepubertal girls (Sainz et al. 1997, Eisman 1999), while in a third study no relation to radius BMD was found in Norwegian boys and girls (Gunnes et al. 1997). An American research team found a relationship between the *ApaI* polymorphism in the *VDR* gene and femoral as well as spine BMD in prepubertal girls (Sainz et al. 1997). Furthermore, in 2002 a Japanese study was published, whereby the *FokI* polymorphism at the start codon site of the *VDR* gene associated with spine BMD in girls (Katsumata et al. 2002). One year after the same was concluded in a study of Swedish boys as well (Strandberg et al. 2003). These findings were, however, quite confusing, as according to an earlier investigation the *FokI* polymorphism does not explain BMD in prepubertal girls (Ferrari et al. 1998). Only one study has been carried out in children to investigate the relationship between the *PvuII* polymorphism in the *ER-α* gene and BMD (Willing et al. 2003). In this study no association was found. The Sp1 polymorphism in the *COL1A1* gene has also attracted paediatric researchers, as its relation to osteoporotic fractures in older adults has been recurrently proven (Mann et al. 2001). Furthermore, this particular polymorphism is thought to express something more like quality than quantity of the bone, and has therefore broaden our view of factors of bone strength and fracture risk. Two studies so far have been conducted to investigate the effect of the *COL1A1* polymorphism to children's bone properties. In the first study the *COL1A1* polymorphism was shown to explain some of the variability in spine BMD in prepubertal girls (Sainz et al. 1999). By contrast, in the other study no relationship was found between the genotype and BMD in a cohort of younger boys and girls (Willing et al. 2003). These discrepant findings might be explained by small sample sizes or by variability in other heritable or environmental factors in these geographically diverse populations.

TABLE 1 Candidate genes for bone metabolism.

Biological classification	Candidate gene	Protein	Chromosome location
Receptors and enzymes	*VDR*	Vitamin D receptor	12q12-14
	ER-α	Estrogen receptor α	6q25
	ER-β	Estrogen receptor β	14q22-24
	AR	Androgen receptor	Xq11-12
	CTR	Calcitonin receptor	7q21
	PTHR1	Parathyroid hormone receptor 1	3p22-21
	GCCR	Glucocorticoid receptor	5q31
	CaSR	Calcium sensing receptor	3q13-21
	LRP5	LDL receptor-related protein 5	11q13
	RANK	Receptor activator of NF-κB	18q22
	CYP17	Cytochrome P-450c17alfa	10q24
	CYP19	Aromatase	15q21
	CYP1A1	Cytochrome P-450	15q22-24
	CYP1B1	Cytochrome P-450	2p22-21
	COMT	Catechol-*O*-methyltransferase	22q11
Cytokines and growth factors	*TNF-α*	Tumor necrosis factor α	6p21
	IL-6	Interleukin 6	7p21
	IGF-I	Insulin-like growth factors I	12q22-24
	TGF-β1	Transforming growth factor β1	19q13
	OPG	Osteoprotegerin	8q24
	Klotho	Klotho protein	13q12
Bone matrix proteins	*COL1A1*	Type I collagen α1	17q21-22
	COL1A2	Type I collagen α2	7q22
	BGP	Osteocalcin	1q25-31
	MGP	Matrix Gla protein	12p13-12

2.3 Genetic polymorphisms and failures

Single nucleotide polymorphisms (SNPs) are a group of genetic markers that are extensively utilized in association studies. They allow the investigation of the relationships between selected allelic variants and skeletal traits. However, the great number of them insists a special consideration when selecting the variants for further analysis. Several SNPs reported in the public SNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/) have been identified as candidates by aligning clone overlaps from available finished and unfinished public human genomic sequences and by detecting high quality base discrepancies. Usually, these have not been validated and should therefore be considered as suspicious. Two hundred and twenty six SNPs altogether have been found in the *COL1A1* gene and entered in to the public database. Most of them exist in the intronic regions, few in the untranslated, and only eighteen in the exonic regions, half of them synonymous and the other half non-synonymous. When the current work started, one common SNP, namely Sp1 binding site polymorphism in the regulatory region of the *COL1A1* gene, had

been found to associate with severe osteoporosis and vertebral fractures in elderly women (Grant et al. 1996). Concerning the *COL1A2* gene, one hundred and fifty two SNPs have been entered in to the public database. One hundred and sixteen of them exist in the intronic regions, seven in the untranslated, and twenty nine in the exonic regions, thirteen synonymous and sixteen non-synonymous. Only one common SNP in the exon 25 of the *COL1A2* gene had been observed in relation to clinical manifestation mild enough characteristic of osteoporosis (Constantinou et al. 1990). Event though this nucleotide variation does not affect the encoded proline residue at codon 392, it lies relatively close to three non-synonymous SNPs: valine to leusine SNP at codon 344, valine to alanine SNP at codon 483, and alanine to proline SNP at codon 549, that may be in linkage disequilibrium with the synonymous SNP. One thousand, five hundred and seventeen SNPs have been found in the *ER-α* gene and entered in to the public database. Most of them exist in the intronic regions, few in the untranslated, and only twelve in the exonic regions, nine of them synonymous and three non-synonymous. A B-myb binding site polymorphism in the regulatory region of the *ER-α* gene has been the most extensively studied *ER-α* SNP in relation to osteoporosis, but has also been found to affect the response to hormone replacement therapy (Herrington et al. 2002a, Herrington et al. 2002b). Another SNP in the *ER-α* gene, also in the first intron, as well as a TA dinucleotide repeat polymorphism 5´ upstream of the first exon has been investigated for a lesser amount and found in linkage disequilibrium with the SNP mentioned above (Becherini et al. 2000). Regarding the *COMT* gene, two hundred and seventy four SNPs have been entered in to the public database. Most of them exist in the untranslated and intronic regions, while only fifteen reside in the exonic regions, eight of them synonymous and seven non-synonymous. Even though originally recognized as a significant genetic contributor for diverse neurophysiologic and psychological features, a valine to methionine SNP in the second exon of the *COMT* gene has been recently found to affect the estrogen metabolism as well as the response to hormone replacement therapy (Worda et al. 2003). In contrast to these genetic variations described above with relatively slight consequences at most on the human physiology and that may partly explain the normal variation of bone growth in healthy children some patients can have so serious genetic defects that their consequences on bone metabolism are far more pronounced.

2.3.1 Type I collagen and brittle bones

Osteogenesis imperfecta, also known as brittle bone disease, is a genetic disorder of connective tissue characterized by bones that fracture easily, often from little or no apparent trauma (Fig. 4). It is the most common bone disease caused by a single gene defect with roughly half million patients in the world. Inheritance, in nearly all cases, follows an autosomal dominant pattern, although sporadic cases are common as well. When there is no evidence of family history, OI is mostly caused by new dominant mutations that took place

in the egg or sperm near the time of conception. Their risk of recurrence in subsequent pregnancies is approximately equal to the risk of OI in the general population (Thompson et al. 1987). However, some few cases of OI result from mosaicism for the mutation in one parent (Lund et al. 1999). A mosaic parent has the mutation in some of the cells of her/his body, including some of the egg or sperm cells as well. The mosaic parent usually appears to be unaffected or experience only mild symptoms. The prevalence of OI is approximately 1 in 20 000 (Kuurila et al. 2000). Because some infants die at birth and would not be included in these surveys, the birth incidence is slightly higher, perhaps 1 in 15 000-18 000 births. The clinical hallmark of OI is skeletal deformity and low bone mass, causing recurrent fractures. Moreover, patients with OI often suffer from muscle weakness, joint laxity, thin skin, blue sclera, and hearing loss, as type I collagen is present in a wide variety of connective tissues (Rauch & Glorieux 2004). Also, respiratory and cardiac complications, thermal instability, blood vessel fragility, as well as neurological manifestations, such as hydrocephalus, may be seen in some patients (McAllion & Paterson 1996). Altogether, severity of OI is extremely variable and ranges from stillbirth to perhaps lifelong absence of symptoms. Moreover, the manifestations of OI can vary markedly within and between families who have identical mutations in the collagen genes, presumably due to the influence of other genes on bone fragility (Sippola et al. 1984).

The most widely used clinical classification for OI devised in the late 1970s by Sillence is based on disease severity (Sillence et al. 1979). Three degrees of deforming OI, types II, III, and IV, are associated with decreasing severity of growth retardation and limb deformity, and result from mutations affecting the structure of type I collagen molecule. In contrast, most cases of non-deforming OI, type I, are the result of mutations affecting the production of an otherwise normal type I collagen molecule. Furthermore, three additional groups of patients with a clinical diagnosis of OI but with clearly distinct features have been described (Glorieux et al. 2000, Glorieux et al. 2002, Ward et al. 2002). Interestingly, no evidence of type I collagen abnormality has been found in these patients and the underlying genetic defects are thus waiting to be discovered. Even though the range of clinical severity in OI is a continuum, categorisation of patients into separate types can be useful to evaluate the prognosis and help assess the effects of therapeutic interventions. However, for optimal care, each patient should be treated individually rather than by the "label" of her/his Sillence type or specific mutation.

Mutations causing deforming OI usually act in a dominant negative manner, meaning that the presence of the abnormal gene product causes the disease (Sakai et al. 1996). The three-dimensional structure of the collagen fibril can be altered by a substitution for glycine in the collagen (Gly-X-Y) triplet, in-frame deletion, in-frame insertion, or by exon skipping. The helical location of the mutation is essential regarding the clinical outcome. Since the helix assembles from the C-terminal propeptide, mutations in the C-terminal region result in greater instability of the collagen molecule and more severe disease

than those located at the mid-helical region (Tsipouras 1993). However, there are exceptions for this rule suggesting that subdomains within the helix are critical for function beyond just contributing to an intact helical structure (Marini et al. 1989, Pack et al. 1989). Mutations located at the N-terminal domain of either chain can be extremely mild and fall into the category of type I OI (Starman et al. 1989). The most common defect is a glycine substitution in the helical domain of the col1a1 chain. Glycine, as the smallest amino acid, is a critical component that must fit in a sterically restricted space where the three chains of the triple helix come together. Therefore, substitution with an amino acid with a bulkier side chain will adversely change the conformation of the collagen helix. Mutations in the consensus donor or acceptor site can lead to exon skipping producing a shortened helix, which has the same effect on helical stability as a glycine substitution, since exons encoding the helical domain maintain the reading frame of the gene (Byers et al. 1983). Much less common are mutations that delete a portion of the gene or insert a segment of intron that remains in-frame with the entire transcript (Mundlos et al. 1996, Wang et al. 1996). In the latter case, a non-helical segment is inserted within the helical domain disrupting the structure of the collagen helix. In addition to the dominant negative mutations, a null mutation of the *COL1A2* gene can result in a deforming OI (Nicholls et al. 2001). Formation of the normal heterotrimeric collagen molecule requires a correct ratio of available α1 and α2 chains at the time of molecule assembly. However, if this ratio is disturbed, either because of underproduction of the α2 chain or overproduction of the α1 chain, abnormal homotrimeric molecules with impaired mechanical properties will be formed (McBride et al. 1997). There is a spectrum of disease severity from type III OI, when both *COL1A2* alleles are affected, to an osteoporosis due to the Sp1 polymorphic alteration of the *COL1A1* gene, which can be explained by the recessive manner of the mutation in which gene dosage contributes to the severity of symptoms.

The most common mutation causing non-deforming, type I OI reduces the output of otherwise normal type I collagen. This will happen when the transcription of the *COL1A1* allele is reduced and the unincorporated α2 chains are degraded intracellularly (Barsh et al. 1982). The severity of osteopenia relates to the degree of underproduction of α1 chain. Complete inactivation of one allele results in haploid insufficiency and a clinical phenotype of type I OI, while homozygosity for this state leads to embryonic lethality (Lohler et al. 1984, Bonadio et al. 1990). Furthermore, more subtle underproduction of *COL1A1* allele due to polymorphic variations may contribute to the development of an even milder phenotype characteristic for osteoporosis (Garcia-Giralt et al. 2005). The most frequent causes for a null *COL1A1* allele are mutations introducing a premature stop codon, which lead to a rapid destruction of the truncated transcript by a process called nonsense mediated decay (Redford-Badwal et al. 1996). Less frequently a null allele may result from retention of an intron within the mature transcript. Failure in the exon skipping

can result when a mutation of a splice donor site is located in a small intron (Stover et al. 1993).

The presence of intracellular mutant procollagen molecules and response to the abnormal extracellular matrix may have a great impact on the function of osteoblast. For instance, it has been shown that the secretion of such procollagen is impaired (Fitzgerald et al. 1999, Lamande & Bateman 1999), which may lead to posttranslational overmodification of the lysine residues in the helical domain. This, in turn, may further affect the quality of the collagen fibrils. Electron microscopy imaging has, indeed, shown that the collagen fibrils of OI are disorganized and have wide variation in fibre diameter (Eyden & Tzaphlidou 2001). Abnormalities in the organic matrix may well affect the interactions of collagen molecules with other proteins and components of the bone matrix, as well as the mineralization of the osteoid. Even though bone from patients with OI often shows a higher average mineralization density compared to age-matched controls (Boyde et al. 1999), the disorder is characterised by an insufficient amount of bone. Both cortical thickness and the amount of trabecular bone are low (Rauch et al. 2000b). Also, the quality of the mineral may be impaired, as evident in the murine model of OI (OIM) that has smaller and less well aligned mineral crystals than normal mice (Fratzl et al. 1996). Altogether, disturbances in the organic and mineral components of bone greatly alter its biomechanical behaviour. For instance, collagen from OIM mice has reduced tensile strength (Misof et al. 1997). Mineralized OI bone may be harder at the material level (Grabner et al. 2001) but it breaks more easily than normal bone when deformed, and fatigue damage accumulates much faster on repetitive loading (Jepsen et al. 1997). Such abnormalities altogether likely account for the brittleness of OI bone.

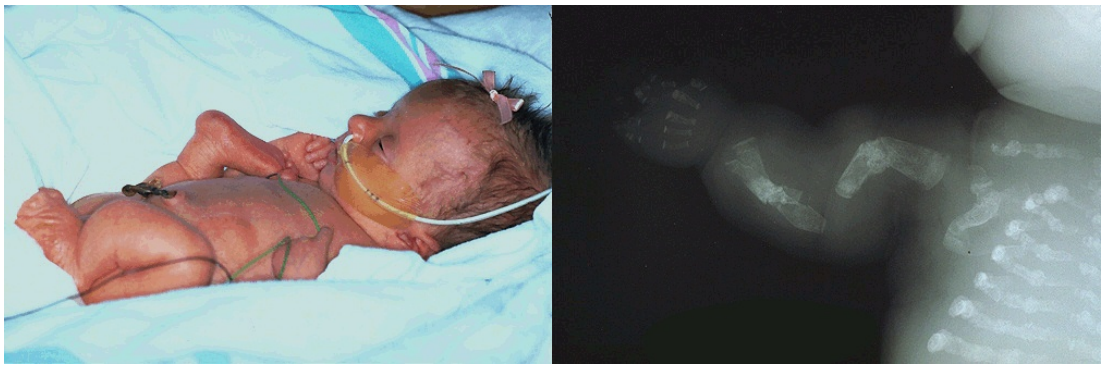

FIGURE 4 Baby with osteogenesis imperfecta. Reproduced with permission from Elsevier: http://medgen.genetics.utah.edu/.

2.3.2 Estrogen receptor α and resistance to estrogen

In 1994, Smith with his colleagues described a 28-year old man with a disruptive homozygous mutation in exon 2 of the *ER-α* gene (Smith et al. 1994). A cytosine to thymidine transition at codon 157 resulted in a stop codon and a severely truncated ER-α unable to bind estrogen. His own serum concentration

of estrogen was markedly above the normal and he had no detectable response to administration of large doses of exogenous estrogen. He was extremely tall (204 cm) without acromegalic features and still growing with open epiphyses and genu valgum. His body proportions were eunuchoid and trabecular bone density extremely low, corresponding to 2 standard deviations below an average for a 15-year old boy.

In addition to the rude example given above, the well-known connection between the estrogen and skeleton corroborates the importance of ER-α for bone metabolism. For a long time past it has been established that estrogen is the major sex steroid affecting the growth, remodelling, and homeostasis of the skeleton. It regulates the processes of osteoblast-mediated bone formation and osteoclast-mediated bone resorption at multiple levels, such as progenitor cell recruitment, proliferation, differentiation, and programmed cell death (Oursler 1998). After diffusion into the cell estrogen is reversibly bound with high affinity to estrogen receptor (ER), a zinc finger containing nuclear hormone receptor that functions as a transcriptional regulator (Beato et al. 1995) (Fig. 5). This triggers a receptor activation that induces a conformational change, dissociation of accessory proteins, receptor dimerization, and posttranslational modifications, such as phosphorylation (Brosens et al. 2004). The activated receptor binds either directly to regulatory DNA elements [estrogen response elements (EREs)] in the promoters of target genes or indirectly with other DNA binding transcription factors [e.g. activator protein 1 (AP-1) factors] via protein-protein interactions, which then bind to the promoter. Once bound to the DNA the activated receptor complex recruits coregulator proteins that interact with components of the transcription apparatus, carry out histone acetylation, and interact with other multi-protein complexes that facilitate chromatin remodelling (Brosens et al. 2004). To induce transcription of a gene, the receptor-coactivator complex must facilitate loosening of the histone-DNA interactions that repress the transcription in chromatin, and stimulate or stabilize formation of a transcription complex at the promoter (Li et al. 2004). In addition, a membrane-associated ER mediates non-genomic actions of estrogen, which can lead both to altered functions of proteins in the cytoplasm and to regulation of gene expression (Moss et al. 1997). This enables a broader range of genes to be regulated compared with the classical mechanism of ER action alone. Signal transduction pathways may further connect the non-genomic actions of estrogen to genomic responses. Given that the functions of many transcription factors are regulated through phosphorylation, they may thereby be targets for non-genomic actions of estrogen. For instance, estrogen may modulate the functions of ERs themselves, as they are targets of phosphorylation by the mitogen-activated protein kinase (MAPK) signalling pathway (Kato et al. 1995), and in this way augment the classical mechanism of ER action. This type of non-genomic-to-genomic signalling is a distinct mechanism by which ER can regulate transcription at alternative response elements (Kushner et al. 2000).

The development of mice exhibiting targeted gene deletion (knock-out) in the *ER-α* has further advanced our understanding of the actions and significance of estrogen in the skeletal physiology. In such knock-out female

mice responsiveness to estrogen is reduced, as higher estrogen concentrations are required to prevent bone loss compared to ovariectomized wild-type mice (Ederveen & Kloosterboer 1999). It has also been shown that deletion of the *ER-α* results in reduced cortical bone density and cortical bone formation in both male and female mice (Korach et al. 1997), suggesting that estrogen stimulates cortical bone formation via ER-α. The effect of *ER-α* disruption on trabecular bone is less certain, however, some evidences suggest a lack of marked trabecular bone loss (Korach et al. 1997). Moreover, linear growth of long bones is generally reduced, especially in females, even though the knock-out mice experience a delayed closure of the epiphyseal growth plate (Korach et al. 1997, Smith et al. 1999). An issue that has become apparent from the numerous gene disruption studies is a collection of caveats to be considered when evaluating phenotypical data from a knock-out model. One of the most relevant concerns is that the mutant phenotype of a knock-out animal may result from multiple influences that occur as a consequence of the targeted mutation, and therefore the mechanisms responsible for the phenotype may not be restricted solely to that particular mutation. For instance, disturbing ER-α signalling in all tissues by gene ablation may destabilize the feedback loops regulating the synthesis of other sex steroids in addition to estrogen, thereby leading to changes in circulating levels of these hormones, some of which also possess major osteotropic activity (Korach et al. 2003). As a further complexity, the other isoform, estrogen receptor β may be able to either compensate for the loss of ER-α or suppress the activity of ER-α (Mosselman et al. 1996).

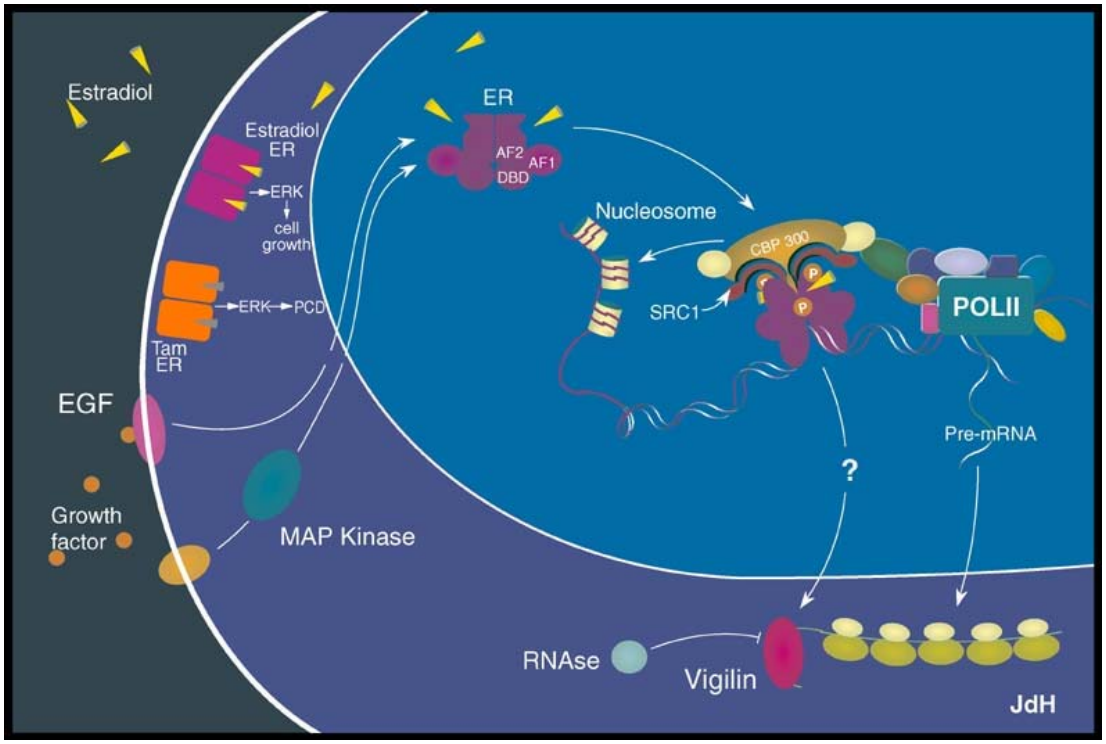

FIGURE 5 Classical mechanism of estrogen receptor signalling. Reproduced with permission from David Shapiro: http://www.life.uiuc.edu/shapiro/.

2.3.3 Estrogen receptor α and impaired response to mechanical strain

Bones are able to withstand functional loads without extensive damage, because they have evolved a homeostatic capacity to adapt their mass and architecture in relation to changes in their habitual loading environment. As a consequence, higher than optimal functional strains, such as those induced by high impact exercise, stimulate an increase in bone mass that re-establishes optimal strains. Conversely, when functional loading is reduced to the extent that optimal strains are not achieved, e.g. due to long-term bed rest, cast immobilization, or microgravity conditions, bone loss occurs to restore them. Mechanical loading of bone induces various stimuli while deforming the bone tissue and engendering movement of extracellular fluid through the bone's lacuno-canalicular system (Suva et al. 2005). Such fluid flow may stimulate bone cells via streaming potentials, wall shear stress, as well as via chemo-transport-related effects (Donahue et al. 2003, Knothe Tate et al. 2004). Osteocytes are likely to be the primary mechanosensitive cells in the bone, as their extensive physical connections with surrounding cells permit the exchange of mechanical and metabolic signals (Han et al. 2004). Mechanical strains are known to directly or indirectly increase the release of several signallers, such as prostanoids (Rawlinson et al. 1991), nitric oxide (Pitsillides et al. 1995), and insulin-like growth factors (Cheng et al. 1999), that stimulate cell proliferation and matrix formation, ultimately leading to changes in bones´ mass and morphology. While tremendous individual responses to mechanical loading have been presently acknowledged, a comprehensive picture of the signalling pathway is still lacking.

The similarities between the effect of estrogen withdrawal and that associated with reduction in mechanical load have led several investigators to suggest that the early responses of bone cells to mechanical strain and estrogen share a common mechanistic pathway that involves ER-α (Wronski et al. 1993, Takata & Yasui 2001). In fact, in 2000 Damien with his colleagues made a remarkable finding that the proliferation of osteoblasts caused by strain can be blocked by estrogen antagonists tamoxifen and ICI 182,780 (Damien et al. 2000). Several discoveries supporting this hypothesis have been made afterwards. For instance, stimulation by mechanical strain was shown to result in upregulation of the extracellular-regulated kinase (ERK) pathway, ERK-mediated phosphorylation of the ER-α (Jessop et al. 2001), and upregulation of ERE activity (Zaman et al. 2000). Perhaps one of the strongest evidence came from the observation that mice lacking functional ER-α produce three times less new cortical bone in response to the same mechanical stimulus as their wild-type littermates (Lee et al. 2003) (Fig. 6). Moreover, osteoblasts derived from these *ER-α* knock-out mice failed to proliferate in response to mechanical stimulation; however, the response was restored after transfecting the mutant cells with fully functional human *ER-α*. An interesting signal transduction pathway for mechanical strain in osteoblasts has been recently proposed by Lanyon based on available data (Fig. 7). According to the model, the initial mechanosensor is

the transmembrane integrin, residing in conjunction with the insulin-like growth factor-1 (IGF-1) receptor (IGF1-R) within focal adhesions. Mechanical stimuli may destabilize the binding interaction between the integrin and its extracellular matrix ligand, such as collagen or osteopontin, causing a conformational change in the integrin. This integrin activation initiates a cascade of events, including activation of the MAPK/ERK pathway and subsequent hormone-independent phosphorylation of the ER-α, which results in both classical activation of gene transcription within the nucleus, necessary for the cell proliferation and matrix formation, as well as ER-α participation in non-genomic signalling events, such as enabling the interaction between IGF and IGF1-R. Since ER-α is involved at more than one stage in this signalling pathway after mechanical strain, it is not surprising that reduction in ER-α function, either quantitative or qualitative, may reduce the effectiveness of the response to mechanical loading. This could also contribute to the reduced bone density observed in the man with a disruptive mutation in his *ER-α* gene.

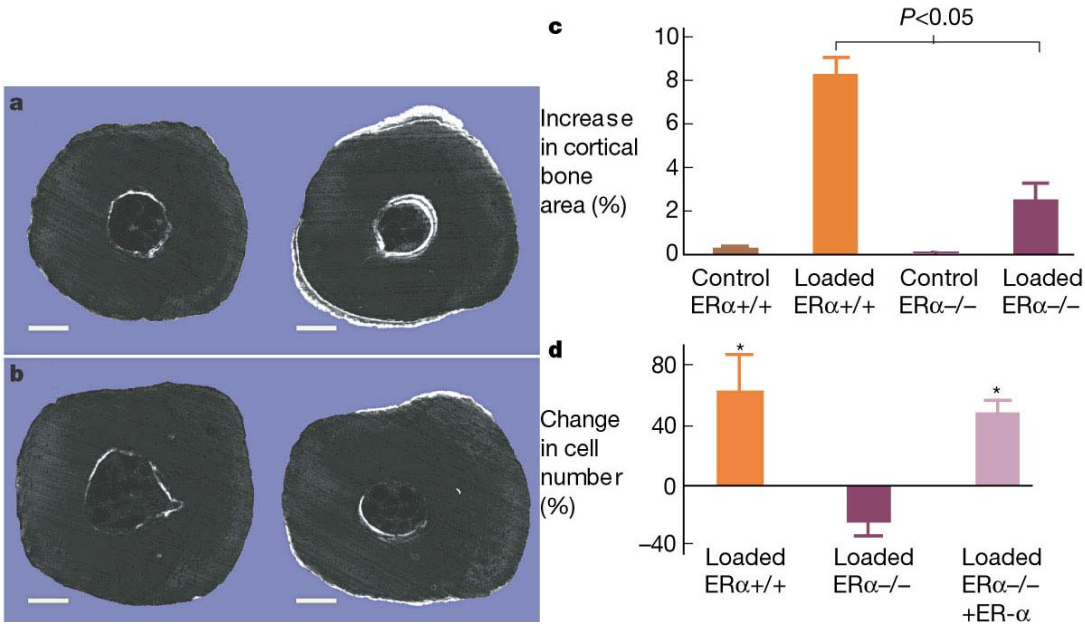

FIGURE 6 a, b, Transverse sections from control (left) and loaded (right) ulnae from ER-α +/+ (a) and ER-α -/- (b) mice. New bone formation is labelled with fluorochrome calcein (shown in white). Scale bars, 100 μm. c, Adaptive increase in cortical bone area of ulnae in response to loading from ER-α +/+ and ER-α -/- mice. d, Mechanical strain induced proliferation of osteoblasts derived from ER-α +/+ as well as ER-α -/- mice before and after transfection with competent ER-α. Reproduced with permission from Macmillan Publishers: Lee, K., Jessop, H., Suswillo, R., Zaman, G. & Lanyon, L. 2003. Endocrinology: bone adaptation requires estrogen receptor- alpha . Nature 424: 389.

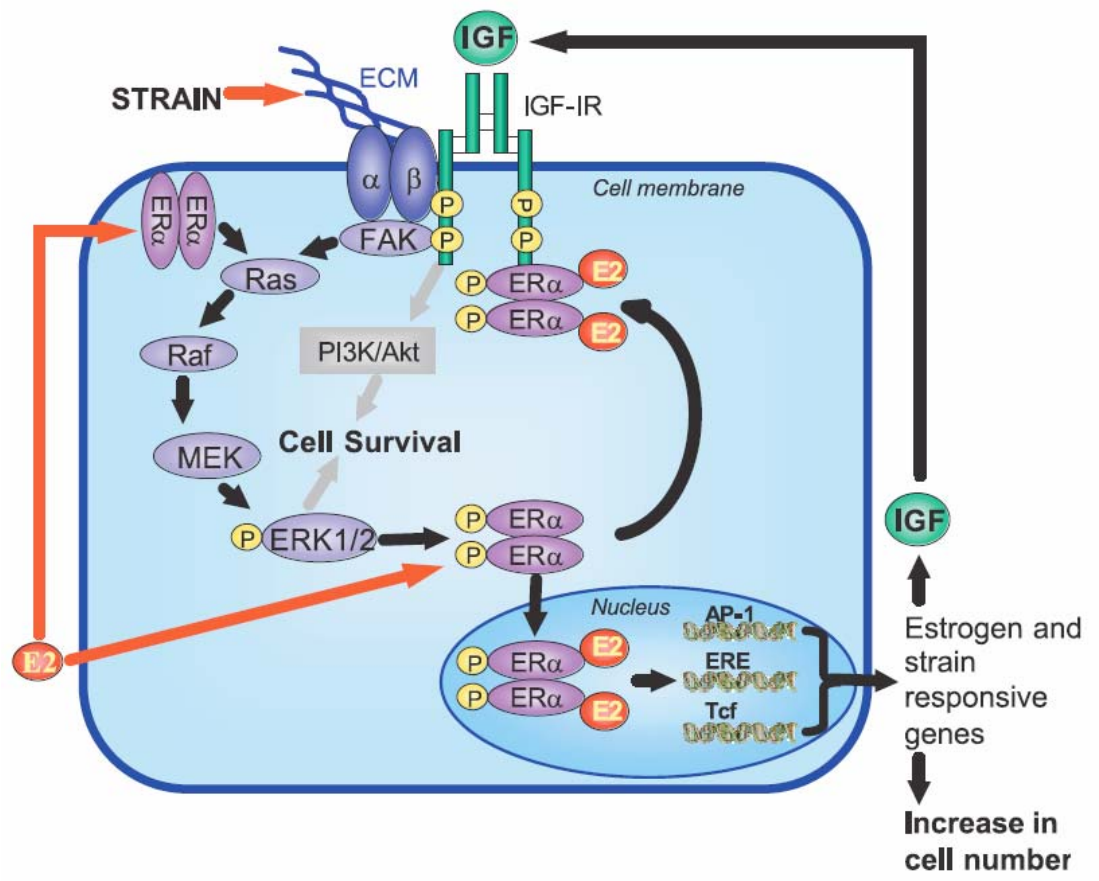

FIGURE 7 Signal transduction pathways for mechanical strain in osteoblasts. Reproduced with permission from Society for Endocrinology: Lanyon, L., Amstrong, V., Ong, D., Zaman, G. & Price, J. 2004. Is estrogen receptor α key to controlling bones´ resistance to fracture? J. Endocrinol. 182: 183-191.

2.3.4 Catechol-*O*-methyltransferase and fast estrogen catabolism

Catechol-*O*-methyltransferase is an enzyme that catalyzes the transfer of a methyl group from S-adenosylmethionine to catecholamines, including the neurotransmitters dopamine, epinephrine, and norepinephrine, as well as to catecholestrogens (Axelrod et al. 1958). This *O*-methylation results in one of the major degradative pathways of the catecholamine transmitters and estrogens. In addition to its role in the metabolism of endogenous substances, COMT is important in the metabolism of catechol drugs used in the treatment of hypertension, asthma, and Parkinson disease (Gordin et al. 2004, Schwartz & Turner 2004). The general function of COMT is the elimination of biologically active or toxic catechols and some other hydroxylated metabolites. For instance, COMT acts as an enzymatic detoxicating barrier between the blood and other tissues shielding against the detrimental effects of xenobiotics (Minn et al. 1991). It may also serve some unique or indirect functions in the kidney and intestine tract by modulating the dopaminergic tone (Meister et al. 1993). In the brain COMT activity may regulate the amounts of active dopamine and norepinephrine in various parts of the brain and therefore associate with mood

and other mental processes (Reenilä & Männistö 2001). Catechol-*O*-methyltransferase has been found in practically all mammalian tissues investigated. In all tissues examined thus far, COMT activity is found almost exclusively in the cytosol, but some activity is also found in a membrane-bound form (Guldberg & Marsden 1975, Bertocci et al. 1991). The highest COMT activity is in the liver, followed by the kidneys and gastrointestinal tract (Nissinen et al. 1988, Schultz & Nissinen 1989, Männistö et al. 1992).

The level of COMT activity is genetically polymorphic in human tissues with a trimodal distribution of low (COMT-LL), intermediate (COMT-LH), and high (COMT-HH) activities (Weinshilboum & Raymond 1977). This single nucleotide polymorphism is caused by autosomal codominant alleles and leads to 3-4 fold differences in enzyme activity (Lachman et al. 1996). Some diseases seem to be associated with this valine to methionine polymorphism located at codon 158 of the *COMT* gene. For example, an obsessive-compulsive disorder with various anxiety reducing rituals (Karayiorgou et al. 1997), aggressive and highly antisocial impulsive schizophrenia (Strous et al. 1997), as well as the late-onset alcoholism (type 1) (Tiihonen et al. 1999) seem to be related to the *COMT-LL* allele. The low activity allele is also found more frequently in patients with breast cancer than in healthy controls, particularly in women with menopausal symptoms (Thompson et al. 1998). This phenomenon may relate to the decreased metabolism of catecholestrogens that COMT also metabolizes. Furthermore, the link of this polymorphism has been found with bipolar manic-depressive illness (Kirov et al. 1998) as well as with depression (Ohara et al. 1998) in some patients. Interestingly, polysubstance abusers have been reported to have more commonly the *COMT-HH* allele than the controls (Vandenbergh et al. 1997). The association of the *COMT* SNP with Parkinson's disease has been extensively studied, but usually no association has been found (Hoda et al. 1996).

Catechol-*O*-methyltransferase participates in the catabolism of estrogens after their initial hydroxylation to catecholestrogens (Fig. 8). The resultant methylated metabolites, the methoxyestradiols, do not bind to the ERs, whereas the intermediate hydroxylated metabolites do to some extent (Zhu & Conney 1998). However, 2-methoxyestradiol inhibits the proliferation of several cancer cell lines and is one of the most potent endogenous inhibitors of angiogenesis known (Fotsis et al. 1994). The first study focusing on the relationship between the *COMT* polymorphism and estrogen levels in postmenopausal women was published in 2003 (Worda et al. 2003). The authors demonstrated that women with the *COMT-LL* allele had significantly higher serum concentrations of estrogen, both before as well as after a single oral dose of estradiol valerate, than those with the *COMT-HH* allele. Furthermore, these women had experienced an early onset of menarche. Since estrogens exert several important effects on bone metabolism and development, COMT activity may have implications on bone health. The idea is rather new and has not been investigated much so far. Theoretically, individuals with the *COMT-LL* allele and thereby decreased COMT activity should show less efficient degradation of

estrogen compared to those with the *COMT-HH* allele. They should therefore have elevated serum concentrations of estrogen as well, which clearly is an important factor contributing to bone density (Ettinger et al. 1998). The first study to investigate the *COMT* SNP in relation to bone failed to show any association between the genotype and bone measures of lumbar spine and femoral neck in postmenopausal women (Tofteng et al. 2004). At the same year, a Swedish group was able to show an association between the low activity allele and high serum concentration of estrogen in middle-aged men (Eriksson et al. 2004). A little later, the same research team found something unexpected, as the COMT-LL genotype associated with low peak bone density in young men (Lorentzon et al. 2004). Unfortunately, the timing of puberty of these men was not reported in that study. It may well be that men with the COMT-LL genotype had experienced an early pubertal development which could result in an early cessation of growth and thereby limit the ability of bone acquisition during the late adolescence. However, it is not feasible to draw comprehensive conclusions concerning the relationships between the COMT activity, estrogen metabolites, pubertal timing, and peak bone mass based on such a few studies.

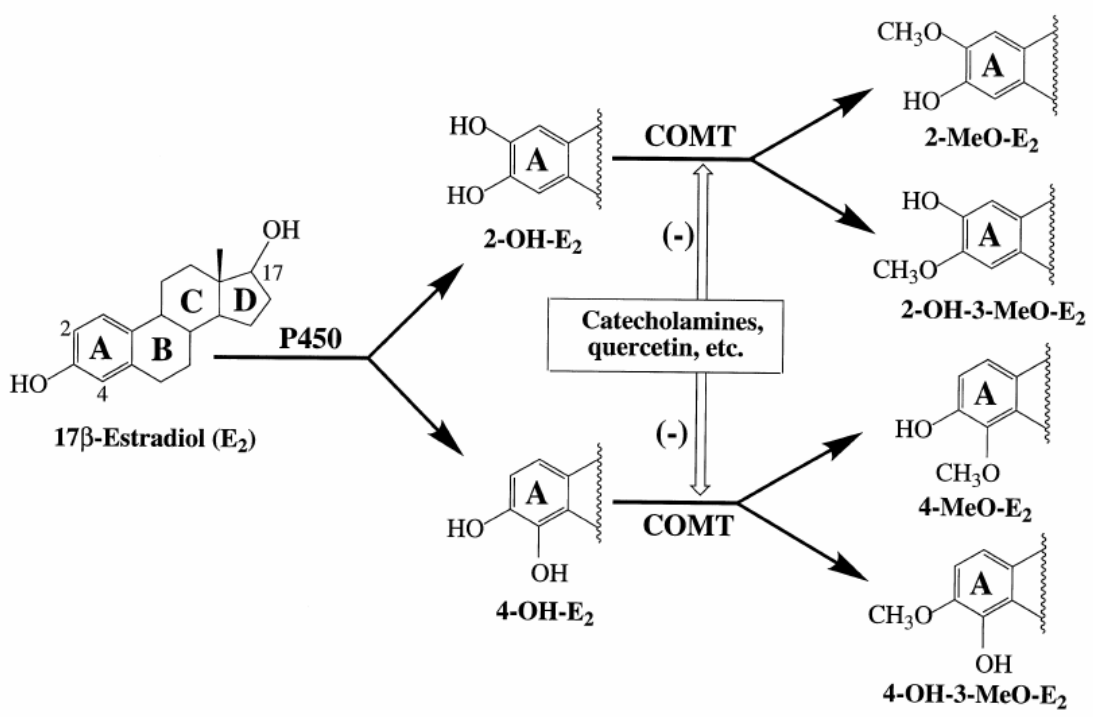

FIGURE 8 Catabolism of estrogens. Reproduced with permission from Oxford University Press: Zhu, B. T. & Conney, A. H. 1998. Functional role of estrogen metabolism in target cells: review and perspectives. Carcinogenesis 19: 1-27.

3 AIMS OF THE STUDY

Childhood and adolescence are critical periods for bone mass acquisition. Impaired bone growth during puberty may contribute to an increased risk for osteoporosis and fractures later in adulthood. Twin studies suggest that heredity plays a major role in the determination of peak bone mass, accounting for up to 80 % of the variance (Sambrook et al. 1996). However, current understanding of the genetic basis of paediatric bone mass and growth process is far from complete. Therefore, the object of this work was to increase our knowledge on the role of selective candidate genes involved in skeletal growth during puberty. The specific aims of the present study were:

1. To investigate the effects of selective polymorphisms of bone organic matrix –related genes (*COL1A1* and *COL1A2*) on bone properties and fractures in pubertal girls (I, II).

2. To investigate the effects of selective polymorphisms of estrogen metabolism –related genes (*ER-*α and *COMT*) on sexual and musculoskeletal development in pubertal girls (III, V).

3. To investigate the effect of selective polymorphism of *ER-*α on the skeletal responsiveness to exercise during puberty (III, IV).

4 MATERIALS AND METHODS

4.1 Subjects

The study subjects were healthy Finnish schoolgirls, who were recruited for a 2-year intervention trial (the Calex-study), in order to evaluate the effects of calcium, vitamin D, and dairy products´ supplementation on bone mass accrual (Cheng et al. 2005). The children were first contacted via class teachers teaching 4th to 6th grades in 61 schools (96 % of the total schools in these areas) in the city of Jyväskylä and its surroundings in Central Finland. Of the 1379 girls 312 fulfilled the inclusion criteria of the study, which were dietary calcium intake below 900 mg/day, age of 10-12 years, sexual development of Tanner stage I-II, and no histories of serious medical condition or medication known to affect bone metabolism. Furthermore, four months later 61 girls with dietary calcium intake above 900 mg/day were randomly selected from the screening to serve as a reference group in the study. Among these 61 girls 12 had become 13-year old and developed into Tanner stage III at the time of baseline assessments. Altogether, 258 (69 %) of those eligible agreed to participate in the study. An informed consent was obtained from all subjects and their parents prior to the assessments. The investigational protocol was approved by the ethical committees of the University of Jyväskylä, Central Hospital of Central Finland, and Finnish National Agency of Medicines.

The trial was originally designed with adequate power (90 %; α level of 0.05) to detect a 3-4 % difference between the treatment groups and the reference group in the primary outcome of bone mineral content over the 2-year period. Studies I-III and V included those 258 girls who participated in the baseline assessments. Two hundred and three girls continued with the 2-year trial until the end point assessments. The main reasons for dropping out from the intervention were either lack of time or interest or aversion to the supplements. Of those 203 girls 134 did not change their physical activity (PA) level over the 2-year period, and were thus available for the Study IV. The

subject numbers during the recruitment and trial are presented in Figure 9 and the variables investigated in the studies are listed in Table 2.

4.2 Questionnaires

Lifestyle characteristics and medical histories were collected via self-administered questionnaires the girls filled with the help of their parents at 6 month intervals. The menarche age was defined as the first onset of menstrual bleeding and collected by a questionnaire and retrospective phone call.

The questionnaire concerning the lifetime fracture history included items on the site, time, and cause of each bone fracture. All fractures were further evaluated and confirmed by an experienced clinician on the basis of hospital medical records and radiographs. Fractures that occurred in less than one year from the baseline assessments as well as those caused by severe trauma (e.g. serious accidents and crushed fingers/toes) were excluded from the analysis.

Food records of three consecutive days (2 week days and 1 weekend day) contained the time of eating, items, and portion of food. The families were given written instructions including an example of how to record food consumption with a help of portion guidebook, and the food records were further checked by nutritionists. Dietary intakes of energy, energy yielding nutrients, calcium, and vitamin D were analyzed using a validated Micro-Nutrica software (Finnish Social Insurance Institute, Turku, Finland).

The PA questionnaire modified from the questions used in the WHO study (Hickman et al. 2000) was designed to evaluate the intensity, type, duration, and frequency of the leisure time (excluding physical education in school) PAs (Wang et al. 2005). The girls were asked to fill their first, second, and third favourite sports they were practising, the duration of exercise in each session, and the frequency of exercises per week. The intensity of each activity was calculated on the basis of energy expenditure (McArdle et al. 1996) and bone loading was defined as whether the activity was weight-bearing or not. For instance, running would be scored as 2 while swimming as 1 according to this definition. A final score of PA was calculated as follows:

$$\text{Score of PA} = \Sigma_{1\text{-}3} \text{ (frequency x intensity index x duration x loading)}$$

In the equation frequency is expressed as times per week, duration as hours, and loading as 1 for non-weight-bearing and 2 for weight-bearing activities. For the purpose of Study III, girls were classified into low (L) and high (H) PA groups according to their baseline scores of PA cut from the median. Another classification into L and H PA groups was made based on the actual level of PA in terms of hours per week cut from the median. Those girls, whose PA level kept low at the baseline, 12, and 24 month follow-up, were further classified into LLPA group (consistently low physical activity), for the purpose of Study

IV. Those girls, whose PA level kept high at the baseline, 12, and 24 month follow-up, were respectively categorized into HHPA group (consistently high physical activity). Those girls, who changed their PA level during the trial by either increasing or reducing, were excluded from the Study IV.

4.3 Anthropometric assessments

Girls underwent a physical examination conducted by a public health nurse at six month intervals. The status of physical maturation was determined by the developmental patterns of breasts and pubic hair according to the Tanner grading system (Tanner 1978). If there was a disagreement between the development of breasts and pubic hair, the final decision was made on the basis of breasts development. Prepubertal was considered stage I (no evidence of breasts or pubic hair development), and early pubertal is indicated by stages II to III (evidence of development). Body weight was measured using an electronic scale and recorded with a precision of 0.1 kg with girls wearing light clothes and bare feet, and body height was determined with a wall-mounted stadiometer to the nearest 0.5 cm. Body mass index (BMI) was calculated as weight (kg) divided by square of height (m). Sitting height was defined from the total body dual-energy X-ray absorptiometry (DXA) scan as the distance between the vertex and a line parallel to the lower edge of the tuber ischiadicum. The length of the forearm was determined using a measuring tape and defined as proceeding from the olecranon to the ulna styloid with the forearm supinated and elbow at 90˚ angle, and the length of the lower leg was defined as the distance between the tuberositas tibia and the medial malleolus with a sitting position and knee at 90˚ angle.

4.4 Bone assessments

Bone measurements were performed at the baseline, 12, and 24 month follow-up, except for DXA scan of the total body that was only taken at the baseline and 24 month follow-up. Calibration of each device was performed daily using the manufacturer's internal standard.

Bone area (BA), bone mineral content (BMC), and areal bone mineral density (aBMD) of the total body, spine L2-L4, total left femur, and hip including femoral neck and trochanter, as well as the fat (FM) and lean tissue (LTM) masses of the total body were assessed using Lunar Prodigy DXA (GE Lunar Corporation, Madison, WI, USA). Girls wore light underwear and removed all metal items before being scanned. They remained motionlessly in the supine position during the posterior-anterior projections that were made using the default scan mode automatically selected by the Prodigy software.

Each scan was analyzed by the same technician according to standard protocol described by the manufacturer. The coefficients of variation (CVs) for repeated measurements were 0.6-1.2 % for BMC, 0.9-1.3 % for aBMD, 2.2 % for FM, and 1.0 % for LTM.

Cross-sectional area (CSA), BMC, volumetric bone mineral density (vBMD), and moment of inertia (Ipolar) of the left distal radius and tibia shaft, as well as cortical thickness (CTh) of the left tibia shaft, were assessed using peripheral quantitative computed tomography (pQCT, XCT 2000, Stratec Medizintechnik, GmbH, Pforzheim, Germany). A 2 mm thick single tomographic slice with a pixel size 0.59 × 0.59 mm was scanned from the transverse plane at 4 % of the forearm length proximal to the joint surface of the distal radius using the standard mode, and 60 % of lower leg length up from the lateral malleolus using the research mode. Thresholds of 169 mg/cm^3 and 280 mg/cm^3 were used to define the outer borders of the radius and tibia, respectively, and a threshold of 710 mg/cm^3 to specifically locate the cortical bone. Image processing and analysis were performed using a validated Geanie software (Bonalyse Oy, Jyväskylä, Finland). The CVs between two consecutive measurements with repositioning were 1.1-3.0 % for CSA and < 1.0 % for vBMD.

Broadband ultrasound attenuation (BUA) through the left calcaneus was measured using a gel coupling scanning quantitative ultrasonometer (QUS-2, Quidel Corporation, Santa Clara, CA, USA). Ultrasonic waves of 200-800 kHz were laterally transmitted through the posterior part of the calcaneus. The region of interest was calculated from the edges of the bone and automatically defined by the device after scanning an area of 1400 mm^2. The BUA values were calculated based on proprietary transient analysis algorithms that evaluate the heel's low-pass filtering characteristics from analysis of the dominant early period. Each measurement was performed twice without repositioning, and the final result was obtained by averaging these two values. The CV for BUA was < 1.2 %.

Speed of sound (SOS) along the long axis of the surface of the distal third of radius (medial surface) and the midshaft of tibia (anteromedial surface) were determined by a mobile ultrasound instrument (Omnisense, Sunlight Technologies Ltd, Rehovot, Israel). For acoustic coupling water soluble ultrasound gel (Aquasonic, Parker Laboratories) was applied to the skin and the manufacturer's largest handheld probe (CM-probe). The probe, transmitting ultrasonic signals of 1.25 MHz, was placed on the skin along the bone, and a scan was made by carefully moving the probe face circumferentially around the bone. While scanning, an SOS was determined once every 0.1 s to provide an SOS profile of the bone being scanned, and the device provided an average SOS of the single scan. An average of three to five successive measurement cycles with statistically consistent SOS values gave the final SOS result. The CV for SOS was < 1.6 %.

4.5 Biochemical analyses

Blood samples were taken in the morning between 7:30 and 9:00 after an overnight fast at the baseline, 12, and 24 month follow-up. For each girl, the date when the samples were taken, which ranged from the middle of December to the end of February, was kept as similar as possible across the three measurement time points. If the girl began menstruation, the blood sample was taken between the 2nd and 5th bleeding day. Venous blood was collected into vacuum tubes, the blood was allowed to clot for 10 to 20 minutes, and serum was finally separated by centrifugation for 10 minutes at 1500 x g. The serum samples were stored as aliquots at –75°C until analyzed.

Serum growth hormone (GH), IGF-1, and insulin-like growth factor binding protein-3 (IGFBP-3) were measured using commercial radioimmunoassays (Nichols Institute Diagnostics, San Juan Capistrano, CA, USA). The intra- and inter-assay CVs were 3.5 % and 5.4 % for GH, 4.0 % and 12.6 % for IGF-1, and 5.7 % and 5.8 % for IGFBP-3, respectively.

Serum 17β-estradiol (E2), testosterone (T), and sex hormone binding globulin (SHBG) were measured using time-resolved fluoroimmunoassays (Wallac Oy, Turku, Finland). The intra- and inter-assay CVs were 5.1 % and 5.2 % for E2, 9.2 % and 9.4 % for T, and 1.1 % and 1.1 % for SHBG, respectively. Non-SHBG-bound free testosterone (FT) was estimated using the following formulas (Ankarberg & Norjavaara 1999):

$$\text{Proportion of FT (FT\%)} = 2.28 - 1.38 \times \log (\text{SHBG nmol} - 1/10)$$

$$\text{FT (pmol/L)} = \text{FT\%} \times \text{T (nmol/L)} \times 10$$

Serum free 17β-estradiol (FE2) was estimated as follows (Ankarberg & Norjavaara 1999):

$$\text{FE2 (pmol/L)} = \text{E2 (pmol/L)} / (K \times \text{SHBG (nmol/L)} + 1),$$

with an equilibrium constant (K) for SHBG of 0.68×10^9 L/mol

Serum amino-terminal propeptide of type I procollagen (P1NP) was measured using a competitive radioimmunoassay (Orion Diagnostica, Mountain View, Espoo, Finland); the intra- and inter-assay CVs were 3.6 % and 1.9 %, respectively. Serum osteocalcin (OC) was measured using an in-house immunoassay (Käkönen et al. 2000); the intra- and inter-assay CVs were 2.4 % and 0.6 %, respectively. Serum bone-specific alkaline phosphatase (B-ALP) activity was measured using an immunoassay (Metra Biosystems Incorporation, Mountain View, CA, USA); the intra- and inter-assay CVs were 1.9 % and 1.8 %, respectively. Serum bone-specific tartrate-resistant acid phosphatase isoform 5b (TRACP 5b) activity was measured using an immunoassay (SBA-Sciences, Oulu, Finland); the intra- and inter-assay CVs were 2.7 % and 5.7 %, respectively. In addition, following bone turnover indexes were calculated for the purpose of Study I:

Type I collagen formation / bone resorption = P1NP / TRACP 5b

Bone formation / bone resorption = (OC + B-ALP) / TRACP 5b

4.6 Genetic analyses

Genomic DNA was extracted and purified from EDTA blood samples using QIAmp Blood Kit (Qiagen GmbH, Hilden, Germany). Deoxyribonucleic acid concentration was estimated by ultraviolet (UV) absorption spectrophotometry at a wavelength of 260 nm, and the samples were stored as aliquots at –75°C until analyzed. The genetic polymorphisms investigated in the studies are listed in Table 3.

A G to T variation located at the transcription factor Sp1 consensus site in the first intron of the *COL1A1* gene (refSNP ID: rs1800012) was directly analyzed by sequencing (I). Three hundred and ninety base pairs (bp) covering the polymorphic site were amplified by polymerase chain reaction (PCR) using the forward primer 5'-AAA GTG ACC TGG AGG CAT TG-3' and reverse primer 5'-CTT CCA ACT CCA ACC TCA GC-3'. The PCR amplification was carried out in a reaction volume of 15 µl containing 20 ng of genomic DNA, 50 mM KCl, 15 mM TRIS-HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.2 mM each of the four dNTP, 0.2 pmol of each primer, and 0.38 unit of Ampli*Taq* Gold DNA polymerase (Applied Biosystems). The reaction was performed in a DNA thermocycler (PTC-225, MJ Research) with a cycling protocol of 95°C, 60°C, and 72°C for 30 seconds each, for 35 cycles. Prior to the first cycle, initial denaturation was performed at 95°C for 10 minutes, and the last cycle was followed by an extension step of 10 minutes at 72°C. The quantity and quality of the PCR products were checked on a 2 % agarose gel containing ethidium bromide. Before loading onto the gel, the products were mixed with 10 x loading buffer containing glycerol and bromophenol blue. Prior to sequencing, the samples were treated with exonuclease I to degrade the residual PCR primers, and shrimp alkaline phosphatase to dephosphorylate the residual nucleotides. The samples were then sequenced using the PCR primers and BigDyeTerminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Thereafter, the products were further purified by Sephadex G-50 Superfine Multiscreen Plate (Millipore) and run through a capillary gel electrophoresis (3730 DNA Analyzer, Applied Biosystems) in order to assemble the DNA sequences, from which the genotypes were finally determined using a Sequencher 4.1.4 software (GeneCodes).

A A to C variation located at the restriction enzyme *PvuII* consensus site in exon 25 of the *COL1A2* gene was detected by PCR followed by restriction fragment length polymorphism (RFLP) analysis (Constantinou et al. 1990) (II). Seven hundred and seventy one bp covering the polymorphic site were amplified by PCR using the forward primer 5´-GGG ATA TAA GGA TAC ACT AGA GG-3´ and reverse primer 5´-GAA ATA TCG GCC CCG CTG GAA-3´. The PCR amplification was carried out in a reaction volume of 20 µl containing

50-200 ng of genomic DNA, 50 mM KCl, 10 mM TRIS-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.2 mM each of the four dNTP, 10 pmol of each primer, and 1.5 unit of *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania). The reaction was performed in a DNA thermocycler (Biometra T3 Combi-Block, Göttingen, Germany) with a cycling protocol of 94°C, 59°C, and 72°C for one minute each, for 30 cycles. Prior to the first cycle, initial denaturation was performed at 94°C for 5 minutes, and the last cycle was followed by an extension step of 7 minutes at 72°C. The PCR products were digested with 10 units of *PvuII* restriction enzyme (Fermentas, Vilnius, Lithuania) in a buffer containing 10 mM TRIS-HCl (pH 7.5), 50 mM NaCl, and 10 mM $MgCl_2$ at 37°C for 4 hours, and then electrophoresed on a 1.5 % agarose gel containing ethidium bromide, from which the genotypes were finally determined by visualizing the bands under UV illumination using a Quantity One software (Bio Rad). The samples containing *p* alleles were cleaved by *PvuII*, resulting in two bands of 541 and 239 bp, while the uncleaved samples containing *P* alleles resulted in a single band of 771 bp. The heterozygotes containing both *p* and *P* alleles resulted in three bands of 771, 541, and 230 bp.

A C to T variation located at the restriction enzyme *PvuII* consensus site in the first intron of the *ER-α* gene (refSNP ID: rs2234693) was detected by PCR followed by RFLP analysis (Salmen et al. 2000) (III, IV). One thousand, three hundred and seventy four bp covering the polymorphic site were amplified by PCR using the forward primer 5´- CTG CCA CCC TAT CTG TAT CTT TTC CTA TTC ACC-3´ and reverse primer 5´- TCT TTC TCT GCC ACC CTG GCG TCG ATT ATC TGA-3´. The PCR amplification was carried out in a reaction volume of 20 µl containing 50-200 ng of genomic DNA, 50 mM KCl, 10 mM TRIS-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.08 % Nonidet P-40, 0.2 mM each of the four dNTP, 1.0 µM of each primer, and 1.2 unit of *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania). The reaction was performed in a DNA thermocycler (Biometra Uno Thermoblock, Göttingen, Germany) with a cycling protocol of 94°C, 58°C, and 72°C for one minute each, for 31 cycles. Prior to the first cycle, initial denaturation was performed at 94°C for 4 minutes, and the last cycle was followed by an extension step of 5 minutes at 72°C. The PCR products were digested with 10 units of *PvuII* restriction enzyme (Fermentas, Vilnius, Lithuania) in a buffer containing 10 mM TRIS-HCl (pH 7.5), 50 mM NaCl, and 10 mM $MgCl_2$ at 37°C for 4 hours, and then electrophoresed on a 2 % agarose gel containing ethidium bromide, from which the genotypes were finally determined by visualizing the bands under UV illumination using a BioDoc II software (Biometra). The samples containing *p* alleles were cleaved by *PvuII*, resulting in two bands of 936 and 438 bp, while the uncleaved samples containing *P* alleles resulted in a single band of 1374 bp. The heterozygotes containing both *p* and *P* alleles resulted in three bands of 1374, 936, and 438 bp.

A G to A variation located at codon 158 in the second exon of the *COMT* gene (refSNP ID: rs4680) was detected by PCR followed by dynamic allele-specific hybridization (DASH) analysis (Howell et al. 1999) (V). Forty seven bp covering the polymorphic site were amplified by PCR using the forward primer

5´-AGT GGA TGC TGG ATT TCG CTG-3´ and reverse primer 5´-biotin-AGG CAC GCA CAC CTT GTC CTT-3´. The PCR amplification was carried out in a reaction volume of 20 µl containing 10-25 ng of genomic DNA, 50 mM KCl, 15 mM TRIS-HCl (pH 8.0), 10 % DMSO, 1.5 mM $MgCl_2$, 0.2 mM each of the four dNTP, 20 pmol of forward primer, 4 pmol of reverse primer, and 0.6 unit of Ampli*Taq* Gold DNA polymerase (Applied Biosystems). The reaction was performed in a DNA thermocycler (Multiblock Hybaid, Middlesex, UK) with a cycling protocol of an initial denaturation at 94°C for 10 minutes, following 40 cycles of 94°C for 15 seconds and 54°C for 30 seconds. The biotinylated PCR products were immobilized to streptavidin-coated microtiter 96-well plates, and surface binding was allowed to proceed for a minimum of 1 hour at room temperature. Thereafter, the non-biotinylated strands were removed through several alkali rinses. An allele-specific oligonucleotide probe (5´-TCG CTG GCG TGA AGG AC-3´ for Val allele and 5´-TCG CTG GCA TGA AGG AC-3´ for Met allele) was annealed to the immobilized target in the presence of Sybr Green I dye (Molecular Probes). The samples were then run on a DASH instrument (Thermo Hybaid Ltd, UK), and fluorescence was measured while raising the temperature from 40°C to 85°C at 0.03°C / second. The genotypes were determined from the presence and order of the observed peaks in a plot of the negative first derivative of fluorescence values versus temperature.

4.7 Statistical analyses

Statistical analyses were carried out using the SPSS (versions 9.0 and 12.0) and MLwiN (version 1.1) softwares.

One-way analysis of variance (ANOVA) and covariance (ANCOVA) were used to test differences in the assessed variables between the different genotype groups (I-V). Adjustments were made for the effects of Tanner stage and BMI or body size index (BSI) that was reduced from body weight and height using a principal components factor analysis. For multiple comparisons least significant difference (LSD) and Bonferroni post hoc tests or, when assuming heterogeneous variances, Dunnett's T3 post hoc test were used. Homogeneity of variances was confirmed with a Levene test. When needed, the distributions of biochemical data were normalized by transformation to achieve the assumption of normal distribution of residuals. Nominal F-test for the PA score arm by genotype product term was used to test for evidence of exercise-gene interaction (III).

Likelihood ratio was used to test for the genotype distribution in girls with and without bone fractures. Odds ratios (OR) with 95 % confidence intervals (CI) were calculated by multi-variate logistic regression analysis after stepwise adjustment for potential confounding factors such as Tanner stage, BMI, aBMD of the total body, and PA to estimate the relative risk of fracture by the genotypes (II).

Regression analysis using FE2 or muscle area as covariates was used to estimate the relative influence of the genotypes and these variables on physical

maturation and skeletal parameters. Bi-variate correlations were calculated using Pearson's coefficient of correlations (V).

Hierarchical linear model with random effects was employed to explore the effects of growth on bone variables of the tibia shaft (IV). In this model, the time relative to menarche age was entered as the explanatory variable in the form of polynomial spline functions to explain the change of these variables over time.

All significant tests were two-sided. A *p* value of less than 0.05 was considered statistically significant, and a trend was set at a *p* value of less than 0.10.

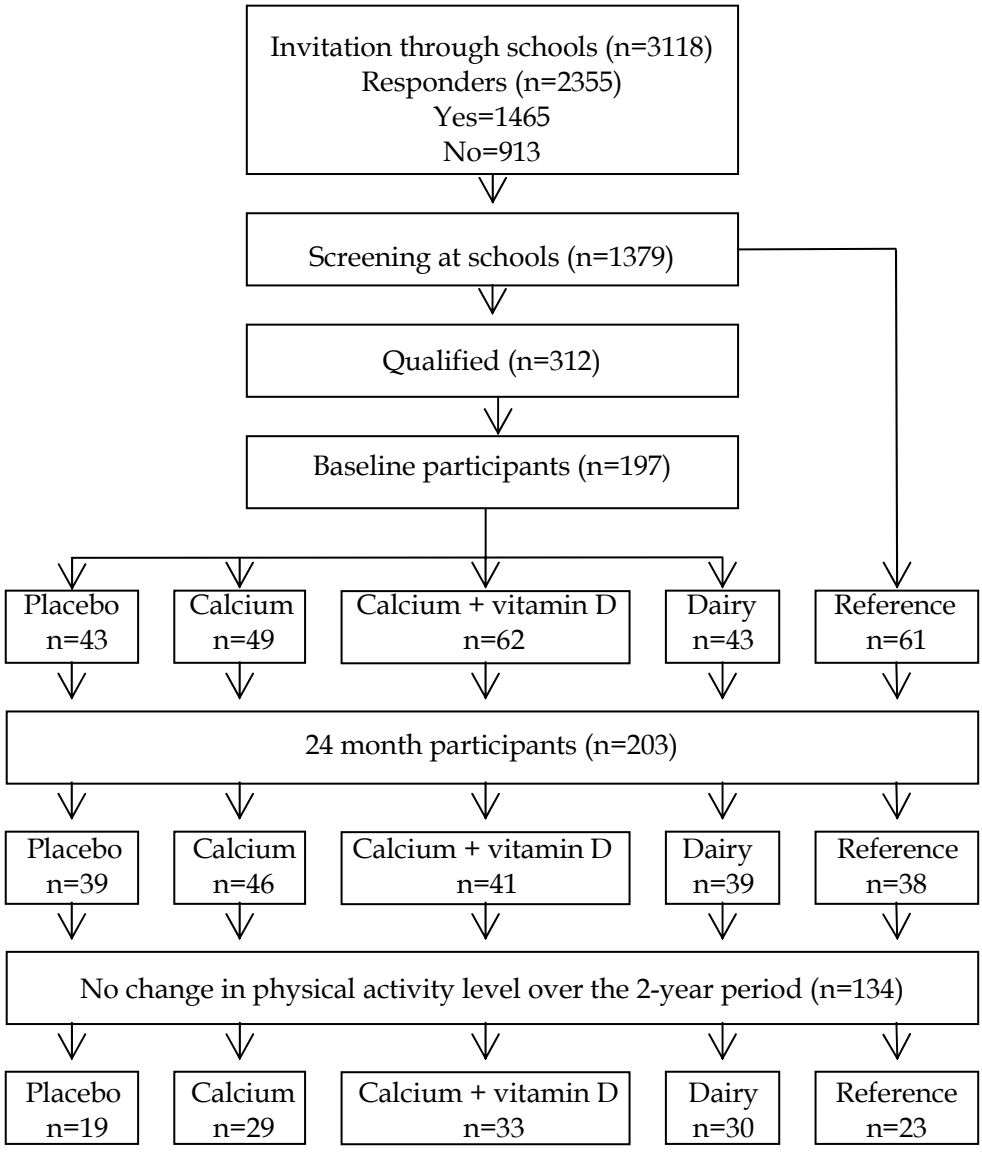

FIGURE 9 Subject numbers during the recruitment and trial.

TABLE 2 Variables assessed in the studies and methods used.

Variable	Measurement site	Method
Basic information:		
Health and fracture history		Questionnaire
Food record		Questionnaire
Physical activity		Questionnaire
Anthropometry:		
Physical maturation		Tanner grading
Body weight		Electronic scale
Body height		Stadiometer
Sitting height		DXA
Forearm length		Measuring tape
Lower leg length		Measuring tape
Bone densitometry:		
Body fat mass		DXA
Body lean mass		DXA
Bone area	Total body/spine/hip	DXA
Bone mineral content	Total body/spine/hip	DXA
Areal bone mineral density	Total body/spine/hip	DXA
Cross-sectional area	Distal radius/tibia shaft	pQCT
Bone mineral content	Distal radius/tibia shaft	pQCT
Volumetric bone mineral density	Distal radius/tibia shaft	pQCT
Moment of inertia	Distal radius/tibia shaft	pQCT
Cortical thickness	Tibia shaft	pQCT
Broadband ultrasound attenuation	Calcaneus	QUS
Speed of sound	Distal radius/tibia shaft	QUS
Serum biochemistry:		
GH		RIA
IGF-1		RIA
IGFBP-3		RIA
E2		DELFIA
T		DELFIA
SHBG		DELFIA
P1NP		RIA
OC		In-house IA
B-ALP		ELISA
TRACP 5b		ELISA
Genetic:		
COL1A1		Sequencing
COL1A2		RFLP
ER-α		RFLP
COMT		DASH

TABLE 3 Genetic polymorphisms assessed in the studies.

Gene	Chromosome	SNP ID	Location	Function
COL1A1	17q21-22	rs1800012	Intron 1	Gene expression
COL1A2	7q22	Nonexistent	Exon 25	Unknown
ER-α	6q25	rs2234693	Intron 1	Gene expression
COMT	22q11	rs4680	Exon 2	Amino acid change

5 RESULTS

5.1 *COL1A1* SNP associates with poor skeletal traits (I)

Two hundred and forty seven healthy schoolgirls, with a mean age of 11.2 years (range 10.0-13.2 years) at the time of the baseline assessments, had a successful base calling by sequencing. One hundred and seventy five (70.9 %) girls were homozygous for the *G* allele (GG) of the *COL1A1* SNP, 68 (27.5 %) were heterozygotes (GT), and 4 (1.6 %) girls were homozygotes for the *T* allele (TT). Further mutation analyses were performed by screening 390 bp around the *COL1A1* SNP for previously published SNPs (rs2696252, rs2696253, rs2696254, and rs2696255). However, no such sequence variations were observed in any of the samples, while one novel heterozygote mutation was identified 134 bp towards the 5'-end from the Sp1 polymorphic site. This 12-year old girl was 148.5 cm tall and weighted 40.3 kg. Moreover, her bone properties seemed fairly normal suggesting the SNP to be either a silent or recessive one.

Next, the COL1A1 genotypes were evaluated with respect to the clinical findings. No significant differences in developmental status, anthropometric measures, dietary intakes, or PA were found between the different genotypes (I, Table 1). The genotype frequencies did not differ significantly between girls who had experienced a non-traumatic fracture and those who had not, as 21 (12 %) of the GG girls, 13 (19 %) of the GT girls, but none of the 4 TT girls had a positive fracture history. However, significant associations were found between the SNP and several bone properties, even after taking into account the slight difference in body size. Girls with the TT genotype had significantly smaller BA of the total body and femoral neck than those with the GT (p = 0.011 and 0.042, respectively) and GG (p = 0.014 and 0.047, respectively) genotype (I, Table 2). Furthermore, the TT girls had significantly lower BMC of the total body, spine L2-L4, and total femur than the GT (p = 0.001, 0.044, and 0.022, respectively) and GG ($p \leq 0.001$, 0.032, and 0.031, respectively) girls. Also, association between the SNP and aBMD of each of the measured bone site was evident (p = 0.002–0.032). Volumetric density and geometrical properties of the distal radius and tibia

shaft were slightly lower in the TT girls compared to the others, though the differences did not reach the statistical significance (I, Table 3). Only trabecular vBMD of the distal radius showed a suggestive association with the SNP, as it tended to be lower in girls with the TT genotype than those with the GT (p = 0.079) and GG (p = 0.059) genotype. To reveal possible association of the SNP with bone material properties analyses were performed concerning the ultrasound propagation. While SOS at the tibia shaft and distal radius were similar between the different genotypes, BUA through the calcaneus was significantly lower in the TT girls compared to the others (p = 0.008 and 0.016) (I, Table 4).

Finally, the biochemical data was evaluated. Of the bone formation markers, no significant difference was found in serum P1NP between the genotypes, while serum OC tended to be lower in girls with the TT genotype than those with the GT (p = 0.076) and GG (p = 0.089) genotype (I, Table 5). Furthermore, serum B-ALP was significantly lower in the TT girls compared to the others (p = 0.037 and 0.027), while no difference was observed in the bone resorption marker, serum TRACP 5b, among the girls. In order to elucidate the bone metabolism in more detail two bone turnover indexes were subsequently used. The ratio of formation of type I collagen and resorption of bone, expressed as P1NP / TRACP 5b, was significantly lower in girls with the TT genotype than those with the GT genotype (p = 0.034), while the difference between the TT and GG genotype was at borderline (p = 0.061). In addition, the ratio of formation and resorption of bone, expressed as (OC + B-ALP) / TRACP 5b, was significantly lower in the TT girls compared to the others (p = 0.016 and 0.031).

5.2 *COL1A2* SNP associates with paediatric fractures (II)

Two hundred and forty four girls had a successful genotyping for the *COL1A2* SNP. Thirty eight (15.6 %) of them were homozygous for the *P* allele (PP), 129 (52.9 %) were heterozygotes (Pp), and 77 (31.5 %) girls were homozygotes for the *p* allele (pp). After the clinical evaluations associations between the genotypes and phenotypes were tested. No significant differences in developmental status or anthropometric measures were found between the different genotypes (II, Table 1). However, girls with the *P* allele tended to have slightly higher BMI than those with the *p* allele (p = 0.07). As developmental status and body size are strong contributors for bone properties, the comparisons of the skeletal and biochemical results were performed by controlling for Tanner stage and BMI. No significant effects of the SNP were found on bone size, mass, density, or material properties (II, Table 2). Also, serum concentrations of biochemical markers for bone turnover were similar between the genotypes (II, Table 3). Considering the fact that Tanner stage I and II clearly differ from each other when it comes to the skeletal age and growth speed, further testing were made by considering girls at different stages

separately. Bone properties were still similar among the genotypes when only girls at their most intensive growth phase, Tanner stage II, were considered. However, an association was found between the SNP and axial bone density (II, Fig. 1) as well as material properties of appendage bones (II, Fig. 2) in prepubertal girls. Girls at Tanner stage I with the PP genotype had significantly lower BMC (p = 0.010) and aBMD (p = 0.013) of the spine L2-L4, as well as SOS at the tibia (p = 0.036) and a tendency toward lower SOS at the radius (p = 0.092) compared to girls with the pp genotype.

Thirty seven girls had experienced a bone fracture as a result of minimal to moderate trauma (by convention, the equivalent of a fall from standing height or less) during their life. The most frequent site of fracture was arm including wrist (31 cases), and the most common age of occurrence was 9-11 years (19 cases). Then, the genotype and allele frequencies were compared between the fractured and non-fractured girls. The distribution of fractures was significantly different in the genotypes (p = 0.023) (II, Table 5). Pooling of the PP and Pp genotypes under a dominant inheritance model showed a clear relationship between the *P* allele and the occurrence of fractures (p = 0.007). The odds ratios showed that the relative risk of fracture among girls with the *P* allele (either PP or Pp genotype) was 4.0 times higher than among girls with the pp genotype (II, Table 5). The crude risk of fracture was 4.9 for the PP genotype and 3.8 for the Pp genotype in relation to the pp genotype. Furthermore, the risk did not change essentially after adjustments for potential confounding factors such as Tanner stage, BMI, aBMD of the total body, and PA in the multivariate regression analysis. Stepwise adjustments for the aforementioned parameters yielded similar results as adjustment for all the factors together.

5.3 *ER-α* SNP acts on responsiveness to exercise (III, IV)

Two hundred and forty five girls had a successful genotyping for the *ER-α* SNP. Forty two (17.2 %) of them were homozygous for the *P* allele (PP), 115 (46.9 %) were heterozygotes (Pp), and 88 (35.9 %) girls were homozygotes for the *p* allele (pp). Girls were further classified into L and H PA groups according to their PA scores cut from the median. The PA levels in terms of hours per week for the L and H groups were 1.5 and 4.4 at the baseline, 2.1 and 4.0 at the 12 month follow-up, and 1.6 and 4.6 at the 24 month follow-up, respectively. No major effects of the SNP were found on bone size, mass, or density. However, there was an interaction between the genotype and exercise with respect to BMC and aBMD of the total body (p = 0.030 and 0.012, respectively), and further comparisons between the L and H groups were thus made within each of the three genotype groups.

At the baseline, girls with the Pp genotype and L PA had significantly lower BMC (p = 0.001) and aBMD (p < 0.001) of the total body (III, Fig. 1) as well as spine L2-L4 (p = 0.011 and 0.002, respectively) (III, Fig. 2) than their H PA

counterparts. This was observed at the total femur (p = 0.001) (III, Fig. 3) and femoral neck (p = 0.040 and 0.006, respectively) (III, Fig. 4) as well. Moreover, the Pp girls with L PA had significantly thinner cortex (p < 0.001) and lower vBMD (p = 0.001), but no difference in CSA of the tibia shaft, than those with H PA (III, Fig. 5). No such differences were found in bone properties of the distal radius neither in muscle CSA of the lower leg between the different groups. Noteworthily, none of the bone properties differed between the L and H PA groups within either of the homozygote groups (PP or pp).

To further explore this observation girls with consistently L PA (LLPA) were compared to those with consistently H PA (HHPA) through the two year. Girls with the Pp genotype and LLPA tended to have the lowest, while those with HHPA tended to have the highest values of all bone variables (IV, Table 4). Particularly, aBMD (p = 0.030) of the total femur, BMC (p = 0.022) and aBMD (p = 0.024) of the femoral neck (IV, Table 4), as well as BMC (p = 0.029) and CTh (p = 0.009) of the tibia shaft (IV, Fig. 1), were significantly lower in the Pp girls with LLPA compared to their HHPA counterparts at the 24 month follow-up. Bone properties of the distal radius, however, were similar between the different groups. No major effect of PA on bone was found within either of the homozygote groups (PP or pp), consistent with the baseline findings.

Finally, a hierarchical linear model was used to explore the growth processes of the tibia shaft longitudinally by synchronizing the girls´ menarche age. In the homozygote groups, the growth processes of tibial BMC, vBMD, CSA, and CTh were comparable and close to each other between LLPA and HHPA (IV, Fig. 2). Meanwhile, in the heterozygote group, girls with HHPA tended to have constantly higher tibial BMC than their LLPA counterparts (p = 0.069), while no differences were observed in the growth processes of the other tibial characteristics.

5.4 *COMT* SNP associates with early pubertal development (V)

Two hundred and forty six girls had a successful genotyping for the *COMT* SNP. Eighty five (34.5 %) of them were homozygous for the low activity allele (LL), 118 (48.0 %) were heterozygotes (HL), and 43 (17.5 %) girls were homozygotes for the high activity allele (HH). No significant difference in chronological age was found between the different genotypes (V, Table 1). However, girls with the LL genotype were significantly more matured (p = 0.003), as they were more likely to be at Tanner stage II and III than girls with the LL genotype (V, Table 5). This was likely to be a consequence of their higher FE2, as the association between the SNP and developmental status vanished after including FE2 in the linear regression model (SNP: p = 0.183 and FE2: p < 0.0001). The LL girls were significantly taller (p = 0.001) and heavier (p = 0.009) as well than the HH girls (V, Table 1). To further clarify the connection between the SNP and body mass DXA and pQCT data were evaluated. It was found that

the greater weight of the LL girls compared to the HH girls was rather due to their larger amounts of LTM than FM. The LL girls had also significantly larger CSA of muscle (p = 0.008), but not of subcutaneous fat, of the lower leg compared to the HH girls, supporting the notion (V, Table 1).

Next, the COMT genotypes were evaluated with respect to the bone measures. Girls with the LL genotype had significantly larger BA (p = 0.002–0.033) of each of the measured bone site, except of femoral neck, as well as higher BMC (p = 0.003–0.032) of each site, except of femoral trochanter, compared to those with the HH genotype (V, Table 2). Meanwhile, no major differences were found in aBMD, except of spine L2-L4 (p = 0.013), between the different genotypes. To distinguish between the effects on skeletal size and volumetric density more clearly pQCT measurements of cortical and trabecular bone were performed at the tibia shaft and distal radius, respectively. Girls with the LL genotype had significantly higher cortical BMC (p = 0.008) than those with the HH genotype (V, Table 3). This was evidently due to their larger cortical CSA (p = 0.010) compared to the HH girls, as cortical vBMD was similar between the genotypes. The endocortical circumference (p = 0.022) was wider in the LL girls compared to the HH girls, though not as much as the periosteal circumference (p = 0.005). As a result they also had wider CTh (p = 0.006) than the HH girls. Moreover, Ipolar, an indicator of bone bending resistance, was significantly higher in the LL girls compared to the HH girls (p = 0.007). The characteristics of trabecular bone at the distal radius did not, however, associate with the SNP, as did those of cortical bone at the tibia shaft.

Thereafter, the hormonal data was evaluated. Serum E2 did not differ significantly when all three genotypes were compared (V, Table 4). However, pooling of the LL and HL genotypes under a dominant inheritance model showed a clear relationship between the *L* allele and elevation of serum E2 (p = 0.031). Furthermore, girls with the LL genotype had significantly higher serum FE2 than those with the HH genotype (p = 0.010). They also had higher serum IGF-1 compared to the HH girls (p = 0.029). To further investigate the relationships between *COMT* SNP, serum FE2, and skeletal traits regression analysis was performed. The genotype proved to be an important predictor of height (p < 0.001) and length of the lower leg (p = 0.001). After including FE2 in the linear regression model *COMT* SNP still retained some of its associations with height (p = 0.024) and length of the lower leg (p = 0.021), suggesting that the effect of the genotype on longitudinal growth is partly exerted via elevated E2 and partly via mechanisms independent of E2. The genotype associated with BMC of the total body (p = 0.001), femoral neck (p = 0.008), and cortical bone at the tibia shaft (p = 0.002) as well. However, this was likely to be a consequence of elevated E2, as the association between the SNP and BMC barely existed after including FE2 in the model. Finally, regression analysis was used to find out whether the association between the SNP and cortical BMC of the tibia shaft was mediated via larger muscles and thus via increased mechanical load. The hypothesis proved to be true, as the association between them vanished after including muscle CSA in the model (SNP: p = 0.192 and CSA: p < 0.001).

6 DISCUSSION

6.1 Why to study children?

The bones of children need to be of the size and shape appropriate for their age and strong enough to support current functional requirement. Too rapid as well as too slow growth may contribute to the incidence of bone fracture during childhood, which in turn can detrimentally affect the skeletal growth and mineral acquisition (Williams et al. 2000, Goulding et al. 2005, Julian 2005). However, in addition to these immediate needs, at the completion of growth the bones must be able to meet the lifetime load-bearing demands during the whole adulthood. Optimal bone growth during childhood and adolescence is thus necessary for the health, not just of the present child, but of the future adult. As the population is aging, our growing concern is the anticipated higher occurrence of fractures with related morbidity and mortality, as well as escalating health care costs. Hence our aim should be the prevention rather than treatment of the fragility fractures to make osteoporosis a disease of the past. However, without understanding the genetic and molecular mechanisms underlying the normal growth processes of children's bones, the diagnosis, prevention, and treatment of skeletal disorders and bone fragility is not feasible, at least not reasonable. The purpose of the work presented in this thesis was to increase our knowledge on the genetic basis of skeletal growth during puberty.

6.2 Why to study genetics?

Much of the vast diversity we see in people is governed by genetic factors that have quantitative effects on phenotype. Single nucleotide polymorphisms are major contributors to this genetic variation, comprising approximately 80 % of all know polymorphisms (The International SNP Map Working Group 2001). They are just single base changes in DNA sequence, with a usual alternative of two

possible nucleotides at a given position. It has been estimated that there are approximately 7 million common SNPs with a minor allele frequency of at least 5 %, and an additional 4 million SNPs with a minor allele frequency between 1 and 5 %, across the entire human population (Kruglyak & Nickerson 2001). Furthermore, there are innumerable very rare single base variants, most of which exist in only a single individual. The four SNPs selected into this work reside in genes whose biological function essentially relate to the structure and metabolism of bone. Furthermore, they are very common across the western population with a minor allele frequency of 15 % (*COL1A1*) and 42 % (*COL1A2, ER-α*, and *COMT*), making them suitable research objects in this relatively small cohort.

Single nucleotide polymorphisms that associate with a phenotype character may have a direct effect on the function of the gene in which they are located. A variant may result in an amino acid change or may alter exon–intron splicing, thereby directly modifying the relevant protein, or it may exist in a regulatory region, altering the level of expression or the stability of the mRNA. Alternatively a SNP may be in linkage disequilibrium with the true functional variant. Overall, it is estimated that each person is heterozygous for 24 000–40 000 substitutions that alter the amino acid (Cargill et al. 1999). It is the combined effect of several SNPs in key genes and environmental factors that determine which kind of skeleton an individual develops. Therefore, the contribution of each gene is likely to be relatively small, perhaps accounting for no more than a few percent of the overall variability of the trait. Considering such a modest genetic effect, sample sizes at the scale of thousands are generally needed to generate a reliable and robust conclusion. Very few study published so far has reach this level, and this is probably the major limitation of the present work as well (Liu et al. 2003). However, being historically isolated and genetically comparatively homogenous makes the Finnish population particularly useful for genetic studies (de la Chapelle 1993). The genetic homogeneity of such an isolated founder population should allow the utilization of smaller sample sizes than would be needed when studying more mixed outbred populations.

6.3 What is the bone phenotype?

Before we can relate the genetics with bone physiology we need to know what it comprises. Bone phenotype is a broad concept with several components. It can be roughly divided into structural, material, and metabolic properties. The structural properties of bone include its geometry, such as size and shape, as well as its micro architecture, such as trabecular architecture and cortical thickness and integrity. Trabecular architecture can be understood as the orientation, thickness, and spacing of the trabeculae, as well as the extent to which the trabeculae are interconnected. The material properties of bone include its mineral and collagen composition, but may be extended to cover the vast array of structural as well as catalytic proteins, hormones, and other

regulatory molecules present in the bone tissue either intra- or extracellularly. For example, the degree of mineralization, crystal size, the quality of the collagen matrix, and the mineral-to-matrix ratio all contribute to the overall phenotype of the skeleton. The metabolic properties of bone include all the activities occurring in the bone from the cell and molecular level, such as responses to different external as well as internal signals either chemical or mechanical by nature, up to the tissue level, such as bone turnover. Indeed, bone is not a static material, but a complex living tissue that undergoes constant renewal to repair the micro damage that occurs on daily basis. Osteoclasts resorb old and damaged bone tissue, which results in a resorptive cavity that is subsequently filled with new bone by osteoblasts. The new bone undergoes first a rapid primary and then a slower secondary phase of mineralization. As long as the rates of bone resorption and bone replacement are balanced, neither a net loss of bone nor an increase in damaged bone occurs. All these components are connected to each other in several ways, as the rate of bone turnover determined by several signals combined affect the material properties, which in turn contribute to the overall structure of bone. Given that most if not all of these components ultimately originate from the genes, we can understand that different genetic factors may well have varying implications on bone physiology and phenotype. That was found in the present work as well. However, a word of caution needs to be remarked that statistical evidence of association does not necessarily imply a causal relationship between the clinical manifestation and SNP at issue, as the functional impact and mechanistic pathways were not investigated in this work. Furthermore, well designed and carefully performed independent replications of the present findings needs to be addressed in future studies.

Even though the *COL1A1* and *COL1A2* genes both code for the chains of type I collagen, SNPs investigated associated with rather differing clinical manifestation. While the *COL1A1* SNP (I) seemed to have much stronger influence on bone that could be detected by radiographic, ultrasonic, as well as by biochemical methods, only a slight measurable effect was contributed by the *COL1A2* SNP (II). However, it is important to note that they both associated with material properties of bone as measured by ultrasound, suggesting an effect on bone quality, such as elasticity. With this regard, subtle changes in the organic matrix have previously been found to exert profound influences on bone elasticity and ultrasound velocity *in vitro* (Mehta et al. 1998). In contrast to previous studies, no association between the *COL1A1* SNP and fractures was found (Grant et al. 1996, Bernad et al. 2002). This may be, however, a fair adversity arising from the small number of girls with the TT genotype. Altogether, both genotypes may weaken the bone and increase the risk of fractures, as early as in the childhood.

As the environmental background may confound the interpretation of genetic findings, it may happen other way around as well. Indeed, the *ER-α* SNP was found to modulate the association between exercise and bone density (III, IV). The heterozygote girls seemed to be particularly vulnerable to the

adverse effects of physical inactivity and benefit most from the effect of exercise, while the homozygote girls may not gain any additional benefit from exercise. Another possibility is that the threshold for the effect to become evident is much lower in the homozygotes compared to the heterozygotes. In other words, the heterozygote girls may need to exercise more vigorously to gain the same positive effects for their bones as the homozygote girls obtain from far less intensive activity. As yet, there are few previous studies alike to compare with. Most of them are animal studies, such as mice and chicken studies, whose findings, however, are in agreement with the present ones (Kodama et al. 1999, Pitsillides et al. 1999, Robling & Turner 2002). Even though the individual responsiveness to exercise training is certainly influenced by several still undiscovered genes, these findings may contribute to this variation. Sometimes even unexpected can be found, such as the role of a unique mutation (Gly171Val) in the low density lipoprotein receptor-related protein 5 (LRP5) in bone biology (Boyden et al. 2002). It was recently found that transgenic mice expressing this particular mutation showed a greater sensitivity to loading resulting in an overadaptation of the skeleton to weight-related forces compared to their non-transgenic littermates (Akhter et al. 2004).

The functional *COMT* SNP was found to associate with elevated concentration of biologically active estrogen and thereby with earlier pubertal development (V). Early skeletal development does not necessarily mean rapid growth, but if so, the LL girls may face the associated complications such as increased risk of pubertal fractures. Due to their advanced maturation, girls with the low activity genotype were over 5 cm taller and had larger bones as well than those with the high activity genotype. The true implication of this, however, remains to be established, as there are contradictory opinions concerning the effects of pubertal timing on height gain. On one hand, late puberty with a longer period of childhood growth before the adolescent growth spurt may result in a taller adult stature (Garn et al. 1986). On the other hand, girls who begin menstruation early may grow much more and for a longer period after the menarche than girls with later onset of puberty (Iuliano-Burns et al. 2001). Since the skeletal growth has not yet terminated, conclusions can not be drawn at this moment concerning the relationships between the *COMT* SNP and adult stature and peak bone mass.

6.4 How to find the needles in a haystack?

Once we have described the various genotypes and phenotypes, we need to recognize which children may have problems with the bone growth as well as which genetic factors are clinically relevant markers for either skeletal disturbances or future risk of osteoporosis. We have to consider the frequencies of particular alleles and genotypes among different populations as well as the heterogeneity of linkage disequilibrium in case of non-causal mutation when

ranking the candidate genes for future clinical use. Too much can not be emphasized how important it is to ultimately discover the causal nucleotide variation and the molecular mechanism behind to produce the clinical manifestation. So far, no satisfactory answer can be given to the question of what phenotype or combination of phenotypes is the best for gene mapping of osteoporosis. It is also unclear what constitutes a "fracture threshold" in children. Based on the present as well as previous findings the *COL1A1* SNP may be one of the relevant markers for skeletal fragility and low bone mass both in childhood and adulthood. Without any further replication it would be too risky and hasty to suggest the three other SNPs investigated in the current work for the clinical use as markers for osteoporosis. However, the *COL1A2* SNP may be considered as a potential marker for bone fragility, especially if future studies will find it to relate with geriatric fractures as well. The *ER-*α SNP may have clinical implications in certain special cases. For instance, it could help to find such immobile patients who may be particularly vulnerable to bone loss due to their limited movement. Furthermore, the *COMT* SNP may have a considerable impact on the success of hormone replacement therapy. However, it is obvious that more than just a few genetic markers are needed for any kind of preventive or diagnostic use to preclude groundless judgements. Finally, in spite of the emergence of several highly promising novel methodologies, including the so called gene chips, the technology to perform routine scanning of multiple genes for subtle polymorphic variation in thousands of individuals in a cost-effective manner is still lacking.

Children are challenging research subjects, as they are transforming all the time on their way to the adulthood. Adolescents of a given chronological age may vary widely in physiological development, which, however, is still considered "normal". Knowing the relationship between the milestones of sexual development and physical growth will enable the assessment of the growth processes in individual children at a particular time and give some indication of the extent of future growth. There are different means to monitor the pubertal development, such as by using the weight-for-age, height-for-age, and BMI-for-age charts, as well as by using the Tanner stages. Furthermore, appropriate weights for height, according to age and sex, provide essential information concerning the child's growth. The major cause of short stature during adolescence is genetically late initiation of puberty, although conditions such as chronic diseases as well as skeletal and chromosomal abnormalities also account for certain children being shorter than normal. Hormonal imbalances leading to abnormal growth are rare (Strasburger & Brown 1991). However, it should be remembered that not all short children have abnormal growth or problems that may have long-lasting effects on health if not treated. On the contrary, normal growth in height does not necessarily imply normal accumulation of bone mass. Careful evaluation of the whole clinical picture should be made in order to differentiate between primary and secondary bone diseases. Most children with chronic diseases are at risk for inadequate accumulation of bone mass, either from the disease process itself, its therapy, or

from associated problems, such as inadequate nutrition or immobility. Moreover, a child may experience fairly normal growth during her/his adolescence, but may still be at risk for osteoporosis in older age. The possibility that different genotypes may associate with bone accumulation than with bone loss must be taken into consideration when assessing skeletal health.

Another challenging issue is the interpretation of the bone assessment data in children. Dual-energy X-ray absorptiometry is the current preferred clinical tool for measuring paediatric bone mass, despite its several limitations. Even though greatly developed and improved its versatility since the first devices, DXA results should be always interpreted in light of several potential confounding factors, such as bone size, skeletal age, pubertal maturation, and body composition. Moreover, well-established paediatric normal values are still lacking in many countries including Finland. Quantitative computed tomography circumvents the artefact of bone size and provides a real three-dimensional measurement of the bone, but requires a higher radiation dose than DXA and is limited only to a very few clinics. Ultrasound, on the other hand, is a less precise technique with limited normative data. Moreover, we still do not know at this moment what it really measures. Assessments of serum and urinary markers of bone turnover can help in the evaluation of children with suspected alterations in the normal dynamics of bone mass accumulation. They reflect current rates of bone formation and bone resorption, and may also reveal dissociated bone disease that is so detrimental to integrity of the skeleton in children. Levels of these markers change more rapidly than bone mass, facilitating short-term longitudinal observation. However, their diurnal and daily variability is significant, especially in growing children (Schonau & Rauch 1997). Because of these limitations, bone turnover markers are useful mainly as a research tool.

When all is said and done, we will found ourselves surrounded by a vast amount of information, both genetic and phenotypic. We will never find disastrous SNPs with high population frequency due to the high evolutionary selection pressure on nucleic acids that may affect protein function. Sequence diversity is much higher among non-coding, synonymous, and conservative regions than among critical sites (Tiret et al. 2002). Furthermore, the mutation frequency is essentially proportional to its age. Rare mutations on average have a more recent origin than common polymorphisms. Therefore, linkage disequilibrium is also more extensive around them, as there has been less time for recombination to break down the SNP couplings. Fortunately, the same holds true for the clinical manifestations, as there is a continuum of skeletal phenotypes with more or less favourable features but still fulfilling the needs of lifelong activities, and only a few with extensive bone abnormalities. However, even healthy children may fall short of optimal bone health because of current lifestyle trends, requiring our attention and hasty action. Hopefully the present work provides some relevant pieces for the big puzzle that will ultimately contribute to the future challenge of dragging out the essential things and bringing the scientific discoveries closer to the clinical practise to truly benefit deserving people.

7 CONCLUSIONS

1. *COL1A1* polymorphism associates with low bone mass and density, as well as with low bone material properties, via imbalanced collagen metabolism and uncoupled bone turnover in pubertal girls (I). *COL1A2* polymorphism associates with increased risk of paediatric fractures independently of bone density. It also associates with low axial bone mass and density, as well as with low material properties of limb bones at prepuberty, before the fast pubertal growth spurt (II).

2. *ER-*α polymorphism by itself has no major effects on pubertal bone development (III). *COMT* polymorphism associates with early sexual and musculoskeletal development, via elevated concentration of estrogen (V).

3. *ER-*α polymorphism modulates the effect of exercise on bone density at loaded bone sites during puberty (III, IV).

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YHTEENVETO (Résumé in Finnish)

Osteoporoosi eli luukato on luuston sairaus, jossa luun määrä vähenee ja rakenne muuttuu huokoiseksi. Luuston haurastumisen myötä kasvaa riski saada luunmurtuma yhä helpommin. Osteoporoosia on pidetty erityisesti ikäihmisten vitsauksena, mutta sitä tavataan myös lapsilla, jolloin se tyypillisesti puhkeaa murrosiän kasvupyrähdyksen aikana. Väestön ikääntymisen ja fyysisen passivoitumisen myötä osteoporoosista on muodostunut merkittävä kansanterveydellinen ongelma niin Suomessa kuin myös muissa hyvinvointivaltioissa. Arvioiden mukaan noin 400 000 suomalaisella eli lähes joka kymmenennellä voidaan todeta osteoporoosia. Koska luuston vahvistaminen lääkkeillä on varsin kallista ja luunmurtumasta toipuminen vaatii pitkän ajan, on osteoporoosin varhainen ehkäisy erityisen keskeistä. Huomio tulisi kiinnittää lapsiin, sillä valtaosa luuston lopullisesta massasta ja rakenteesta luodaan nimenomaan varhaisnuoruudessa. Lapsen kasvu vaikuttaa näin ollen ratkaisevasti hänen koko elinaikaiseen osteoporoosiriskiin.

Perintötekijöiden vaikutus luuston kasvuun on kiistaton. Niiden arvioidaan selittävän keskimäärin 80 % luun huippumassan vaihtelusta. Luuston vahvuus on monen tekijän summa ja siihen vaikuttavat useat eri geenit, joilla todennäköisesti on vielä vuorovaikutuksia niin toistensa kuin myös elintapojen ja ympäristötekijöiden kanssa. Luumassan normaali yksilöidenvälinen vaihtelu ei niinkään johdu yksittäisten geenien vahingollisista mutaatioista, vaan niiden yleisistä monimuotoisuuksista eli polymorfioista, jotka aiheuttavat lieviä muutoksia geenien ilmenemiseen tai niiden tuottamien proteiinien rakenteeseen. Aikuisväestössä geenitutkimuksia on toteutettu runsain määrin, mutta lapsitutkimuksia on vielä valitettavan vähän.

Tämän tutkimuksen tavoitteena oli parantaa käsitystämme lasten luuston kasvuun vaikuttavista perintötekijöistä. Tarkoituksena oli selvittää neljän luuston rakenteen ja aineenvaihdunnan kannalta olennaisen geenin monimuotoisuuden yhteyttä murrosikäisten tyttöjen luusto-ominaisuuksiin sekä lapsuuden luunmurtumiin. Lisäksi haluttiin selvittää, onko perintötekijöillä mahdollisesti vuorovaikutuksia luustolle suotuisten elintapojen, tässä tapauksessa liikuntaharjoittelun kanssa. Tutkimukseen valituista geeneistä kaksi tuottaa luun elollisen aineksen pääproteiinia, tyypin I kollageenia (*COL1A1* ja *COL1A2*) ja kaksi liittyy luustolle tärkeän hormonin, estrogeenin toimintaan (*ER-α*) sekä aineenvaihduntaan (*COMT*). Kollageenisäikeet antavat luustolle vetolujuutta ja sitkojoustavuutta. Aminohappomuutoksiin johtavat mutaatiot tyypin I kollageenia tuottavissa geeneissä lähes poikkeuksetta aiheuttavat vakavaa synnynnäistä luustosairautta, osteogenesis imperfektaa. Sairauden oireet voivat olla hyvinkin lieviä, mutta vaikeasti hauras luusto voi johtaa jopa vauvan kuolemaan pian syntymän jälkeen. Oireiden vakavuusaste riippuu niin aminohappomuutoksen laadusta kuin myös mutaation sijainnista geenissä. Luuston kasvua ja aineenvaihduntaa säätelee voimakkaasti estrogeeni. Sen ansiosta muun muassa luiden kasvulevyt sulkeutuvat, jolloin nuoren pituuskasvu päättyy. Mutaatio *ER-α*-

geenissä voi aiheuttaa estrogeeniresistenssiä, jolloin hormonin sitoutuminen reseptoriin estyy ja elimistön kyky käyttää estrogeeniä heikentyy. Tällaisille henkilöille on ominaista alhainen luuntiheys sekä aikuisikään saakka jatkuva pituuskasvu. Tuoreiden tutkimustulosten mukaan ER-α paitsi välittää estrogeenin vaikutuksia solun sisään, myös osallistuu siihen viestintäreittiin, johon luusolujen kyky aistia mekaanista kuormitusta perustuu. Hiirikokein on voitu osoittaa, että toimiva ER-α on välttämätön, jotta luusto pystyy mukautumaan aina kulloiseenkin ulkopuolelta kohtaamaansa kuormitukseen. Katekoli-*O*-metyylitransferaasi on entsyymi, joka hajottaa katekoliamiineja, kuten välittäjäaineina toimivia dopamiinia, adrenaliinia ja noradrenaliinia, minkä vuoksi *COMT*-geeniä on paljon tutkittu erilaisten hermostollisten häiriötilojen ja psykiatristen sairauksien yhteydessä. Kyseinen proteiini hajottaa myös katekoliestrogeenia, joten sen aktiivisuus vaikuttaa veren estrogeenipitoisuuteen ja täten mahdollisesti myös luuston aineenvaihduntaan.

Tässä tutkimuksessa osoitettiin *COL1A1*-geenin polymorfian olevan yhteydessä tyttöjen luuston alhaiseen massaan ja tiheyteen kuin myös heikentyneeseen materiaaliominaisuuteen (I). Biokemialliset löydökset viittasivat kollageenin epäsuhtaiseen aineenvaihduntaan ja luuston häiriintyneeseen vaihtuvuuteen, mikä saattaa haitallisesti vaikuttaa luuston hienorakenteeseen ja vahvuuteen. Tämä saattaa selittää myös aikaisemmissa tutkimuksissa tehtyjä havaintoja kyseisen polymorfian ja osteoporoottisten luunmurtumien välisistä yhteyksistä. Tutkimuksessa löydettiin merkittävä yhteys myös *COL1A2*-geenin polymorfian ja luuntiheydestä riippumattoman murtumariskin välillä (II). Lisäksi polymorfia liittyi esimurrosikäisten tyttöjen selkärangan alhaiseen massaan ja tiheyteen sekä raajaluiden heikkoon materiaaliominaisuuteen. Kyseistä yhteyttä ei voitu havaita varhaismurrosikään edenneillä tytöillä, mahdollisesti heidän voimakkaan kasvupyrähdyksen takia. Vaikka *ER-α*-geenin polymorfialla itsellään ei havaittu olevan merkittävää vaikutusta luuston kasvuun (III), se muunsi tyttöjen liikuntaharjoittelun ja luuntiheyden välistä yhteyttä (III, IV). Eriperintäiset tytöt näyttivät olevan erityisen herkkiä ruumiillisen joutilaisuuden aiheuttamille epäsuotuisille vaikutuksille ja hyötyvän parhaiten liikunnasta, kun taas samaperintäiset tytöt eivät näyttäneet saavan lisähyötyä liikuntaharjoittelusta. On myös mahdollista, että kynnys havaittaville luustovaikutuksille on samaperintäisillä tytöillä matalampi kuin eriperintäisillä tytöillä. Tämä tarkoittaisi sitä, että eriperintäisten tulisi liikkua rivakammin saavuttaakseen samat hyödyt kuin samaperintäiset saavat paljon keveämmästä liikunnasta. Tutkimuksessa osoitettiin lopuksi *COMT*-geenin polymorfian vaikuttavan veren estrogeenipitoisuuteen ja tätä kautta aikaistavan murrosiän puhkeamista. Tytöt, joilla oli heikon entsyymiaktiivisuuden tuottava geeni, olivat merkittävästi pidempiä sekä ylipäätään lihaksistoltaan ja luustoltaan kehittyneempiä verrattuna tyttöihin, joiden geeni tuotti tehokkaasti toimivan entsyymin. Nähtäväksi jää, onko tällä varhaistuneella sukukypsyydellä ja kehittymisellä merkittävää vaikutusta siihen, kuinka vahvaksi nuoren luusto lopulta kehittyy hänen saavuttaessaan aikuisiän.

Näiden tulosten pohjalta ymmärrämme perintötekijöiden merkitystä lasten luuston kasvussa hieman aiempaa paremmin. Osteoporoosin sukurasitus ei rajoitu koskemaan pelkästään vanhuusiän perinnöllistä luukatoa, vaan vaikutus saattaa ilmetä jo lievinä kasvuhäiriöinä lapsuusiässä. Tietyt perintötekijät saattavat myös altistaa lapsuuden luunmurtumille, jotka omalta osaltaan voivat hidastaa nuoren kasvua aiheuttaen mahdollisesti jopa pysyviä luustovaurioita. Vaikka kunkin yksittäisen geenin vaikutus luuston ilmiasuun on ehkä vain muutaman prosentin luokkaa, geenitutkimuksien hyödyt tulevaisuuden terveydenhuollolle ja lääketeollisuudelle ovat korvaamattomia, sillä niistä voi olla apua niin lasten luustosairauksien kuin myös vanhuuden osteoporoosin diagnosoinnissa, ennaltaehkäisyssä ja hoitovasteessa.

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ORIGINAL PAPERS

I

COL1A1 Sp1 POLYMORPHISM ASSOCIATES WITH BONE DENSITY IN EARLY PUBERTY

by

Suuriniemi, M., Kovanen, V., Mahonen, A., Alén, M.,
Wang, Q., Lyytikäinen, A. & Cheng, S

(submitted)

COL1A1 Sp1 polymorphism associates with bone density in early puberty

Miia Suuriniemi,[1,2] Vuokko Kovanen,[2] Anitta Mahonen,[3] Markku Alén,[4] Qingju Wang,[2] Arja Lyytikäinen,[2] and Sulin Cheng[2]

[1]Department of Cell Biology, University of Jyväskylä, Finland
[2]Department of Health Sciences, University of Jyväskylä, Finland
[3]Department of Medical Biochemistry, University of Kuopio, Finland
[4]Kuopio Research Institute of Exercise Medicine, Finland

Running title: *COL1A1*, bone density, and children

Corresponding author:
Miia Suuriniemi
University of Jyväskylä
Department of Health Sciences
P.O. Box 35
FI-40014 University of Jyväskylä, Finland

Tel: +358-14-260 2068
Fax: +358-14-260 2011
E-mail: miisu@sport.jyu.fi

ABSTRACT

Optimal acquisition of bone mass in puberty is a key determinant of the lifetime risk of osteoporosis and has a strong genetic basis. We investigated the relationship between the *COL1A1* Sp1 polymorphism and BMD in early puberty, and how the genotypes relate to bone size and geometry as well as bone turnover and material properties in 247 10-13 yr-old girls. Bone properties were measured using DXA, pQCT, and ultrasound. Also, serum P1NP, OC, B-ALP, and TRACP 5b were assessed. Our results showed that girls with the TT genotype had significantly lower BMC and BMD of the total body, lumbar spine, and proximal femur, as well as BUA at the calcaneus, than those with the GT and GG genotype. They also had significantly lower B-ALP, as well as P1NP/TRACP 5b and (OC+B-ALP)/TRACP 5b, compared to the others. These findings indicate that the *COL1A1* polymorphism is associated with low bone properties in early puberty, and suggest a possible physiological effect on collagen metabolism and bone turnover. This information may contribute to the identification of children that may be at risk for suboptimal acquisition of peak bone mass, and may ultimately be of value in the planning of early preventive strategies for osteoporosis.

Key words: COL1A1, Sp1 SNP, bone mineral density, bone turnover, fracture, puberty

INTRODUCTION

Optimal acquisition of bone mass in puberty is a key determinant of the lifetime risk of osteoporosis. Approximately 60-80% of the variance in peak bone mass has been attributed to heritable factors (Sambrook et al. 1996), which has challenged researchers to search for the candidate genes. However, studies in older adults outnumber those in young people, despite the likelihood that genetic influences have a greater effect on bone mineral acquisition than on loss (Eisman 1999). One of the most important genes for predisposition to osteoporosis is the *COL1A1* gene, which encodes the α1(I) protein chain of type I collagen, the major protein of bone. There is a single nucleotide polymorphism (SNP) affecting an Sp1 binding site within the first intron of the *COL1A1* gene, which has functional effects on collagen gene regulation. This polymorphism leads to abnormal production of the α1(I) collagen chain relative to α2(I) (Mann et al. 2001), which raises the possibility that some of the collagen may be present in the form of [$\alpha1(I)_3$] homotrimers, instead of the normal [$\alpha1(I)_2\alpha2(I)$] heterodimers. Previous studies have shown the association between the presence of type I collagen homotrimers in bone and impaired mechanical strength of bone (Chipman et al. 1993; Saban et al. 1996; McBride et al. 1998), and homotrimers have been found from osteogenesis imperfecta (Deak et al. 1985) and osteoarthritis (Bailey et al. 2004) patients. Moreover, there is an increasing body of evidence to suggest that the *COL1A1* polymorphism is related to reduced bone mineral density (BMD) and an increased risk of osteoporotic fractures in old age (Grant et al. 1996; Garnero et al. 1998; Langdahl et al. 1998; Roux et al. 1998; Uitterlinden et al. 1998; Keen et al. 1999; McGuigan et al. 2000; Weichetova et al. 2000; Mann et al. 2001; Bernad et al. 2002). However, there is only two studies conducted in children, and they ended up in rather contrasting conclusions (Sainz et al. 1999; Willing et al. 2003). To determine whether the association between the *COL1A1* polymorphism and BMD is manifested in early puberty, and how the genotypes relate to bone size and geometry as well as to bone turnover and material properties, we conducted a study with diversified assessments in pre- and early pubertal Finnish girls.

SUBJECTS AND METHODS

Subjects

The study subjects were 258 (247 for the genetic analysis) healthy pre- and early pubertal Finnish girls, aged 10-13 years, who were recruited for an intervention study (the Calex-study) to evaluate the effects of calcium, vitamin D, and milk products´ supplementation on bone mass accrual (Cheng et al. 2003; Suuriniemi

et al. 2003; Suuriniemi et al. 2004; Wang et al. 2004). To be eligible for the study, the participants had to have no history of serious medical conditions and no history of medication known to affect bone metabolism. The sexual development was determined by a public health nurse using the Tanner grading system (Tanner 1978).

Energy and nutrient intakes were analyzed from a 3-day food diary by the Finnish Micro-Nutrica PC-software. Level of physical activity was assessed by a self-reported questionnaire including items on the frequency, duration, and types of exercise done during the subject's leisure time (Hickman et al. 2000). The data of physical activity is provided in terms of hours per week. Fracture history was clarified by a parents-reported questionnaire including items on the site, time, and cause of each fracture, and confirmed by hospital medical records. Girls with a fracture history of less than one year were excluded from the study. Fractures caused by severe trauma (e.g. serious accidents and crushed fingers/toes) were excluded from this analysis. The investigational protocol was approved by the ethical committees of the University of Jyväskylä, the Central Hospital of Central Finland, and the Finnish National Agency of Medicines. An informed consent was obtained from all subjects and their parents prior to the assessments. The results presented in this report are from the baseline assessments, from those girls who have the genetic data available (n = 247).

Body composition assessments

Body weight was determined (± 0.1 kg) using a calibrated scale and body height (cm) was measured with a fixed-scale measuring device. Sitting height was defined from the total body dual-energy X-ray absorptiometry (DXA) scan as the distance between the vertex and a line parallel to the lower edge of the tuber ischiadicum. The results of body weight and height were used to determine body mass index (BMI), expressed as [weight (kg) / height^2 (m)]. Body weight and height were highly correlated with each other, and they were therefore reduced to one body size index using principal components factor analysis. This index was used for adjustment, instead of body weight and height, to avoid double-control. The fat and lean tissue masses of the total body were analyzed from the total body DXA scan.

Bone measurements

Bone area (cm), bone mineral content (BMC, g), and areal bone mineral density (aBMD, g/cm^2) of the total body, lumbar spine (L2-L4), and left proximal femur (total femur and femoral neck) were measured using DXA (Prodigy, GE Lunar Corp., Madison, Wisconsin, USA). The percentage coefficient of variation (%CV) for repeated measurements ranged from 0.6 to 1.2 for BMC and from 0.9 to 1.3 for aBMD at the different bone sites.

Volumetric BMD (vBMD, mg/cm^3), cortical thickness (CTh, mm), and cross-sectional area (CSA, mm^2) were measured using peripheral quantitative

computed tomography (pQCT, XCT 2000, Stratec Medizintechnik, GmbH, Pforzheim, Germany). We scanned the left distal radius (4% of the total length of the forearm medial to the reference line; the length of the forearm was defined as proceeding from the olecranon to the ulna styloid of the subject's arm with the elbow at 90° and fingers held up) and tibia shaft (60% of the lower leg length between the tuberositas tibia and the medial malleolus). Data were then analysed using BonAlyse software (BonAlyse Oy, Finland) (Cheng et al. 2000). The %CV was < 3% for CSA and < 1% for vBMD.

Broadband ultrasound attenuation [BUA, dB/MHz (n = 217)] of the left calcaneus was measured using a gel coupling scanning quantitative ultrasonometer (QUS-2, Quidel Corporation, Santa Clara, CA, USA) (Cheng et al. 1999). The speed of sound [SOS, m/s (n = 217)] at the distal third of radius (medial surface) and the midshaft of tibia (anteromedial surface) were determined by a mobile Omnisense instrument (Sunlight Technologies Ltd, Rehovot, Israel) (Barkmann et al. 2000). The %CV was < 1.2% for BUA and < 1.6% for SOS.

Biochemical assessments

Fasting morning blood samples were collected for determinations of bone formation [aminoterminal propeptide of type I procollagen (n = 100), osteocalcin (n = 217), and bone-specific alkaline phosphatase (n = 217)] and bone resorption [bone-specific tartrate-resistant acid phosphatase (n = 217)] markers. The serum aminoterminal propeptide of type I procollagen (P1NP) level was measured by a competitive radioimmunoassay (P1NP, Orion Diagnostica, Mountain View, Espoo, Finland) (Melkko et al. 1996); the intra- and inter-assay %CVs were 3.6% and 1.9%, respectively. The serum osteocalcin (OC) level was measured by an in-house immunoassay (Käkönen et al. 2000); the intra- and inter-assay %CVs were 2.7% and 8.1%, respectively. The serum bone-specific alkaline phosphatase (B-ALP) activity was measured by an immunoassay (Alkphase-B, Metra Biosystems Inc., Mountain View, CA, USA) (Gomez et al. 1995); the intra- and inter-assay %CVs were 1.9% and 1.8%, respectively. The serum bone-specific tartrate-resistant acid phosphatase (TRACP 5b) activity was measured by a specific immunoassay (BoneTRAP, SBA-Sciences, Oulu, Finland) (Halleen et al. 1996; Halleen et al. 1998; Halleen et al. 2000); the intra- and inter-assay %CVs were 2.7% and 5.7%, respectively. In addition, the ratio of formation of type I collagen and resorption of bone was expressed as P1NP/TRACP 5b and the ratio of formation and resorption of bone was expressed as (OC+B-ALP)/TRACP 5b.

Genetic analysis

Genomic DNA was extracted and purified from EDTA blood samples using QIAmp Blood Kit (Qiagen GmbH, Hilden, Germany). For a capillary cycle sequencing reaction (CCSR), the *COL1A1* gene carrying the polymorphic site

(refSNP ID: rs1800012) was amplified by polymerase chain reaction (PCR) using the forward primer 5′-AAAGTGACCTGGAGGCATTG-3′ and reverse primer 5′-CTTCCAACTCCAACCTCAGC-3′. The 15 µl PCR reaction mixture consisted of 20 ng genomic DNA, 50 mM KCl, 15 mM TRIS-HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.2 mM each of the four deoxyribonucleotides, 0.2 pM of each primer, and 0.38 unit of Ampli*Taq* Gold DNA polymerase (Applied Biosystems). Amplification was carried out in a DNA thermocycler (PTC-225, MJ Research). After amplification, the PCR products were purified according to *ExoI*-SAP protocol and combined with the primers and BigDyeTerminator v3.1 Cycle Sequencing Kit (Applied Biosystems). The amplified CCSR products of 390 bp length were further purified by Sephadex G-50 Superfine Multiscreen Plate (Millipore) and run through a capillary gel electrophoresis (3730 DNA Analyzer, Applied Biosystems) in order to assemble the DNA sequences, from which the genotypes were determined using a Sequencher 4.1.4 software (GeneCodes).

Statistical analysis

Statistical analyses were carried out using the Statistical Package (SPSS) version 12.0 for Windows. The conformance of the *COL1A1* allele frequencies to Hardy–Weinberg equilibrium proportions was tested by using a χ^2 test (Weir 1990). One-way analysis of variance (ANOVA) was used to test the differences in physical characteristics, except fat and lean mass, as well as in nutrient intakes and physical activity with respect to the genotypes. Differences in bone properties, fat and lean mass, and bone turnover markers among the different genotypes were tested using analysis of covariance (ANCOVA), controlling for the effect of body size index. The distributions of P1NP, OC, TRACP 5b, P1NP/TRACP 5b, and (OC+B-ALP)/TRACP 5b were normalized by transformation to their natural logarithm to achieve the assumption of normal distribution of residuals. For multiple comparisons least significant difference (LSD) post hoc test or, when assuming heterogeneous variances, Dunnett's T3 post hoc test was used. Homogeneity of variances was confirmed with a Levene test. A p value of less than 0.05 was considered statistically significant, and a trend was set at a p value of less than 0.10.

RESULTS

General characteristics

The genotype distribution was found to be in Hardy-Weinberg equilibrium, suggesting that the subjects represented a homogenous genetic background. The frequencies of the GG, GT, and TT genotypes were 70.9, 27.5, and 1.6%. No significant differences in physical characteristics, developmental status, nutrient intakes, or physical activity were found between the COL1A1 genotypes (Table

1). There were 34 girls who had sustained a fracture resulted from minimal to moderate trauma (by convention, the equivalent of a fall from standing height or less). Twelve percent of the GG girls and nineteen percent of the GT girls had a positive fracture history.

Bone mass and areal density

Bone mass and density parameters of the subjects are shown in Table 2. Girls with the TT genotype had significantly lower bone areas of the total body and femoral neck than those with the GT (p = 0.011 and 0.042, respectively) and GG (p = 0.014 and 0.047, respectively) genotype. No differences were found in the bone areas of the total femur or lumbar spine between the genotypes. However, girls with the TT genotype had significantly lower BMC of the total body, total femur, and lumbar spine than those with the GT (p = 0.001, 0.022, and 0.044, respectively) and GG ($p \leq$ 0.011, 0.031, and 0.032, respectively) genotype. In addition, areal BMD of each of the measured bone site was significantly lower in the TT girls compared to the others (p = 0.002 – 0.032).

Bone geometry and volumetric density

Bone geometrical and density parameters of the subjects are shown in Table 3. No significant differences were found in CSA, CTh, or vBMD of the tibia shaft or distal radius between the genotypes. However, trabecular bone density (tr. vBMD) of the distal radius tended to be lower in girls with the TT genotype than those with the GT (p = 0.079) and GG (p = 0.059) genotype.

Bone material properties

Bone ultrasound parameters of the subjects are shown in Table 4. While SOS at the tibia shaft and distal radius was similar between the different genotypes, BUA at the calcaneus was significantly lower in girls with the TT genotype compared to the others (p = 0.008 and 0.016).

Bone turnover markers

Bone turnover parameters of the subjects are shown in Table 5. Of the bone formation markers, no significant differences were found in serum P1NP concentration between the genotypes. However, girls with the TT genotype tended to have lower serum concentration of OC than those with the GT (p = 0.076) and GG (p = 0.089) genotype. Furthermore, serum concentration of B-ALP was significantly lower in the TT girls compared to the others (p = 0.037 and 0.027). Concentration of the bone resorption marker, serum TRACP 5b, was similar in the different genotype groups. However, the ratio of formation of type I collagen and resorption of bone, expressed as P1NP/TRACP 5b, was significantly lower in girls with the TT genotype than those with the GT genotype (p = 0.034). The difference between the TT genotype and GG genotype

was at borderline (p = 0.061). Accordingly, the ratio of formation and resorption of bone, expressed as (OC+B-ALP)/TRACP 5b, was significantly lower in the TT girls compared to the others (p = 0.016 and 0.031).

DISCUSSION

This is the first study to show a striking association between the Sp1 polymorphism in the *COL1A1* gene and bone mass and density as well as bone material properties in early puberty. The TT genotype was associated with a decreased BMC, BMD, and BUA, while the bone areas were only a slightly smaller compared to the other genotypes. Furthermore, bone volumetric density and geometrical properties were slightly lower in girls with the TT genotype compared to the others. The differences between the genotypes did not, however, reach the statistical significance, which suggests that the *COL1A1* polymorphism affects more bone mass than bone size, and does not dramatically disturb the accurate and fine biological control system for keeping bone volumetric density fairly stable over time, regardless of large changes in bone size and mass during growth.

The results of the present study are even more interesting when considering the fact that the TT girls were a little older as well as heavier and taller than the other girls, which let us to expect them to have bigger bones as well. Meanwhile, the extra weight of the body of the TT girls seemed to derive from a slightly increased fat mass, not from muscle or bone mass. Moreover, if the comparisons between the genotypes were made ignoring the effect of the body size, the differences disappeared, except in trabecular bone density of the distal radius, B-ALP, P1NP/TRACP 5b, and (OC+B-ALP)/TRACP 5b (p = 0.036 – 0.094). Our results are in agreement with a rather small study of 6-12 yr-old Mexican-American girls (n = 109; GG = 86, GT = 22, TT = 1), where Sainz and co-workers found the *COL1A1* polymorphism to explain some of the variability in spine BMD (Sainz et al. 1999). On the other hand, Willing et al. did not identify any association between the *COL1A1* polymorphism and BMC/BMD in a cohort of 428 non-Hispanic white girls and boys of ages 4.5-6.5 years (Willing et al. 2003). It has been suggested that differences in dietary calcium intake may explain the controversial findings observed in these studies. A study of Brown et al. found that the COL1A1 genotypes influence BMD through effects on rate of bone loss, and those effects varied depending on the dietary calcium intake (Brown et al. 2001).

Bone quality assessed by ultrasound and a potential association to collagen polymorphisms has been studied to a lesser extent, despite the theoretical attractiveness of collagen as a major contributing factor to bone material properties. Kann and co-workers reported an association between the *COL1A1* polymorphism and SOS at the calcaneus in 740 women of ages 55-80 years (Kann et al. 2002), while three studies (Ashford et al. 2001; Gerdhem et al.

2003; Pluijm et al. 2004), each of elderly women, found no association at all. Interestingly, a meta-analysis of 16 studies reported the COL1A1 genotype-dependent increased fracture risk to be largely independent of the genotype-dependent differences in BMD (Mann et al. 2001). This strongly suggests an association of the *COL1A1* polymorphism to skeletal factors determining bone strength other than BMD. The results of our study demonstrate an association between the *COL1A1* polymorphism and material properties of bone as measured by ultrasound attenuation at the calcaneus. Importantly, the genotype-dependent differences in BUA values remained significant after adjusting for femoral neck BMD or tibial vBMD. Furthermore, after adjusting for tibial CSA and CTh the differences slightly declined but still remained significant. However, these attributes are not site-matched but still highly correlated with calcaneal BUA. Altogether, these findings evince that the effects of genotypes on BUA is partly independent from their effects on bone density. On the other hand, SOS values of the tibia shaft and distal radius were rather similar between the different genotypes, which may be a consequence of several factors. The SOS was measured at 1.25 MHz, corresponding to a wavelength of approximately 3 mm in bone, and thus prevents the signal from penetrating any deeper into the cortical layer (Sievänen et al. 2001). Therefore, SOS can be expected to depend mostly on cortical vBMD, as found indeed, which was not related to the *COL1A1* polymorphism.

Consistent with the bone properties girls with the TT genotype had lower levels of bone formation markers as well as higher levels of bone resorption markers, even though, most of them did not reach the statistical significance, probably due to the small sample size. The difference in the levels of biomarkers can not be explained by the slight difference in age of the TT girls, since they were still almost two years ahead of their menarche age (13.4 ± 0.8), when the biomarkers are known to start to decline until they reach the values observed in adults (Blumsohn et al. 1994). A possible functional significance associated with the *COL1A1* polymorphism is suggested by the observation that the relative formation of bone compared to its resorption was significantly lower in the TT girls compared to the others. P1NP/TRACP 5b of the TT girls was only some half of those in the other girls, suggesting the existence of some kind of imbalanced collagen metabolism and uncoupled bone turnover associated with the TT homozygosity. This, in turn, seems to disturb the mineralization process by causing a decreased B-ALP activity and ultimately, the lower bone density as presented before. This is not surprising when considering the fact that it is the collagen matrix that serves as a ground for attachment of mineral crystals in the bone. The lower serum OC concentration of the TT girls may indicate a decrease in bone radial growth as manifested by their rather small diameter. Interestingly, bone longitudinal growth seems to be unaffected by the genotype, since the body height as well as sitting height were fairly normal. However, interpretation of the results of bone turnover markers is very difficult because they depend on age, pubertal status, growth velocity, mineral accrual, hormonal regulation, and day-to-day variation. Moreover, how

each of the biomarkers reflects the stages of linear growth and mineral accrual, is far from understood.

In contrast to several previous studies (Grant et al. 1996; Langdahl et al. 1998; Keen et al. 1999; Bernad et al. 2002) we were unable to find an association between the *COL1A1* polymorphism and fractures. On the other hand, in some studies patients with fractures did not differ from controls with regard to the *COL1A1* polymorphism (Liden et al. 1998; Uitterlinden et al. 1998; Hustmyer et al. 1999; Välimäki et al. 2001). One important thing to be born in mind is that our subjects were healthy children while the previous studies have focused on older, mainly postmenopausal women with osteoporotic fractures, which make it very difficult to compare these studies. Another explanation for the discrepancy may reside in the limited power of the present study. There were only four girls with the TT genotype, none of them having a fracture, while 21 of the 174 girls with the GG genotype and 13 of the 68 girls with the GT genotype had a fracture. This may be a fair adversity and larger cohorts are required to identify possible association between the genotypes and childhood fractures.

We chose a direct DNA sequencing for the determination of the COL1A1 genotypes to overcome the difficulties associated with the traditional methods for their detection. The apparent frequency of the *T* allele of 0.154 is somewhat lower than the 0.18 – 0.22 observed for other European populations (Grant et al. 1996; Garnero et al. 1998; Langdahl et al. 1998; Uitterlinden et al. 1998), but much similar to the 0.136 frequency reported earlier for Finnish population (Vinkanharju et al. 2001). According to public SNP database, there is supposed to be four other SNPs (rs2696252, rs2696253, rs2696254, and rs2696255), in addition to the Sp1 SNP, within the 390 bp product that we sequenced. However, they have been identified as candidate SNPs by aligning clone overlaps from available finished and unfinished public human genomic sequences and by detecting high quality base discrepancies. Furthermore, they have not been validated, and should therefore be considered as suspicious. In fact, we found a homologous DNA region elsewhere which may have led on to a misalignment of the different clones, and most importantly, we found none of these four sites to be polymorphic among our study sample, which strongly impugns their existence. On the other hand, we identified a novel SNP 134 bp towards the 5'-end from the Sp1 polymorphic site. However, only one subject in our study sample had a differing genotype which appeared as a heterozygote, and the bone properties of that particular girl seemed fairly normal suggesting the SNP to be either a silent or recessive one.

The major limitation of the present study is the small number of the TT genotypes, which may have led on to a systematic error as well as expose to heterogeneous variances of the measured characters. In fact, we found the variances of CTh and TRACP 5b not to be equal among the genotypes, so we used Dunnett's T3 post hoc test for multiple comparisons as well. However, the results were comparable with those when LSD post hoc test was used, suggesting that this is not a major concern undermining the reliability of our study.

In conclusion, the results of this study indicate that the *COL1A1* Sp1 polymorphism is associated with low BMC and BMD as well as with low BUA in pre- and early pubertal girls. Furthermore, the results of bone biomarkers suggest a possible physiological effect toward imbalanced collagen metabolism and uncoupled bone turnover. This information may contribute to the identification of a subset of children that may be at risk for suboptimal acquisition of peak bone mass, and may ultimately be of value in the planning of early preventive strategies for osteoporosis.

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Table 1. Characteristics according to COL1A1 genotypes

	GG (n = 175)	GT (n = 68)	TT (n = 4)
Age (yr)	11.2 ± 0.8	11.2 ± 0.8	11.5 ± 0.7
Weight (kg)	38.9 ± 8.4	38.7 ± 8.5	44.2 ± 7.6
Height (cm)	145.3 ± 8.0	145.7 ± 8.2	151.8 ± 6.1
Sitting height (cm)	74.5 ± 4.2	74.5 ± 4.2	77.1 ± 2.2
BMI (kg/m^2)	18.3 ± 2.8	18.1 ± 2.8	19.1 ± 2.3
Tanner stage (1/2/3 %)	50 / 45 / 5	59 / 37 / 4	25 / 75 / 0
Energy intake (MJ/day)	6.5 ± 1.7	6.5 ± 1.4	6.9 ± 0.3
Calcium intake (mg/day)	816 ± 364	807 ± 340	806 ± 157
Vitamin D intake (μg/day)	2.7 ± 1.8	2.9 ± 1.8	2.5 ± 1.0
Physical activity (h/week)	2.9 ± 2.1	2.8 ± 2.1	2.4 ± 1.9
Fractures (n)	21	13	0

Values are mean ± SD. None of the differences are significant.

Table 2. Estimated bone mass and density according to COL1A1 genotypes

	GG (n = 175)	GT (n = 68)	TT (n = 4)	TT - GG*	TT - GT*
Total body					
Area (cm)	1480 ± 5	1484 ± 8	1396 ± 33	0.014	0.011
BMC (g)	1413 ± 8	1407 ± 13	1211 ± 56	< 0.001	0.001
Areal BMD (g/cm^2)	0.949 ± 0.003	0.943 ± 0.005	0.875 ± 0.023	0.002	0.004
Fat mass (g)	10590 ± 292	9925 ± 465	11554 ± 1928	NS	NS
Lean mass (g)	27143 ± 135	27382 ± 215	25962 ± 890	NS	NS
Total femur					
Area (cm)	23.8 ± 0.1	23.9 ± 0.2	23.7 ± 0.7	NS	NS
BMC (g)	20.1 ± 0.2	20.2 ± 0.3	17.6 ± 1.1	0.031	0.022
Areal BMD (g/cm^2)	0.835 ± 0.006	0.843 ± 0.010	0.742 ± 0.040	0.022	0.014
Femoral neck					
Area (cm)	3.85 ± 0.03	3.87 ± 0.05	3.40 ± 0.22	0.047	0.042
BMC (g)	3.26 ± 0.03	3.30 ± 0.04	3.03 ± 0.17	NS	NS
Areal BMD (g/cm^2)	0.815 ± 0.006	0.829 ± 0.009	0.730 ± 0.039	0.032	0.013
Spine L2-L4					
Area (cm)	27.9 ± 0.2	28.0 ± 0.3	27.9 ± 1.1	NS	NS
BMC (g)	23.5 ± 0.3	23.3 ± 0.4	19.6 ± 1.8	0.032	0.044
Areal BMD (g/cm^2)	0.832 ± 0.006	0.825 ± 0.010	0.711 ± 0.040	0.003	0.006

Values are estimated mean, adjusted for body size, ± SE.
* *p* values were determined by ANCOVA, with body size index as a covariate.
None of the differences between GG and GT are significant.
NS, not significant.

Table 3. Estimated bone geometry and density according to COL1A1 genotypes

	GG (n = 175)	GT (n = 68)	TT (n = 4)
Tibia			
CSA (mm^2)	370 ± 2	377 ± 4	352 ± 16
CTh (mm)	3.46 ± 0.02	3.43 ± 0.04	3.23± 0.16
vBMD (mg/cm^3)	667 ± 4	658± 6	654 ± 24
Radius			
CSA (mm^2)	227 ± 3	228 ± 5	219 ± 20
vBMD (mg/cm^3)	288 ± 3	286 ± 4	264 ± 18
tr. vBMD (mg/cm^3)	226 ± 2	225 ± 3	199 ± 14

Values are estimated mean, adjusted for body size, ± SE.
None of the differences are significant.

Table 4. Estimated bone material properties according to COL1A1 genotypes

	GG (n = 154)	GT (n = 59)	TT (n = 4)	TT - GG*	TT - GT*
BUA at heel (db/MHz)	65.3 ± 0.6	66.5 ± 0.9	56.5 ± 3.6	0.016	0.008
SOS at tibia (m/s)	3488 ± 10	3479 ± 16	3555 ± 62	NS	NS
SOS at radius (m/s)	3663 ± 8	3655 ± 13	3737 ± 49	NS	NS

Values are estimated mean, adjusted for body size, ± SE.
* *p* values were determined by ANCOVA, with body size index as a covariate.
None of the differences between GG and GT are significant.
NS, not significant.

Table 5. Estimated bone turnover according to COL1A1 genotypes

	GG (n = 154 / 69*)	GT (n = 59 / 28*)	TT (n = 4 / 3*)	TT - GG†	TT - GT†
Formation					
P1NP (μg/L) ‡	664 ± 26	691 ± 42	575 ± 129	NS	NS
OC (ng/mL) ‡	45.5 ± 1.0	46.3 ± 1.6	36.8 ± 6.2	NS	NS
B-ALP (U/L)	121 ± 3	120 ± 4	83 ± 17	0.027	0.037
Resorption					
TRACP 5b (U/L) ‡	12.9 ± 0.3	12.5 ± 0.5	13.9 ± 1.8	NS	NS
Turnover index					
P1NP/TRACP 5b ‡	51 ± 2	55 ± 4	32 ± 11	NS	0.034
(OC+B-ALP)/TRACP 5b ‡	13 ± 0	14 ± 1	10 ± 2	0.031	0.016

Values are estimated mean, adjusted for body size, ± SE.
* Others / P1NP and P1NP/TRACP 5b.
† *p* values were determined by ANCOVA, with body size index as a covariate.
‡ Differences in means were examined after ln transformation of the data.
None of the differences between GG and GT are significant.
NS, not significant.

II

RELATION OF *PvuII* SITE POLYMORPHISM IN THE *COL1A2* GENE TO THE RISK OF FRACTURES IN PREPUBERTAL FINNISH GIRLS

by

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Relation of *Pvu*II site polymorphism in the COL1A2 gene to the risk of fractures in prepubertal Finnish girls

Miia Suuriniemi,[1,4,2] Anitta Mahonen,[3] Vuokko Kovanen,[4] Markku Alén,[5] and Sulin Cheng[4]

[1]*Department of Cell Biology, University of Jyväskylä, and* [2]*LIKES-Foundation for Sport and Health Sciences, Jyväskylä FIN-40014;* [3]*Department of Medical Biochemistry, University of Kuopio, Kuopio FIN-70211, Finland;* [4]*Department of Health Sciences, University of Jyväskylä, Jyväskylä FIN-40014; and* [5]*PEURUNKA-Medical Rehabilitation Center, Jyväskylä FIN-41340, Finland*

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children; genetics; osteoporosis; type I collagen

ALTHOUGH MUCH IS KNOWN regarding the incidence and pattern of fractures during growth, the pathogenesis of the increased fracture rate during childhood has not yet been defined. Low bone mass is an important component of the risk of fracture, but other abnormalities arise in the skeleton that contribute to skeletal fragility. In addition to the inability of the mineralization process to keep pace with the growth of the long bones, an imperfection in the alignment of collagen fibers with the principal directions of loading may play a role. Furthermore, various nonskeletal factors, such as the liability to fall, contribute to fracture risk. There is, therefore, a distinction to be made between diagnosis of osteoporosis and assessment of fracture risk.

Bone mass and strength are under strong genetic control. Most efforts toward understanding the genetics of bone density have focused on association studies of candidate genes known to be involved in bone metabolism in adult populations (1). During the past years, a few researchers have examined the influence of candidate genes, reported to be associated with adult bone mass, on the phenotypic variability of skeletal development in children. Defining the genetic and environmental factors responsible for variations in bone properties and fracture rate during skeletal growth should help identify children at risk for fractures also later in life (12).

Besides bone mass, the strength and mechanical properties of bone also depend on the architecture and molecular structure of inorganic and organic components (23). Type I collagen is the major structural protein in bone and consists of a heterotrimeric complex of two α1-polypeptides and one α2-polypeptide. Polymorphism in the Sp1 binding site of the collagen type I α1 gene (COL1A1) has been found to be related to decreased bone mass and osteoporotic fractures in women (15, 41). More recently, the COL1A1 gene alleles have been found to be associated with the normal variations in the apparent density of cancellous bone in the axial skeleton of prepubertal girls (34). Virtually all mutations that result in osteogenesis imperfecta (OI) affect the genes that encode the chains of type I procollagen, especially the COL1A1 gene. Nevertheless, OI patients with mutations in the collagen type I α2 gene (COL1A2) gene have been characterized (6, 32, 38, 39, 42). The well-described strain of mice with a nonlethal recessively inherited mutation in the

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Address for reprint requests and other correspondence: S. Cheng, Dept. of Health Sciences, Univ. of Jyväskylä, PO Box 35, FIN-40014 Jyväskylä, Finland (E-mail: cheng@sport.jyu.fi).

COL1A2 gene (oim) that results in phenotypic and biochemical features that simulate moderate-to-severe human OI is living evidence of the importance of the collagen type I α2 polypeptide to the bone structure and strength (10, 25, 31, 33). In this study, we examined the relationship between a single nucleotide polymorphism (A→C) in codon 392 in the COL1A2 gene (11) and various bone properties, bone turnover markers, and the retrospective occurrence of fractures in early and prepubertal girls.

METHODS

Subjects. The study subjects were 258 (244 for the genomic analysis) healthy early and prepubertal Finnish girls, aged 10–12 yr, who were recruited for an intervention study to evaluate the effects of calcium, vitamin D, and milk product supplementation on bone accrual (the CALEX study). To be eligible for the study, the participants had to have no history of serious medical conditions and no history of medication known to affect bone metabolism. For determination of sexual development by a public health nurse, the Tanner grading system was used (40). Only those girls at Tanner stage I–II (prepuberty and early puberty) were included in the study. Level of physical activity was assessed by a self-reported questionnaire including items on the frequency, duration, and types of exercise done during the subject's leisure time (19). The data of physical activity is provided in terms of hours per week. Fracture history was clarified by a parents-reported questionnaire including items on the site, time, and cause of each fracture and was confirmed by hospital medical records. Girls with a fracture history of less than 1 yr were excluded from the study. Fractures caused by severe trauma (e.g., serious accidents and crushed fingers/toes) were excluded from this analysis. The investigational protocol was approved by the ethical committee of the University of Jyväskylä, the Central Hospital of Central Finland, and the Finnish National Agency of Medicines. An informed consent was obtained from all subjects and their parents prior to the assessments. The results presented in this report are from the baseline assessments.

Body composition assessments. Body height was measured with a fixed-scale measuring device. Weight was determined (±0.5 kg) using a calibrated scale. The results of height and weight were then used to determine body mass index (BMI), expressed as weight in kilograms divided by the square of the height in meters (in kg/m^2). The fat and lean tissue mass of the total body were analyzed from a total body dual-energy X-ray absorptiometry (DXA) scan.

Bone property assessments. Bone mineral content (BMC, g) and areal bone mineral density (aBMD, g/cm^2) of the total body, left proximal femur (total femur and femoral neck), and lumbar spine (L2–L4) were measured using DXA (Prodigy; GE Lunar, Madison, WI). The percentage coefficient of variation (%CV) for repeated measurements ranged from 0.6 to 1.2 for BMC and from 0.86 to 1.3 for aBMD at the different bone sites. Cross-sectional area (CSA, mm^2), cortical thickness (CTh, mm), and volumetric BMD (vBMD, mg/cm^3) were measured using peripheral quantitative computed tomography (pQCT) (model XCT 2000; Stratec Medizintechnik, Pforzheim, Germany). We scanned the left distal radius (4% of the total length of the forearm medial to the reference line) and tibia shaft (60% of the lower leg length between the tuberositas tibia and the medial malleolus). Data were then analyzed using BonAlyse software (BonAlyse Oy, Jyväskylä, Finland) (8). The %CV was <3% for CSA and <1% for vBMD. Broadband ultrasound attenuation (BUA, dB/MHz) of the left calcaneus was measured using a gel coupling scanning quantitative ultrasonometer (QUS-2; Quidel, Santa Clara, CA) (7). The speed of sound (SOS, m/s) at the distal third of radius (medial surface) and the midshaft of tibia (anteromedial surface) were determined by a mobile ultrasound bone sonometer (Omnisense; Sunlight Technologies, Rehovot, Israel) (2).

Biochemical markers of bone turnover. After an overnight fast, blood was drawn in the morning between 7–9 AM for determinations of bone formation [osteocalcin, bone-specific alkaline phosphatase (BAP), and amino-terminal propeptide of type I procollagen (P1NP) in a subgroup, $n = 102$] and bone resorption [bone-specific tartrate-resistant acid phosphatase (TRAP 5b) in a subgroup, $n = 213$] markers. The serum intact osteocalcin level was measured by a competitive immunoassay (NovoCalcin; Metra Biosystems, Mountain View, CA) (4); the intra-assay and interassay %CV were 2.4% and 0.62%, respectively. The serum BAP activity was measured by an immunoassay (Alkphase-B; Metra Biosystems, Mountain View, CA) (14); the intra- and interassay %CV were 2.1% and 15.3%, respectively. The serum P1NP level was measured by a competitive radioimmunoassay (Orion Diagnostica, Espoo, Finland) (28); the intra- and interassay %CV were 3.6% and 1.85%, respectively. The serum TRAP 5b activity was measured by a specific immunoassay (16, 17); the intra-assay and interassay %CV were 2.7% and 24.5%, respectively.

Analysis of collagen type I α2 gene polymorphism. Genomic DNA was isolated from EDTA-stabilized blood, using a blood kit (QIAamp; Qiagen, Hilden, Germany). The polymorphic *Pvu*II site of the COL1A2 gene was detected by polymerase chain reaction (PCR) followed by enzymatic digestion (11). The upstream primer was 5′-GGGATATAAGGATACACTAGAGG-3′, and the downstream primer was 5′-GAAATATCGGCCCCGCTGGAA-3′. The reaction mixture of 20 μl contained 50–200 ng genomic DNA, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each primer, and 1.5 U of *Taq* DNA polymerase (Fermentas, Vilnius, Lithuania). The reactions were performed in a DNA thermocycler (T3 combi-block; Biometra, Göttingen, Germany) with a cycling protocol of 94°C, 59°C, and 72°C for 1 min each, for 30 cycles. Prior to the first cycle, initial denaturation was performed at 94°C for 5 min, and the last cycle was followed by an extension step of 7 min at 72°C. The PCR products were digested with 10 U of *Pvu*II restriction enzyme and a buffer, containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, and 10 mM $MgCl_2$, by incubation for 4 h at 37°C. The digested products were analyzed on an 1.5% agarose gel. The PCR products containing *p* alleles were cleaved by *Pvu*II, resulting in bands of 541 bp and 239 bp compared with the uncleaved *P* alleles band of 771 bp.

Statistical analysis. Statistical analyses were carried out using the Statistical Package (SPSS) version 9.0 for Windows. Differences in bone properties (CSA, CTh, BMC, aBMD, vBMD, BUA, and SOS) and serum bone turnover markers among the three genotypes as well as between the fracture and nonfracture groups were tested using analysis of covariance, controlling for the effects of BMI and Tanner stage. The likelihood ratio was used to test for the genotype distribution in girls with and without fractures. Odds ratios [with 95% confidence intervals (CI)] were calculated by multivariate logistic regression analysis after stepwise adjustment for potential confounding variables such as Tanner stage, BMI, aBMD of the total body, and physical activity to estimate the relative risk of fracture by COL1A2 genotypes. Under a possible dominant inheritance model, analysis was

Table 1. *Physical characteristics in relation to the COL1A2 genotype*

	PP	*Pp*	*pp*	*P* Value
n	38	129	77	
Age, yr	11.1 ± 0.7	11.2 ± 0.8	11.2 ± 0.7	0.43
Weight, kg	39.3 ± 9.8	39.7 ± 8.3	37.3 ± 7.6	0.12
Height, cm	146.0 ± 8.7	145.9 ± 8.2	144.8 ± 7.4	0.61
BMI	18.2 ± 3.3	18.5 ± 2.9	17.6 ± 2.4	0.07
Physical activity, h/wk	2.9	2.8	3.0	0.88
Tanner stage (1/2%)	49/51	49/51	57/43	0.62
Total body fat mass, g	10,734 ± 6,477	11,067 ± 5,480	9,072 ± 4,453	0.40[a]
Total body lean mass, g	27,120 ± 4,471	27,390 ± 4,249	26,843 ± 4,134	0.84[a]

Values are means ± SD. BMI, body mass index. *P* values were determined by analysis of variance. [a]Adjusted for Tanner stage and BMI.

subsequently performed on the combined *PP* and *Pp* genotype groups. To control potential influence of bone density on the SOS, we used site-matched volumetric BMD (vBMD of radius in SOS at radius and vBMD of tibia in SOS at tibia) in addition to Tanner stage and BMI as a covariate in the covariance analysis of SOS in fracture vs. nonfracture girls. All significant tests were two-sided. A *P* value of less than 0.05 was considered statistically significant. Genotype distribution was studied by the Hardy-Weinberg equilibrium (29).

RESULTS

Physical characteristics. Table 1 shows physical characteristics according to the COL1A2 genotype. The overall prevalence of the genotypes in this study was 15.6% *PP*, 52.9% *Pp*, and 31.6% *pp*. The genotype distribution was found to be in Hardy-Weinberg equilibrium, suggesting that the subjects represented a homogenous genetic background. There were no significant differences in developmental status or physical activity among the girls in the different COL1A2 genotype groups. The mean values for weight, height, and BMI, as well as the values for total body fat and lean mass were similar in the different COL1A2 genotype groups.

Bone measurements and bone turnover. We found no significant effect of the COL1A2 gene polymorphism on BMC or aBMD of the total body, total femur, femoral neck, or lumbar spine (L2–L4) (Table 2). No significant differences were found between the different genotype groups in CSA or vBMD in the radius or tibia, or in the CTh of the tibia, BUA of the calcaneus, or SOS at the radius and tibia. Serum concentrations of biochemical markers for bone turnover were independent of the COL1A2 genotypes (Table 3). No clear relationship was found between the *P* allele and aforesaid parameters by pooling of the two genotype groups (*PP* and *Pp*) (data not shown). However, when Tanner stage I girls were analyzed separately, individuals with the *PP* genotype had significantly lower BMC ($P = 0.010$) and aBMD ($P = 0.013$) of the L2–L4 (Fig. 1), as well as SOS at the tibia ($P = 0.036$) and a trend toward lower SOS at the radius ($P = 0.092$), compared with individuals with the *pp* genotype (Fig. 2). No significant effect of the COL1A2 gene polymorphism on bone properties was found at Tanner stage II (data not shown).

Fracture data. Among our study sample, there were 46 girls (18%) who had sustained at least one fracture.

Table 2. *Bone measurements in relation to the COL1A2 genotype*

	PP	*Pp*	*pp*	*P* Value
n	38	129	77	
BMC, g				
Total body	1411 ± 320	1425 ± 267	1373 ± 258	0.99
Total femur	20.1 ± 4.5	20.2 ± 3.8	19.6 ± 3.8	0.93
Spine L2–L4	22.9 ± 7.19	23.6 ± 5.81	23.1 ± 4.84	0.77
Areal BMD, g/cm^2				
Total body	0.943 ± 0.057	0.950 ± 0.057	0.939 ± 0.059	0.88
Total femur	0.834 ± 0.094	0.843 ± 0.092	0.822 ± 0.097	0.80
Spine L2–L4	0.818 ± 0.128	0.833 ± 0.105	0.825 ± 0.092	0.63
CSA, mm^2				
Distal radius	225 ± 53.63	230 ± 39.97	222 ± 39.23	0.67
Tibia shaft	279 ± 42.66	283 ± 37.72	278 ± 40.85	0.68
Volumetric BMD, mg/cm^3				
Distal radius	291 ± 41	288 ± 37	285 ± 34	0.73
Tibia shaft	859 ± 35	858 ± 38	853 ± 43	0.61
Cortical thickness, mm	3.49 ± 0.41	3.48 ± 0.36	3.39 ± 0.41	0.72
Ultrasound				
BUA of heel, dB/MHz	64.8 ± 8.46	66.7 ± 8.08	63.8 ± 8.10	0.41
SOS of radius, m/s	3,619 ± 98	3,636 ± 103	3,655 ± 90	0.27
SOS of tibia, m/s	3,441 ± 138	3,460 ± 128	3,478 ± 121	0.54

Values are means ± SD. BMC, bone mineral content; BMD, bone mineral density; CSA, cross-sectional area; SOS, speed of sound; BUA, broad-band ultrasound attenuation. *P* values were determined by analysis of covariance, with Tanner stage and BMI as covariates.

Table 3. *Bone turnover in relation to the COL1A2 genotype*

	PP	*Pp*	*pp*	*P* Value
n	20	48	34	
Formation				
Osteocalcin, ng/l	41.3 ± 10.0	43.1 ± 9.42	42.0 ± 8.69	0.66
BAP, U/l	109 ± 21.8	123 ± 33.2	119 ± 39.0	0.27
P1NP, μg/l	669 ± 253	665 ± 221	676 ± 223	0.99
Resorption*				
TRAP 5b, U/l	11.4 ± 3.2	11.3 ± 2.8	11.0 ± 2.3	0.36

Values are means ± SD. BAP, bone-specific alkaline phosphatase; P1NP, amino-terminal propeptide of type I procollagen; TRAP 5b, bone-specific tartrate-resistant acid phosphatase. *P* values were determined by analysis of covariance, with Tanner stage and BMI as covariates. *Total subject number is 213 (*PP, n* = 35; *Pp, n* = 112; *pp, n* = 66).

One girl had sustained three fractures over a 2-yr period, and eight girls had had two fractures. The age when the fracture occurred ranged from newborn to 11 yr. Four of the fractures had happened at birth, ten occurred at the ages of 1–5 yr, thirteen at the ages of 6–8 yr, and nineteen at the ages of 9–11 yr. The most frequent fracture site was arm (31 cases). There were 11 collarbone, 7 finger, and 6 toe fractures, and 1 skull fracture. All the fractures, except those that happened at birth and one due to a knock against the bookshelf, were caused by falling down (e.g., from chair, sofa, or bed; or during walking, running, or bicycling). In the analyses we only included those girls ($n = 37$) who had had a fracture resulting from minimal to moderate trauma (by convention, the equivalent of a fall from standing height or less).

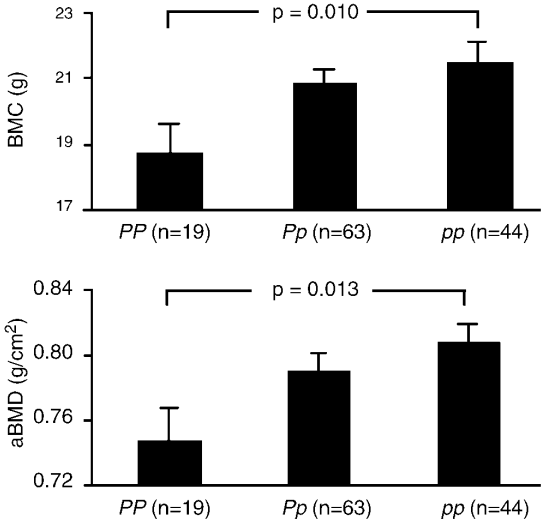

Fig. 1. Bone mineral content (BMC, *top*) and areal bone mineral density (aBMD, *bottom*) of the lumbar spine (L2–L4) in relation to the COL1A2 genotype at Tanner stage I. Data are adjusted means ± SE. *P* values were determined by analysis of covariance, with body mass index (BMI) as a covariate.

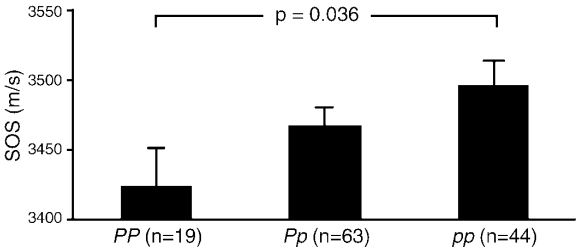

Fig. 2. Speed of sound (SOS) at tibia in relation to the COL1A2 genotype at Tanner stage I. Data are adjusted means ± SE. *P* values were determined by analysis of covariance, with BMI as a covariate.

There were no significant differences in physical characteristics, physical activity, or developmental status between the girls with fractures and their counterparts (Table 4). No significant differences were found between the fracture and nonfracture groups in BMC or aBMD of the total body, total femur, or femoral neck, CSA or vBMD in the radius or tibia, CTh of the tibia, or BUA of the calcaneus. The biochemical markers of bone turnover were similar between girls with and without fracture. However, aBMD of the L2–L4 was significantly lower in the fracture group compared with the nonfracture group ($P = 0.04$). Also, the fracture group had significantly lower SOS values at the radius ($P = 0.029$) as well as at the tibia ($P = 0.036$) compared with the nonfracture group after adjusting for site-matched vBMD and Tanner stage as well as BMI (Fig.

Table 4. *Characteristics in relation to fracture history*

	Fracture	Nonfracture	*P* Value
n	37	221	
Age, yr	11.2 ± 0.7	11.2 ± 0.8	0.80
Weight, kg	40.0 ± 7.9	39.0 ± 8.7	0.52
Height, cm	146.7 ± 8.5	145.5 ± 8.0	0.41
BMI	18.4 ± 2.3	18.3 ± 3.0	0.75
Physical activity, h/wk	2.3	2.9	0.09
Tanner stage (1/2%)	51/49	50/50	0.97
Fat mass, g	11,089 ± 5,217	10,498 ± 5,731	0.39[a]
Lean mass, g	27,694 ± 4,163	27,195 ± 4,288	0.36[a]
BMC of total body, g	1,409 ± 245	1,412 ± 278	0.88[a]
aBMD of total body, g/cm²	0.936 ± 0.05	0.948 ± 0.06	0.18[a]
BMC of total femur, g	19.9 ± 3.4	20.0 ± 4.0	0.79[a]
aBMD of total femur, g/cm²	0.823 ± 0.07	0.838 ± 0.10	0.30[a]
BMC of L2–L4, g	22.4 ± 4.6	23.6 ± 5.9	0.14[a]
aBMD of L2–L4, g/cm²	0.801 ± 0.08	0.835 ± 0.11	0.04[a]
CSA of distal radius, mm²	230 ± 36	226 ± 44	0.56[a]
CSA of tibia shaft, mm²	286 ± 39	281 ± 40	0.31[a]
vBMD of distal radius, mg/cm³	279 ± 34	289 ± 37	0.12[a]
vBMD of tibia shaft, mg/cm³	855 ± 43	857 ± 38	0.75[a]
CTh of tibia shaft, mm	3.50 ± 0.37	3.44 ± 0.40	0.35[a]
BUA of heel, dB/MHz	65.9 ± 7.40	65.5 ± 8.06	0.66[a]

Values are means ± SD. aBMD, areal BMD; vBMD, volumetric BMD; CTh, cortical thickness. *P* values were determined by analysis of variance. [a]Adjusted for Tanner stage and BMI.

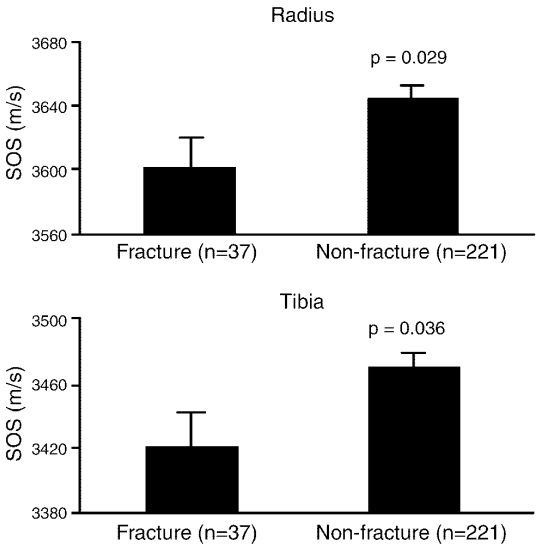

Fig. 3. SOS in relation to fracture history, for radius (*top*) and tibia (*bottom*). Data are adjusted means ± SE. *P* values were determined by analysis of covariance, Tanner stage, BMI, and volumetric bone mineral density (vBMD) of radius and tibia, respectively, as covariates.

3). When all fractures ($n = 46$) were included in the analysis, the differences were even stronger (BMC of the L2–L4, $P = 0.071$; aBMD of the L2–L4, $P = 0.028$; SOS at the radius, $P = 0.003$; SOS at the tibia, $P = 0.039$) (data not shown).

Fracture risk according to the COL1A2 genotype. The distribution of fractures was significantly different in the genotype groups ($P = 0.023$) (Table 5). Pooling of the *PP* and *Pp* genotype groups under a dominant inheritance model showed a clear relationship between the *P* allele and the occurrence of fractures ($P = 0.007$). Of the girls who had sustained at least one fracture before the laboratory assessments, 88% had at least one copy of the *P* allele, in comparison to 65% of the girls who had not had fractures during their lives. The odds ratios showed that the relative risk of fracture among girls with the *P* allele (either *PP* or *Pp* genotype) was 4.0 times higher than among the girls with the *pp* genotype (Table 5). The crude risk of fracture was 3.8 for the *Pp* genotype group and 4.9 for the *PP* genotype group in relation to the *pp* genotype group. The risk did not change essentially after adjustments for potential confounding factors such as Tanner stage, BMI, and aBMD of the total body in the multivariate regression analysis (Table 5). Stepwise adjustments for the aforementioned parameters yielded similar results as adjustment for all the five factors together (data not shown). When the results were additionally adjusted for physical activity, the risks of fracture still remained similar.

DISCUSSION

We were able to show a significant association between the COL1A2 polymorphism and the retrospective occurrence of fractures in early and prepubertal girls. To the best of our knowledge, this is the first study reporting the genotypic effect of *Pvu*II site polymorphism in the COL1A2 gene on bone properties and the occurrence of fractures.

Differences among COL1A2 gene alleles may be more difficult to demonstrate during puberty, when large increases in skeletal size, bone mass, and bone density occur over a brief period of time. In fact, we found no effect on bone properties in relation to the genotype when girls at Tanner stage I (prepuberty) and II (early puberty) were considered together, but there was an association of the COL1A2 polymorphism with spine BMC and BMD, as well as with SOS when girls at Tanner stage I were considered separately. This finding is consistent with the hypothesis that COL1A2 genotype contributes to bone strength in part by an effect on bone density and in part by an effect on bone quality. As in our work, earlier studies had shown that the genetic effect associated with polymorphism in the type I collagen gene appeared stronger at the spine (15, 20).

We found no effect on the bone properties studied except for the aBMD of the lumbar spine (L2–L4) and the SOS at the radius and tibia in relation to fracture history. Theoretically, the SOS is a measure of BMD and elasticity combined. Mehta et al. (26) found that the organic matrix exerts a profound influence on bone elasticity and that the subtle changes in the organic matrix have effect on ultrasound velocity in vitro. Our previous study in females with diseases related to collagen mutations (Ehlers-Danlos syndrome and systemic sclerosis) showed that collagen abnormalities may impact on bone mass measurements differently depending on skeletal site, modality of the assessment, and the source and nature of collagen defects. Ultra-

Table 5. *Fracture risk related to the pp genotype of the COL1A2*

Genotype	Fractures/Total, %	Odds Ratio, 95% CI		
		Crude	Adjusted[a]	Adjusted[b]
pp	4/77(5.2)*			
Pp	22/129(17.1)*	3.8(1.2–11.3)*	3.9(1.3–12.0)*	3.9(1.3–11.9)*
PP	8/38(21.1)*	4.9(1.4–17.4)*	4.9(1.4–17.7)*	5.1(1.4–18.5)*
PP/Pp	30/167(18.0)*	4.0(1.4–11.8)*	4.2(1.4–12.4)*	4.1(1.4–12.4)*

Fourteen subjects missed the genotype data from the total sample ($n = 258$). [a]Adjusted for Tanner stage, BMI, and aBMD of the total body. [b]Adjusted for all above ([a]) and physical activity. *$P < 0.05$.

sound assessment was able to detect the differences between patients and matched controls (9). We found that those girls who had sustained a fracture previously had significantly lower SOS values than girls who had not. After adjusting for site-matched vBMD, the significant differences remained. Our results indicate that differences in SOS might be derived from the differences in bone elasticity between the fracture and nonfracture groups.

Girls with the *PP* genotype had 4.9 times higher relative risk of fracture compared with girls with the *pp* genotype. In addition, after adjusting for Tanner stage, BMI, and aBMD of the total body, no change in fracture risk was found between the COL1A2 genotype groups. Thus we may assume that COL1A2 genotype predisposes to fracture mostly by an effect on bone quality, such as bone structure or matrix composition, rather than by an effect on bone quantity. The increased risk of fracture could not be a consequence of higher physical activity, since the physical activity did not differ in the COL1A2 genotype groups, and adjustment for physical activity did not affect the association between the COL1A2 polymorphism and the risk of fracture. Even though the fractures were not osteoporotic, the fact of their occurrence was due to minimal to moderate trauma may be related to the structure and strength of bones. The involvement of BMD in bone strength is well established, although the correlation between these two parameters is only partial (23). Conversely, the existence of an association between type I collagen structure and bone strength has been well documented in OI and other clinical syndromes attributable to mutations of type I collagen genes encoding for both α1- and α2-chains (35–37). Although most OI patients have subnormal BMD, some of them have decreased bone strength but normal BMD (30). This provides evidence that type I collagen structure independently of BMD may be related to changes in the mechanical strength of bone.

The association between COL1A1 gene Sp1 polymorphism and the risk of fractures has recently been found to be independent of BMD (3). Garnero and coworkers have shown more recently that different age-related forms of the COOH-terminal cross-linking telopeptide of type I collagen are associated with increased fracture risk independently of BMD (13). Because the type I collagen molecule consists of two α1-chains and one α2-chain, mutations in the α2-chain are considered to have a smaller potential for deleterious consequences than α1 mutations. Our results support these findings and show that the association between the COL1A2 polymorphism and the fracture risk is independent of BMD. We conclude that the main effect of the COL1A2 genotype on the fracture risk is mediated by the effect of the genotype on the elastic properties of bone assessed by the quantitative ultrasound, which is believed to provide information about bone quality. Nevertheless, bone density at least at the spine may also be affected by the COL1A2 genotype during the fast pubertal growth period.

The mechanisms by which the different COL1A2 alleles affect fracture risk are not yet known. The sequence change detected is a CpA to CpC transversion in exon 25 which does not affect the encoded proline residue at position 392 of the α2(I) chain (22). Constantinou et al. (11) observed the same polymorphism in the amplified products of cDNA from a patient with OI and several patients with osteoporosis (11). Our results raise the possibility that this polymorphism may be in linkage disequilibrium with a causal mutation in the same gene or in genes nearby. Another possibility could be a direct influence on the gene regulation. For example, synonymous single nucleotide polymorphisms located in coding regions (cSNPs), although seemingly translationally silent, can have a profound influence on splicing. In fact, cSNPs can disrupt (or eventually create) exonic splicing enhancers and silencers; create new splice sites or strengthen cryptic ones; alter pre-mRNA secondary structures important for exon definition; and, conceivably, modify the pausing architecture of a gene, provoking changes in RNA *Pol*II processivity, which might in turn affect splice site choice (5). Approximately 15% of mutations that cause genetic disease affect pre-mRNA splicing (21). An important fact is that Nicholls et al. (29) found an association between the absence of this *Pvu*II polymorphic site (denoting the *P* allele) in exon 25 and a splice site mutation (G→A) causing deletion of exon 21 from the pro-α2(I) chain of type I collagen in a patient with very mild OI. The mutant pre-mRNA was alternatively spliced, yielding both full-length and deleted transcripts. Therefore, further investigation with other polymorphisms within the COL1A2 gene and nearby markers may be useful. If individuals, due to their altered collagen production, have reduced trabecular thickness, then they would be at higher risk of trabecular perforations. This would reduce bone strength proportionally more than the accompanying loss of bone density. This could be an explanation for the finding that carriage of the *P* allele predicts fractures independently of bone density.

In conclusion, our study demonstrated a significant association between *Pvu*II site polymorphism in the COL1A2 gene and fractures in early and prepubertal girls. This information may contribute to the identification of a subset of the population of normal girls that may be at risk of developing fractures later in life and may ultimately be of value in the planning of early preventive strategies for osteoporosis.

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DISCLOSURES

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III

THE ASSOCIATION BETWEEN EXERCISE AND PUBERTAL BONE DENSITY IS MODULATED BY ESTROGEN RECEPTOR α GENOTYPE

by

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Association Between Exercise and Pubertal BMD Is Modulated by Estrogen Receptor α Genotype

Miia Suuriniemi,[1,2,3] Anitta Mahonen,[4] Vuokko Kovanen,[2] Markku Alén,[5] Arja Lyytikäinen,[2,3] Qingju Wang,[2,3] Heikki Kröger,[6] and Sulin Cheng[2]

ABSTRACT: Genetic and environmental factors contribute to bone mass, but the ways they interact remain poorly understood. This study of 245 pre- and early pubertal girls found that the *Pvu*II polymorphism in the *ER-α* gene modulates the effect of exercise on BMD at loaded bone sites.

Introduction: Impaired achievement of bone mass at puberty is an important risk factor for the development of osteoporosis in later life. Genetic, as well as environmental, factors contribute to bone mass, but the ways they interact with each other remain poorly understood.
Materials and Methods: We investigated the interaction between a *Pvu*II polymorphism at the *ER-α* gene and physical activity (PA) on the modulation of bone mass and geometry in 245 10- to 13-year-old pre- and early pubertal Finnish girls. Level of PA was assessed using a questionnaire. Bone properties were measured using DXA and pQCT. The analyses were controlled for the effects of Tanner stage and body size index.
Results: Girls with heterozygote ER-α genotype (Pp) and high PA had significantly higher bone mass and BMD, as well as thicker cortex, at loaded bone sites than their low-PA counterparts. No differences were found in bone properties of the distal radius, which is not a weight-bearing bone. Bone properties did not differ in either homozygote groups (PP and pp) regardless of the PA level.
Conclusions: These findings suggest that the *Pvu*II polymorphism in the *ER-α* gene may modulate the effect of exercise on BMD at loaded bone sites. The heterozygotes may benefit most from the effect of exercise, whereas neither of the homozygote groups received any significant improvement from high PA. Furthermore, high PA may hide the genetic influence on bone. Indeed, it seems that one may compensate one's less favorable Pp genotype by increasing leisure PA at early puberty.
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Key words: single nucleotide polymorphism, estrogen receptor, exercise, BMD, puberty

INTRODUCTION

Impaired achievement of bone mass at puberty is an important risk factor for the development of osteoporosis in later life. Being the time period of greatest acquisition of bone mass, puberty is the ideal time for bone growth stimulation.[1,2] Accordingly, the available evidence suggests that the skeleton may be most responsive to exercise during growth.[3–6] As a multifactorial trait, a number of genes, as well as environmental factors, contribute to bone mass, but the ways in which they interact with each other have remained poorly understood. Explicitly, the genome is not an isolated source of fixed, one-way information, but its effects on phenotype are constantly being modulated by several environmental factors.

As a mediator of estrogen hormone action, the gene encoding estrogen receptor α (*ER-α*) has been considered an important candidate for the determination of osteoporotic risk. A *Pvu*II polymorphism residing in the first intron of the *ER-α* gene was first found to be associated with BMD variation in the Japanese population,[7] but subsequent studies searching for a segregation of *Pvu*II restriction fragment length polymorphism (RFLP) with BMD in other populations have yielded conflicting results.[8–19] To date, only one published study regarding the effects of *ER-α* polymorphism on bone properties has been directed on children, and that was carried out in adolescent boys.[20] The molecular mechanism by which the *Pvu*II polymorphism may influence the gene function and ultimately the phenotype is yet unclear because it lies in an intronic and apparently nonfunctional area of the gene.

It has been well established from both human[21,22] and animal studies[23–26] that mechanical loading is an important

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[1]Department of Cell Biology, University of Jyväskylä, Jyväskylä, Finland; [2]Department of Health Sciences, University of Jyväskylä, Jyväskylä, Finland; [3]LIKES-Foundation for Sport and Health Sciences, Jyväskylä, Finland; [4]Department of Medical Biochemistry, University of Kuopio, Kuopio, Finland; [5]PEURUNKA-Medical Rehabilitation Center, Jyväskylä, Finland; [6]Department of Surgery, Kuopio University Hospital, Kuopio, Finland.

regulator of skeletal mass and architecture. Recent data have led several authors to suggest that the early responses of resident bone cells to mechanical strain and estrogen share a common mechanistic pathway, which involves *ER-α*.[27–31] Importantly, Lee et al.[32] recently showed that the adaptive response of bones to mechanical loading requires functional ER-α.

Several studies have separately focused on either the influence of genetic polymorphisms or environmental factors on bone properties, but unfortunately, few have explored gene–environment interactions. Studies in the field of exercise physiology have highlighted the genetic variability of individual differences in response to training.[33] Interestingly, Blanchet et al.[34] found an interaction between leisure physical activity (PA) and a *Bsm*I polymorphism at the *vitamin D receptor* gene on the modulation of BMD in postmenopausal women. Undoubtedly, part of the controversy over the potential association between *ER-α* polymorphism and BMD could be explained by differences in environmental factors between subjects recruited from different study designs and gene–environment interactions. Therefore, the aim of this study was to investigate the interaction between a *Pvu*II polymorphism at the *ER-α* gene and PA on the modulation of bone mass and geometry in pre- and early pubertal Finnish girls.

MATERIALS AND METHODS

Subjects

The study subjects were 258 (245 for the genetic analysis) healthy pre- and early pubertal Finnish girls, 10–13 years of age, who were recruited for an intervention study (the Calex study) to evaluate the effects of calcium, vitamin D, and milk product supplementation on bone mass accrual.[35,36] The subjects were first contacted through class teachers teaching fourth to sixth grades in 61 schools (96% of the total schools in these areas) in the city of Jyväskylä and its surroundings in central Finland. Of the 1379 girls, 312 passed through the screening procedure based on dietary calcium intake (<900 mg Ca/day), age (10–12 years old), and sexual development (Tanner stages I-II), determined by a public health nurse using the Tanner grading system.[37] In addition, 5 months later, we randomly selected 61 girls from the screening, whose dietary calcium intake was >900 mg Ca/day. Among these 61 girls, there were 12 who were now 13 years old and had developed into Tanner stage III. To be eligible for the study, the participants had to have no history of serious medical conditions and no history of medication known to affect bone metabolism. Among those eligible, 258 (69%) agreed to participate in the study. Energy and nutrient intakes were analyzed from a 3-day food diary by the Finnish Micro-Nutrica PC-program. The investigational protocol was approved by the ethical committee of the University of Jyväskylä, the Central Hospital of Central Finland, and the Finnish National Agency of Medicines. Informed consent was obtained from all subjects and their parents before the assessments. The results presented in this report are from the baseline assessments from those girls who had genetic data available ($n = 245$).

PA questionnaire

A self-administrated PA questionnaire modified from the questions used in a WHO study[38] was filled out by the subject with their parents' help. The questionnaire was designed to evaluate the intensity, type, duration, and frequency of leisure time (after school) PAs. The questionnaire asked the girls what were the first, second, and third favorite sports they were practicing, the duration of exercise in each session, and the frequency of exercise per week. The intensity of each activity was calculated based on the energy expenditure.[39] Bone loading was based on whether the activity was weight bearing or not. A score of PA was calculated as follows: Score of physical activity = Σ_{1-3}(frequency × intensity index × duration × loading), where frequency = times/week, duration = hours, loading is non–weight bearing = 1 and weight bearing = 2. For the purpose of the analysis, subjects were classified into low (L) and high (H) PA groups according to the scores of PA cut from the median. Another classification into low and high PA groups was made based on the actual level of PA (hours/week) cut from the median.

Genetic analysis

Genomic DNA was extracted and purified from EDTA blood samples using the QIAmp Blood Kit (Qiagen, Hilden, Germany). The polymorphic *Pvu*II site of the *ER-α* gene was detected by PCR followed by enzymatic digestion.[40] PCR was performed with the following steps: 94°C for 4 minutes and 94°C for 1 minute, 58°C for 1 minute, 72°C for 1 minute for 31 cycles, and 72°C for 5 minutes. The product contains parts of intron 1 and exon 2 of the *ER-α* gene. After amplification, the PCR product was digested with *Pvu*II restriction endonuclease (Fermentas) and electrophoresed in a 2.0% agarose gel. Capital and lowercase letters refer to the absence and presence of the restriction site, respectively.

Body composition assessments

Body height was measured with a fixed-scale measuring device. Weight was determined (±0.1 kg) using a calibrated scale. The results of height and weight were used to determine body mass index (BMI), expressed as weight (kg) divided by height squared (m^2). Because body weight and height were highly correlated with each other, they were reduced to one body size index (BSI), different than BMI, using principal components factor analysis. This index was used for adjustment instead of body weight and height. The fat and lean tissue masses of the total body were analyzed from a total body DXA scan.

Bone mass/BMD and geometry assessments

BMC (g) and areal BMD (aBMD, g/cm^2) of the total body, lumbar spine (L_2–L_4), and left proximal femur (total femur and femoral neck) were measured using DXA (Prodigy; GE Lunar, Madison, WI, USA). The percentage CV (%CV) for repeated measurements ranged from 0.6 to 1.2 for BMC and from 0.86 to 1.3 for aBMD at the different bone sites. Volumetric BMD (vBMD, mg/cm^3), cortical thickness (CTh, mm), and bone, as well as lower leg muscle cross-sectional areas (CSA, mm^2), were measured using

Table 1. Physical Characteristics According to Combined ER-α Genotypes and PA Levels

	ER-α-PP and low PA (n = 24)	*ER-α-PP and high PA (n = 18)*	*ER-α-Pp and low PA (n = 56)*	*ER-α-Pp and high PA (n = 59)*	*ER-α-pp and low PA (n = 43)*	*ER-α-pp and high PA (n = 45)*
Age (years)	11.4 ± 0.7	11.0 ± 0.7	11.1 ± 0.8	11.4 ± 0.8	11.0 ± 0.7*	11.3 ± 0.7
Weight (kg)	41.4 ± 9.6	39.3 ± 6.5	37.8 ± 8.4	39.8 ± 8.3	37.1 ± 7.9†	39.2 ± 8.5
Height (cm)	149.3 ± 7.6‡	146.0 ± 6.2	143.9 ± 7.9	146.5 ± 8.3	143.5 ± 8.9	146.1 ± 7.1
BMI (kg/m^2)	18.4 ± 3.4	18.4 ± 2.4	18.1 ± 2.8	18.4 ± 3.0	17.8 ± 2.3	18.2 ± 2.9
Tanner stage (1/2/3%)	46/46/8	44/56/0	52/39/9	44/49/7	70/28/2§	51/49/0
Total body fat mass (g)	11445 ± 6333	11000 ± 4203	10002 ± 5215	10400 ± 5634	9950 ± 4882	10463 ± 5810
Total body lean mass (g)	28364 ± 4603	27158 ± 3056	26455 ± 4232	28049 ± 4499	25988 ± 4162	27362 ± 3925
PA score	133 ± 53	305 ± 115	141 ± 41	324 ± 91	140 ± 42	300 ± 94
PA (hours/week)	2.7 ± 2.1	3.1 ± 1.5	2.3 ± 2.0	3.3 ± 2.1	2.1 ± 1.8	3.7 ± 2.1

Data represent means ± SD.
* $p < 0.05$, significant difference compared with high PA and Pp or pp genotype or low PA and PP genotype.
† $p < 0.05$, significant difference compared with low PA and PP genotype.
‡ $p < 0.05$, significant difference compared with low PA and Pp or pp genotype.
§ $p < 0.05$, significant difference compared with low PA and PP or Pp genotype or high PA and Pp genotype.

pQCT (XCT 2000; Stratec Medizintechnik, Pforzheim, Germany). We scanned the left distal radius (4% of the total length of the forearm medial to the reference line; the length of the forearm was defined as proceeding from the olecranon to the ulna styloid of the subject's arm with the elbow at 90° and fingers held up) and tibia shaft (60% of the lower leg length between the tuberositas tibia and the medial malleolus). Data were analyzed using BonAlyse software (BonAlyse Oy, Finland).[41] The %CV was <3% for CSA and <1% for vBMD.

Statistical analysis

The conformance of the *ER-α* allele frequencies to Hardy-Weinberg equilibrium proportions[42] and differences in genotypes between the PA groups were tested using a χ^2 test. One-way ANOVA was used to test the differences in physical characteristics, except fat and lean mass, as well as in nutrient intakes with respect to ER-α genotype and PA level. Differences in bone and muscle properties (BMC, aBMD, CSA, vBMD, and CTh) as well as in fat and lean mass among the different groups were tested using analysis of covariance, controlling for the effects of Tanner stage and BSI. For multiple comparisons, the least significant difference (LSD) posthoc test was used. A nominal *F*-test for the PA score arm by genotype product term was used to test for evidence of exercise–gene interaction between PA and the *Pvu*II polymorphism at the *ER-α* gene. A *p* value of <0.05 was considered statistically significant. Statistical analyses were carried out using the Statistical Package (SPSS) version 12.0 for Windows.

RESULTS

General characteristics

The genotype distribution was found to be in Hardy-Weinberg equilibrium, suggesting that the subjects represented a homogenous genetic background. No significant differences in genotype and allele frequencies were found between the low and high PA groups in the total sample ($p = 0.61$). No significant differences in physical characteristics or nutrient intakes were found between the low and high PA groups or between the three ER-α genotypes separately. Physical characteristics of the subjects according to combined ER-α genotype and PA are presented in Table 1. Girls with low PA and pp genotype were younger compared with girls with high PA and Pp ($p = 0.007$) or pp ($p = 0.042$) genotype, or low PA and PP ($p = 0.044$) genotype. They were also lighter compared with girls with low PA and PP ($p = 0.043$) genotype and less matured compared with girls with low PA and PP ($p = 0.046$) or Pp ($p = 0.040$) genotype or high PA and Pp ($p = 0.011$) genotype. Girls with low PA and PP genotype were taller compared with girls with low PA and Pp ($p = 0.006$) or pp ($p = 0.005$) genotype. The mean values for BMI, total body fat, and lean masses were similar in the different groups. Nutrient intakes of the subjects are presented in Table 2. Girls with low PA and pp genotype had lower energy intake compared with girls with high PA and PP ($p = 0.034$) or pp ($p = 0.024$) genotype or low PA and PP ($p = 0.022$) genotype. They had also lower intakes of protein and vitamin D compared with girls with low PA and PP genotype ($p = 0.035$ and 0.045, respectively).

Bone mass and aBMD

High PA girls had significantly higher BMC and aBMD of the total body ($p = 0.025$ and 0.033, respectively), lumbar spine ($p = 0.011$ and 0.003, respectively), total femur ($p = 0.003$ and 0.013, respectively), and femoral neck ($p = 0.037$ and 0.003, respectively) compared with low PA girls (data not shown). BMC and aBMD of the total body, lumbar spine, total femur, and femoral neck were not significantly different between the three ER-α genotypes. However, we found an interaction between exercise and ER-α genotype with respect to total body BMC and aBMD (p for interaction = 0.030 and 0.012, respectively). After grouping the subjects according to their PA levels, we found a significant association between the *ER-α* polymorphism and total body BMC ($p = 0.007$) and aBMD ($p = 0.003$), as well as femoral aBMD (total femur: $p = 0.003$; femoral

TABLE 2. NUTRIENT INTAKES ACCORDING TO COMBINED ER-α GENOTYPES AND PA LEVELS

	ER-α-PP and low PA (n = 24)	*ER-α-PP and high PA (n = 18)*	*ER-α-Pp and low PA (n = 56)*	*ER-α-Pp and high PA (n = 59)*	*ER-α-pp and low PA (n = 43)*	*ER-α-pp and high PA (n = 45)*
Energy (kJ/day)	6919 ± 1829	6938 ± 1555	6495 ± 1307	6423 ± 1585	6001 ± 1587*	6763 ± 1681
Protein (g/day)	61 ± 15	61 ± 17	56 ± 14	57 ± 17	52 ± 13†	58 ± 14
Calcium (mg/day)	889 ± 325	926 ± 502	803 ± 325	807 ± 363	764 ± 320	781 ± 339
Vitamin D (μg/day)	3.2 ± 1.4	3.3 ± 2.6	2.6 ± 1.4	2.7 ± 1.8	2.3 ± 1.3†	2.9 ± 2.2

Data represent means ± SD.
* $p < 0.05$, significant difference compared with high PA and PP or pp genotype or low PA and PP genotype.
† $p < 0.05$, significant difference compared with low PA and PP genotype.

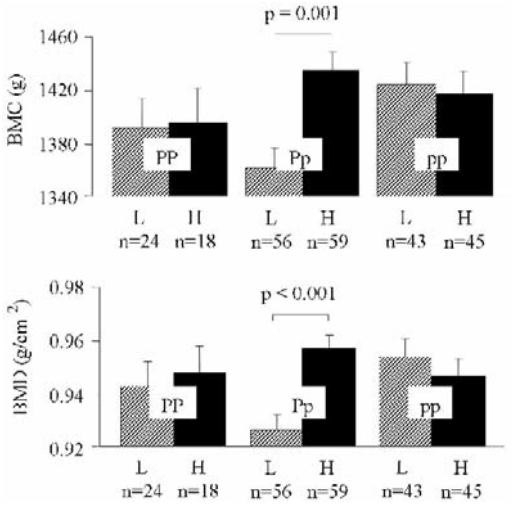

FIG. 1. Bone mass and BMD of the total body according to combined ER-α genotypes and PA levels. Data represent adjusted means ± SE. *p* values were determined by analysis of covariance with Tanner stage and BSI as covariates.

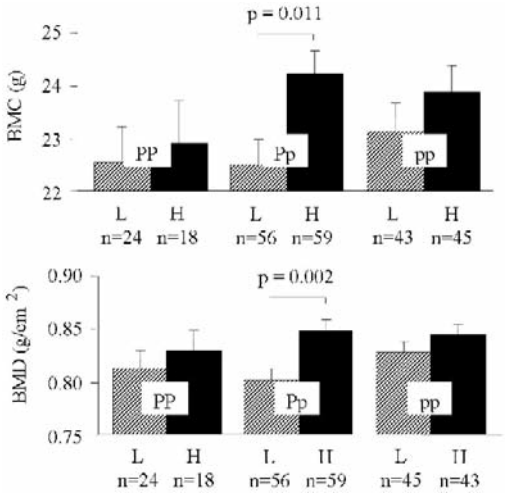

FIG. 2. Bone mass and BMD of the lumbar spine (L_2–L_4) according to combined ER-α genotypes and PA levels. Data represent adjusted means ± SE. *p* values were determined by analysis of covariance with Tanner stage and BSI as covariates.

neck: $p = 0.038$), but only in low PA girls. No such result was observed in high PA girls. Girls with heterozygote ER-α genotype (Pp) who had high PA (H) had significantly higher values of BMC ($p = 0.001$) and aBMD ($p < 0.001$) of the total body (Fig. 1) as well as lumbar spine ($p = 0.011$ and 0.002, respectively; Fig. 2) than their low PA counterparts (L). The same result was observed at the total femur ($p = 0.001$; Fig. 3) and femoral neck ($p = 0.040$ and 0.006, respectively; Fig. 4). No difference was found between girls with different PA levels within either homozygote groups (PP and pp) at any measured bone site. When the classification into low and high PA groups was made according to the level of PA (hours/week), the differences became smaller and did not reach statistical significance.

Volumetric BMD and geometry

High PA girls had significantly higher values of CTh (high versus low: 3.51 ± 0.4 versus 3.38 ± 0.4 mm, respectively, $p = 0.023$) and vBMD (high versus low: 671 ± 47 versus 658 ± 49 mg/cm^3, respectively, $p = 0.034$) of the tibia shaft than low PA girls. Bone properties did not differ between the three ER-α genotypes. However, when subjects were grouped according to their PA level, low PA girls bearing the Pp genotype had significantly thinner cortex of the tibia ($p = 0.023$) compared with the two homozygote groups. No such result was found in high PA girls. Girls with high PA and heterozygote ER-α genotype (Pp) had significantly thicker cortex ($p < 0.001$) and higher vBMD ($p = 0.001$) but no difference in bone CSA of the tibia compared with their low PA counterparts (Fig. 5). Bone properties did not differ in either homozygote groups (PP and pp), regardless of the PA level. No differences were found in bone properties of the distal radius between separate or combined ER-α genotypes and PA levels. In addition, muscle CSA of the lower leg was similar in the different groups. When the classification into low and high PA groups was made according to the level of PA (hours/week), the differences between the low and high PA groups became smaller but was still significant in the case of CTh of the tibia shaft within the heterozygote (Pp) group (low

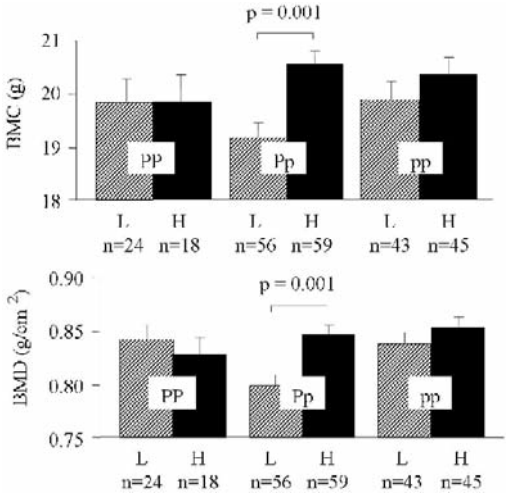

FIG. 3. Bone mass and BMD of the total femur according to combined ER-α genotypes and PA levels. Data represent adjusted means ± SE. *p* values were determined by analysis of covariance with Tanner stage and BSI as covariates.

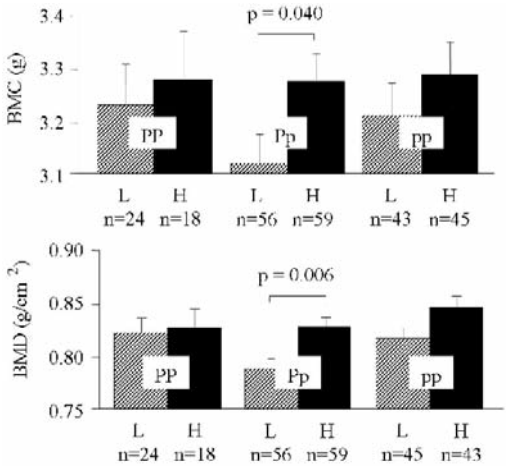

FIG. 4. Bone mass and BMD of the femoral neck according to combined ER-α genotypes and PA levels. Data represent adjusted means ± SE. *p* values were determined by analysis of covariance with Tanner stage and BSI as covariates.

versus high: 3.37 ± 0.4 versus 3.58 ± 0.4 mm, respectively, $p = 0.040$).

DISCUSSION

There are no previously published studies that have investigated the possible modifying roles of the *ER-α* gene polymorphism to exercise-induced effects on bone properties in pubertal girls. Our results suggest that ER-α genotype

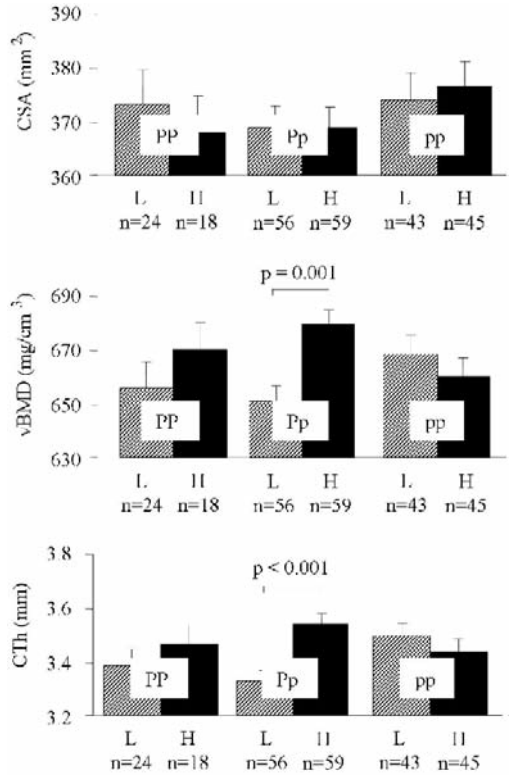

FIG. 5. Bone geometry and vBMD of the tibial shaft according to combined ER-α genotypes and PA levels. Data represent adjusted means ± SE. *p* values were determined by analysis of covariance with Tanner stage and BSI as covariates.

may influence the relationship between PA level and bone mineral mass/BMD at the loaded bone sites, such as the lumbar spine, femur, and tibia, during the fast growing period. On the other hand, no difference was observed in bone mass or geometry of the distal radius, which is not a weight-bearing bone, among girls with the different genotypes and PA level. The most popular kinds of sports the girls were practicing were running, swimming, and biking during summertime and skating, cross-country, and downhill skiing during wintertime, most of which mainly load the lower leg. Thus, different kinds of sports may create osteogenic strains site-specifically in the skeleton, depending on the loading action of the exercise. A possible benefit of PA was observed only in girls carrying the Pp genotype, whereas no strong benefit was observed in girls with the PP or pp genotype. This suggests that the heterozygote group may benefit most from the effect of exercise at loaded bone sites. However, this interpretation must be taken with caution. Low PA girls bearing the Pp genotype had lower values of bone properties compared with the other groups, whereas both homozygote groups with low PA had bone values as high as those with high PA. Therefore, it seems

that the heterozygote girls really need to exercise to reach the bone density of the others.

Generally, the measurement of PA in children is difficult. In this study, two different kinds of PA classification were made. The first one was calculated based on the intensity, type, duration, and frequency of the PAs, and the other classification was based only on the duration and frequency of PA. After calculation without the intensity and type of PA, the differences in bone properties became smaller, which suggests that the type of PA, whether weight bearing or not, is an important determinant of BMD. This is consistent with a previous Swedish study, in which the authors concluded that weight-bearing PA has great osteogenic potential.[43] Furthermore, they also found the bone area to be largely determined by body size and not by the mechanical loading from PA, as we did also. To control the effect of body size on bone size and mass, we made adjustment for BSI. In both homozygote groups, the bone size, seen as CSA, reflected the characteristics of body size, and was inversely related to BMD. However, there is no evidence of body size dependency in the bone size of the Pp girls. In addition, the compensatory coupling of bone size and BMD cannot be seen in the heterozygote girls. Therefore, the heterozygote girls seem to constitute a risk group, which would irreplaceably benefit from the effect of exercise.

It has been presumed that the mechanical load applied to bone is transduced through the skeleton through a mechanical signal that is detected by certain cells. Two types of cells are candidates for detecting these mechanical signals: bone-lining cells (of osteoblastic origin) and osteocytes. Osteocytes seem the most mechanosensitive cells in bone. These cells are connected to each other, to osteoblasts, and to bone-lining cells by means of cell processes residing in the canaliculi of unmineralized bone matrix, and these conduits allow communication between cells. Changes in the flow of interstitial fluid through osteocytic canaliculi, either by an electrical potential or a direct mechanical effect over the surface, will produce a signal that modulates bone formation (osteoblasts) or resorption (osteoclasts).[44] A very recent study suggests that mechanical strain can induce hormone-independent activation of ER-α,[29] through activation of extracellular regulated kinase-1 (ERK-1), a member of the mitogen-activated protein kinase (MAPK) family.[45] ERK-1 in turn activates ER-α through phosphorylation within the amino terminus of the receptor.[29,46] Consequently, our data evoke an interesting aspect when the effects of mechanical strain on ER-α regulation are concerned.

The mechanisms by which the different *ER-*α alleles might affect bone properties are not clear. Deficient ER-α protein expression in osteoblasts and osteocytes has been reported in males with idiopathic osteoporosis.[47] In addition, Herrington et al.[48] found that the *P* allele produces a functional binding site for the transcription factor B-myb and is capable of augmenting transcription in vitro. Thereby, the biological significance of the polymorphism in the *ER-*α gene may be in the regulation of the *ER-*α mRNA levels and protein expression. However, we have to keep in mind that individual single nucleotide polymorphisms are usually linked to each other, and the causal polymorphism may thus be elsewhere in the same gene or in genes nearby. To this regard it has been well established that the *Pvu*II polymorphism is in linkage disequilibrium with an upstream TA repeat polymorphism in the promoter region of the *ER-*α gene.[49] Our results found differential response between homozygotes and heterozygotes (a heterosis effect), which differs from the allele dosage effect that Herrington et al.[48,50] found. However, the effect of their hormone replacement therapy at the molecular level is dependent of estrogen action, whereas the effect of physical loading on ER-α activation may be hormone-independent. Therefore, the important characteristics of the ER-α protein in light of estrogen action and mechanical strain induction may well differ from each other. Molecular heterosis is common in humans and may occur in up to 50% of all gene associations.[51] It can also be gene-, phenotype-, gender-, and organ-specific. Importantly, Ushiyama et al.[52] found a heterosis effect examining the potential association between the *Pvu*II polymorphism in the *ER-*α gene and generalized osteoarthritis (GOA): the heterozygote genotype was significantly related to GOA, whereas neither homozygote genotype was a risk for GOA. There are several mechanisms of molecular heterosis, of which an interaction at the level of protein subunits would account for our findings. It is not difficult to visualize an allosteric effect in which two identical ER-α subunits would function more or less efficiently in the mechanical strain mediation than structurally different subunits. Such a mechanism has also been described in humans for the *TIGR* gene and steroid-induced glaucoma.[53] Another possibility suggests that when two functionally disparate alleles are present, the range of expression of *ER-*α gene products is greater in Pp heterozygotes, thus providing for a greater range of plasticity and response to change in mechanical strain than in PP or pp homozygotes.

We found no differences in bone properties with regard to ER-α genotype. On the other hand, according to PA level, high PA girls had significantly higher bone mass and BMD than low PA girls. However, after taking the genotypes into account, the associations persisted in the low PA girls, but disappeared in the high PA girls. Thus, high PA may hide or compensate the genetic effects on bone properties. Our results are in concordance with a recent exercise intervention trial in middle-aged men conducted by Remes et al.,[54] which found that the ER-α genotype may modulate the exercise-induced effects on BMD. However, they found no differences in aBMD values between the genotype groups in different measurement points in the exercise and reference groups; only the change (%) in lumbar aBMD was significantly associated with the ER-α genotype in the exercise group. Moreover, they speculated that the increase in the lumbar aBMD may reflect age-related degenerative changes in the spine. The difference in gender and age of the subjects as well as the difference in study design and statistical approach makes it difficult to compare the results between the studies. Nonetheless, both of them suggest that the association between PA level, either pubertal or life-time, and BMD may have genetic components, and these parameters could share a common genetic basis. Furthermore, they could help clarify conflicting results observed in earlier

studies investigating the effect of exercise training on BMD, as well as contradictory findings on the association between the ER-α genotype and BMD.

This study has a number of potential limitations. First, the cross-sectional design and the use of questionnaire to assess PA level may misclassify activity levels. However, high PA girls had significantly higher bone mass and BMD than low PA girls, suggesting that this is not a major problem. It still does not exclude the possibility of selection bias, i.e., that more physically active girls may be genetically stronger at the beginning than less physically active girls. Another limitation is the small sample size for evaluating gene–environment interactions, which may lead to a false positive conclusion.

The uniqueness of this study is the careful assessments of bone properties at weight-bearing and non–weight-bearing skeletal sites and the use of pQCT to assess vBMD and geometry. There has also been limited work on the genetic determinants of bone-related traits in pre- and early pubertal children. Most studies of childhood activity and BMD have been undertaken in elite sporting populations and therefore cannot be generalized to normal children. Consequently, a strength of this study is in focusing on girls partaking in a wide range of exercise to a normal extent.

In conclusion, these findings suggest that the *Pvu*II polymorphism in the *ER-*α gene may modulate the effect of exercise on BMD at loaded bone sites. The heterozygotes may benefit most from the effect of exercise, whereas neither of the homozygote groups received any significant improvement from high PA. Furthermore, high PA may hide the genetic influence on bone. Indeed, it seems that one may compensate one's less favorable Pp genotype by increasing leisure PA at pre- and early puberty.

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Address reprint requests to:
Miia Suuriniemi, MSc
Department of Health Sciences
University of Jyväskylä
PO Box 35
Jyväskylä FIN-40014, Finland
E-mail: miisu@sport.jyu.fi

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IV

THE EFFECT OF ESTROGEN RECEPTOR α POLYMORPHISM ON THE SKELETAL RESPONSIVENESS TO EXERCISE DURING PUBERTY

by

Suuriniemi, M., Suominen, H., Mahonen, A., Alén, M. & Cheng, S

(submitted)

The effect of estrogen receptor α polymorphism on the skeletal responsiveness to exercise during puberty

Miia Suuriniemi,[1,2] Harri Suominen,[2] Anitta Mahonen,[3] Markku Alén,[2] and Sulin Cheng[2]

[1]Department of Cell Biology, University of Jyväskylä, Finland
[2]Department of Health Sciences, University of Jyväskylä, Finland
[3]Department of Medical Biochemistry and Bone and Cartilage Research Unit, University of Kuopio, and Department of Surgery, Kuopio University Hospital, Finland

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Corresponding author:
Miia Suuriniemi
University of Jyväskylä
Department of Health Sciences
P.O. Box 35
FI-40014 University of Jyväskylä, Finland

Tel: +358-14-260 2068
Fax: +358-14-260 2011
E-mail: miisu@sport.jyu.fi

ABSTRACT

Mechanical loading affects bone mass and structure through a cascade of cellular events that involve estrogen receptor α (ER-α). We recently showed in pre- and early pubertal girls that the *PvuII* polymorphism in the *ER-α* gene modulates the effect of exercise on bone density at loaded bone sites. To further explore this observation, we compared girls with consistently low physical activity (LLPA) to those with consistently high physical activity (HHPA) in the range of 4 years when the girls underwent through their puberty. One hundred and thirty four girls, aged 10-12 years at the baseline, were measured using dual-energy X-ray absorptiometry and peripheral quantitative computed tomography at the baseline, 12, and 24 month follow-up. The skeletal benefits of exercise were observed only in girls carrying the Pp genotype, whereas no strong benefits were observed in girls with the PP or pp genotype. Furthermore, in the homozygotes, the growth processes of bone mass, density, and size of the tibia were comparable between LLPA and HHPA, while in the heterozygotes, girls with HHPA tended to have constantly higher bone mass of the tibia than those with LLPA, even after menarche. The heterozygote girls seem to be particularly vulnerable to the adverse effects of physical inactivity and may benefit most from the effect of exercise, while the homozygote girls may not gain any additional benefit from exercise. This genetic effect could also contribute to the failure of bone's adaptation to functional loading and thus to postmenopausal osteoporosis as well.

Key words: Estrogen receptor, polymorphism, exercise, bone mineral mass, puberty

INTRODUCTION

Mechanical loading through exercise builds strong bones and this effect is most pronounced during skeletal growth and development. Over the past few years, there have been observations to suggest that estrogen receptor α (ER-α) plays an important role in mediating the responses of the skeleton to mechanical loading. The idea of ER-α as one of the skeletal mechanotransducers is supported by several findings from *in vitro* studies. For example, exposure of osteoblasts to mechanical strain has shown to stimulate cell proliferation and activity of the estrogen response elements (ERE), both of which were blocked by the ER antagonist ICI 182,780.[1] Furthermore, mechanical strain was found to activate ER-α by extracellular-regulated kinase (ERK) –dependent phosphorylation of residue Ser^{122}, within the activation domain at the amino-terminus of the ER-α.[2] More recently, Lee et al. showed that mice homozygous for deletion in the *ER-α* gene had an impaired osteogenic response to mechanical strain.[3]

Considering such a crucial role for the ER-α in the mechanotransduction it is justified to take one step forward and ask whether genetic polymorphisms in the *ER-α* have any effects in the skeletal responsiveness to mechanical loading during exercise. If this turns out to be correct, it would denote a true clinical implication of the findings for human populations, and help explain why some people are less responsive to exercise than others. In fact, we were recently able to show in pre- and early pubertal girls that the *PvuII* polymorphism in the *ER-α* gene modulates the effect of exercise on bone density at loaded bone sites.[4] The heterozygotes benefited most from the effect of exercise, while neither of the homozygote groups got any significant improvement from high physical activity (PA). To further explore this observation longitudinally, we compared girls with consistently low physical activity (LLPA) to those with consistently high physical activity (HHPA) in the range of 4 years centred by menarche when the girls underwent through their puberty. The aim of the present study was thus to investigate the influence of the *ER-α PvuII* polymorphism on the skeletal responsiveness to exercise during rapid growth, and clarify whether the effects sustain after the menarche.

SUBJECTS AND METHODS

Subjects

The study subjects were part of a cohort of 258 healthy pre- and early pubertal Finnish girls, aged 10-12 years at the baseline, who were recruited for a 2-year intervention study (the Calex-study) to evaluate the effects of calcium, vitamin D, and milk products´ supplementation on bone mass accrual.[4-8] To be eligible

for the study, the participants had to have no history of serious medical conditions and no history of medication known to affect bone metabolism. The investigational protocol was approved by the ethical committee of the University of Jyväskylä, the Central Hospital of Central Finland, and the Finnish National Agency of Medicines. An informed consent was obtained from all subjects and their parents prior to the assessments. This report uses longitudinal data of those girls who participated in the bone assessments and did not change their PA level over a 2-year period (n = 134).

Lifestyle and behavioural characteristics as well as medical history were collected via a self-administered questionnaire at 6-month interval during the study. Girls filled the questionnaire with their parents' assistance and the questionnaires were checked by a study nurse. Energy and nutrient intakes were analyzed from a 3-day food diary by the Finnish Micro-Nutrica PC-program. The menarche age was defined as the first onset of menstrual bleeding and collected by a questionnaire and retrospective phone call. Height and weight were determined with the subjects wearing light clothing and no shoes every 6 month. Body mass index (BMI) was calculated as weight (kg) / height^2 (m).

The method for the determination of PA level has been described in more detail in our previous report.[4] Briefly, a self-administrated PA questionnaire regarding the intensity, type, duration, and frequency of the leisure time (after school) physical activities was collected at the baseline, 6, 12, 18, and 24 month follow-up. A score of PA was calculated as follows: Score of PA = $\Sigma_{1\text{-}3}$ (frequency * intensity index * duration * loading), where frequency = times/week, duration = hours/week, loading: non-weight bearing = 1 and weight bearing = 2. For the purpose of the analysis, girls were classified into low and high PA groups according to the scores of PA cut from the median. Those girls, whose PA level kept low at the baseline, 12, and 24 month follow-up, were then categorized into LLPA group (consistently low physical activity). Those girls, whose PA level kept high at the baseline, 12, and 24 month follow-up, were respectively categorized into HHPA group (consistently high physical activity). Those girls, who changed their PA level (either increased or reduced) during the study, were excluded from this report.

Genetic analysis

Genomic DNA was extracted and purified from EDTA blood samples using QIAmp Blood Kit (Qiagen GmbH, Hilden, Germany). Genotyping for the polymorphic *PvuII* site of the *ER-α* gene was carried out using polymerase chain reaction – restriction fragment length polymorphism –based method, as previously described.[9]

Hormonal analysis

Blood samples were taken in the morning between 7:30 and 9:00 after an overnight fast at the baseline, 12, and 24 month follow-up. For each subject, the date when the samples were taken, which ranged from the middle of December to the end of February, was kept as similar as possible across the 3 measurement time points. If the girl began menstruation, the blood sample was taken between the 2nd and 5th bleeding day. Serum samples were stored at –75°C until analyzed.

Serum 17β-estradiol (E2) and sex hormone binding globulin (SHBG) were assessed using time-resolved fluoroimmunoassays (Delfia, Wallac Oy, Turku, Finland), and serum insulin-like growth factor-1 (IGF1) by an immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, California, USA). Between and within assay coefficients of variation (CVs) were 2.9-8.3 % and 0.1-15.1 % for E2, 0.7-1.6 % and 0.1-3.2 % for SHBG, and 9.3-15.8 % and 3.3-4.6 % for IGF1, respectively.

Bone assessments

Bone area (BA), bone mineral content (BMC), and areal bone mineral density (aBMD) of the spine L2-L4, total femur, and femoral neck were assessed at the baseline, 12, and 24 month follow-up, and BA, BMC, aBMD, lean tissue mass (LTM), and fat mass (FM) of the total body were assessed at the baseline and 24 month follow-up using dual-energy X-ray absorptiometry (DXA, Prodigy, GE Lunar Corp., Madison, Wisconsin, USA). The CVs for repeated measurements were 0.6-1.2 % for BMC, 0.9-1.3 % for aBMD, 1.0 % for LTM, and 2.2 % for FM.

Cross-sectional area (CSA), BMC, volumetric bone mineral density (vBMD), and moment of inertia (Ipolar) of the distal radius and tibia shaft, as well as cortical thickness (CTh) of the tibia shaft, were assessed at the baseline, 12, and 24 month follow-up using peripheral quantitative computed tomography (pQCT, XCT 2000, Stratec Medizintechnik, GmbH, Pforzheim, Germany) with an in-plane pixel size of 0.59 mm and analyzed using a validated software (Geanie®CE, BonAlyse Oy, Jyväskylä, Finland), as previously described.[7,8] The CVs between two consecutive measurements with repositioning were 1.1-3.0 % for CSA and < 1.0 % for vBMD.

Statistical analysis

The distributions of hormonal data were normalized by transformation to achieve the assumption of normal distribution of residuals. One-way analysis of variance (ANOVA) and covariance (ANCOVA) were used to test differences in the assessed variables between the different groups at the baseline, 12, and 24 month follow-up. For multiple comparisons Bonferroni post hoc test was used. A p value of less than 0.05 was considered statistically significant. Statistical analyses were carried out using the Statistical Package (SPSS) version 12.0 for Windows.

In addition, a hierarchical linear model with random effects was employed to explore the effects of growth on bone variables of the tibia shaft. In this model, the time relative to menarche was entered as the explanatory variable in the form of polynomial spline functions to explain the change of these variables over time in the different genotype groups with LLPA and HHPA (MLwiN 1.1 software, Multiple Project, Institute of Education, University of London, UK).

RESULTS

Physical characteristics of the subjects according to combined ER-α genotype and PA at the 24 month follow-up are presented in Table 1. No significant differences in maturational status or anthropometric data were observed between the six different groups. The same was true when comparing abovementioned characteristics at the baseline and 12 month follow-up as well (data not shown). The PA levels in terms of hours per week for each group were as follow: PP-LLPA: 1.8; PP-HHPA: 4.2; Pp-LLPA: 1.7; Pp-HHPA: 4.3; pp-LLPA: 1.7; pp-HHPA: 4.4. Dietary intakes over the 2-year period were similar among the different groups. However, girls with the PP genotype and HHPA had significantly higher calcium intake than those with the PP and LLPA ($p = 0.024$), Pp and LLPA ($p = 0.039$), and Pp and HHPA ($p = 0.041$) (Table 2, data shown at the 24 month follow-up).

The serum concentrations of E2, SHBG, and IGF1 did not differ significantly between the different groups during the 2-year period (Table 3, data shown at the 24 month follow-up).

Girls with the heterozygote genotype (Pp) and LLPA tended to have the lowest, while those with HHPA tended to have the highest bone values of all (Table 4, data shown at the 24 month follow-up). Particularly, aBMD ($p = 0.030$) of the total femur, BMC ($p = 0.022$) and aBMD ($p = 0.024$) of the femoral neck (Table 4), as well as BMC ($p = 0.029$) and CTh ($p = 0.009$) of the tibia shaft (Figure 1), were significantly lower in the Pp girls with LLPA compared to their HHPA counterparts. After controlling for the effects of maturational or E2 status and body weight or muscle CSA of the lower leg, the differences still remained significant (data not shown). The bone properties of the distal radius were, however, similar between the different groups. No differences were found between girls with different PA level within either homozygote group (PP or pp) at any measured bone site.

We further compared girls within each genotype group with LLPA to those with HHPA using a hierarchical linear model. This enables us to explore the growth processes of the tibia shaft longitudinally by synchronizing the girls´ maturational status. In the PP and pp groups, the growth processes of tibial BMC, vBMD, CSA, and CTh were comparable and close to each other between LLPA and HHPA (Figure 2). However, in the Pp group, girls with HHPA tended to have constantly higher tibial BMC than their LLPA counterparts ($p =$

0.069), while no differences were observed in the growth processes of the other tibial characteristics.

DISCUSSION

The results of the present longitudinal study confirm our previous cross-sectional findings[4] and suggest that the ER-α polymorphism may influence the skeletal responsiveness to exercise at the loaded bone sites, such as femur and tibia, during the fast growing period, and the effects sustain even after the menarche. On the other hand, no differences were observed in bone mass or geometry of the distal radius, which is not a weight bearing bone, between girls with different genotypes and PA level. The skeletal benefits of exercise were observed only in girls carrying the Pp genotype, whereas no strong benefits were observed in girls with the PP or pp genotype. Furthermore, the differences remained significant after adjusting for muscle CSA, suggesting a primary effect on bone. These results are in agreement with previous studies that have demonstrated a lack of association between ER-α genotypes and muscle mass and strength.[10-13] However, physically low active girls bearing the Pp genotype had lower values of bone properties compared to the other groups, whereas both homozygote groups with low PA had as high bone values as those with high PA. Therefore, the heterozygote girls seem to constitute a risk group, which would irreplaceably benefit from the effect of exercise, while the homozygote girls may not gain any additional benefit by increasing their habitual PA. This appears to be the case in chicks and mice as well. The more responsive chicken breed exhibits lower bone mass than the less responsive breed.[14] Also, long bones in mice of the C3H/He strain are largely unresponsive to mechanical loading, yet they have very high bone density.[15] These mice must generate more strain in their bones before an anabolic response is initiated, which may hold true in the homozygote girls of the present study as well. Moreover, in addition to being less sensitive to enhanced loading conditions, the C3H/He mice are also less sensitive to reduced loading conditions,[16] a finding similar to ours for girls.

We further explored the growth of the tibia in more detail, in terms of bone mass, density, and geometry. The growth rates in the range of 4 years were pretty similar among the girls. This is likely a reflection of the normal pubertal development, strongly driven by the high hormonal output, which may more or less hide the effect of PA. The differences in the bone characteristics between LLPA and HHPA groups were most evident in girls with the Pp genotype, and they still existed after the menarche, suggesting that they may last up to adulthood as well. However, we do not know the consequences of reduction in PA level for the HHPA girls. The differences likely emerged when the HHPA girls started their active lifestyle earlier in their childhood, and imperceptibly continued to sharpen thereafter. The stunning

similarity in the vBMD between LLPA and HHPA well arises from the fact that these girls are fairly healthy, lacking any major defects of bone mineralization, but may also indicate that exercise do not make the bones denser.

The extent to which endogenous hormone adequacy limits the skeletal response to mechanical loading is not clear, although models of skeletal adaptation suggest that responsiveness may be modulated by hormonal milieu. Taking this into consideration we made adjustment for the serum concentration of E2 while performing further analysis of covariance. The differences in the femoral and tibial characteristics between the Pp girls with LLPA and HHPA still remained significant, suggesting that the genetic effect on the skeletal responsiveness to PA is independent of estrogen action. Furthermore, we did not find any major differences in the serum concentrations of E2, SHBG, or IGF1 between the different groups. The hormonal status observed in this study probably represents the overall systemic state, since the effects of mechanical strain on the hormonal fluctuations are probably transient and local as well. These findings are relevant given the known cross-talk between the E2 and IGF1 signaling pathways and the effects of E2 on the ER-α expression,[17] which could confound our results. Unfortunately, we know nothing about the ER-α number and activity in the bone tissue of these girls, neither do we know the local hormonal status in the skeleton.

It is well established that skeletal loading triggers a cascade of cellular responses in osteoblasts which ultimately induce adaptive bone modeling and remodeling, leading to changes in bones´ morphology and mass. The process, by which bone cells sense mechanical stimuli in their external environment and then translate the information into a signal that can potentially elicit the responses to skeletal loading, is, however, poorly understood. One of the few certainties about mechanotransduction is that it likely occurs by means of more than one stimulus and through multiple mechanisms. Accumulating evidence support the involvement of ER-α in the bone cells´ early responses to mechanical strain, and have shown diminished or eliminated strain-adaptive responses of the skeleton, if ER-α is non-functional or absent.[3] Lanyon and colleagues have proposed an interesting signal transduction pathway for mechanical strain in osteoblasts based on available data.[18] According to their model, the initial mechanosensor is the transmembrane integrin, residing in conjunction with the IGF1 receptor (IGF1-R) within focal adhesions. Mechanical stimuli may destabilize the binding interaction between the integrin and its extracellular matrix ligand, such as collagen or osteopontin, causing a conformational change in the integrin. This integrin activation initiates a cascade of events, including activation of the mitogen-activated protein kinase (MAPK)/ERK pathway and subsequent phosphorylation of the ER-α, which results in both classical activation of gene transcription within the nucleus, necessary to the cell proliferation and bone matrix formation, as well as ER-α participation in extranuclear signalling events, such as enabling the interaction between IGF and IGF1-R. Since ER-α is involved at more than one stage in this signalling pathway after mechanical strain, it may well be that reduction in ER-

α function, either quantitative or qualitative, due to genetic polymorphisms may reduce the effectiveness of the response to exercise.

Functional polymorphisms that alter protein structure, gene expression, or mRNA processing appear to play a critical role in shaping human phenotypic variability, including responsiveness to environmental factors. Thousands of single nucleotide variants in our genome altogether form the basis why we look different from each other, and explain why some of us may response more vigorously to external stimuli than others. Certain single polymorphic locus may be sufficient to cause a certain degree of resistance or unresponsiveness for example to drug or exercise. Moreover, epistatic effects (multiple interacting polymorphisms) are likely to play a role as well. The most commonly studied polymorphisms, nonsynonymous changes that alter amino acid coding, appear in many cases insufficient to account for interindividual differences in disease etiology and response to therapies. Further, it is estimated that functional polymorphisms that are *cis*-regulatory in the human genome outnumber those that alter protein sequence, and they provide a rich substrate for evolutionary selection.

The mechanisms by which the different *ER-α* alleles might affect bone responsiveness are not clear. Possible explanations for the heterosis effect observed in this study include that both of the alleles confer an independent but cumulative advantage for the subject; for example, the receptor subunit produced by one allele may be more readily phosphorylated and thus activated, while the other one may be better in terms of transactivation. Therefore, the hybrid receptor combines the best traits of both parental alleles, and is thus more efficient in translating the mechanical signal into skeletal responses than the homodimeric receptors. Another explanation suggests a greater responsiveness in the heterozygotes because they show a broader range of gene expression than the homozygotes. External stimuli may also alter the genomic imprinting, the phenomenon by which the two alleles of certain genes are differentially expressed according to their parental origin, or the various mechanisms, such as DNA methylation or histone deacetylation, which induce more random allelic silencing or activation. Recently, epigenetic alterations have been firmly demonstrated to play a role in tumor initiation, development, and progression, as well as to contribute to progression to a drug-resistant state.[19] This would have a particular impact evident only in heterozygotes, if the two alleles functionally differ from each other. For example, if the more active allele is silent in the situations deprived of mechanical loading, which is able to switch on the active allele and repress the less active one, we could see a more powerful response to exercise in the heterozygote individuals than in either of the homozygotes.

This study has some limitations. Firstly, there was no exercise intervention included that would have allowed us to explore the responsiveness to exercise more correctly. Meanwhile, we made the classification according to the persistent PA level during a 2-year period that likely reflects the overall PA during the whole childhood, and the study is thus cross-sectional in that regard.

Therefore, the possibility of selection bias can not be ignored. However, if such exists, we may well assume it to affect all the genotypes evenly. The existence of about 2.5 hours difference in the weekly PA between the LLPA and HHPA girls and considering the type of PA, whether weight bearing or not, when classifying the girls into the different PA groups, should provide a clear and fairly reliable image with respect to their loading conditions. It has to be remembered that these girls are not athletes but normal children partaking in a wide range of exercise to a normal extent, and therefore nicely represent the mass of the population. Another limitation is the small sample size, which may lead to a false positive conclusion.

In conclusion, the present findings suggest that the *PvuII* polymorphism in the *ER-α* gene may affect the skeletal responsiveness to exercise during puberty. The heterozygote girls seem to be particularly vulnerable to the adverse effects of physical inactivity and may benefit most from the effect of exercise, while the homozygote girls may not gain any additional benefit by increasing their PA. Importantly, the effects of exercise in the heterozygotes may sustain beyond the menarche. Altogether, these findings may partly explain the genetic basis of human variation in responsiveness to exercise training and the failure of bone's adaptation to functional loading in postmenopausal osteoporosis as well.

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FIGURE LEGENDS

Fig. 1. Tibial characteristics according to combined ER-α genotypes and PA levels at the 24 month follow-up. Data represent means. ANOVA with Bonferroni for multiple comparisons. * p=0.029, LLPA vs. HHPA. † p=0.009, LLPA vs. HHPA.

Fig. 2. Growth patterns of the tibia shaft according to PA levels within ER-α genotypes during puberty.

Table 1. Physical characteristics according to combined ER-α genotypes and PA levels at the 24 month follow-up

	ER-α-PP LLPA (n=12)	ER-α-PP HHPA (n=12)	ER-α-Pp LLPA (n=32)	ER-α-Pp HHPA (n=30)	ER-α-pp LLPA (n=21)	ER-α-pp HHPA (n=27)
Age (yr)	13.2 (0.8)	13.1 (0.6)	13.1 (0.6)	13.3 (0.7)	13.0 (0.8)	13.2 (0.7)
Menarche age (yr)	13.1 (0.9)	13.0 (0.9)	13.0 (0.7)	12.7 (0.8)	12.8 (0.8)	13.1 (0.8)
Weight (kg)	53.0 (12.5)	49.6 (8.7)	47.7 (10.8)	50.9 (8.3)	47.5 (6.1)	46.9 (9.0)
Height (cm)	160.1 (6.0)	158.2 (5.0)	155.7 (7.5)	157.4 (6.0)	156.2 (5.5)	155.8 (8.3)
BMI ($kg \cdot m^{-2}$)	20.5 (4.1)	19.8 (3.2)	19.5 (3.4)	20.5 (3.0)	19.4 (2.1)	19.2 (2.8)
FM (kg)	15.1 (9.2)	13.9 (5.8)	13.3 (6.6)	14.1 (6.2)	12.4 (4.7)	11.8 (5.8)
LTM (kg)	35.2 (5.3)	34.1 (3.6)	32.8 (4.7)	35.1 (4.1)	33.2 (3.6)	33.5 (4.1)

Data represent means (SD).
None of the differences are significant.

Table 2. Dietary intakes according to combined ER-α genotypes and PA levels at the 24 month follow-up

	ER-α-PP LLPA (n=12)	ER-α-PP HHPA (n=12)	ER-α-Pp LLPA (n=32)	ER-α-Pp HHPA (n=30)	ER-α-pp LLPA (n=21)	ER-α-pp HHPA (n=27)
Energy (MJ/day)	6.7 (1.4)	7.9 (1.4)	7.2 (1.9)	7.2 (2.1)	7.8 (1.8)	7.3 (1.8)
Protein (g/day)	63 (12)	76 (17)	63 (18)	64 (19)	67 (18)	68 (20)
Calcium (mg/day) *	873 (346)	1459 (458)	1001 (360)	998 (344)	1169 (466)	1231 (559)
Vitamin D (µg/day)	3.1 (1.2)	3.5 (2.8)	2.6 (1.3)	3.5 (2.2)	3.1 (1.4)	3.9 (4.1)

Data represent means (SD).
ANOVA with Bonferroni for multiple comparisons.
* Girls with ER-α-PP and HHPA had significantly higher calcium intake than those with ER-α-PP and LLPA (p=0.024), ER-α-Pp and LLPA (p=0.039), and ER-α-Pp and HHPA (p=0.041).

Table 3. Hormonal data according to combined ER-α genotypes and PA levels at the 24 month follow-up

	ER-α-PP LLPA (n=12)	ER-α-PP HHPA (n=12)	ER-α-Pp LLPA (n=32)	ER-α-Pp HHPA (n=30)	ER-α-pp LLPA (n=21)	ER-α-pp HHPA (n=27)
E2 (pmol/L)	172 (95)	169 (72)	149 (40)	183 (108)	177 (93)	165 (50)
SHBG (nmol/L)	60 (31)	57 (19)	58 (21)	62 (27)	76 (33)	69 (27)
IGF1 (ng/mL)	358 (75)	349 (109)	368 (81)	339 (94)	347 (86)	351 (88)

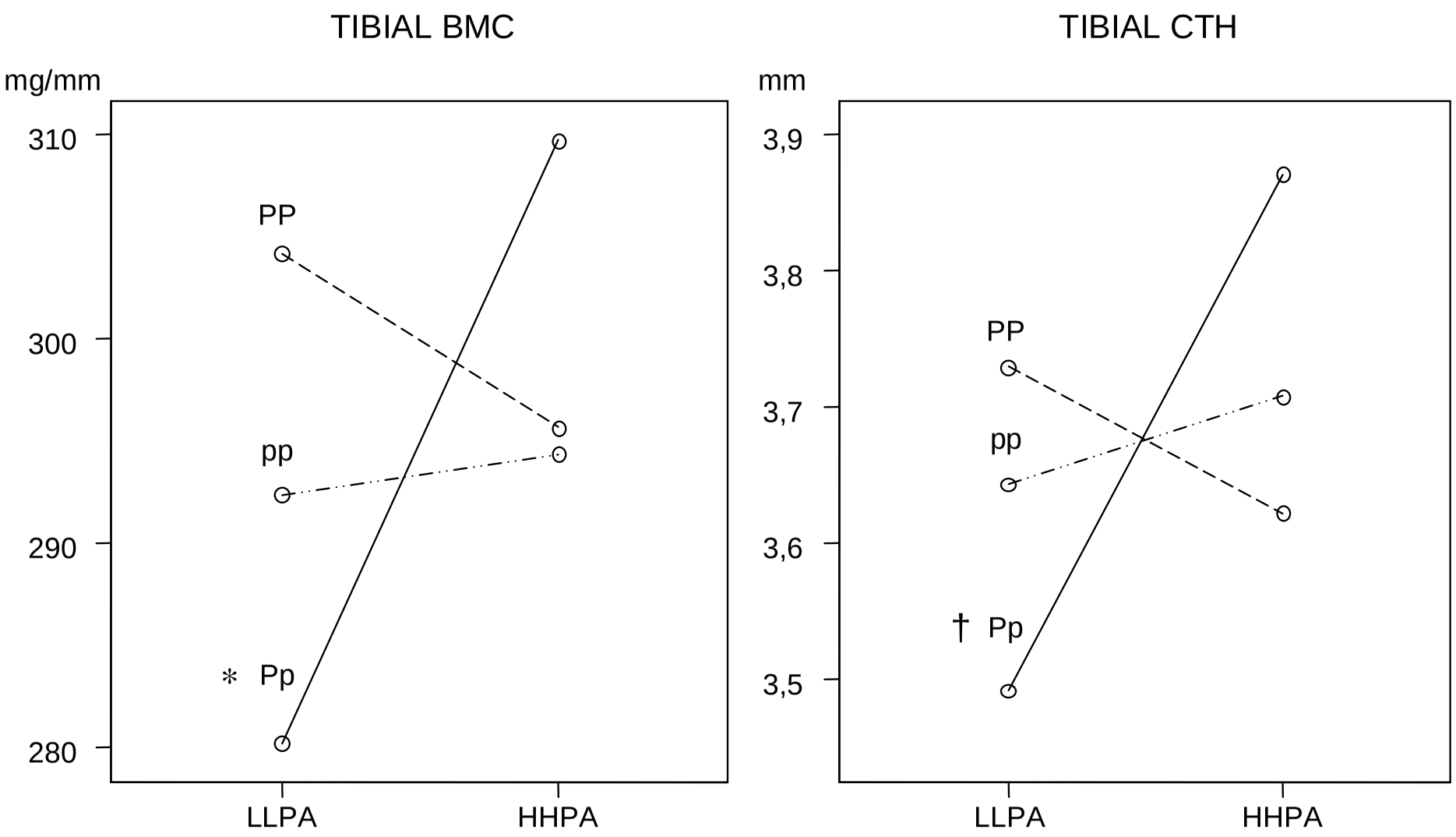
Data represent means (SD).
None of the differences are significant.

Table 4. Bone properties according to combined ER-α genotypes and PA levels at the 24 month follow-up

	ER-α-PP LLPA (n=12)	ER-α-PP HHPA (n=12)	ER-α-Pp LLPA (n=32)	ER-α-Pp HHPA (n=30)	ER-α-pp LLPA (n=21)	ER-α-pp HHPA (n=27)
Total body						
BA (cm)	1846 (190)	1840 (188)	1746 (253)	1842 (211)	1777 (169)	1750 (219)
BMC (g)	1905 (262)	1920 (306)	1771 (373)	1953 (330)	1809 (295)	1802 (284)
aBMD (g/cm^2)	1.030 (0.068)	1.039 (0.069)	1.006 (0.077)	1.055 (0.075)	1.013 (0.081)	1.027 (0.057)
Spine L2-L4						
BA (cm)	34.2 (3.1)	34.4 (2.7)	33.3 (4.4)	35.0 (3.8)	34.0 (3.8)	33.3 (4.2)
BMC (g)	33.6 (5.7)	33.9 (6.1)	31.4 (8.4)	36.2 (7.8)	32.1 (7.1)	32.8 (7.7)
aBMD (g/cm^2)	0.980 (0.109)	0.983 (0.129)	0.931 (0.147)	1.022 (0.131)	0.937 (0.122)	0.977 (0.130)
Total femur						
BA (cm)	28.0 (2.0)	28.0 (1.9)	27.3 (2.6)	28.0 (2.1)	26.9 (2.0)	26.7 (2.2)
BMC (g)	26.7 (3.4)	26.6 (4.9)	24.4 (4.9)	27.4 (4.5)	24.7 (3.8)	25.3 (3.0)
aBMD (g/cm^2) *	0.955 (0.096)	0.945 (0.132)	0.886 (0.121)	0.977 (0.127)	0.916 (0.097)	0.949 (0.092)
Femoral neck						
BA (cm)	4.25 (0.54)	4.40 (0.33)	4.29 (0.31)	4.32 (0.43)	4.25 (0.27)	4.10 (0.53)
BMC (g) †	3.98 (0.52)	4.12 (0.65)	3.71 (0.62)	4.20 (0.61)	3.78 (0.54)	4.02 (0.45)
aBMD (g/cm^2) †	0.916 (0.092)	0.937 (0.125)	0.867 (0.110)	0.953 (0.111)	0.892 (0.088)	0.939 (0.094)
Distal radius						
CSA (mm^2)	275 (43)	278 (65)	262 (46)	261 (53)	260 (58)	268 (47)
BMC (mg/mm)	78 (10)	78 (10)	74 (13)	80 (14)	77 (17)	77 (12)
vBMD (mg/cm^3)	288 (32)	287 (48)	287 (50)	314 (58)	300 (49)	290 (42)
Ipolar (mg·cm)	438 (103)	438 (136)	394 (112)	422 (145)	410 (162)	409 (119)

Tibia shaft						
CSA (mm^2)	425 (41)	418 (47)	391 (55)	419 (53)	410 (49)	412 (58)
vBMD (mg/cm^3)	716 (43)	709 (64)	718 (54)	741 (46)	713 (53)	720 (54)
Ipolar ($mg \cdot cm$)	2794 (551)	2741 (650)	2391 (664)	2829 (699)	2614 (621)	2641 (674)

Data represent means (SD).
ANOVA with Bonferroni for multiple comparisons.
* Girls with ER-α-Pp and LLPA had significantly lower aBMD (p=0.030) of the total femur than those with ER-α-Pp and HHPA.
† Girls with ER-α-Pp and LLPA had significantly lower BMC (p=0.022) and aBMD (p=0.024) of the femoral neck than those with ER-α-Pp and HHPA.


TIBIAL BMC
mg/mm
310
300
290
280
PP
pp
* Pp
LLPA
HHPA
TIBIAL CTH
mm
3,9
3,8
3,7
3,6
3,5
PP
pp
† Pp
LLPA
HHPA

V

THE *COMT* val158met POLYMORPHISM IS ASSOCIATED WITH EARLY PUBERTAL DEVELOPMENT, HEIGHT, AND CORTICAL BONE MASS IN GIRLS

by

Eriksson, A. L., Suuriniemi, M., Mahonen, A., Cheng, S. & Ohlsson, C. 2005

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