Children's Bone Health

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Children's Bone Health

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Voor Allard

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Chapter 1

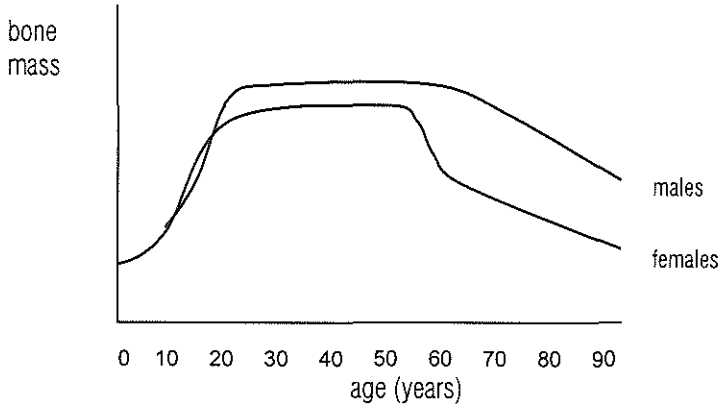
GENERAL INTRODUCTION

GENERAL INTRODUCTION

DEFINITIONS

Osteoporosis is defined as a systemic skeletal disease, characterised by low bone mass and microarchitectural deterioration of bone tissue with a consequent increase in bone fragility and susceptibility to fracture[1]. It has been shown that the risk of fractures increases as bone density declines. Each standard deviation decrease in BMD has been shown to be associated with a doubling of fracture risk, in adults as well as in children[2-4]. In 1994, a working group of the World Health Organisation (WHO) has proposed guidelines for the interpretation of bone mass measurements in Caucasian women[5]. BMD is compared to the average of young healthy individuals and expressed as T-score. A T-score below –2.5 is categorised as osteoporosis, and a T-score between –1 to –2.5 as osteopenia. In adults different cut-off points has been suggested in case of corticosteroid-induced osteoporosis[6]. In children, age- and sex-adjusted Z-scores are used to describe bone mass measurements. No consensus on cut-off points for intervention in children has been reached yet.

Figure 1. Bone mass in males and females during life.



BONE MASS IN CHILDHOOD

Osteoporosis in adulthood is a world-wide problem causing high morbidity and high costs. The lifetime risk of fractures of hip, distal radius and spine is 40% for white women and 13% for white men from 50 years and older. The incidence of fractures will increase fourfold world-wide during the next fifty years due to increased life expectancy[7]. Bone mineral density in later life largely depends on peak bone mass achieved in adolescence or young adulthood and on the subsequent bone loss[8] (Figure 1). Dent already postulated in 1973 that 'senile osteoporosis is a paediatric disease'[9]. Therefore, the perception that osteoporosis is a disease of the elderly should be altered. Fortunately, more and more paediatricians are becoming aware of their task and responsibility for children's and adult's bone health. Juvenile idiopathic osteoporosis and

osteogenesis imperfecta are examples of primary osteoporosis in childhood. However, osteoporosis is more frequently a complication of a chronic disease or its treatment. Some of these diseases are listed in Table 1.

Table 1. Causes of osteoporosis in childhood

Primary	
	Idiopathic juvenile osteoporosis
	Osteogenesis imperfecta
Secondary	
Endocrine	Hypopituitarism
	Cushing's syndrome
	Hyperthyroidism
	Hypogonadism
Nutritional deficiencies	Calcium
	Vitamin D
	Malabsorption
	Malnutrition
Immunologic and inflammatory	Inflammatory bowel disease
	Rheumatoid arthritis
Drugs	Corticosteroids
	Anticonvulsants
	Anticoagulants
	Methotrexate
Neoplastic	Leukemia
	Neuroblastoma
Immobilisation	Cerebral palsy
	Paraplegia

DETERMINANTS OF BONE MASS

Over the past decade, observed differences in BMD between races[10], and twin and family studies[11] strongly suggest that genetic factors play a major role in the pathogenesis of osteoporosis. It has been estimated that up to 80% of the variation in BMD is (poly)genetically determined[11,12]. Polymorphisms in several candidate genes have been investigated to analyse their contribution to aspects of osteoporosis, for example polymorphisms in the 3' end of the vitamin D receptor gene[13,14], and a G to T substitution in the Sp1 binding site of the collagen type Iα1 (COLIA1) gene[15-17]. However, results have been conflicting. The number of candidate genes is still growing and genetic studies in large populations are needed to evaluate their effect on peak bone mass.

Additional determinants of peak bone mass are hormonal status (puberty), physical activity, calcium intake and weight[18-21]. In a previous study reference values for lumbar spine and total

body BMD were collected. In this study, the major determinant of BMD during childhood appeared to be weight in boys and pubertal development in girls[19]. The mean calcium intake in Dutch children was relatively high compared to recommended dietary allowances (RDA)[19]. Recently, new RDA for calcium and vitamin D have been published by the Health Council of the Netherlands[22] (Table 2). Furthermore, physical activity stimulates bone formation. Especially weight-bearing physical activities with high peak strain, such as jumping, basketball, volleyball etc, positively affect bone mass[23].

Table 2. Recommended dietary allowances for calcium and vitamin D[22]

Age	Calcium (mg/day)	Vitamin D (IU/day)[a]	
		Without sun exposure	With sun exposure[b]
6-12 months	450	400	200
1-3 years	500	200	100
4-8 years	700	200	100
9-18 years	1200 (boys)	200	100
	1100 (girls)	200	100
19-30 years	1000	200	100

[a] 400 IU=10 μg vitamin D_3

[b] 15 minutes/ day in the open air with at least hands and face uncovered

BONE & BONE TURNOVER

Bone is composed of cells, mineral and organic matrix. The latter can be divided in collagen and non-collagenous proteins. Osteoblasts synthesise the matrix proteins and mineralise it. The mineral in calcified bone is mostly in the form of needle –shaped carbonate containing calcium phosphate crystals called hydroxyapatite. The crystals lie along the collagen fibrils[24]. Two types of bone can be distinguished in the skeleton: trabecular and cortical bone. Trabecular bone is also called cancellous or spongious bone, which is present for example in vertebrae, ultradistal radius, calcaneus and Ward's area (a calculated region of low density in the femoral neck). Cortical bone is also described as compact bone and is predominantly present in the femoral neck and distal radius. Eighty percent of the total skeleton exists of cortical bone[25]. Trabecular bone has a higher metabolic rate and therefore changes in bone mass may be detectable earlier in trabecular bone than in sites that are predominantly cortical[26].

Bone is an active tissue, which is constantly regenerated throughout life. Bone remodelling occurs in small packets of cells called basic multicellular units (BMUs). First *activation* occurs, the bone surface is converted from resting lining cells, to an activated bone surface on which circulating mononuclear cells of the haematopoetic lineage fuse and form differentiated osteoclasts. Subsequently, osteoclasts *resorb* bone, and in the following *reversal phase* preosteoblasts appear in the resorption cavity and formation is coupled to resorption. In this phase a cement line is formed, which marks the limit of resorption and acts as glue between the old and new bone. Finally, in the *formation phase,* osteoblasts fill the cavity with new bone[27].

However, in addition to the remodelling, children grow, whereby bone modelling is achieved by appositional growth along periostal surfaces and by the calcification of cartilage adjacent to the growth plate[28].

MARKERS OF BONE TURNOVER

Bone metabolism is a strongly coupled mechanism of bone formation by osteoblasts and bone resorption by osteoclasts. Biochemical markers of bone turnover are divided in markers of bone formation and markers of bone resorption (Table 3). They can be measured in urine and serum. In contrast to serum markers, urinary assessments need to be corrected for urinary creatinine or require 24-hour sampling.

Table 3.

Biochemical markers of bone resorption

Marker		Assayed in	Notes
ICTP	Carboxy terminal cross-linked telopetide of type I collagen	Serum	Released during the degradation of type I collagen
Ntx	Collagen type I cross-linked N-telopeptide	Serum & Urine	Breakdown product of the type I collagen derived from bone
Hydroxyproline		Urine	Amino-acid found in collagenous proteins of bone and other soft connective tissue Released when bone matrix is broken down
Calcium		Urine	Released when bone matrix is broken down

Biochemical markers of bone formation

Marker		Assayed in	Notes
ALP	Alkaline phosphatase	Serum	Enzyme secreted by osteoblasts and other cells (liver, intestines, kidneys). In children ± 80% of ALP is derived from bone. Bone specific ALP exists and can be assayed
Osteocalcin		Serum	Small non-collagenous protein synthesised by osteoblasts and chondrocytes and deposited in the extracellular bone matrix. A small amount enters the circulation. The precise function is unknown
PICP	Carboxyterminal propeptide of type I procollagen	Serum	Set free from type I procollagen before the collagen molecules are incorporated into collagen fibres
PINP	Amino-terminal propeptide of type I procollagen	Serum	Set free from type I procollagen before the collagen molecules are incorporated into collagen fibres

Biochemical markers of bone turnover might be valuable tools to evaluate efficacy of treatment for osteoporosis[29-32]. Furthermore, they may provide insight into the pathophysiology of bone disorders. However, no single marker fulfils all the criteria for an ideal marker. When one interprets the results, a few limitations should be considered, such as the tissue specificity of the marker and sources of variability (stability of the parameter, diurnal variation[33], day-to-day[34] and circannual variation[35]). Markers of bone turnover are difficult to interpret especially in children since markers of bone turnover reflect growth (skeletal modelling) and remodelling. In studies assessing whether cross-sectional bone markers predict bone gain in the following year only weak associations have been reported in peripubertal girls[36] and no association in young adults[37]. However, measurements of markers of bone turnover and BMD in adults have been shown to be independent predictors of the risk of developing osteoporosis and fractures[38]. In addition, bone markers were especially valuable in predicting response of treatment with bisphosphonates in adults[30,39]. This may be of potential use in monitoring response to treatment schedules in children as well, which may help to determine the frequency of the treatment cycles in individual patients.

DUAL ENERGY X-RAY ABSORPTIOMETRY

Dual energy X-ray absorptiometry (DXA) measures bone mineral density in the spine, femur and various other regions. Bone mineral density is the ratio between the amount of mineral measured and the projected area. By combining measurements at various sites one gains information about the bone density in cortical as well as in trabecular bone. DXA has low radiation dose, great precision, and accuracy[40]. These qualities make DXA suitable for children and for our research.

An important shortcoming of DXA is that it measures an areal density (g/cm^2), as a consequence DXA may underestimate BMD in short stature[41]. Correction for bone area removes some of the dependence on bone size. Mathematical models are used to correct for bone size and calculate bone mineral apparent density (BMAD) or volumetric density. A widely used validated model for lumbar spine is $BMAD = BMD \times [4/(\pi \times width)]$[42]. Such corrections are required especially in children with short stature.

Besides bone mineral density, DXA measurement of total body also provides estimates of body composition as lean body mass and fat mass. DXA has been shown to be an accurate and precise method to measure body composition[43,44].

GROWTH HORMONE, BONE AND BODY COMPOSITION

Growth hormone (GH) affects longitudinal growth, bone density and body composition. It is well known that adults and children with growth hormone deficiency (GHD) have decreased bone density, increased fat mass and decreased lean body mass[45-47].

GH stimulates insulin-like growth factor I (IGF-I) production in the liver and in skeletal cells. IGF-I enhances bone collagen and matrix synthesis, and stimulates replication of osteoblasts. However, the direct effect of GH on bone is limited. Osteoclasts are indirectly activated via paracrine factors derived from the osteoblasts[24]. Furthermore, renal 1-α-hydroxylase activity is increased by IGF-I[48]. This enzyme is needed to transform 25-hydroxyvitamin D in the active form 1,25-dihydroxyvitamin D, which consequently will increase intestinal calcium absorption and will

accomplish other stimulatory effects on bone formation[24]. In addition, GH has an indirect effect by increasing muscle mass and muscle strength, which stimulate bone formation. GH replacement therapy improves growth, body composition, and bone density[47,49].

AIMS

Osteoporosis is one of the major public health problems in postmenopausal women and the elderly of either sex. Due to ageing of the population it has been expected that fracture risk will increase fourfold in the next fifty years. The attendant costs could have devastating effects on health care systems[7]. Thus, reducing the incidence of fractures by preventing osteoporosis might alleviate individual burden and social costs. However, most studies have been performed in (postmenopausal) women. Because bone mineral density in later life largely depends on peak bone mass achieved in adolescence or young adulthood and on the subsequent bone loss[8], one of the prevention measures should be optimising bone mass acquisition during childhood. Knowledge of determinants of bone mass acquisition in childhood will therefore be required.

As the major part of the variation in bone mass has been explained by genetic determinants, the *first aim* of our study was to identify genetic determinants of bone mass in childhood, which might help us to identify subjects at risk for developing osteoporosis.

The *second aim* was to assess reference data for biochemical parameters of bone turnover and expand our reference values for bone density and body composition, because knowledge of normal physiological variation is necessary to identify pathological changes.

The *third aim* was to study bone density and bone metabolism in various diseases, because bone density might change due to the disease itself or its treatment. Our specific research questions were:

- What are the effects on bone density and fracture rate on the long- and short-term in childhood acute lymphoblasic leukemia which treatment protocol contains high dose dexamethasone and methotrexate, but no cranial irradiation?
- How is bone density development in children with chronic renal failure and what are the effects of growth hormone treatment?
- What are the long-term effects on bone density in growth hormone deficient children treated with GH replacement therapy?
- Finally, does therapy with gonadotrophin releasing hormone agonists in precocious puberty have negative effects on bone density in the long-term?

STRUCTURE AND SCOPE OF THE THESIS

The thesis can be divided in two main parts. In the first part (*Chapter 2 to 5*) bone mineral density, bone metabolism and body composition in healthy children and young adults have been evaluated, while in the second part (*Chapter 6 to 10*) these issues were studied in children with various diseases.

Healthy children were studied to gain references for parameters of bone turnover, and to extend our reference data for bone density and body composition. Furthermore, the effect of polymorphisms in two candidate genes, e.g. polymorphisms in the vitamin D receptor gene and

the collagen Iα1 gene, were studied. For a proper interpretation of our findings in diseased children, it is essential to have, preferably own reference data for serum markers of bone turnover, bone density, and body composition. Results of our studies in healthy children are presented in *Part I (Chapter 2 to 5)*.

The long-term survival of acute lymphoblastic leukemia (ALL) has improved dramatically during the last decades, consequently more emphasis is being placed on the long- and short-term side effects of ALL and its treatment. Some of these side-effects, such as osteoporosis, growth retardation and adiposity, have been investigated cross-sectionally in children ten years after diagnosis (*Chapter 6*). Furthermore, we longitudinally studied children who were newly diagnosed and followed during and after cessation of chemotherapy (*Chapter 7*).

In *Chapter 8 and 9* treatment with growth hormone will be discussed. Growth-retarded children with chronic renal failure were treated with growth hormone. The effects of GH treatment on bone density and body composition were compared to children with chronic renal failure but without growth hormone treatment (*Chapter 8*).

Bone density, body composition and serum lipid levels in growth hormone deficient children, and the longitudinal effects of growth hormone treatment on these parameters will be described in *Chapter 9*.

Puberty is considered to be a crucial period for bone mass acquisition. It is therefore important to know whether children with a disorder in pubertal development will achieve an adequate peak bone mass. The effects of gonadotrophin-releasing hormone-agonist (GnRH-a) on bone density and body composition were evaluated in children with precocious or early puberty (*Chapter 10*). Patients were studied before, during and after cessation of GnRH-a.

In *Chapter 11 & 12* results will be summarised and discussed, and recommendations for future research are made.

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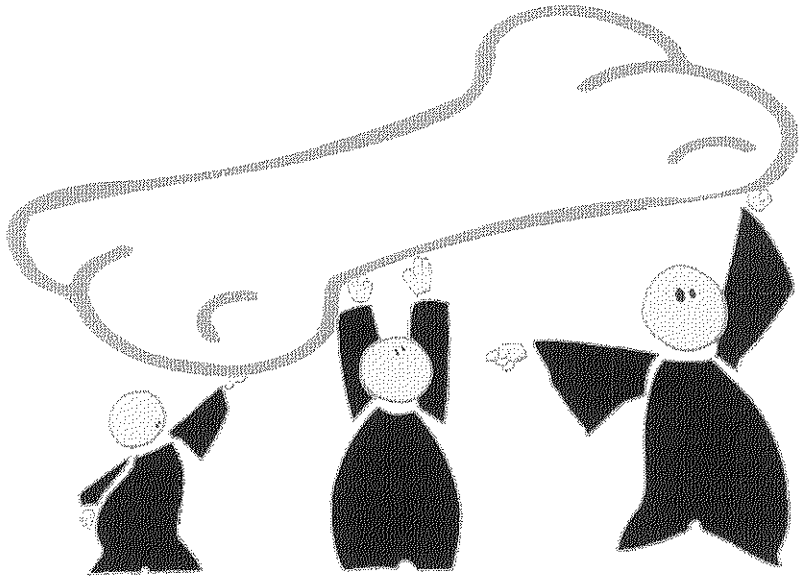
Part One

BONE MINERAL DENSITY IN HEALTHY CHILDREN AND ADOLESCENTS



Chapter 2

COLLAGEN Iα1 POLYMORPHISM IS ASSOCIATED WITH BONE CHARACTERISTICS IN CAUCASIAN CHILDREN AND YOUNG ADULTS

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ABSTRACT

A large proportion of the variation in bone mass can be explained by genetic factors. We analysed the G to T substitution in the Sp1 binding site in the first intron of the collagen type Iα1 (COLIA1) gene in relation to bone mass. The genotypes GG, GT and TT were determined in 148 Caucasian children and young adults. We performed dual energy X-ray absorptiometry twice, while 'speed of sound' (SOS) was assessed by tibial ultrasonometry, at follow-up. Genotype distribution was 104 (70%) GG, 40 (27%) GT and 4 (3%) TT. Carriers of the T-allele had a 0.5 SDS (standard deviation score) decreased bone mineral content (BMC) of total body ($p=0.001$), and a 0.4 SDS decreased bone mineral density (BMD) both for lumbar spine ($p=0.04$) as for total body ($p=0.05$). The genotype effect on BMD and BMC decreased after adjustment for height or body mass index. When we calculated apparent BMD, these differences diminished to 0.1 SDS and were no longer significant. T-allele carriers had shorter stature (0.4 SDS; $p=0.04$) and had smaller bones (0.5 SDS lower width of the lumbar vertebral body; $p=0.01$). The T-allele was also associated with lower SOS ($p=0.03$), independent of BMD and BMC, and lower lean body mass. Similar associations were found at follow-up. The change in BMD and BMC SDS between the first and second measurement did not differ between the GG and GT&TT group. In conclusion, the COLIA1 polymorphism in children and young adults is associated with several bone characteristics. However, at least a part of the COLIA1 effect on bone mass may be related to differences in frame size.

INTRODUCTION

Osteoporosis is a common disorder in the elderly with an increasing incidence world-wide. It is characterised by reduced bone mineral density, deterioration of the micro-architecture of bone tissue, and increased fracture risk[1]. Variation in the attainment of peak bone mass plays an important role in the development of osteoporosis in later life[2].

Over the past decade, observed differences in BMD between races[3], and twin and family studies[4] strongly suggest that genetic factors play a major role in the pathogenesis of osteoporosis. It has been estimated that up to 80% of the variation in BMD is (poly)genetically determined[4,5]. Therefore, genetic research on osteoporosis is worthwhile to identify subjects with an increased risk of developing osteoporosis, at an early stage.

Polymorphisms in several candidate genes have been investigated to analyse their contribution to aspects of osteoporosis, including polymorphism at the 3' end of the vitamin D receptor gene[6,7] and a G to T substitution in the Sp1 binding site at the collagen type Iα1 (COLIA1) gene[8-10]. Most studies have been performed in elderly women, who had undergone substantial bone loss. However, candidate gene polymorphisms may play a role in the attainment of peak bone mass, as well. Children also have had shorter exposure to lifestyle and environmental factors which can influence the overall effect of genetic factors on bone mass. A few studies in paediatric populations have been performed, however, with conflicting results. For example, Sainz et al. found an association of bone density with COLIA1[11], and the VDR gene[12] in prepubertal girls. Berg et al.[13], however, could not confirm this association for the COLIA1 polymorphism in a group of healthy children and young adults.

We hypothesise that COLIA1 genotype might affect anthropometric or bone characteristics in children and young adults. Therefore, we investigated the association between COLIA1

polymorphism and several bone characteristics in a group of healthy Dutch children and young adults. We chose to recruit the same population as used for our reference study on bone density and body composition, which was performed approximately 4 years ago. So, we were able to evaluate bone gain as well as cross-sectional data.

SUBJECTS

For this study we recruited 176 Dutch children and young adults (65 boys and 111 girls) from the Rotterdam Region in the Netherlands. All but six subjects participated in our previous study (1994-1995) to assess normative values for bone density and body composition measured by dual energy X-ray absorptiometry. The cross-sectional results of this first study have been presented previously[14,15]. The mean follow-up time was 4.4 years (range 3.2-6.7 years). The ethnicity was Caucasian for 142 children (53 males), Black for 7 children (3 males), Hispanic for 9 children (3 males), Asian for 6 children (1 male), and mixed ethnicity 6 (1 male). The six children who participated only at follow-up were all Caucasian (4 males). This study was approved by the medical ethics committee of the University Hospital Rotterdam, and written informed consent was obtained from the parents or guardians and from all children aged 12 years and over.

MATERIAL AND METHODS

Anthropometry

Height was assessed using a fixed stadiometer and expressed as standard deviation scores (SDS)[16]. Weight was measured without shoes on a standard clinical balance. The questionnaire to determine calcium intake, physical activity, medical history, menarche, use of oral contraceptives, was identical tot the one used in the previous study[14]. Body mass index was calculated (kg/m^2) and lean body mass (LBM, gram) and percentage body fat (%fat) were measured by total body DXA (Lunar DPX-L, Madison, WI), all expressed as SDS[15,17]. As validated previously[18], pubertal development was evaluated by self-assessment of breast and pubic hair stage in girls and genitalia and pubic hair stage in boys, according to Tanner[19].

Bone characteristics

Bone mineral density (BMD, $grams/cm^2$) was determined by DXA of lumbar spine (L2-L4) and total body($_{TB}$). DXA measurements were performed twice (in 1994-1995 and in 1998-1999). For children with weight below 30 kilograms paediatric software was used. To account for differences in bone size we calculated apparent BMD of lumbar spine($_{LS}$) with the model $BMAD_{LS} = BMD_{LS} \times [4/(\pi \times \text{width})]$, in which width is the mean width of the second to fourth lumbar vertebral body. This model was validated by in vivo volumetric data obtained from magnetic resonance imaging of lumbar vertebrae[20]. The coefficient of variation has been reported to be 1.04% for lumbar spine BMD and 0.64% for total body BMD[21]. Total body DXA also measures Bone Mineral Content (BMC, grams). BMD, BMAD, BMC and width of the lumbar vertebrae were compared to our age- and sex-matched Dutch reference values and expressed as SDS[14,15].

In 102 subjects we also performed tibial ultrasonometry once (in 1998-1999), using the SoundScan® Compact (Myriad Ultrasound Systems LTD, Rehovot, Israel). Following standard

operating procedures, all bone assessments were done on the right tibia at the mid-tibial point. The results (speed of sound; SOS in m/s) are compared to healthy age matched Dutch controls, and expressed as SDS[22].

Genotyping

DNA was isolated from blood according to standard procedures. The Sp1 polymorphism of the COLIA1 gene was detected by the polymerase chain reaction (PCR) with a mismatched primer that introduces a di-allelic restriction site as described previously[8,9]. This test discriminates the two alleles represented as G and T, previously described as 'S' and 's', respectively. 'G' represents guanine and 'T' thymidine as the first bases in the Sp1-binding site in the first intron of the gene for COLIA1. The polymorphism results in three genotype groups, designated GG, GT, and TT.

Statistical Analysis

Because of the known difference in allele frequency and BMD between different races[23], we limited the analysis to the Caucasian children. Allele dose was defined as the number of copies of a certain allele in the genotype group. To quantify the strength of association we performed linear regression analysis, in which genotype groups were designated as 0, 1 or 2, corresponding with the number of T-alleles. We used regression analysis to adjust for possible confounders. Independent samples T-tests were performed to test for differences in bone density, body composition and SOS between the COLIA1 genotypes. Firstly, we analysed the results of the 1994-1995 study. Secondly, the same analyses were performed on the data of the 1998-1999 study. Thirdly, the absolute change between follow-up and baseline measurements, and the association with genotype was analysed.

In childhood, and especially during puberty, bone mineral density and body size change markedly. We therefore separately analysed the allele-dose effects before, during and after puberty. To correct for the age differences between the genotype groups, bone characteristics were expressed in age- and sex-matched standard deviation scores (SDS). P-values of less than 0.05 (two-tailed) were considered to be significant.

RESULTS

Subjects

The genotype distribution was 104 (70.3%) GG, 40 (27.0%) GT and 4 (2.7%) TT. There was no difference in genotype distribution between boys and girls. The allelic frequencies were G=0.84 and T=0.16. The distribution of genotypes was in Hardy-Weinberg equilibrium (p=0.95). Mean age at follow-up was 15.6 years (range: 7.6-25.3). Baseline characteristics of all Caucasian subjects and according to COLIA1 genotype are reported in Table 1. The three genotype groups did not differ significantly in age, calcium intake and physical activity. Because of the small numbers in the TT group we pooled the GT and TT group and compared this pooled group with the GG group. Lean body mass (LBM) SDS and height SDS were significantly higher in GG-group compared to the combined GT&TT (p=0.02 and p=0.04, respectively). No genotype effect on % fat was observed. The change in height SDS, BMI SDS, and LBM SDS between first and second measurement did not differ between the GG and GT&TT group.

Table 1. Characteristics of Caucasian boys and girls by COLIA1 Sp1 genotype at baseline*.

	All	COLIA1 Sp1 genotype				p-value
		GG	GT	TT	GT&TT	GG vs
N	(142)	(99)	(39)	(4)	(43)	GT&TT
Age (years)	11.4	11.8 (4.3-19.9)	10.7 (4.3-17.9)	8.2 (5.0-14.8)	10.4 (4.3-17.9)	0.08
Height SDS	0.15	0.26 (-2.39-2.72)	-0.13 (-2.11-1.73)	-0.03 (-1.37-1.36)	-0.11 (-2.11-1.73)	0.04
Weight (kg)	43.7	46.3 (17.0-115.0)	38.5 (16.0-69.0)	30.5 (18.0-51.0)	37.8 (16.0-69.0)	0.10
Body Mass Index SDS	0.24	0.32 (-1.70-3.50)	0.05 (-2.80-2.60)	0.10 (-1.30-1.50)	0.05 (-2.80-1.50)	0.15
% fat SDS	0.02	0.01 (-2.13-2.26)	0.02 (-1.98-1.73)	0.07 (-0.48 -0.73)	0.03 (-1.98 –1.73)	0.93
Lean body mass SDS	0.28	0.41 (-1.72-3.52)	-0.01 (-1.17-1.96)	-0.02 (-0.53-0.79)	-0.01 (-1.17-1.96)	0.02

* Values presented are the mean and range

The mean age at menarche was 13.1 years range (11 –16 years), similar to normal age of menarche in the Netherlands[16]. At baseline 68 children were 'prepubertal' (Tanner 1); 49 were 'pubertal' (Tanner stage 2-4) and 25 were 'postpubertal' (Tanner 5). At follow-up, 31 children had Tanner stage 1; 48 had Tanner stage 2, 3 or 4, and 69 had Tanner stage 5. Nine % of the girls used oral contraceptives, at baseline (35.2% at follow-up). No difference in oral contraceptive use between the genotype groups was found.

Bone measurements by genotype

Table 2 shows the results of bone measurements according to COLIA1 genotype. At baseline, we observed carriers of the T-allele to have a 0.5 SDS lower total body BMC ($p=0.001$). The T-allele was also associated with a 0.4 SDS lower BMD both at the lumbar spine ($p=0.04$) as well as for total body ($p=0.05$). When differences in bone size were taken into account by calculating apparent BMD (BMAD) for the lumbar spine these differences diminished to 0.1 SDS and were no longer significant. T-allele carriers had a 0.5 SDS decreased width of the lumbar vertebral body ($p=0.01$).

After adjustment for height SDS or BMI SDS, the COLIA1 genotype effect on lumbar spine BMD SDS, and total body BMD SDS disappeared. Only the genotype effect on BMC SDS adjusted for BMI SDS, and height SDS remained significant. When we adjusted the genotype effect for lean body mass all associations decreased and were no longer significant.

When we performed the same analyses for the bone density measurements at follow-up similar differences between the genotypes were found (Table 2). Again, the T-allele was strongly associated with a 0.5 SDS decreased BMC of total body ($p=0.01$), while carriers of the T-allele had a 0.4 SDS decreased BMD both for lumbar spine ($p=0.06$) as for total body ($p=0.05$). Also at baseline these differences diminished to 0.15 SDS ($p=0.45$) for apparent BMD (BMAD). T-allele carriers had a 0.35 SDS lower width of the lumbar vertebral body ($p=0.07$).

The change in BMD and BMAD SDS of lumbar spine and BMD and BMC SDS of total body between the second and first measurement did not differ between the GG and GT&TT group. The correlation coefficients of the first and second bone density and body composition measurements varied from 0.70 to 0.82 ($p<0.001$).

Table 2. Bone measurements expressed as standard deviation scores of Caucasian boys and girls by COLIA1 Sp1 genotype*.

	All	COLIA1 Sp1 genotype				p-values
		GG	GT	TT	GT & TT	GG vs GT&TT
BASELINE	(n=142)	(n=99)	(n=39)	(n=4)	(n=43)	
Lumbar spine						
BMD	0.04 (0.98)	0.15 (1.03)	-0.22 (0.79)	-0.21 (1.16)	-0.22 (0.81)	0.04
BMAD	-0.07 (1.01)	-0.04 (1.04)	-0.18 (0.90)	0.13 (1.43)	-0.15 (1.02)	0.57
Width	0.19 (0.99)	0.33 (1.04)	-0.09 (0.84)	-0.40 (0.52)	-0.13 (0.12)	0.01
Total body						
BMD	-0.11 (0.98)	0.01 (1.02)	-0.40 (0.86)	-0.09 (0.68)	-0.37 (0.85)	0.05
BMC	0.10 (0.97)	0.27 (1.04)	-0.27 (0.68)	-0.29 (0.75)	-0.27 (0.68)	0.001
FOLLOW-UP	(n=148)	(n=104)	(n=40)	(n=4)	(n=44)	
Lumbar spine						
BMD	0.21 (1.08)	0.32 (1.09)	-0.04 (0.95)	-0.03 (1.81)	-0.04 (1.02)	0.06
BMAD	0.22 (1.08)	0.27 (1.10)	0.12 (0.98)	0.13 (1.63)	0.12 (1.03)	0.45
Width	-0.01 (1.05)	0.10 (1.05)	-0.25 (1.04)	-0.24 (0.79)	-0.25 (1.01)	0.07
Total body						
BMD	0.24 (1.01)	0.35 (1.06)	-0.04 (0.80)	0.30 (1.51)	-0.01 (0.86)	0.05
BMC	0.32 (1.06)	0.46 (1.09)	-0.01 (0.78)	0.12 (1.56)	-0.00 (0.85)	0.01
Tibia						
SOS	0.02 (0.96)	0.16 (0.97)	-0.24 (0.93)	-0.62 (0.66)	-0.28 (0.90)	0.03

* values are presented as mean standard deviation score (SD). *BMD* bone mineral density; *BMAD* bone mineral apparent density; *BMC* bone mineral content; *SOS* speed of sound; *width* width of the lumbar vertebral body.

We found lower SOS SDS in T-allele carriers (Table 2), with evidence for an allele dose effect of –0.4 SDS per T-allele copy ($p=0.02$). After correcting for height and BMI the association remained significant ($\beta=-0.40$, $p=0.02$). The allele dose effect on SOS did not change after adjustment for BMD, BMC or lean body mass.

When the COLIA1 genotype effect on BMD and BMC was compared in boys and girls similar trends were observed although somewhat stronger in boys. When we considered the pubertal stage, the COLIA1 genotype effect on BMD and BMC appeared to be somewhat stronger in the prepubertal stage (data not shown). Allele frequency of all volunteers, irrespective of race was G=0.85 and T=0.15. No TT genotype was found in the other races.

DISCUSSION

We found that in healthy Dutch children the T-allele of the G to T substitution in the Sp1 binding site of the COLIA1 gene was associated with lower bone mineral density (BMD) and lower bone mineral content (BMC). These associations were demonstrated in two bone density measurements with an interval of approximately 4 years. An obvious frame size effect was observed: the association was most apparent for BMC, less for BMD of total body and lumbar spine, while no significant association between BMAD and genotype was found. Moreover, the COLIA1 polymorphism was associated with height and vertebral body width. In line with this, the T-allele also tends to be associated with lower mean lean body mass and BMI SDS. A significant allele dose effect on tibial speed of sound (SOS) was found, which was independent of BMD or BMC.

With regard to our study population some characteristics should be noted. We separately analysed the Caucasians, who account for 82% of our population, so it was an ethnically homogeneous group. The allele frequencies of the COLIA1 variants were similar to those reported for other Caucasian populations[8,9,24]. In contrast, in other races, especially Asians, the T-allele frequency is much lower[23,25]. It is clear that such ethnical differences may contribute to the differences between various studies. To account for (non-significant) age differences between the three genotypes, we used standard deviation scores. Interestingly, we observed that the SD scores for bone density and body composition were somewhat higher at follow-up than at baseline, while these differences were only significant for total body BMD. This could imply that relatively healthy children were included. At the first measurement, four years earlier, the mean SDS for the same group did not differ from zero. However, since we observed the genetic effect both at baseline and at follow-up we believe that the healthy responder bias did not seriously affect our results. The difference in BMD, BMAD, and BMC SDS between baseline and follow-up did not differ between the GG and pooled GT&TT group, indicating that the COLIA1 Sp1 polymorphism does not influence the rate of increment in any of the performed measurements. Although the baseline and follow-up measurements are correlated, the observation of an association in both measurements would argue against a chance finding. However, because only four subjects had the TT genotype, results must be interpreted with caution and larger studies will be required.

With respect to our findings it is important to realise what various DXA parameters mean. Firstly, in BMC (grams) no correction for bone size is made. Secondly, a correction for measured bone area is made resulting in a BMD value (g/cm^2). Thirdly, the volumetric density or true density is the amount of mineral per cubic centimetre. This can be measured directly by quantitative computed tomography, but can also be derived from a DXA measurement by calculating BMAD. These subsequent corrections to adjust for differences in bone size imply that for a constant true bone density, the measured BMD or BMC in a small vertebra, as in short stature, will be lower than in a large vertebra (tall stature). In the present study, the association between genotype and BMD much diminished after correction for bone size. Moreover, a direct association between genotype and the width of the vertebral body was found.

Several studies demonstrated a higher fracture risk for T-allele carriers but this risk was mostly independent of BMD in postmenopausal women[9,26,27]. This suggests that the COLIA1 gene variants might be associated with differences in bone quality, rather than in bone mineral density. Indeed we observed the genotype effect on SOS to be stronger than on the 'mineral' measurements such as BMC and BMD. Ultrasound transmission velocity is considered to be

dependent on density, microarchitecture, and elasticity, but also on macrostructure[28,29]. Furthermore, the genotype effect on SOS was found to be independent of BMD and BMC. Our findings with DXA and ultrasound strongly support the hypothesis that Sp1 COLIA1 polymorphism is not so much associated with mineralisation, but appears to be associated more in particular with bone morphology and quality in children and young adults.

Previous studies on this polymorphism in children differ in design, in age and gender of the subjects, in methods to measure bone mass or the expression of data on bone mass (e.g. two or three dimensions), in the expression of data (SDS versus absolute values), and in ethnic background, making it very difficult to establish concordance among them. Moreover, the differences may be small and can easily be missed in small samples. Sainz et al.[11] studied a homogeneous group of 109 prepubertal girls from Mexican descent. In this study bone density of cancelous vertebral bone was assessed by computed tomography. T-allele carriers had lower bone density, while no allele effect on bone size was found. In concordance with our longitudinal results, Berg and colleagues[13] did not find an association between genotype and radial bone gain measured with single photon absorptiometry. As in our study, the follow-up period of 4 years might have been too long, because substantial changes in bone density occur during puberty. Thus large differences can be expected between prepubertal children who were pubertal at follow-up, and children who were already postpubertal at baseline. With regard to the cross-sectional DXA measurements, however, they did not find a genotype effect on bone density in boys and girls aged 12 to 20 years.

In postmenopausal women, Uitterlinden et al.[9] showed that the association between COLIA1 genotype and bone density may be mediated in part by a genetic effect of COLIA1 on body weight. Garnero et al.[24] found in premenopausal French women that the TT group was shorter than the GG group. In this study group the COLIA1 Sp1 polymorphism was associated with BMD of lumbar spine and total body, and BMC of total body. However, after correction for height the differences decreased, and were no longer significant, suggesting that part of the effect on bone mass may result from differences in bone size. Also in our study, the T-allele showed a tendency towards a decreased height SDS and the genotype effect on BMD and BMC decreased substantially after adjustment for height or BMI. Moreover, a direct association between genotype and vertebral body size was observed. Together, these results suggest a consistent effect of the COLIA1 Sp1 polymorphism on several aspects related to frame size, both at young and old age. The effects on bone appear to be most apparent in the elderly and during growth in children and less apparent during the fourth or fifth decade. Therefore, we hypothesise that when COLIA1 production is stressed, as in periods of growth in children or increased bone turnover in the elderly, genotype differences will become more important. Indeed, in elderly postmenopausal women the genotype dependent differences were found to increase with age[9], while in another group of elderly women increased rates of bone loss have been reported in the TT genotype[30]. In a somewhat younger population, however, Heegaard et al.[31] did not find an association between rate of bone loss and COLIA1.

The mechanism by which different COLIA1 alleles affect bone characteristics, is not completely elucidated yet, although there is strong evidence to suggest the Sp1 COLIa1 polymorphism to be functional. The COLIA1 gene and the COLIA2 gene together code for the two polypeptides that in a 2:1 ratio constitute the collagen type I fibril. The T-allele has been shown to bind the transcription factor (Sp1 protein) with a higher affinity than the G-allele, which was found

to result in higher mRNA and protein expression levels. As a consequence, a disturbance in the synthesis ratio of COLIA1/COLIA2 occurs in T-allele carriers resulting in a different structure of the collagen type I bone matrix with most likely an excess of COLIA1 homotrimers[10]. Decreased mineralisation and small body size are also found in the *oim/oim* mouse model of osteogenesis imperfecta[32,33] and in mild osteogenesis imperfecta patients[34]. In those cases the phenotype is supposedly due to excess formation of COLIA1 homotrimers.

In our study carriers of the T-allele showed a tendency towards lower BMI, caused by a decrease in lean body mass, because % fat did not differ. Lean body mass measured by DXA is fat free soft tissue, and reflects mainly muscle mass. The associations between genotype and BMC or BMD decreased markedly after adjustment for lean body mass. It is well known that muscle mass plays an important role in the stimulation of bone formation[35]. These data suggest that a part of the genotype effect of the COLIA1 gene may be mediated by differences in muscle mass.

In conclusion, the T-allele of the Sp1 COLIA1 polymorphism in children and young adults is associated with lower SOS, BMC and BMD of total body, and BMD of lumbar spine. An allele dose effect was found for SOS, whereas this association was independent of BMD or BMC. The associations for BMD diminished after adjustments for height or BMI. In addition, no genotype effect on BMAD was found. Moreover, T-allele carriers had shorter stature and smaller bone size. Together these findings suggest that a part of the effect on bone mass may be mediated by a genetic effect of COLIA1 related to bone morphology and bone quality. The associations between the COLIA1 Sp1 polymorphism and bone mass, SOS, and lean body mass, both at young and at old age, raises the possibility that the COLIA1 polymorphism can be used at an early stage to explain at least a part of the risk for osteoporosis later on in life.

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Chapter 3

VITAMIN D RECEPTOR GENE POLYMORPHISM PREDICTS HEIGHT AND BONE SIZE, RATHER THAN BONE DENSITY IN CHILDREN AND YOUNG ADULTS

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ABSTRACT

Peak bone mass is considered to be under strong genetic control. We studied the association between anthropometry, bone density and vitamin D receptor (VDR) genotype in an ethnically homogeneous group of 148 Caucasian children and young adults. Bone density was measured by dual energy X-ray absorptiometry and VDR genotype was determined by a direct haplotyping procedure of the *BsmI, ApaI,* and *TaqI* restriction fragment length polymorphisms. A second DXA measurement was performed after approximately 4 years. Results are expressed as age- and sex-adjusted standard deviation scores (SDS). Previously, the collagen IA1 Sp1 polymorphism was studied in this population.

We found VDR genotype to be associated with a 0.4 SDS increased height per allele copy of haplotype '3' ($p=0.04$) and a 0.4 SDS increased width of the lumbar vertebral body in the haplotype '3' allele carriers ($p=0.05$). We observed a trend towards a 0.3 SDS decreased bone mineral apparent density of lumbar spine (BMAD) per copy of haplotype '3' allele ($p=0.10$). In contrast, no association with areal bone mineral density (BMD) was observed. In the follow-up analyses, no differences in height- or bone gain between the VDR genotypes were demonstrated. By combining the risk alleles of VDR and collagen IA1 Sp1 genotype an additive genotype effect on height ($p=0.006$) and vertebral body width ($p=0.001$) was found.

In conclusion, we found VDR genotype to influence frame size and BMAD. The VDR genotype effects on stature and bone size seem to neutralize the effect on areal BMD.

INTRODUCTION

Peak bone mass is considered to be under strong genetic control[1-3]. Bone mass acquisition in childhood is also associated with weight, height, hormonal status, and lifestyle factors such as physical activity and calcium intake[4-7]. Some of these determinants might be under genetic control as well. Identification of the genes mediating effects on bone mass may lead to better understanding of the pathogenesis of osteoporosis, and might help us to identify subjects at risk.

In the extensive search for candidate genes, which are associated with osteoporosis, the vitamin D receptor (VDR) polymorphisms have been most frequently studied[5,8-10]. The hormone 1,25-dihydroxyvitamin D_3 is required for the mineralisation of bone, intestinal calcium absorption, control of calcium and phosphate homeostasis, and the regulation of parathyroid hormone secretion. The effects of vitamin D are mediated by the VDR. These characteristics make the VDR gene a logical candidate gene to analyse for effects on variations in bone mass. The VDR gene maps to chromosome 12q13-14 and several sites of sequence variation in the VDR gene have been described to date. For example, a cluster of linked sites exists near exon 9 and the 3' UTR (untranslated region) and are detected by *BsmI, ApaI, and TaqI* as restriction fragment length polymorphisms (RFLPs)[10]. The majority of the association studies have been performed in pre- and postmenopausal women, because the skeleton undergoes many changes in this period of life and most fractures occur in the elderly. However, variation in the attainment of peak bone mass plays an important role in the development of osteoporosis in later life[11]. In this respect, polymorphisms of the VDR gene may also play a role in the attainment of peak bone mass.

Few pediatric populations have been studied, but so far with conflicting results, similar to what was found for adult populations. Sainz et al.[12] found an association between bone density measured with quantitative computed tomography (QCT) and the *BsmI* and *ApaI* polymorphism in

prepubertal girls. Others could not confirm this finding in a group of healthy children and young adults[13,14]. Some studies reported an association between the *BsmI* polymorphism and frame size, i.e. height at birth and final height[15,16]. Therefore, possible effects of VDR polymorphism on bone might be mediated by other frame size factors. We therefore investigated the association between VDR polymorphism, anthropometry and bone density characteristics in a group of healthy Dutch children and young adults. In view of the known influence of vitamin D on collagen synthesis[17,18] and previous observations on collagen Iα1 (COLIA1) Sp1 genotype effects on frame size in this cohort (see *Chapter 2*), we also studied possible interaction between both genotype effects.

MATERIALS AND METHODS

Subjects

In 1994-1995, 500 healthy Dutch children (403 Caucasian) from the Rotterdam region participated in a study to obtain reference values for dual energy X-ray absorptiometry (DXA) measurements. Children with diseases or using drugs known to affect bone metabolism were excluded. The cross-sectional results of this first study have been presented previously[4,19]. All participants were approached to volunteer in a follow-up study. One hundred forty-eight Caucasian children and young adults (57 boys and 91 girls) agreed, six children participated only at follow-up. Blood samples were taken at follow-up. The mean follow-up period was 4.4 years (range 3.2-6.7 years). The study was limited to Caucasian children, 28 children of other races were excluded. This study was approved by the medical ethics committee of the University Hospital Rotterdam. From the parents and all children older than 12 years of age written informed consent was obtained.

Anthropometric and bone density measurements

Height was measured with a fixed stadiometer. Weight was measured without shoes on a standard clinical balance. Body mass index (BMI) was calculated as weight /height^2. Height and BMI were expressed as age- and sex-matched standard deviation score (SDS)[20,21]. As validated previously[22] pubertal development was evaluated by self-assessment of breast and pubic hair stage in girls and genitalia and pubic hair stage in boys, according to the method of Tanner[23]. We classified Tanner stage 1 as prepubertal, Tanner stage 2, 3, 4 as pubertal and Tanner stage 5 as postpubertal. A questionnaire was administered to determine calcium intake, physical activity, medical history, and menarche.

Bone mineral density (BMD, grams/cm^2) of lumbar spine (LS) and total body (TB) were determined by DXA (Lunar DPX-L, Madison, WI). For children with weight below 30 kilograms pediatric software was used. To correct for bone size we calculated apparent BMD (BMAD, grams/cm^3) of lumbar spine with the model $BMAD_{LS} = BMD_{LS} \times [4/(\pi \times \text{width})]$. 'Width' represents the mean width of the second to fourth lumbar vertebral bodies. This model was validated by in vivo volumetric data obtained from magnetic resonance imaging of lumbar vertebrae[24]. Lean body mass was assessed by total body DXA. Bone density and body composition were expressed as age- and sex-adjusted standard deviation scores (SDS) using our own reference data[4,19]. We used the same data to obtain reference values for vertebral width, allowing us to calculate SD scores.

Genotyping procedure

DNA was isolated from lymphocytes according to standard procedures as described previously[8]. Three clustered anonymous RFLPs in the 3' end of the VDR gene were determined by PCR and enzymatic digestion of the products with *BsmI*, *ApaI*, and *TaqI* using a direct haplotyping PCR procedure. The clustered restriction site polymorphisms at the VDR gene locus were monitored simultaneously and individually, in this procedure. The direct haplotyping procedure has been described in detail previously[8]. The alleles were named as described earlier[8,10] for alleles defined by individual RFLPs; in haplotypes as for example "BAt", capital letters denote absence and lowercase letters the presence of the site for the restriction enzymes *BsmI* (B/b), *ApaI* (A/a) and *TaqI* (T/t) on each of the alleles. in the direct haplotyping number 1 stands for baT, 2=BAt, 3=bAT, 4=BAT, 5=bAt. We determined the G to T substitution in the polymorphic Sp1 binding site in the first intron of the COLIA1 gene as described previously (see *Chapter 2*).

Statistical analysis

The analysis was limited to the ethnically homogeneous group of Caucasian children. To take possible age and sex differences between the genotype groups into account we calculated age- and sex-matched standard deviation scores. We grouped subjects by allele copy number (0,1,2) for the most common haplotype alleles 1,2 and 3 and individual RFLPs *BsmI* and *ApaI*. Because there is a 99% concordance between b and T (and B and t)[8], we do not present the results for *TaqI*. Hardy Weinberg Equilibrium was calculated according to standard procedures using Chi square analysis. Genotype effects were only tested when consistent effects at baseline and follow-up were found. We allowed for three possible genetic models to explain differences between groups, i.e., an allele dose effect, a dominant effect or a recessive effect. Allele dose was defined as the number of copies of a certain allele in the genotype. In case of a consistent trend reflected as an allele dose effect, which showed no significant deviation of linearity, a linear regression analysis was performed to quantify the association. In case of a dominant or recessive effect of the test allele, independent sample T-tests were performed to test for differences between two genotype groups. For dominant alleles we compared test allele carriers versus non-carriers, while for recessive effects homozygous subjects for the test allele were compared to heterozygous carriers combined with non-carriers. We searched for possible interaction between risk alleles of the VDR gene and the polymorphic Sp1 site in the COLIA1 gene. Non-haplotype '3' alleles and the T-allele were assigned as risk alleles. As a consequence the non-risk genotypes of VDR were [1,3], [2,3] or [3,3] and of COLIA1 this was [GG]; whereas the risk genotypes of VDR were [1,1],[1,2],[1,4] or [2,2] and of COLIA1 they were [GT] or [TT]. We compared three risk groups, i.e. those not carrying any risk genotype (=0), those carrying one risk genotype, either from VDR or from COLIA1 (=1), and those carrying risk a genotype from both VDR as well as COLIA1 (=2). In case of an additive effect, we performed a linear regression analysis. P-values ≤ 0.05 were considered to be significant.

RESULTS

Subjects

Baseline characteristics are presented in Table 1. SD scores of height, vertebral body width, and bone density parameters showed a normal distribution and the means did not differ from the expected zero. No significant gender differences in baseline characteristics were found.

Table 1. Baseline characteristics

	Boys	Girls
Number	53	89
Age (years)*	10.6 (4.3-18.5)	11.8 (4.3-19.9)
Pubertal stage:		
Prepubertal	31 (59%)	37 (42%)
Pubertal	15 (28%)	34 (38%)
Postpubertal	7 (13%)	18 (20%)
Calcium intake (mg/day)*	1208 (460-1897)	1187 (302-4356)
Physical activity (hours/week)*	9.3 (1.5-23)	7.7 (1.8-22)

*Mean (range)

The mean age at menarche was 13.1 years (range 11–16 yrs), which is the mean age of menarche in Dutch girls[20]. At follow-up, 31 children were prepubertal, 48 pubertal and 69 postpubertal. The VDR haplotype and genotype frequencies are given in Table 2. The distribution of the genotypes was found to be in Hardy Weinberg equilibrium in boys and girls.

Table 2. VDR haplotype and genotype distribution*

Haplotype	Code	Boys (%)		Girls (%)		Total (%)	
baT	1	60	(57)	81	(46)	141	(49.6)
BAt	2	33	(31)	70	(39)	103	(36.3)
bAT	3	12	(11)	26	(15)	38	(13.4)
BAT	4	1	(0.9)	1	(0.6)	2	(0.7)
bAt	5	0		0		0	
Total		106		178		284	
Genotype							
baT-BAt	1,2	17	(32)	36	(40)	53	(37.2)
baT-baT	1,1	17	(32)	14	(16)	31	(21.8)
baT-bAT	1,3	6	(11)	15	(17)	21	(14.8)
BAt-BAt	2,2	6	(11)	12	(13)	18	(12.7)
BAt-bAT	2,3	4	(8)	10	(11)	14	(9.8)
bAT-bAT	3,3	2	(4)	1	(1)	3	(2.1)
baT-BAT	1,4	1	(2)	1	(1)	2	(1.4)
Total		53		89		142	(100)
HWE p-value		*0.84*		*0.65*		*0.86*	

*ranking according to frequency in the Caucasian population

HWE = Hardy Weinberg Equilibrium

Table 3. Antropometric characteristics and bone density by VDR haplotype allele at baseline*.

	Number of VDR allele copies				
Haplotype allele	**0**	**1**	**2**		
1	35	76	31		
2	57	67	18		
3	104	35	3		
Anthropometry					
Height				ß	p-value
1	0.28 (1.00)	0.03 (1.07)	0.27 (0.80)	-	-
2	0.36 (0.97)	-0.09 (1.03)	0.35 (0.81)	-	-
3	0.06 (0.94)	0.33 (1.15)	1.17 (0.75)	0.35	0.04
Vertebral width					
1	0.07 (0.81)	0.21 (1.11)	0.28 (0.91)	-	0.41**
2	0.53 (1.09)	-0.10 (0.88)	0.19 (0.82)	-	-
3	0.08 (0.89)	0.51 (1.27)	0.41 (0.40)	-	0.05**
Bone density					
$BMAD_{LS}$					
1	0.07 (0.93)	-0.13 (1.09)	-0.11 (0.90)	-	0.32**
2	-0.23 (0.92)	-0.01 (1.06)	0.18 (1.54)	0.22	0.09
3	0.00 (1.05)	-0.23 (0.86)	-0.79 (0.61)	-0.28	0.10
BMD_{LS}					
1	0.09 (0.83)	-0.01 (1.11)	0.11 (0.80)	-	-
2	0.10 (0.97)	-0.07 (1.01)	0.26 (0.92)	-	-
3	0.06 (0.99)	0.04 (0.98)	-0.52 (1.03)	-	-
BMD_{TB}					
1	-0.08 (0.89)	-0.09 (1.09)	-0.19 (0.80)	-	-
2	-0.05 (1.01)	-0.22 (0.95)	0.16 (0.98)	-	-
3	-0.12 (0.94)	-0.03 (1.14)	-0.47 (0.76)	-	-
BMC_{TB}					
1	0.19 (0.92)	0.07 (1.09)	0.09 (0.70)	-	-
2	0.26 (1.07)	-0.12 (0.83)	0.43 (1.00)	-	-
3	0.05 (0.84)	0.25 (1.29)	0.36 (1.06)	-	-

* Mean standard deviation score (SD); ß is the regression coefficient= the increment or decrement in SDS per allele copy

** Independent sample T-test in case of a dominant test allele effect (0 versus 1&2 allele copies)

vertebral width mean width of the lumbar vertebral body L2-4; *BMAD* bone mineral apparent density; *BMD* bone mineral density; *BMC* bone mineral content; *LS* lumbar spine; *TB* total body

Anthropometry and bone characteristics

We first analyzed the subjects grouped by their VDR genotype based on copy number for the three most frequent haplotype alleles, i.e. haplotype 1, 2, and 3. The genotype effects by VDR haplotype allele are reported in Table 3. At baseline, height SDS was significantly increased in VDR haplotype '3' allele carriers with evidence of an allele dose effect (β=0.35, p=0.04), while a similar trend was found at follow-up (β=0.28 p=0.10).

Interestingly, we observed a similar trend when we analyzed vertebral body width as another parameter of frame size. At baseline, vertebral body width was 0.4 SDS increased in haplotype '3' carriers vs non-carriers (p=0.05), although this did not reach significance at follow-up (p=0.47). No significant associations between the VDR genotypes and BMI and LBM were found.

When we analyzed parameters of bone density no genotype effects on BMD and BMC were observed at baseline and follow-up (Table 3). However, we observed a consistent association between haplotype allele '3' carriers and decreased BMAD, at baseline as well as at follow-up. Although obvious trends were found, the differences did not reach significance. At baseline, BMAD decreased 0.3 SDS per copy of VDR haplotype allele '3' (p=0.10) and at follow-up (p=0.11). An opposite trend in BMAD was observed for carriers of haplotype 2 (p=0.09).

No association between the VDR genotypes and physical activity was observed.

Longitudinal data

We went on to compare baseline with follow-up measurements. The change in height and vertebral body width SDS between baseline and follow-up was not associated with VDR genotype. No correlation was found between VDR genotype and bone gain, expressed as ΔBMC, ΔBMD, and ΔBMAD SDS (data not shown).

Individual RFLPs

We also analyzed the association between bone density and VDR genotype as defined by the individual *BsmI* and *ApaI* restriction site polymorphisms. No consistent effects were found for height, vertebral body width, BMD, or BMC. In concordance with the results of the direct haplotyping, a B-allele dose effect on BMAD was found at baseline and follow-up (β=0.25, p=0.05). No differences in BMAD between the *ApaI* genotype groups were observed.

Interaction of VDR and COLIA1 genotype

The above-described results identified non-haplotype '3' alleles as risk alleles for decreased height and vertebral body width. In this cohort, we previously found the T-allele of the G to T substitution in the polymorphic Sp1 binding site of the collagen IA1 gene to be associated with decreased height and vertebral body width SDS, as well (*Chapter 2*). We therefore analyzed height and vertebral body width SDS according to VDR genotype combined with COLIA1 genotype (Figure 1). This risk genotype score analysis revealed a dose-dependent decrease in height (β=-0.4 SDS; p= 0.006), as well as in vertebral body width (β=-0.4 SDS; p=0.001). We performed multiple linear regression analysis with VDR haplotype '3', COLIA1 and an interaction term (VDR3xCOLIA1) as independent variables and height and vertebral body width as dependent variable. The interaction term was not significant in the model for height (p=0.63) nor for vertebral body width SDS (p=0.11).

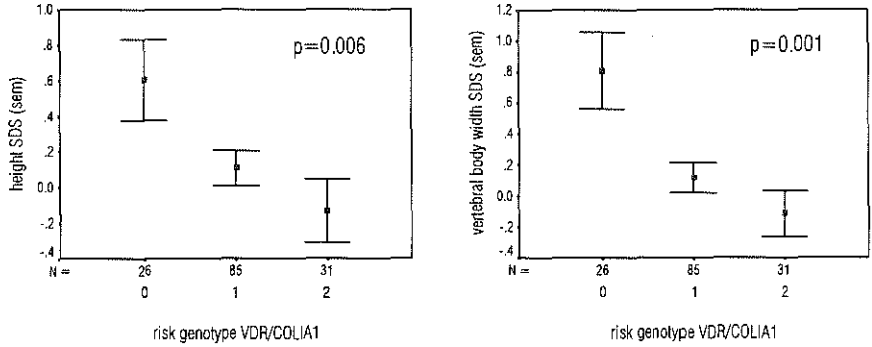

Figure 1. Interaction of effects of VDR genotype and COLIA1 genotype on height and vertebral body width. The combined genotypes were analysed as a risk score: '0' those not carrying a risk genotype of VDR nor COLIA1, '1' those carrying a risk genotype of either VDR or COLIA1, and '2' those carrying a risk genotype of VDR as well as COLIA1. P-values of regression analyses are given.

DISCUSSION

In this study we investigated the relationship between VDR genotype, anthropometry and bone density in children and young adults. We found a significantly increased height and lumbar vertebral body width SDS in carriers of a particular haplotype of three adjacent polymorphic sites (haplotype '3'=bAT). In addition, we found this haplotype to be associated with decreased BMAD, although this trend did not reach significance. This suggests that VDR polymorphism affects several frame size characteristics, in addition to an effect on apparent BMD, that may influence areal bone density.

Our observations of the effect of VDR genotype on aspects of frame size are in line with the previously reported associations of VDR genotype with birth weight, height, growth to final height and bone area[15,16,25]. Taken together these observations suggest a role of the vitamin D endocrine system in regulating growth of the skeleton, which is reflected in genotype dependent differences in frame size. Vitamin D is likely to affect growth as chondrocytes in the growth plate have receptors for 1,25-dihydroxyvitamin D_3[26,27] and vitamin D is one of the regulators of chondrocyte proliferation in the growth plate[28,29]. Indeed, growth retardation is a well-known clinical feature of rickets[30,31]. Vitamin D-dependent rickets (VDDR) type I or pseudo-vitamin D-deficiency rickets is caused by a mutation in the gene coding for P450c1α (which catalyzes 1α hydroxylation of 25-hysroxyvitamin D), while VDDR type II is an autosomal recessive disease caused by loss of function mutations of the VDR. Furthermore, studies with animal models of VDDR type I[32,33] and type II[34,35] also show growth retardation. So, clinical and experimental findings strongly suggest that variations in vitamin D levels or VDR influence growth.

With respect to interpretation of our results we must note that the polymorphisms used are anonymous. They are likely to act as markers through linkage disequilibrium for truly functional sequence variation elsewhere in the gene. One approach to partly overcome this drawback is to increase genetic resolution by combining informative alleles in multi-allelic haplotype markers. Uitterlinden et al.[8] developed a direct molecular haplotyping PCR test to monitor three clustered

RFLPs at the VDR gene locus simultaneously. This method was used in a study of 1782 elderly Caucasian men and women[8] and in a cohort of 814 young Canadian women[5]. We found similar VDR haplotype frequencies to those reported in these Caucasian populations. Uitterlinden et al.[8] reported an association between haplotype '3' and a mildly decreased BMD, whereas in women haplotype '2' showed a weak trend towards higher BMD. Rubin et al.[5] showed that haplotype '1' and '2' were significantly correlated with BMD, but did not found an association for haplotype '3'. Together with our findings these results suggest that haplotype '3' may be assigned as a risk allele, while haplotype '2' may be assigned as a protective allele with respect to bone characteristics.

The VDR haplotype alleles can be dissected to the frequently used *BsmI* and *ApaI* alleles. The effects of the *ApaI* alleles can be tested by comparing haplotype '1' (baT) with haplotype '2' (BAt) combined with '3' (bAT), reflecting 'a' vs 'A'. Comparing haplotype '2' with haplotype '1' and '3' reflects the effects of the 'B' vs 'b' alleles. Sainz et al.[12] reported that VDR gene alleles predict bone density in prepubertal girls of Mexican descent. The genotypes 'aa' and 'bb' had significantly higher volumetric BMD than 'AA' and 'BB'. In 75 young adult Finns the bb genotype was also associated with higher BMD[36]. We demonstrated an inverse relationship; BMAD ('volumetric BMD') was the highest in the BB and AA group (=haplotype 2), which is in concordance with trends observed in the Rotterdam study[8] and the Canadian study[5]. These results are different, however, from Gunnes et al.[13] and Lorentzon et al.[15] who both found no association between VDR polymorphism and BMD. In these cases, areal BMD was assessed by single photon absorptiometry and DXA, while no correction for bone size was made. Although the trend is weak and the power is low, we found that VDR polymorphism was particularly associated with apparent ('volumetric') BMD and not with areal BMD. Indeed, Sainz et al.[12] used volumetric density assessed by quantitative computed tomography when they found an association with VDR polymorphisms. Furthermore, SPA and DXA will overestimate BMD in tall stature. Therefore, the fact that haplotype '3' allele carriers are taller, while their BMAD is lower, might neutralize the effect on areal BMD. These findings suggest that the association might be more evident with volumetric density in growing individuals. Thus, areal BMD may be a less suitable end-point to study effects of VDR genotype.

More in general, differences between studies examining BMD in relation to VDR genotype might be explained in various ways. Firstly, VDR genotype is not involved in determining BM(A)D and the findings are due to chance. Secondly, the association is true but there may be 'allelic heterogeneity' between populations. Thirdly, environmental and genetic influences might interact, and such influences might be different in various developmental stages, and may also be site-dependent according to physical loading and bone composition.

The change in BMAD or BMD SDS between baseline and follow-up did not associate with the VDR genotypes in the present study. Gunnes et al.[13] also studied bone gain and did not find an association between the individual *BsmI* polymorphism and bone gain in the forearm. This suggests that children follow their own 'BMD accrual' curve, similar to their growth curve.

By combining risk alleles, we found additive effects of the VDR haplotype '3' and COLIA1 Sp1 polymorphisms, which both affect height and bone size. Either carrying the VDR risk allele or the COLIA1 risk allele had an effect on frame size while carrying risk alleles at both loci further enhanced the effect. The interaction term, however, was not significant, indicating an additive effect rather that a 'true' interactive effect. While these are epidemiological observations, this

notion is supported by molecular biological experimental evidence. The VDR is a transcription factor that amongst others regulates the expression of the COLIA1 gene[17,18]. It can thus be hypothesized that VDR–regulated expression of the COLIA1 gene differs across VDR and COLIA1 alleles. Recently, a similar interaction was observed between the VDR gene and COLIA1 gene in the susceptibility for fracture in an elderly population[37]. Although the exact mechanism remains to be elucidated, together these results suggest this intergenic interaction to have effects at different stages in life.

With regard to our study population some characteristics should be noted. The group of children we studied was a random sample of the previous study population. We only analyzed Caucasian children, so it was an ethnically homogenous group. We found VDR genotypes to be in Hardy Weinberg Equilibrium suggesting that no severe selection bias has occurred. The effects we observed are considerable in terms of effect size but most were borderline significant. This reflects the limited statistical power we could develop given the limited sample size. For example, only three children were homozygous for haplotype '3', so results should be interpreted carefully, and larger study populations are needed. For the same reason no conclusion could be drawn from our study, whether the strength of the VDR genotype effect depends on pubertal stage.

In conclusion, VDR genotype, as defined by haplotypes constructed of the *BsmI, ApaI* and *TaqI* polymorphisms, is associated with height and vertebral body width in Caucasian children and young adults. We observed additive interaction between genotype effects of VDR polymorphism and the COLIA1 Sp1 polymorphism on frame size. VDR is weakly associated with bone mineral apparent density and not with areal bone density. The VDR genotype effect on areal BMD is neutralized by increased height and bone size, therefore only effects on BMAD were found.

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Chapter 4

A CROSS-SECTIONAL STUDY ON BIOCHEMICAL PARAMETERS OF BONE TURNOVER AND VITAMIN D METABOLITES IN HEALTHY DUTCH CHILDREN AND YOUNG ADULTS

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ABSTRACT

Objective: To provide reference data of biochemical markers of bone turnover and vitamin D metabolites for children and young adults. *Methods:* Blood samples were taken in 176 healthy Dutch children and young adults (7.6-25.3 years) to assess serum calcium, alkaline phosphatase (ALP), inorganic phosphate, osteocalcin, collagen type I cross-linked N-telopeptide (NTx), N-terminal propeptide of type I procollagen (PINP), 25-hydroxyvitamin D_3, and 1,25-dihydroxyvitamin D_3 levels were measured. Cross-linked telopeptide of type I collagen (ICTP) and carboxyterminal propeptide of type I procollagen (PICP) were assessed in 286 subjects (1.4-25.3 years). *Results:* Calcium and vitamin D levels were independent of age. The peak concentrations for NTx, ICTP, PICP, PINP, ALP and osteocalcin were found during puberty, in girls approximately 2.5 years earlier than in boys. Strong correlations were found between the markers of bone turnover, while no correlation was found between the markers of bone turnover and bone mineral density measured by dual energy X-ray absorptiometry. *Conclusions:* Single measurement of bone markers cannot predict bone density. Reference data according to gender, age and Tanner stage are given, which allow calculating standard deviation scores adjusted for age and gender.

INTRODUCTION

Biochemical markers of bone turnover might be valuable tools to evaluate efficacy of treatment for osteoporosis[1-3]. Furthermore, they may provide insight into the pathophysiology of bone disorders. Studies are most frequently performed in adults, mainly in postmenopausal women. However, during childhood and adolescence significant changes in bone turnover occur, especially during puberty[4]. Reference data of biochemical markers for these particular age groups are needed to interpret values found in patients. Combining various markers of bone formation and bone resorption, and longitudinal measurements may help the physician and researcher.

Bone formation markers

Alkaline phosphatase (ALP) is for 80% derived form bone, however many isophorms are produced by the liver, intestines, and kidneys[5]. Bone specific ALP is an enzyme solely produced by osteoblasts and therefore more specific for the formation of bone[6]. Bone specific ALP represents approximately 75-90% of the total ALP in children over the age of four years, declining to 50% after puberty[7]. The type I collagen constitutes approximately 90% of the organic matrix of bone[8]. Carboxyterminal propeptide of type I procollagen (PICP) and amino-terminal propeptide of type I procollagen (PINP) are set free from type I procollagen before the collagen molecules are incorporated into collagen fibres. Thus, serum concentrations of both PICP and PINP are directly related to the amount of newly formed collagen laid down in the bone. Risteli et al.[9] reported that the clearance of PINP by the scavenger receptor of endothelial liver cells is not affected by hormone levels, whereas PICP clearance by the mannose receptor of endothelial liver cells might be influenced by hormones as estrogens and IGF-I. Therefore, PINP is potentially more specific for monitoring changes in bone turnover. Osteocalcin is a small noncollagenous protein, which is synthesised by osteoblasts and chondrocytes and deposited in the extracellular bone matrix. A small amount enters the circulation[10] where it is rapidly cleared by the kidneys[11]. The function of osteocalcin however is not fully known yet. It might be interpreted as marker of bone turnover

rather than bone formation, since the absence of osteocalcin appeared to result in increased mineralisation[12].

Bone resorption markers

Collagen type I cross-linked N-telopeptide (Ntx) is a marker of bone resorption, which can be measured in urine[13] and serum[14]. Both measurements are strongly correlated, but urine samples need to be corrected for creatinine concentration[14]. Ntx is solely originating from type I collagen from the bone by osteoclast activity due to the unique amino acid sequences and orientation of the cross-linked alpha-2 (I) N-telopeptide[14]. Cross-linked telopeptide of type I collagen (ICTP) is released during the degradation of type I collagen. However, high ICTP concentration has been found to be associated with pathological bone degradation as lysis of bone[15], whereas NTx might be more effective in monitoring more subtle changes in osteoclastic activity as osteoporosis and its treatment[14].

In children as well as in adults bone is constantly remodelled. Existing mineralised tissue is resorbed by osteoclasts and newly formed by osteoblasts. However, in addition to the remodelling, children grow, whereby bone modelling is achieved by appositional growth along periostal surfaces and by the calcification of cartilage adjacent to the growth plate[16]. As a result, changes in biochemical markers of bone turnover will also reflect changes in growth velocity[10,17]. So, biochemical markers of bone turnover are not specific for either bone modelling or bone remodelling.

The aim of the present study was to provide reference data for biochemical parameters of bone turnover and vitamin D metabolites in children and young adults, and to study the interrelationships between these serum markers and between the markers and bone density.

SUBJECTS

In 1994-1995, 500 healthy Dutch children and adolescents from the Rotterdam Region, the Netherlands, participated in our study to assess reference values for bone density and body composition measured by dual energy X-ray absorptiometry (DXA). These cross-sectional results[18,19] have previously been presented. In 1998 -1999, 88 boys and 123 girls agreed to have a second DXA-measurement, of whom 63 boys and 113 girls (83%) also agreed to a blood test to assess biochemical parameters of bone turnover. For PICP and ICTP 90 samples (56 boys and 34 girls), that were collected and assayed earlier, were added. The medical ethics committee of the University Hospital Rotterdam approved this study, and written informed consent was obtained from the parents or guardians and all children aged 12 years and over.

METHODS

As validated previously[20], pubertal development was evaluated by self-assessment of breast and pubic hair stage in girls and genitalia and pubic hair stage in boys, according to Tanner[21]. In case of discrepancies between variables, emphasis was placed on the breast development in girls and genital development in boys. None of the subjects was taken medication or suffered diseases known to affect bone mass. Bone mineral density (BMD, g/cm^2) was determined by DXA of the

lumbar spine (LS) and total body (TB). For children with weight below 30 kilograms paediatric software was used. We calculated volumetric density (bone mineral apparent density, BMAD) of the lumber spine with the model $BMAD_{LS} = BMD_{LS} \times [4/ (\pi \times width)]$[22].

In the afternoon, blood samples were taken to assess serum calcium, ALP, inorganic phosphate, osteocalcin, NTx, ICTP, PICP, PINP, 25-hydroxyvitamin D_3, and 1,25-dihydroxyvitamin D_3. Serum calcium, ALP and inorganic phosphate were analysed immediately after blood sampling. Serum was stored at -80 °C until analysis[23]. All samples were analysed in duplicate. Serum calcium, ALP and organic phosphate were measured on the Dimension® clinical chemistry system (Dade Behring Inc, Newark USA). For ALP, the test temperature was 37 °C, the method responds to all ALP isoenzymes. For calcium, ALP, and phosphate the intra-assay Coefficient of Variation (CV) was 0.9, 1.4, 1.4% and the interassay CV 1.9, 3.1, 3.6%, respectively. Assessment of intact osteocalcin was performed by immunoradiometric assay (IRMA) (DiaSorin, Stillwater, USA). The intra-assay CV was 6.3 % and the interassay was CV 9.5 %. 25-Hydroxyvitamin D_3 was assessed by radioimmunoassay (RIA) (DiaSorin, Stillwater, USA). The intra-assay CV was 12.5 % and the interassay CV was 11 %. 1.25-dihydroxyvitamin D_3 was assessed by RIA of Immuno Diagnostic System (Boldon, UK); intra- and interassay CV of 8% and 16.8%. ICTP, PICP, and PINP were measured by RIA (Orion Diagnostica, Espoo, Finland). Intra-assay CV is 6.2, 3.2, 13.7 % and the inter-assay CV is 7.9, 6.6, 8.2 %, respectively. Serum levels of NTx were measured by ELISA (Osteomark, Ostex International, Seattle, USA) with an intra-assay CV of 4.6%, and an interassay CV of 6.9 %. The reported intra- and interassay coefficients of variation were the percentages provided by the manufacturer, we presented the highest reported CV.

STATISTICS

Visual inspection of the various parameters plotted versus age showed in most cases a roughly linear increase up to a certain age, followed by a gradual decrease, with a subsequent flattening of data points. Therefore, we chose a piecewise linear regression model or so-called broken stick method to describe the relation with age, thereby avoiding the necessity to quantify the relationship using a smooth curve, whose equation is hard to find. Using this technique breakpoints were defined and their location was calculated[24]. Log-transformed (base 10) data for osteocalcin, PINP, ALP, NTX, and ICTP were used to get a better fit to a normal distribution. The resulting residuals from the equations were analysed to investigate the influence of pubertal stage in addition to age. In case of significant differences in hormone levels and bone turnover markers between boys and girls, they were separately analysed. Kruskall Wallis tests and Mann Whitney tests were used to compare the various Tanner stages, and to analyse seasonal differences in vitamin D levels. Pearson's correlation coefficients were used to correlate variables with normal distributions, otherwise Spearman's correlation coefficients were used. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS version 10.0).

RESULTS

The mean age of the subjects was 15.9 years (range: 7.6-25.3 years). Thirty-one children had Tanner stage 1 (prepubertal); 15 had Tanner stage 2, 15 children had Tanner stage 3, 30 had Tanner stage 4, and 85 had Tanner stage 5 (postpubertal). Because of the low number of children in Tanner stage 2, 3 and 4, these Tanner stages were combined to one group (pubertal). For PICP and ICTP 90 subjects were added (mean age 5.5 years, range: 1.4-15.6 years). All, but two were prepubertal (1 boy had Tanner stage 2 and 1 girl had Tanner stage 5). Mean age of menarche was 13.2 years (sd 1.2 yrs), similar to the mean age of menarche in the Netherlands[25]. 16 % Of the subjects used multivitamins in the days of evaluation.

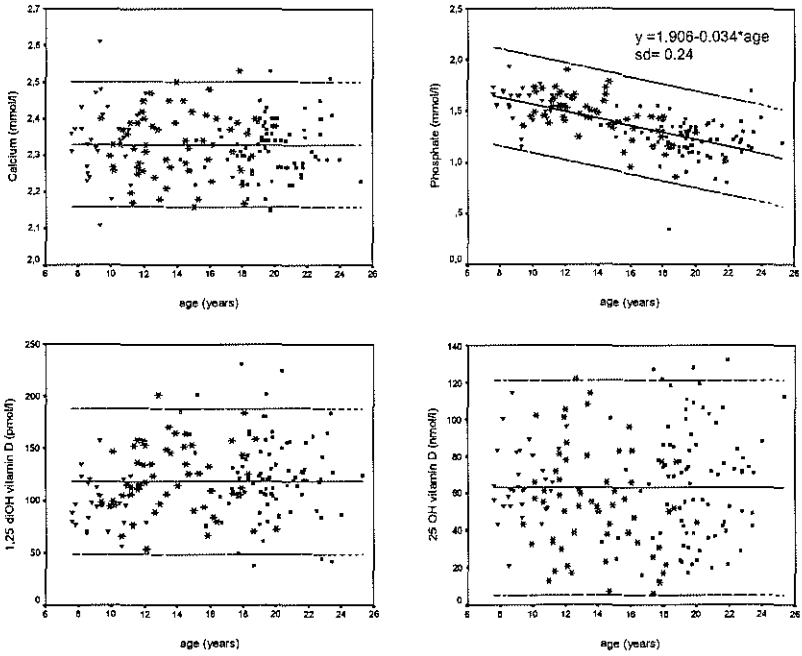

Figure 1. Serum calcium, phosphate, 1,25-dihydroxyvitamin D_3, and 25-hydroxyvitamin D_3 in boys and girls. Solid line = mean and dotted line ± 2 SD. ▼ = Tanner stage 1; * = Tanner stage 2 to 4; and ■ = Tanner stage 5.

No differences in calcium, phosphate, 25-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 levels between boys and girls were found. Of these parameters, only phosphate level depends on age ($r=0.67$ $p<0.001$). Results are presented in Figure 1. Figures 2 and 3 show the age-related changes in parameters of bone formation and bone resorption for boys and girls. The equations used to calculate the fitted curves are presented in Table 1. The equations can be used to calculate age- and sex adjusted SD scores. PICP was normally distributed, but had different standard deviations depending on age in boys. With a large variance until the age of 14.9 years (SD=139), and with a SD of 57 for the age 18.4 years and over. Linear interpolation from 139 μg/l to 57 μg/l between the age of 14.9 to 18.4 years was used to calculate the SD for this age group. Table 2 show the results of the serum levels in prepubertal (Tanner 1), pubertal

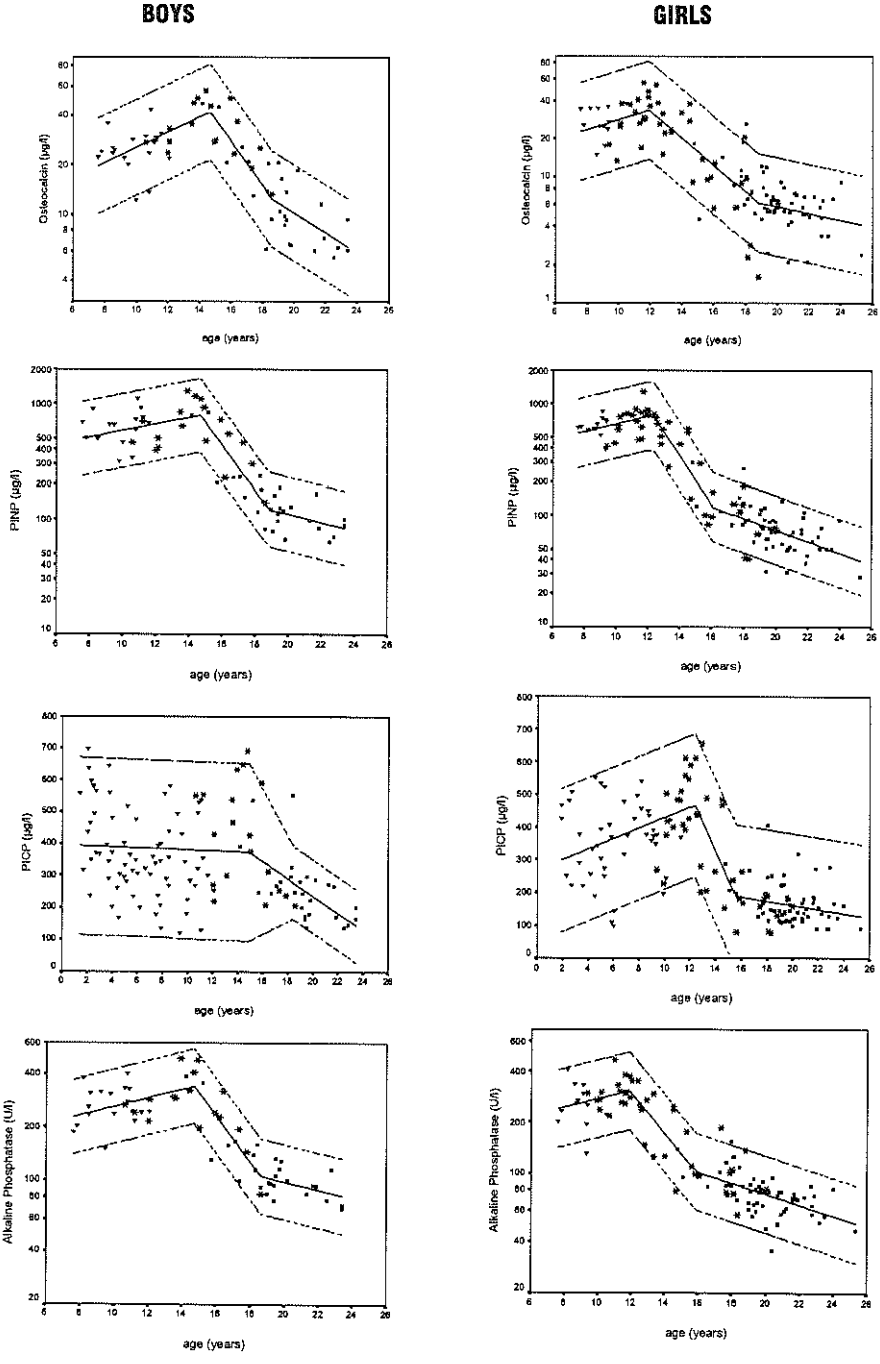

Figure 2. Parameters of bone formation for boys and girls. Osteocalcin, PINP=amino-terminal propeptide of type I procollagen, PICP = carboxyterminal propeptide of type I procollagen, and alkaline phospahatase. Solid line = mean and dotted line ± 2 SD. ▼ = Tanner stage 1; * = Tanner stage 2 to 4; and ■ Tanner stage 5.

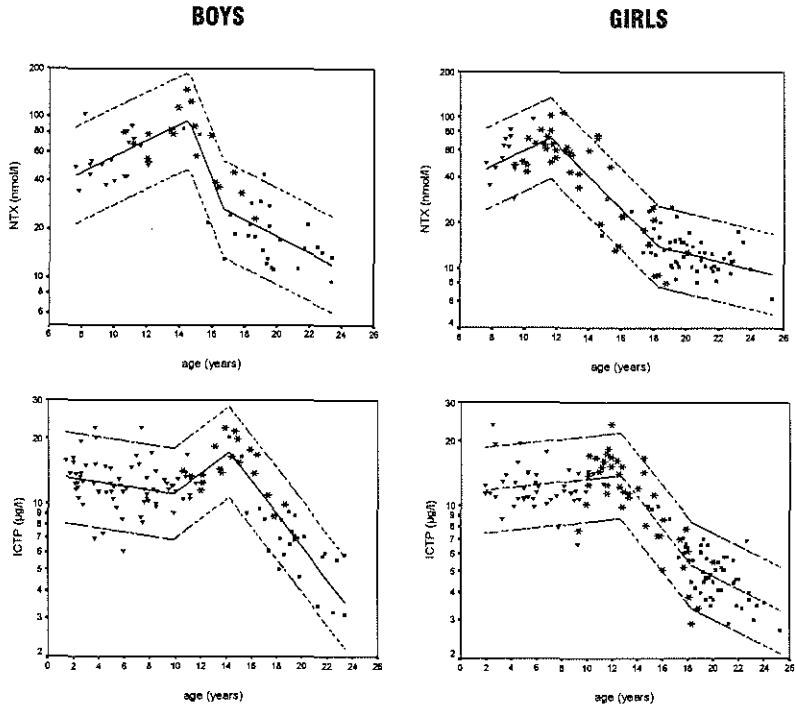

Figure 3. Parameters of bone resorption for boys and girls. *NTx* collagen type I cross-linked N-telopeptide, *ICTP* cross-linked telopeptide of type I collagen. Solid line = mean and dotted line = ± 2 SD. ▼ = Tanner stage 1; * = Tanner stage 2 to 4; and ■ = Tanner stage 5.

(Tanner stage 2-4) and postpubertal (Tanner 5) children by gender. Significant differences between the three pubertal stages were found for phosphate, ALP, osteocalcin, PINP, PICP, ICTP, and NTx (Kruskal Wallis $p<0.001$). Pair-wise comparison with the previous Tanner stage showed that parameters were significantly lower in the postpubertal children compared to the pubertal children. Only osteocalcin and ICTP in boys were significantly higher in the pubertal children compared to the prepubertal children. The analysis of the residuals showed that for all parameters the residuals were independent of pubertal stage. This means that for healthy children it is not necessary to correct for age, sex as well as pubertal stage, but correction for age and sex will be sufficient. The peak concentrations for these parameters were found for boys between the age of 14.2-14.9 years corresponding with genital stage 4-5, and between the age of 11.6-12.7 years in girls corresponding with breast stage 3-4 in healthy Dutch children[26].

25-Hydroxyvitamin D_3 showed the highest levels in summer (July to September) and the lowest levels in January to March (Kruskall Wallis $p=0.001$) (Figure 4). There was seasonal variation in 1,25-dihydrxoyvitamin D_3 levels as well, with the highest level in July until December. No differences in serum vitamin D levels were found between children with or without multivitamin use.

Table 1. Breakpoints (age in years), intercepts and slopes for the fitted curves. Equations derived are given below.

	BP1	BP2	A	B	C	D	SD
Boys							
ALP (U/l)	14.7	18.6	2.17	0.025	-0.131	-0.023	0.11
Osteocalcin (μg/l)	14.7	18.6	0.94	0.046	-0.134	-0.060	0.14
PINP (μg/l)	14.7	18.8	2.48	0.029	-0.197	-0.035	0.16
NTx (nmol/l)	14.6	16.7	1.26	0.049	-0.258	-0.051	0.15
ICTP (μg/l)	9.9	14.2	1.13	-0.009	0.044	-0.075	0.10
PICP (μg/l)	14.9	-	395.7	-1.49	-26.95	-	**
Girls							
ALP (U/l)	11.9	15.9	2.197	0.024	-0.12	-0.033	0.11
Osteocalcin (μg/l)	11.9	18.8	1.058	0.04	-0.11	-0.027	0.19
PINP (μg/l)	12.3	16.1	2.469	0.034	-0.216	-0.052	0.15
NTx (nmol/l)	11.6	18.3	1.27	0.05	-0.11	-0.026	0.13
ICTP (μg/l)	12.7	18.3	1.06	0.006	-0.073	-0.029	0.10
PICP (μg/l)	12.4	15.6	276.7	16.2	-86.9	-6.19	109.7

BP1 first breakpoint; *BP2* second breakpoint; *A* intercept; *B* first slope; *C* second slope; *D* third slope; *SD* standard deviation.

** for age ≤ 14.9: SD= 139; for 14.9 > age ≤ 18.4:SD=139 –23*(age-14.9); for age > 18.4: SD= 57

Fitted curves for log ALP, log osteocalcin, log PINP, log NTx, log ICTP, and $PICP_{girls}$ can be calculated:

Mean =(A+B*AGE)*(AGE<BP1)+(A+B*BP1+C*(AGE-BP1))*(AGE ≥ BP1 AND AGE ≤ BP2)
+(A+B*BP1+C*(BP2-BP1)+D*(AGE-BP2))*(AGE ≥BP2).

Mean ($PICP_{boys}$) = (A+B*AGE)*(AGE<BP1)+(A+B*BP1+C*(AGE-BP1))*(AGE ≥BP1).

In these equations the logical terms, like (AGE<BP1), are 1 if the condition is true and 0 otherwise.

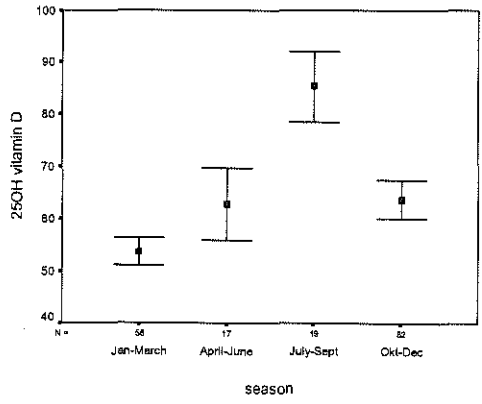

Figure 4. Mean (sem) serum levels of 25-hydroxyvitamin D_3 plotted by season.

Table 2. Median and 10th and 90th percentile in boys and girls by Tanner stage

Tanner stage	**I**	**II-IV**	**V**
Boys (n)	18	17	27
Phosphate (mmol/l)	1.56 (1.40-1.72)	1.46 (1.30-1.81)	1.25 (1.07-1.53)*
ALP (U/l)	250 (183-379)	276 (139-479)	100 (75-205)*
Osteocalcin (μg/l)	24.1 (13.4-36.1)	28.5 (18.5-51.2)*	10.5 (6.1-29.5)*
PICP (μg/l; *n=117)*	345 (199.3-573.8)	426 (209.0-649.0)	247(143.8-418.8)*
PINP (μg/l)	620.5 (330.7-924.1)	519.8 (216.8-1174.0)	121.5 (69.0-345.8)*
ICTP (μg/l; *n=117)*	12.1 (9.2-16.0)	14.1 (9.9-21.6)*	7.4 (3.4-10.8)*
NTx (nmol/l)	50.6 (37.2-88.9)	71.7 (32.7-126.0)	18.0 (11.3-50.4)*
Girls (n)	12	39	57
Phosphate (mmol/l)	1.65 (1.17-1.75)	1.45 (1.11-1.69)	1.22 (1.01-1.41)*
ALP (U/l)	254 (150-386)	238 (79-349)	76 (55-103)*
Osteocalcin (μg/l)	25.3 (15.9-37.1)	24.3 (5.6-42.4)	6.3 (3.4-11.8)*
PICP (μg/l; *n=145)*	371 (192-512)	384 (137-605)	148 (109-268)*
PINP (μg/l)	622.5 (437.4-827.1)	493.3 (80.2-850.0)	75.6 (48.0-123.6)*
ICTP (μg/l; *n=145)*	11.7 (9.9-16.5)	11.8 (5.1-16.9)	4.8 (3.5-7.0)*
NTx (nmol/l)	53.6 (31.2-90.9)	50.2 (12.9-79.8)	12.8 (9.6 –22.2)*

* significant difference with previous Tanner Stage (Mann Whitney test)

Phosphate anorganic phosphate, *ALP* alkaline phosphatase, *PICP* carboxyterminal propeptide of type I procollagen, *PINP* N-terminal propeptide of type I procollagen, *ICTP* cross-linked telopeptide of type I collagen, *NTx* cross linked N-telopeptide of bone collagen.

Correlations

Markers of bone formation as well as bone resorption were highly correlated (Table 3). The markers of bone resorption ICTP and NTx showed a correlation of $r_s = 0.90$. High correlation between the bone formation markers were found, as well as between bone resorption and bone formation markers. All correlation coefficients were significant at a p-level of <0.01.

No associations between markers of bone turnover and cross-sectional bone mineral density (BMD and BMAD) measurements were found.

Table 3. Spearman correlation coefficients for the markers of bone turnover.

	phosphate	osteocalcin	PICP	PINP	ICTP	NTx
ALP	0.70	0.85	0.82	0.90	0.88	0.85
Phosphate	-	0.72	0.67	0.71	0.71	0.72
Osteocalcin	-	-	0.84	0.92	0.89	0.87
PICP	-	-	-	0.88	0.71	0.82
PINP	-	-	-	-	0.91	0.91
ICTP	-	-	-	-	-	0.90

All significant at a p-level of <0.01. *ALP* alkaline phosphatase, *PICP* carboxyterminal propeptide of type I procollagen, *PINP* amino-terminal propeptide of type I procollagen; *ICTP* cross-linked telopeptide of type I collagen, *NTx* collagen type I cross-linked N-telopeptide

Discussion

The present study provides reference values for commonly used markers of bone metabolism and vitamin D metabolites. Alkaline phosphatase, PICP, osteocalcin, PINP, ICTP, and NTX showed peak concentrations in puberty. Concentrations peaked earlier in girls than in boys. Calcium and vitamin D metabolites were independent of age and gender. Various markers of bone formation show highly significant correlation, similar correlations for the bone resorption markers are found. As expected, the correlations between these markers were highly significant, because bone turnover is a strongly coupled mechanism of bone resorption by osteoclasts and bone formation by osteoblasts[8]. No associations between bone markers and bone density measurements were demonstrated.

Peak concentrations were attained during puberty. The peak occurred at the age of 12 in girls and occurred approximately 2.5 years later in boys. Such a pattern reflects gender differences in pubertal growth spurt, sexual development and bone mass acquisition. The increase in markers of bone turnover coincides with the pubertal growth spurt, subsequently the markers decline during late puberty. Blumsohn et al.[4] reported the highest levels of bone turnover in Tanner stages II and III in girls. Similar patterns of peak values during puberty are reported for various bone markers, such as PICP[27,28], bone specific ALP[6], PINP[29], and osteocalcin[29].

Surprisingly, 25-hydroxyvitamin D_3 showed very high variation with also rather low serum levels but sufficient or even relatively high 1,25-dihydroxyvitamin D_3 levels compared to reference data for adults provided by the manufacturers. Part of the high variation and low 25-hydroxyvitamin D_3 levels might be caused by the seasonal variation on vitamin D levels. In concordance with other reports[30,31], we found the highest levels of 25-hydroxyvitamin D_3 in summer. This is the result of higher sun exposure during these months. Of course, evaluation of seasonal variations should be performed in longitudinal studies and was not an aim of our study.

A few limitations of serum bone markers should be kept in mind. Firstly, the tissue specificity of the marker. For example, type I collagen is not only present in bone, but also in skin, cartilage, ligaments and synovia. ICTP is liberated during the degradation of type I collagen, and therefore a product derived from bones, skin, and joints. Furthermore, the production of the enzyme alkaline phosphatase is widely spread in our body[5]. Secondly, nearly all bone markers show a diurnal variation with peak concentrations in the morning and nadir concentrations in the late afternoon[32]. In our study, DXA measurements could only be performed in the afternoon because of logistic reasons. Therefore blood samples were taken in the afternoon as well. Furthermore there is a day-to-day variation[10] and a circannual pattern[30]. Thirdly, another source of variability is the stability of the parameter, for example osteocalcin. After a few hours at room temperature osteocalcin is converted into smaller fragments, resulting in a significant loss of immunoreactivity[33]. It is therefore extremely important to standardise the blood sampling and, in case of osteocalcin, to process the blood immediately after sampling. Markers of bone turnover are difficult to interpret especially in children since markers of bone turnover reflect growth (skeletal modelling) and remodelling. Age-related changes in cross-sectional bone parameters, show high similarity with growth velocity curves. In our study we were not able to correlate height velocity with the bone markers, because the follow-up interval between the first and second height measurement was too long. In studies with shorter intervals high correlation between height velocity and bone markers are reported[34,35].

With regard to our study some limitations should be noted. The long interval between the two BMD and height measurements and the fact that bone markers were assessed cross-sectionally at the second follow-up, made it impossible to study changes in BMD and height, and their possible relation with bone markers. Furthermore, due to the relatively low number of pubertal children willing to participate in our study, we were unable to evaluate the differences between Tanner stage 2,3 and 4.

Many markers of bone turnover show large variation. Correction for age and gender will be helpful and necessary for long term follow-up. However, one single measurement will not be informative for monitoring bone mass. This is underscored by the lack of association between the bone markers and bone density. Furthermore, in studies in order to assess whether cross-sectional bone markers can predict bone gain in the following year only weak associations in peripubertal girls[29] and no association in young adults[36] have been reported. However, measurements of markers of bone turnover and BMD in adults have been shown to be independent predictors of fracture risk[37-39]. In addition, bone markers are especially valuable in predicting BMD response to bisphosphonates[1,40] or hormonal replacement therapy[41] in adults.
Bone markers may play a role in improving the care of children with growth disorders, for instance in monitoring or even predicting the effect of growth hormone treatment. For example, PICP has been shown to decrease shortly after start of glucocorticoid treatment in children[42]. Thus, PICP may provide rapid assessment of the side effects on growth and bone. Recently, Schönau et al.[43] presented a new prediction model for growth response to growth hormone (GH) treatment in growth hormone deficient children (GHD) and included markers of bone turnover in this model. We earlier reported significant increases in markers of bone turnover in GHD children during GH replacement therapy[44], as well as significant decreases during gonadotrophin releasing hormone agonist treatment in children with precocious puberty[45]. These changes in bone turnover reflected the changes in bone mineral density.

Although bone markers are increasingly used in paediatric patients, studies in various patients groups are needed to establish their value in children. The present study provides reference data for various serum bone markers, together with (log-transformed) mean and SD that allow calculation of SD scores. These data are needed for interpretation of values found in paediatric patients.

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Chapter 5

REFERENCE DATA FOR BONE DENSITY AND BODY COMPOSITION MEASURED WITH DUAL ENERGY X-RAY ABSORPTIOMETRY IN CAUCASIAN CHILDREN AND YOUNG ADULTS

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ABSTRACT

Objective: Knowledge of normal physiological variation of bone density and body composition is needed to identify pathological changes. Therefore, we gained reference values for bone density and body composition measured with dual energy x-ray absorptiometry (DXA) for children and young adults. *Methods:* In the present study, cross-sectional results of 444 healthy Caucasian volunteers (4-20 years) from the Netherlands were combined with the results of 198 children who agreed to participate in the follow-up study approximately 4 years later. DXA of lumbar spine and total body was performed to assess bone density and body composition. *Results:* Bone density and lean body mass (LBM) increased with age. Maximal increase in bone density and LBM occurred around the age of 13 years in girls and approximately two years later in boys. Bone density of total body and lumbar spine showed an ongoing slight increase in the third decade. Fat percentage in boys remained 10.5% throughout childhood, but increased in girls. *Conclusions:* Most of the skeletal mass in lumbar spine and total body is reached before the end of the second decade, with a slight increase thereafter. This study provides reference values for bone density and body composition measured with DXA for children and young adults.

INTRODUCTION

Osteoporosis is a world-wide problem causing high morbidity and high costs[1]. Reduced bone mass, instability, elasticity of the bone and muscle strength play a role in fracture risk. It has been shown that for each standard deviation decrease in bone mineral density (BMD) fracture risk doubled to tripled in postmenopausal women[2,3]. Recently, Goulding et al.[4] reported similar fracture risk increments in young girls.

Peak bone mass is generally defined as the highest level of bone mass achieved as a result of normal growth. Peak bone mass is important because bone mass in later life depends on the peak bone mass achieved in young adulthood, and the subsequent bone loss. Thus, other influencing factors being equal, a high peak bone mass provides a larger reserve later in life[5,6]. Paediatricians should play an important role in the early recognition and treatment of impaired bone mass acquisition in childhood, therefore good reference data for bone mineral density are required.

We previously studied 500 healthy Dutch children 4 to 20 years of age to gain reference values for bone density and body composition[7,8]. No conclusions could be drawn regarding the age peak bone mass occurs. The objective of the follow-up study was firstly to extend our reference values for bone mineral density and body composition in children and young adults in the Rotterdam region. Moreover, this study may also provide insight in the age at which peak bone mass is reached.

SUBJECTS AND METHODS

Subjects

In 1994-1995 a study was performed to gain normative values for bone density and body composition measured by dual energy X-ray absorptiometry. In this study 444 Caucasian children participated (188 boys and 256 girls), aged between 4 and 20 years. The cross-sectional results of this first study have been presented previously[7,8].

The follow-up study was performed in 1998-1999. We recruited 198 children and young adults (84 boys and 114 girls) from the Rotterdam Region in the Netherlands. All subjects participated in our previous study to assess normative values. The mean follow-up time was 4.3 years (range 3.2-6.9 years). The results of the first and second study were combined to gain new reference data for Caucasian children and young adults.

The study protocol was approved by the ethics committee of the University Hospital Rotterdam. Written informed consent was obtained from the parents and from patients older than 12 years of age.

METHODS

Bone mineral density (BMD, grams/cm^2) was determined by DXA of lumbar spine($_{LS}$) and total body($_{TB}$). For children with weight below 30 kilograms paediatric software was used. To account for differences in bone size we calculated apparent BMD (BMAD) of lumbar spine with the model $BMAD_{LS} = BMD_{LS} \times [4/(\pi \times \text{width})]$. Width is the mean width of the second to fourth lumbar vertebral body. This model was validated by in vivo volumetric data obtained from magnetic resonance imaging of lumbar vertebrae[9]. The coefficient of variation (CV) has been reported to be 1.04% for lumbar spine BMD and 0.64% for total body BMD[10]. Total body DXA also measures Bone Mineral Content (BMC, grams), lean body mass (LBM), and percentage body fat (%fat). The CV for the paediatric weight range have been reported 1.0% for LBM, 4.1% for fat mass, and1.8% for BMC[11] and for the adult weight 1.05% for LBM, 2.2% for fat mass and 0.64% for BMC[10]. As validated previously, pubertal development according to Tanner was evaluated by self-assessment[12,13].

STATISTICAL ANALYSIS

We used a frequently used non-linear model to describe age-related changes in BMD or body composition[14]. In this regression model the second expression was assigned to pubertal growth and the first part to the slower long-term component of growth. The expression used for BMD, BMAD, BMC, and lean body mass, further referred to as parameters, is a logistic function:

$$\frac{G1}{1+G2 \times e^{-(G3 \times age)}} + \frac{P1}{1+e^{-(P2 \times (age-P3))}}$$

G1 = the asymptotic value of the parameter associated with growth
G2 = a factor influencing the parameter at age 0 and affects the overall shape of growth curve
G3 = a rate constant and is the primary determinant of the shape of the growth curve
P1 = the asymptotic value of the parameter associated with puberty
P2 = a rate constant for the increase in the parameter due to puberty
P3 = the age at which the rate of change in the parameter due to puberty is at a maximum

For BMD, BMAD, BMC and lean body mass the values of G1-3 and P1-3 were determined separately for males and females using non-linear least square regression analysis. The fitting procedure was an iterative process. Initial estimates were provided by visual inspection of the data. Percentage body fat (%fat) had a skewed distribution in boys and girls and did not show the growth curve as the other parameters. Logistic transformation of the data was necessary, while we fit the curve allowing fractional polynomials[15].

For all outcomes we examined whether the variance was depending on age by modelling the absolute residuals[16], again allowing fractional polynomials. Two sample T-tests were used to compare two independent groups. $P<0.05$ was considered to be the limit of significance.

RESULTS

Table 1. Mean bone mineral density values of lumbar spine (BMD_{LS}mean g/cm^2), bone mineral apparent density ($BMAD_{LS}$mean g/cm^3) and bone mineral density of total body (BMD_{TB}mean g/cm^2) and standard deviations (SD) in boys and girls.

Boys						
Age (years)	**$BMAD_{LS}$mean**	**SD**	**BMD_{LS}mean**	**SD**	**BMD_{TB}mean**	**SD**
4-4.9	0.250	0.036	0.592	0.062	0.799	0.029
5-5.9	0.262	0.036	0.631	0.067	0.819	0.034
6-6.9	0.269	0.036	0.665	0.073	0.839	0.038
7-7.9	0.273	0.036	0.694	0.078	0.859	0.043
8-8.9	0.276	0.036	0.719	0.084	0.880	0.048
9-9.9	0.278	0.036	0.742	0.089	0.900	0.053
10-10.9	0.280	0.036	0.764	0.095	0.920	0.057
11-11.9	0.282	0.036	0.791	0.100	0.942	0.062
12-12.9	0.285	0.036	0.828	0.106	0.967	0.067
13-13.9	0.290	0.036	0.886	0.111	1.000	0.072
14-14.9	0.300	0.036	0.968	0.117	1.045	0.076
15-15.9	0.315	0.036	1.064	0.123	1.103	0.081
16-16.9	0.332	0.036	1.152	0.128	1.158	0.086
17-17.9	0.349	0.036	1.214	0.134	1.200	0.091
18-18.9	0.360	0.036	1.251	0.139	1.229	0.096
19-19.9	0.367	0.036	1.271	0.145	1.251	0.100
20-20.9	0.370	0.036	1.281	0.150	1.270	0.105
21-21.9	0.372	0.036	1.286	0.156	1.287	0.110
22-22.9	0.373	0.036	1.289	0.162	1.305	0.115
Girls						
Age (years)	**$BMAD_{LS}$mean**	**SD**	**BMD_{LS}mean**	**SD**	**BMD_{TB}mean**	**SD**
4-4.9	0.280	0.023	0.631	0.055	0.790	0.048
5-5.9	0.284	0.025	0.660	0.063	0.809	0.051
6-6.9	0.288	0.027	0.689	0.070	0.827	0.053
7-7.9	0.293	0.029	0.718	0.078	0.845	0.056
8-8.9	0.297	0.031	0.747	0.086	0.864	0.058
9-9.9	0.302	0.032	0.779	0.094	0.886	0.061
10-10.9	0.309	0.034	0.819	0.102	0.913	0.063
11-11.9	0.319	0.036	0.876	0.109	0.947	0.066
12-12.9	0.335	0.038	0.957	0.117	0.990	0.068
13-13.9	0.355	0.040	1.049	0.125	1.036	0.071
14-14.9	0.372	0.042	1.128	0.133	1.079	0.073
15-15.9	0.383	0.043	1.181	0.141	1.114	0.076
16-16.9	0.391	0.045	1.214	0.148	1.139	0.078
17-17.9	0.396	0.047	1.236	0.156	1.156	0.081
18-18.9	0.401	0.049	1.252	0.164	1.168	0.083
19-19.9	0.406	0.051	1.265	0.172	1.177	0.086
20-20.9	0.410	0.052	1.277	0.180	1.184	0.088
21-21.9	0.415	0.054	1.287	0.187	1.190	0.091
22-22.9	0.419	0.056	1.297	0.196	1.196	0.093

Table 2. Mean lean body mass values (LBMmean gram), bone mineral content of total body (BMC_{T6}mean, gram), and percentage body fat (%fat), and standard deviations (SD) in boys and girls.

Boys						
Age (years)	**LBMmean**	**SD**	**BMCmean**	**SD**	**ln(%fatmean)***	**ln(%fatsd)***
4-4.9	15885	2351	708	69	2.35	0.53
5-5.9	18092	2636	839	99	2.35	0.53
6-6.9	20467	2921	965	129	2.35	0.53
7-7.9	22985	3207	1084	159	2.35	0.53
8-8.9	25616	3492	1197	190	2.35	0.53
9-9.9	28324	3777	1310	220	2.35	0.53
10-10.9	31065	4062	1438	250	2.35	0.53
11-11.9	33802	4347	1599	280	2.35	0.53
12-12.9	36586	4633	1813	310	2.35	0.53
13-13.9	40687	4918	2087	340	2.35	0.53
14-14.9	49492	5203	2406	370	2.35	0.53
15-15.9	55472	5488	2725	400	2.35	0.53
16-16.9	57983	5773	2997	430	2.35	0.53
17-17.9	59943	6059	3200	460	2.35	0.53
18-18.9	61690	6344	3336	490	2.35	0.53
19-19.9	63249	6629	3419	521	2.35	0.53
20-20.9	64628	6914	3469	551	2.35	0.53
21-21.9	65836	7199	3498	581	2.35	0.53
22-22.9	66937	7499	3515	612	2.35	0.53
Girls						
Age (years)	**LBMmean**	**SD**	**BMCmean**	**SD**	**ln(%fatmean)***	**ln(%fatsd)***
4-4.9	15468	2317	714	112	3.22	0.24
5-5.9	17434	2499	832	134	3.24	0.24
6-6.9	19452	2682	939	156	3.26	0.24
7-7.9	21535	2865	1039	179	3.28	0.24
8-8.9	23762	3047	1147	201	3.29	0.24
9-9.9	26297	3230	1275	223	3.31	0.24
10-10.9	29323	3412	1438	246	3.33	0.24
11-11.9	32759	3595	1640	268	3.35	0.24
12-12.9	36070	3778	1871	290	3.37	0.24
13-13.9	38692	3960	2104	313	3.39	0.24
14-14.9	40512	4143	2313	335	3.41	0.24
15-15.9	41736	4325	2477	357	3.42	0.24
16-16.9	42588	4508	2595	379	3.44	0.24
17-17.9	43213	4690	2673	402	3.46	0.24
18-18.9	43692	4873	2723	424	3.48	0.24
19-19.9	44069	5056	2753	446	3.50	0.24
20-20.9	44370	5238	2772	469	3.52	0.24
21-21.9	44611	5421	2783	491	3.53	0.24
22-22.9	44815	5612	2790	514	3.55	0.24

* Because of a skewed distribution, a logarithmic transformation for percentage body fat (%fat) was performed. We showed natural logarithm (ln) of the data. Calculating standard deviation scores(SDS) for boys %fatsds = (ln(%fat)-ln(%fatmean))/ln(%fatsd), and for girls %fatsds = (ln(%fat+10)-ln(%fatmean))/ln(%fatsd). For the other parameters SDS=(measured value-mean)/SD.

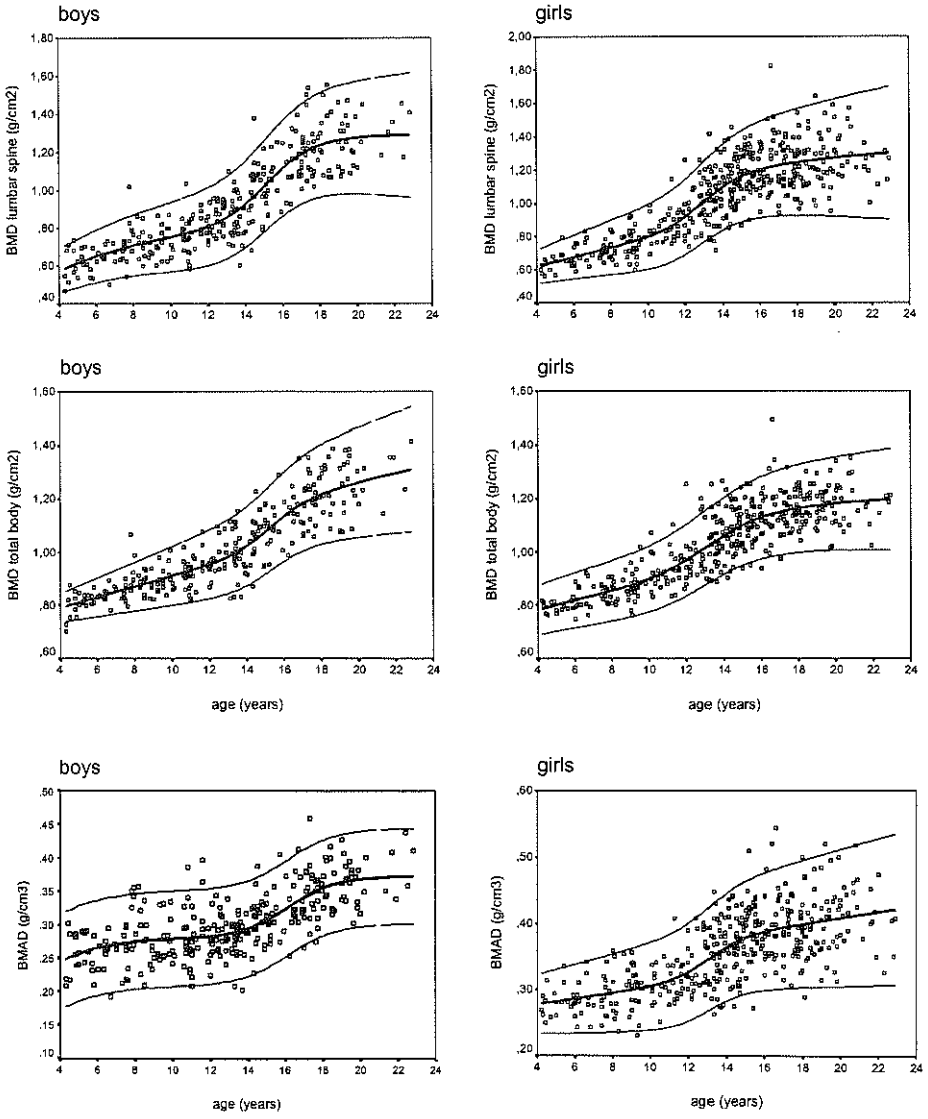

Figure 1. Bone mineral density (BMD) of lumbar spine and total body and bone mineral apparent density (BMAD) of lumbar spine plotted by age in boys and girls. The bold line represents the fitted line, the thin lines represent the ± 2 SD.

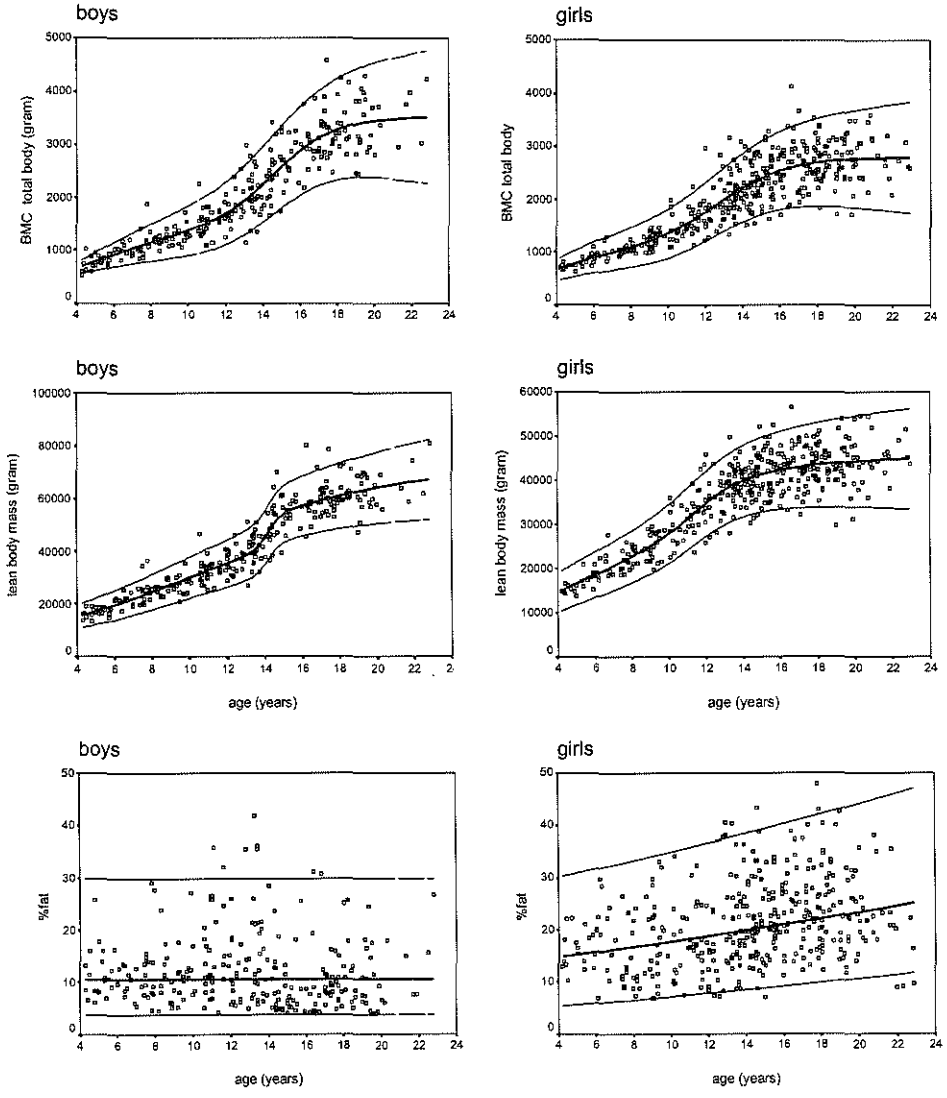

Figure 2. Bone mineral content (BMC), lean body mass (LBM) and percentage body fat (%fat) plotted by age in boys and girls. The bold line represents the fitted line, the thin lines represent the ± 2 SD.

Table 3. Bone mineral content (BMC), bone mineral density (BMD) and bone mineral apparent density (BMAD) per Tanner stage in Caucasian children

Tanner	1	2	3	4	5
Boys (n)	104	35	19	57	56
BMC_{TB}	1178 (341)	1785 (307)*	2099 (508)*	2823 (655)*	3224 (509)*
BMD_{TB}	0.88 (0.07)	0.96 (0.07)*	1.00 (0.09)	1.12 (0.12)*	1.21 (0.11)*
BMD_{LS}	0.71 (0.10)	0.83 (0.09)*	0.88 (0.14)	1.09 (0.17)*	1.21 (0.16)*
BMAD	0.27(0.04)	0.28 (0.03)	0.29 (0.04)	0.32 (0.04)*	0.35 (0.04)*
Girls (n)	84	23	35	91	134
BMC_{TB}	1067 (242)	1539 (296)*	1836(295)*	2369 (477)*	2724 (469)*
BMD_{TB}	0.85 (0.06)	0.94 (0.06)*	0.97 (0.07)	1.09 (0.09)*	1.17 (0.08)*
BMD_{LS}	0.72 (0.09)	0.84 (0.09)*	0.96 (0.14)*	1.14 (0.15)*	1.25 (0.16)*
BMAD	0.30 (0.03)	0.31 (0.02)*	0.33 (0.04)*	0.37 (0.04)*	0.40 (0.05)*

* significant increase compared to previous Tanner stage $p < 0.05$

LS lumbar spine, *TB* total body. Tanner stage was not scored in 4 children.

Percentage body fat remained 10.5% during childhood in boys and increased with age in girls. An age-dependent increase in bone density and lean body mass was found in boys and girls. Reference data for boys and girls are shown in Tables 1 & 2, and Figures 1 & 2
Increases in bone density per Tanner stage are presented in Table 3. In the logistic function parameter 'P3' represents the age at which the rate of change in the parameter due to puberty is at a maximum. These ages are shown in Table 4. Maximal increase in BMD and BMAD occurred around the age of 13 years in girls and approximately two years later in boys.

Table 4. Mean age (sem) at which the rate of change in the bone density or body composition due to puberty is at a maximum

	Boys	**Girls**
BMD_{LS}	15.1 (0.3)	13.0 (0.4) *
BMD_{TB}	15.3 (0.5)	13.1 (0.5)*
BMC_{TB}	14.5 (0.6)	12.6 (1.01)
$BMAD_{LS}$	16.2 (0.5)	13.1 (0.5)*
LBM	14.2 (0.2)	11.5 (0.7)*

* $p<0.001$ boys compared to girls

Discussion

The present study provides reference values for bone mineral density and body composition measured with DXA for Caucasian children and young adults. The mean and standard deviation are given for boys and girls from 4 to 23 years of age, with age categories of one year, which enables calculation of age- and sex-matched standard deviation scores. Besides correction for age and gender, results should be adjusted for height, pubertal stage, or bone age especially in children with delayed or advanced skeletal maturation or growth disorders.

BMD, measured by DXA, is an areal density that varies with bone size. Given a fixed volumetric density, large vertebrae have greater BMD values than small vertebrae[17]. On the other hand, BMD will be underestimated in children with short stature. In order to correct for bone size or height, mathematical models are used to calculate BMAD ('volumetric BMD'). A direct measurement of volumetric BMD is possible with quantitative computed tomography (QCT), but this technique involves high radiation exposure. However, our results showed no substantial increase in BMAD till puberty, similar to what has been reported using QCT[18]. Together with various clinical studies[19,20], this finding underscores the utility of BMAD as an appropriate correction for bone size or height.

With regard to our study population some characteristics should be noted. The 500 children who participated in the first study were recruited from three primary and two secondary schools in Rotterdam. Mean height SDS, BMD SDS and BMI SDS at baseline from the volunteers, who were willing to participate in the follow-up study did not differ from the means of the total group at baseline, indicating that we studied a random sample of the baseline population. Interestingly, we found the bone density values to be somewhat higher at follow-up than at baseline, when we expressed them as SDS using our old reference data. A similar increase was found in a longitudinal study to assess normative data for ultrasound measurements in another group of Caucasian children in Rotterdam[21]. We cannot really explain this finding, which might present a 'secular trend' found in longitudinal data. We found no evidence for a healthy-responder bias, since we studied a random sample and no increase in calcium intake or in physical activities were found between baseline and follow-up measurements.

The increase in BMD and BMAD with age is similar to what has been found in other studies[22-26]. The main increase occurs during puberty. During puberty, growth hormone as well as sex steroids levels increase and both are known to positively influence bone mineralisation[27,28]. Maximal increase in BMD and BMAD occurred around the age of 13 years in girls and approximately two years later in boys. Besides an increase of BMD with age, an increase in lumbar spine BMAD was found as well, suggesting that the increase in lumbar spine BMD does reflect a real increase in mineralisation, and is not merely due to accelerated growth.

Although all bone density parameters showed a clear flattening off after puberty, no accurate conclusion could be drawn using our statistical model whether peak bone mass has been reached. In this model G1 and P1 represent the asymptotic value of the parameter associated with growth and with puberty, respectively. Adding up G1 to P1 would provide the value of peak bone mass. However, an ongoing increase is found in some of the parameters, partly due to the few numbers of volunteers in the older age categories. Nevertheless, our results suggest that most of the skeletal mass in lumbar spine and total body is reached before the end of the second decade.

Most cross-sectional studies reported that peak bone mass is reached at late adolescence[24,29,30], while others found that lumbar spine BMD increased till the mid-30-s[31]. Longitudinal studies in girls show that PBM is reached around the age of 30[32,33]. Furthermore, Matkovic et al.[29] described bone mass acquisition at various skeletal sites in females. BMD in proximal femur and vertebral body reached their peak in late adolescence. Most of the skeletal mass in other sites is accumulated in late adolescence as well. However, a slight gain in bone mass of radius, total body and skull was found with a peak in the late forties. This suggest a slow but ongoing bone accumulation at some skeletal sites.

DXA provides precise body composition analysis with a low radiation dose[34]. DXA measurements performed in adults and children are able to detect small changes in body composition and were highly correlated with bioelectrical impedance analysis, skinfold-thickness measurements and underwater weighing[8,35,36]. In the present study, mean %fat remained stable (10.5%) in boys and increased in girls with age. %Fat showed a wide variance in both sexes. Lean body mass (LBM) or fat-free mass consists mainly of muscles. Especially in boys a steep increase during puberty was found, caused by increased growth hormone and androgen secretion.

DXA devices of the three major manufacturers, e.g. Lunar, Hologic and Norland, will not give identical results, because of differences in calibration and bone-edge detection algorithms. The correlation between the differently measured BMDs, however, is highly significant. Conversion formulas to calculate a standardised BMD have been developed for adults, but not for children. Moreover, even when another DXA machine of the same manufacturer is used, the results may vary slightly[37]. Therefore, to calculate reliable SD scores one has to use at least reference data gained with an identical DXA device. Of course, locally gained reference data are preferred.

Knowledge of normal physiological variation of bone density and body composition is needed to identify pathological changes. This study provides reference values for bone density and body composition measured by DXA for children and young adults. Age-and sex-adjusted Z-scores should be calculated using ethnic–specific data where possible. Most of the skeletal mass in lumbar spine and total body is accumulated before the end of the second decade, with a small ongoing increase thereafter.

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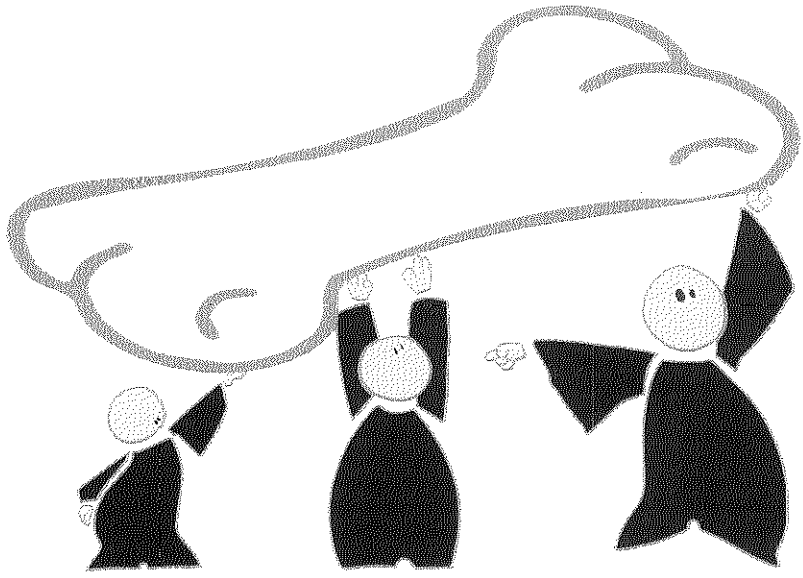
Part Two

BONE MINERAL DENSITY IN VARIOUS DISEASES



Chapter 6

BONE MINERAL DENSITY, BODY COMPOSITION, AND HEIGHT IN LONG-TERM SURVIVORS OF ACUTE LYMPHOBLASTIC LEUKEMIA IN CHILDHOOD

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ABSTRACT

Background: Childhood leukemia has increasing numbers of survivors, so more emphasis is being placed on long-term effects. The ALL-6 protocol of the Dutch Childhood Leukemia Study Group involved high-dose dexamethasone and methotrexate, and no cranial irradiation. Therefore, we studied the long-term effects on Bone Mineral Density (BMD), body composition, and growth in survivors of non-high-risk ALL treated with the ALL-6 protocol. *Procedure*: Twenty-three subjects (12.2-25.4 years) participated in this cross-sectional study. Mean follow-up was 9.6 years (range: 7.9-11.4 years). BMD of lumbar spine ($_{LS}$) and total body ($_{TB}$) and body composition were measured by dual energy X-ray absorptiometry; results are expressed as standard deviation scores (SDS). Bone Mineral Apparent Density ($BMAD_{LS}$) was calculated to correct for bone size. A questionnaire was administered to determine physical activity, calcium intake and medical history. *Results*: Mean SDS for BMD_{LS}, BMD_{TB}, and $BMAD_{LS}$ were normal. None of the subjects had BMD below –2 SDS, one subject had $BMAD_{LS}$ below –2 SDS. Mean SDS for lean body mass, percentage fat, and height were not significantly different from zero. Calcium intake correlated positively with BMD. Nine subjects reported traumatic fractures (eight during or shortly after therapy). *Conclusions:* Ten years after ALL-6 treatment no long-term side effects on height, BMD and body composition were found in this small group of patients, despite high dose dexamethasone and methotrexate. This study suggests that ALL treatment without cranial irradiation might not be associated with long-term side effects on growth and BMD.

INTRODUCTION

Because childhood leukemia has increasing numbers of survivors, more emphasis is being placed on the long-term side effects of this disease and its treatment. From 1984 until 1988, children with non-high-risk acute lymphoblastic leukemia (ALL) were treated according to the ALL-6 protocol of the Dutch Childhood Leukemia Study Group (DCLSG). This moderately intensive treatment protocol without cranial irradiation (CI) proved to be very successful. The event-free survival rate was 81 % and the survival rate was 85 % after 8 years follow-up[1]. Therefore, the protocol was reintroduced in the Netherlands in 1997 as the ALL-9 protocol.

Intensive chemotherapy may affect bone mineral density, height, and body composition. Under healthy conditions, bone mineral density increases in childhood and adolescence until peak bone mass is reached in the second or third decade[2]. A serious disease during this important period of growth and bone accumulation may predispose these children to osteoporosis and growth retardation. Putative causes of decreased bone mineral density in ALL are the leukemic process itself[3], ectopic production of parathyroid hormone[4], paracrine secretion of lymphokines[5,6] and decreased physical activity. Furthermore, the negative effects of the treatment may play a major role, like chemotherapy with steroids[7] and methotrexate (MTX)[8-10], and cranial irradiation[11-13].

Several studies in children with leukemia showed reduced BMD during therapy[6,14,15]. However, comparing patients with different treatment protocols and different risk-groups often makes the results difficult to interpret. Only a few studies described follow-up of long-term survivors with normal, as well as reduced BMD[13,16-18].

The aim of this study was to evaluate bone mineral density, body composition, and growth in a group of long-term survivors of childhood ALL, all treated according to the ALL-6 protocol.

SUBJECTS AND METHODS

Subjects

Twenty-three subjects (13 boys and 10 girls, mean age 17.2 year, range 12.2-25.4 years) participated in this cross-sectional study. All were diagnosed as non-high-risk acute lymphoblastic leukemia (pre B-cell or common ALL, 35 % and 65%, respectively) and were treated in the Sophia Children's Hospital with the ALL-6 protocol of the DCLSG. Mean age at diagnosis was 5.4 year (range 1.9-12.4 years). Non-high-risk ALL was defined as peripheral white blood cell count $< 50 \times 10^9$/l, absence of mediastinal mass, and/or cerebromeningeal leukemia at diagnosis. Systemic chemotherapy involved dexamethasone, MTX, mercaptopurine, asparaginase, and vincristine. None of them received cranial irradiation but received high-dose i.v. methotrexate and prolonged intrathecal triple therapy (MTX, prednisolone, and cytarabine) as CNS prophylaxis. Cumulative planned dose of dexamethasone was 1444 mg/m^2, and of MTX 8250 mg/m^2 (orally and i.v.). The mean received dose of dexamethasone was 1407 mg/m^2 and 8203 mg/m^2 MTX. Dexamethasone therapy during remission induction consisted of 6 mg/m^2 daily divided into three doses for 4 weeks, then tapered off to 0 mg in 10 days. After complete remission was achieved, three weekly courses of i.v. methotrexate (2000 mg/m^2) were administered. Maintenance treatment consisted of MTX 30 mg/m^2/week orally or i.v. for 5 weeks, alternated with dexamethasone in a dose as for induction treatment for 2 weeks. Treatment was completed in approximately 2 years[1]. The mean follow-up period after discontinuation of therapy was 9.6 years (range 7.9-11.4 years).

The study protocol was approved by the ethics committee of the University Hospital Rotterdam. The ALL-6 protocol was a multicentre protocol; 34 patients were treated in the Sophia Children's Hospital. Among them 29 patients are in first remission and 5 patients died. Of 29 eligible subjects, 23 (79%) enrolled in the study after we obtained written informed consent. Two boys were lost to follow-up approximately 8 years after diagnosis, they were in first remission at last follow-up. Four subjects declined to participate for personal reasons.

Anthropometry

Height was measured with a Harpenden stadiometer. Target height (TH) was calculated:
$TH\ (cm) = [(height_{father} + height_{mother} + 12)/2] + 3$ for males and $[(height_{father} + height_{mother} - 12)/2] + 3$ for females. Target height standard deviation scores and target height range (TH SDS ± 1.3 SD) were calculated according to Dutch references[19]. Weight was measured on a standard clinical balance. The body mass index (BMI) was calculated as weight/(height)^2. Height and BMI were compared to age and sex-matched reference values and expressed as SDS[19]. Results on height[20] and BMI[21] until four years after cessation of treatment have been reported previously. Tanner stage was scored and testes volume was measured with an orchidometer according to Prader by one investigator[22]. Pubertal development was compared to Dutch references[19]. Bone age was assessed by one investigator (IvdS) according to the Tanner-Whitehouse radius-ulna-short bones method. Normal range was defined as bone age within 1.3 SD below and above chronological age[23].

Bone mineral assessments

BMD of lumbar spine ($_{LS}$) and total body ($_{TB}$) was measured by dual energy X-ray absorptiometry (DXA; Lunar DPXL, Madison, WI, USA). The lumbar spine is composed mainly of

trabecular bone, whereas 80% of the total body bone consists of cortical bone[24]. BMD (g/cm^2) is an areal density, which varies with bone size. To correct for bone size we calculated apparent BMD of lumbar spine with the model $BMAD_{LS} = BMD_{LS} \times [4/(\pi \times width)]$. This model was validated by in vivo volumetric data obtained from magnetic resonance imaging of lumbar vertebrae[25]. The results were compared to healthy age- and sex-matched Dutch controls[26] and expressed as SDS. The coefficient of variation has been reported to be 1.04% for spine BMD and 0.64% for total body BMD[27].

DEXA of total body also provides estimates of body composition as lean tissue mass and percentage fat. These results are expressed as SDS as well, using our own Dutch reference values (n=403, age: 4-20.5 years)[28].

Questionnaires

During an interview, physical activity, dietary calcium-intake, medical history, previous fractures, use of oral contraceptives and other medication, and age at menarche were determined. Physical activity included physical education classes, organized sports, recreational activity and habitual walking and cycling and was measured in minutes per week[29]. Physical activity was also analyzed using peak strain scores[30]. Calcium intake was assessed by a detailed food frequency questionnaire of dairy products[31].

Statistics

One-sample T-tests were performed to compare mean SDS values with normal and independent-sample T-tests to compare males and females. Pearson's correlation coefficient was calculated to test the association between variables with a normal distribution and Spearman's correlation coefficient in case of a non-normal distribution. P values of <0.05 were considered statistically significant.

RESULTS

Height and puberty

Table 1 shows the clinical characteristics of the subjects. No significant differences in these characteristics between the sexes were found. The mean height SDS was 0.26 (SD 1.07), not significantly different from zero. Twenty (87 %) subjects had height SDS within target height range; two subjects even had height SDS above TH range. All subjects started therapy before the onset of puberty. Normal testicular volumes were found in all boys. At time of evaluation, nine subjects (four boys and five girls) were still in puberty, three subjects showed delayed puberty, and no early puberty was found. Menarche occurred at an early age in one girl, and occurred late in two girls. Three girls used oral contraceptives.

Table 1. Clinical characteristics [mean (SD)]

	Male	Female
Number	13	10
Age at diagnosis (years)	6.0 (3.5)	4.5 (2.6)
Age at study (years)	18.0 (3.8)	16.2 (1.3)
Follow-up (years)	9.7 (1.1)	9.5 (1.1)
Height SDS	0.47 (0.84)	-0.01 (1.30)
BMD_{LS} SDS	0.36 (1.25)	0.28 (0.75)
BMD_{TB} SDS	0.16 (1.17)	0.22 (0.78)
$BMAD_{LS}$ SDS	0.07 (1.30)	0.19 (0.71)
Target height SDS	-0.31 (1.05)	0.31 (0.88)
BMI SDS	0.61 (1.09)	0.45 (1.48)

SDS standard deviation score, *BM(A)D* bone mineral (apparent) density, *LS* lumbar spine, *TB* total body, *BMI* body mass index.

Bone mineral density and body composition

The individual SD scores for BMD of lumbar spine and total body, and $BMAD_{LS}$ are given in Figure 1. The mean BMD SDS for lumbar spine (mean 0.33, SD 1.07) and total body (mean 0.19, SD 1.00) and $BMAD_{LS}$ (mean 0.12, SD 1.04) were not significantly different from reference values. None of the subjects had BMD SDS of lumbar spine and total body below -2.0, and only one boy had $BMAD_{LS}$ below –2.0 SDS. No significant difference in BMD between males and females was found. No association between follow-up time or age at diagnosis and BMD of lumbar spine (Figure 3), total body or $BMAD_{LS}$ was found.

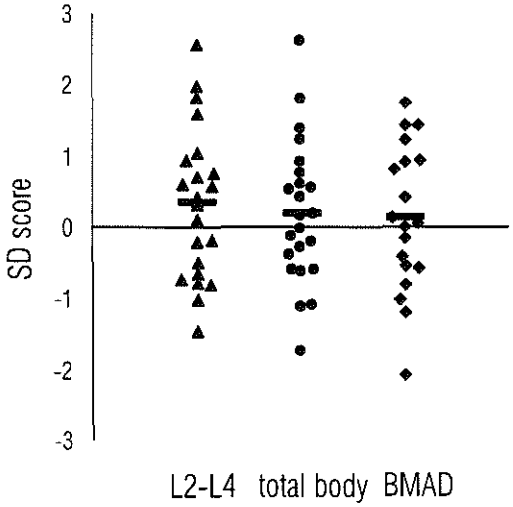

Figure 1. Bone mineral density of lumbar spine (L2-L4) and total body and bone mineral apparent density (BMAD) expressed as SD scores. Bars represent the means.

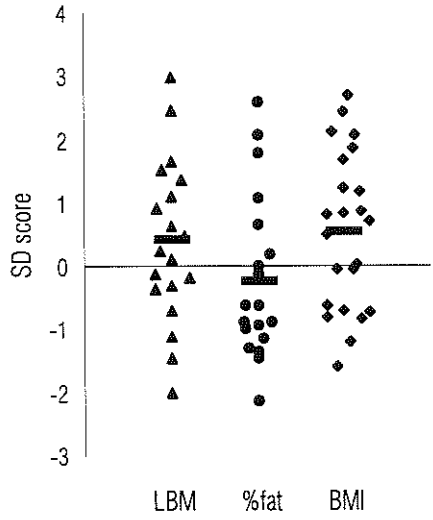

Figure 2. Lean Body Mass (LBM), percentage body fat (% fat) and body mass index (BMI) expressed as SD scores. Bars represent the means.

The individual results of the body composition measurements are shown in Figure 2. BMI SDS (mean 0.54, SD 1.24) was just significantly higher than zero ($p=0.049$). Four subjects had a BMI SDS above 2 SDS. In 19 subjects SD scores for body composition measured by DXA were calculated. Both lean body mass (LBM) and percentage fat did not differ significantly from zero (SDS: 0.38 (SD 1.29) and –0.22 (SD 1.30), respectively). Only two subjects had percentage fat SDS above 2 SDS. Eleven subjects reached final height. Mean bone age was not delayed in the twelve subjects left. Two patients showed a delay in bone age, whereas two patients showed advanced skeletal maturation. BMD corrected for bone age instead of chronological age did not change the results.

After discontinuation of ALL treatment none of the subjects suffered from other serious diseases or used drugs that could affect growth or bone mineralisation. During and after treatment nobody had clinical signs of avascular osteonecrosis.

Calcium intake and physical activity

Three subjects had calcium intake below the recommended intake (800-1200 mg/day)[32]. The mean calcium intake was 1295 mg/day (range 282-3028 mg/day; SD 585). Calcium intake showed a significant positive correlation with BMD of lumbar spine (Figure 3) and total body ($r=0.47$; $p=0.025$) and $BMAD_{LS}$ ($r=0.52$; $p=0.023$).

Physical activity was not significantly associated with BMD or $BMAD_{LS}$. The subjects spent 11.7 hr/week (SD 6.6 h/week) on physical activities. Our healthy controls spent 9.1 and 7.5 hr/week on physical activities for boys and girls, respectively[26]. Taking peak strain scores into account, no association with BMD was seen as well.

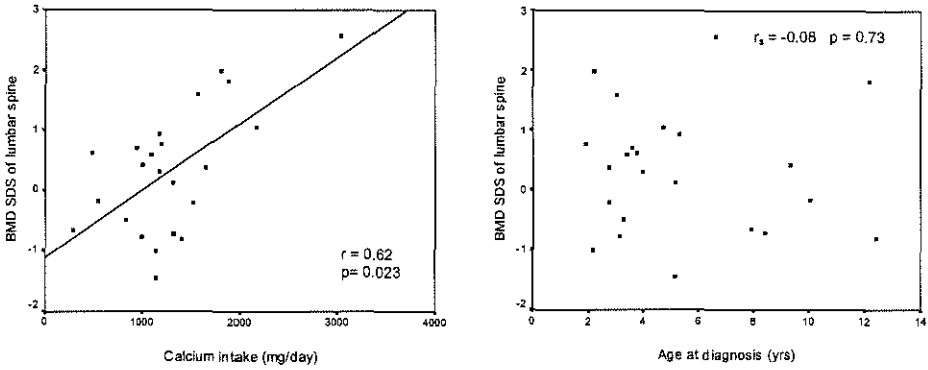

Figure 3. Pearson's correlation coefficient of calcium intake vs. BMD of lumbar spine and Spearman's correlation coefficient of age at diagnosis vs. BMD of lumbar spine.

Fractures

Two subjects had a history of two fractures and seven subjects suffered from one fracture. Eight fractures occurred during or shortly after treatment, all after trauma. Most of the fractures were located in the extremities. No vertebral compression fractures were reported. Previous fractures were not associated with lower BMD or $BMAD_{LS}$ at time of evaluation.

DISCUSSION

Approximately ten years after treatment, no long-term side effects on bone mineral density, height, body composition and bone maturation were found in survivors of non-high-risk ALL in childhood. The number of patients investigated in this study is small; however this is compensated by the fact that the study population is very homogenous regarding malignancy (all pre-B ALL), risk group, treatment schedule and follow-up time.

Concern has been expressed regarding detrimental effects of high dose dexamethasone and MTX on bone mineral density, fat mass and growth[21,33,34]. It is well known that children with ALL show high incidence of bone pain and fractures[3]. Previous studies have reported the leukemic process itself[3], high dose of corticosteroids[7], methotrexate[8,10], and cranial irradiation as important pathogenic factors[13,35]. Corticosteroids are considered the main cause of reduction in BMD by decreasing bone formation and increasing bone resorption[7]. An additional negative effect of MTX is expected, insofar as even low-dose MTX is able to cause significant osteopenia via suppression of osteoblast activity and stimulation of osteoclast recruitment[9].

Recently, a few studies have reported on bone mineral density in long-term survivors of childhood leukemia and other malignancies. Most studies covered heterogeneous groups of patients with regard to malignancy, treatment, and follow-up period. Arikoski et al.[17] showed reduced femoral and lumbar spine BMD after ALL treatment; a history of cranial irradiation appeared to be a risk factor for osteopenia. This risk factor has been reported by others, as well[16]. Henderson et al.[13] found no 'catch-up' in BMD one year after completion of therapy in various malignancies. The observation interval might have been too short in this study. Nysom et al.[36]

reported reduced BMD in irradiated and non-irradiated patients eleven years after diagnosis. The mechanism causing osteopenia after cranial irradiation is not totally elucidated, but growth hormone deficiency as a consequence of an injured hypothalamic-pituitary axis appears to be a major factor[37].

BMD measured by DXA is an areal density (g/cm^2), and short stature can underestimate BMD[38]. Correction for area removes some of the dependency on bone size. We used a validated model to calculate volumetric BMD[25]. Few studies perform this correction. The reduced BMD found after treatment in some studies might partly be explained by short stature. Furthermore, differences in intensity and duration of drug regimens, duration of the follow-up, and homogeneity of the study group regarding malignancy and treatment protocol may account for the reported variations in the severity and incidence of osteopenia in survivors of leukemia.

The many fractures (in 39% of the subjects) during and shortly after discontinuation of chemotherapy indicate reduced BMD during treatment. Preliminary results in patients treated with the current national DCLSG ALL-9 protocol also show this reduction in BMD (data not published). In our healthy control group, 21% of the participants reported previous fractures[26]. Nysom et al.[36] showed significant higher fracture risk only in the youngest children treated for ALL.

Mean calcium intake corresponds to the calcium intake in our reference population (1180 mg/day)[26]. Calcium intake positively correlated with BMD and BMAD. So, dietary recommendations for adequate calcium intake may be required in the follow-up of these patients to prevent osteopenia.

Previously, it has been suggested that activities with peak strain (high loads and few repetitions) have a greater positive effect on BMD than activities with a high number of repetitions and lower loads[30]. No correlation between total time spent on activities and BMD or peak strain scores and BMD was found in our subjects. Caution must be used in interpreting the significance of these correlations. They reflect recent exposure, whereas long-term exposure is expected to have a greater effect on bone density. Therefore, longitudinal data are necessary to study the effects of environmental factors like calcium intake and physical activity.

In our study, children had normal height ten years after discontinuation of chemotherapy. Growth retardation is frequently reported in survivors of childhood leukemia[11,20,39].

Cranial irradiation plays a major role in the pathogenesis. Chemotherapy alone caused growth retardation, but catch-up growth occurred after cessation of therapy[40]. In a long-term follow-up study, other endocrine deficiencies were rare[34]. Normal height and BMD in our patients treated with chemotherapy only suggests normal growth hormone secretion.

We found a tendency towards delayed puberty, but our group of pubertal patients was too small for proper analysis.

High incidence of obesity is reported in young adults after treatment of leukemia in childhood. Overweight was not likely to be due to cranial irradiation, growth hormone deficiency or abnormal timing of puberty, but chemotherapy (especially corticosteroids) may play a major role[21,41]. Another explanation of obesity is the relative physical inactivity of unknown cause in survivors of childhood malignancy[42]. Earlier published data about the same patients with ALL-6 treatment reported significantly increased BMI SDS during treatment (BMI SDS −0.37 at diagnosis; 1.34 at cessation of therapy). Although BMI decreased after cessation of therapy, it was not normalized within four years (BMI SDS 0.74)[21]. Despite high-dose dexamethasone during therapy, lean body mass and percentage body fat were normal in our group. BMI SDS was slightly higher than

normal. BMI correlates with fat mass and lean tissue mass measured with DXA, but cannot differentiate between these components. Therefore, body composition measurements with DXA are preferred over BMI for evaluation of fat mass. In addition, we found a trend towards increased physical activity compared with our healthy control group. In our subjects, treatment with high dose dexamethasone did not result in persisting adiposity, as was suggested earlier[21,41].

In conclusion, ten years after ALL-6 treatment with high-dose dexamethasone and MTX, no long-term side effects on bone mineral density, height and body composition were found. Previous studies on BMD during treatment and the many fractures during and shortly after discontinuation of chemotherapy suggested that leukemia itself and chemotherapy negatively affected BMD. Fortunately, this negative effect appears to be temporary. Future research should be focused on the development of preventive strategies to avoid bone loss during therapy, for example, with calcium and vitamin D supplementation.

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Chapter 7

ALTERED BONE MINERAL DENSITY AND BODY COMPOSITION, AND INCREASED FRACTURE RISK IN CHILDHOOD ACUTE LYMPHOBLASTIC LEUKEMIA

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ABSTRACT

Objective: We evaluated fracture rate and changes in bone mineral density (BMD) and body composition in children with acute lymphoblastic leukemia (ALL) treated with dexamethasone based chemotherapy. *Study design:* 61 Children with ALL participated. At diagnosis, during therapy, and one year after cessation of therapy, BMD and body composition were measured using dual energy X-ray absorptiometry of lumbar spine$_{(LS)}$ and total body$_{(TB)}$. Blood samples were taken to assess markers of bone turnover. *Results:* BMD_{LS} was significantly reduced at diagnosis, and remained low during therapy. Total body BMD was normal at diagnosis, with a fast decrease mainly in the first 32 weeks, in which chemotherapy was relatively intensive. Apparent BMD ('volumetric' BMD) was reduced as well, but this did not reach significance at diagnosis and during follow-up. Bone formation markers were reduced at diagnosis, and formation as well as resorption markers increased during treatment. Fracture rate was 6 times higher in ALL patients compared to healthy controls. Not the BMD SD-score itself, but a decrease in BMD_{LS} in the first six months was associated with higher fracture risk. Lean body mass was decreased at baseline. % Body fat increased significantly during therapy. After ALL treatment was completed, BMD and body composition tended to improve. *Conclusions:* Children with ALL are at risk for osteoporosis due to the disease itself and the intensive chemotherapy. Fracture rate increases substantially, not only during but also shortly after ALL treatment.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most common malignancy in childhood. Musculoskeletal disorders are well-known complications of ALL and its treatment. Children with ALL often develop bone pain, disturbances of gait, and fractures[1,2]. During the last decades, the survival rate after childhood ALL has improved substantially[3], consequently the short-term and long-term side-effects of the disease and its treatment gained importance.

Reduced bone turnover and bone mineral density has been reported at diagnosis and during treatment of ALL[4-6]. Also in long-term survivors of childhood ALL reduced bone density has been found[7]. The cause of reduced bone mineral density is most probably multifactorial. The disease itself as well as its treatment, such as corticosteroids, methotrexate, and radiotherapy, play a role. To date, central nervous system prophylaxis by cranial irradiation has been replaced by intrathecal chemotherapy in most treatment protocols for ALL.

We previously reported normal bone mineral density and body composition in children ten years after ALL treatment[8]. However, fracture rate was almost doubled in these children compared to their healthy peers. Most fractures occurred during or shortly after discontinuation of chemotherapy. The treatment protocol used in our long-term survivors' study was very similar to the currently used protocol, as both contain high dosages of dexamethasone and methotrexate (MTX), avoiding cranial irradiation.

The aim of this study was to evaluate fracture rate and changes in bone mineral density (BMD) and body composition in a homogeneous group of children with ALL, all treated with chemotherapy only. Furthermore, biochemical parameters of bone turnover were assessed in order to provide insight into the mechanisms involved.

PATIENTS

We studied 61 children (37 boys and 24 girls; median age 7.1 years; range 1.6-16.8 years) with ALL, who were referred to the Sophia Children's Hospital and treated according to the current national ALL protocol of the Dutch Childhood Leukemia Study group (DCLSG-ALL9). Patients with peripheral white blood cell counts over 50 x 10^9 /l, T-cell phenotype and/or mediastinal mass, extramedullary leukemia, patients with t(9;22), 11q23 with MLL gene rearrangements and poor responders to induction chemotherapy, were stratified to a high risk treatment schedule (n=18, median age 9.1 years), whereas the other patients received standard (non-high risk) treatment (n=43, median age 5.5 years), both for a total period of 109 weeks. Immunophenotyping revealed a T-ALL in 8 patients and a precursor B-ALL phenotype in 53 patients.

Central nervous system (CNS) prophylaxis was administered by recurrent intrathecal triple therapy, consisting of prednisolone, methotrexate (MTX) and cytarabine (Ara-C) combined with post-remission systemic MTX. No CNS irradiation was used. Total cumulative doses of the chemotherapeutic agents used in the HR and NHR protocol are shown in Table 1.

Table 1. Total cumulative doses of chemotherapeutic agents used in NHR and HR ALL protocol.

	t=½ year		t= 1 year		t=2 years (stop)	
	NHR	**HR**	**NHR**	**HR**	**NHR**	**HR**
DEXA (mg/m^2) p.o.	446	320	698	572	1370	1244
MTX (mg/m^2)#	6450	12000	6900	12450	8100	13650
VCR (mg/m^2) i.v.	24	18	36	30	68	62
L-ASP (U/m^2) i.v.	24000	33000	24000	33000	24000	33000
6-MP (mg/m^2) p.o.	5250	5250	10500	10500	24500	24500
ARA-C (mg/m^2) i.v.	-	1920	-	1920	-	1920
DNR (mg/m^2) i.v.	-	175	-	175	-	175
CP (mg/m^2) i.v.	-	1920	-	1920	-	1920

DEXA dexamethasone; *MTX* methotrexate; *VCR* vincristine; *L-ASP* L-asparaginase (Paronal®); *Ara-C* cytarabine; DNR daunomycine; *CP* cyclophosphamide. # Administration route in HR-protocol intravenously (i.v.) and in NHR orally (p.o.).

METHODS

All assessments were performed at diagnosis (t=0), after 32 weeks (t=½), after 1 year (t=1), two years (t=2, cessation of therapy) and three years (t=3). The time point of 32 weeks was chosen, because differences between the HR and NHR protocol are most obvious in the first 32 weeks of treatment. The study protocol was approved by the ethics committee of the University Hospital Rotterdam. Of 69 eligible subjects, 61 (88 %) enrolled in the study after written informed consent. Eight patients declined to participate. Eight children were excluded from further follow-up because they had a relapse or underwent bone marrow transplantation. One girl died in induction.

Anthropometry

Height was measured with a Harpenden stadiometer and weight was measured on a standard clinical balance. The body mass index (BMI) was calculated as weight/(height)^2. Height and BMI were compared to age and sex-matched reference values and expressed as standard deviation

scores (SDS)[9,10]. Pubertal development was scored according to Tanner[11]. Bone age was assessed by one investigator (IvdS) according to the Tanner-Whitehouse Radius-Ulna-Short bones method[12].

Bone mineral assessments

BMD of lumbar spine ($_{LS}$) and total body ($_{TB}$) was measured by dual energy X-ray absorptiometry (DXA, Lunar DPXL, Madison, WI, USA). Pediatric software was used for children with a weight below 30 kg. The lumbar spine is mainly composed of trabecular bone, whereas approximately 80% of the total body bone consists of cortical bone[13]. BMD (g/cm^2) is an areal density, which varies with bone size. To correct for bone size we calculated apparent BMD of lumbar spine with the model $BMAD_{LS} = BMD_{LS} \times [4/(\pi \times \text{width})]$. This model was validated by in vivo volumetric data obtained from magnetic resonance imaging of lumbar vertebrae[14]. The results were compared to our age- and sex-matched Dutch controls and expressed as SDS[15]. Body composition was measured by total body DXA, lean body mass (LBM) and percentage fat (%fat) were also expressed as SDS[16]. DXA was only performed in patients older than 4 years of age (n=45). However, one child was too young at baseline and in two children DXA was not performed at baseline because of logistic reasons. These three children participated in the follow-up measurements.

Biochemical parameters

Blood samples of all patients were obtained for the assessment of calcium, anorganic phosphate, 1,25-dihydroxyvitamin D, PTH, and insuline-like growth factor I (IGF-I). We measured alkaline phosphatase (ALP), procollagen type I C-terminal propeptide (PICP) as parameters of bone formation, and carboxy terminal telopeptide of type I collagen (ICTP) as a parameter of bone resorption. Intact PTH was measured by immunoradiometric assay (IRMA) (DiaSorin, Stillwater, MN, USA) and 1,25-dihydroxyvitamin D by RIA (Immuno Diagnostic Systems, Boldon, United Kingdom). PICP and ICTP were measured by radioimmunoassay (Orion Diagnostica, Espoo, Finland). IGF-I was measured by non-extraction IRMA (Med-Genix Diagnostics, Belgium) and expressed as age-and sex-adjusted SDS. ALP, phosphate, PICP, ICTP were expressed as sex- and age-matched SDS using our own reference values.

Questionnaires

Questionnaires were administered to determine dietary calcium intake, physical activity and number of fractures. Physical activity included physical education classes, organised sports, recreational activity and habitual walking and cycling and was measured in minutes per week. Calcium intake was assessed by a detailed food frequency questionnaire of dairy products[17] and compared to recommended dietary allowances[18]. Fracture incidence rate was calculated and compared with the incidence rate in our healthy population[16].

Statistical analysis

Statistical analyses were carried out with the SPSS version 10.0 for Windows. One sample T-tests were performed to compare mean SDS values with normal and independent sample T-tests to compare two groups. Within patient changes were tested using a paired sample T-test, for parameters with a skewed distribution Wilcoxon paired sample tests were used and median with

range are given (PTH). Mann-Whitney tests were used to compare two independent samples with a skewed distribution. Pearson's correlation coefficient was calculated to test the association between variables with a normal distribution and Spearman's correlation coefficient in case of a non-normal distribution. To detect differences in fracture rates between ALL patients and healthy children, the incidence rate ratio was tested using Poisson statistics. P-values of less than 0.05 were considered statistically significant.

RESULTS

Anthropometry & body composition

The results of height and body composition are shown in Figure 1. Mean height SDS was -0.19 (SD 1.14, p=0.20) at diagnosis. During treatment height SDS decreased significantly, mainly in the first 32 weeks of treatment. One year after cessation of treatment, there was a significant increase in height SDS (p=0.001; t=3 compared to t=2). BMI SDS was normal at diagnosis (mean –0.31; SD=1.42; p=0.09) and increased significantly. After finishing treatment, there was a non-significant decrease in BMI SDS (p=0.09; t=3 compared to t=2).

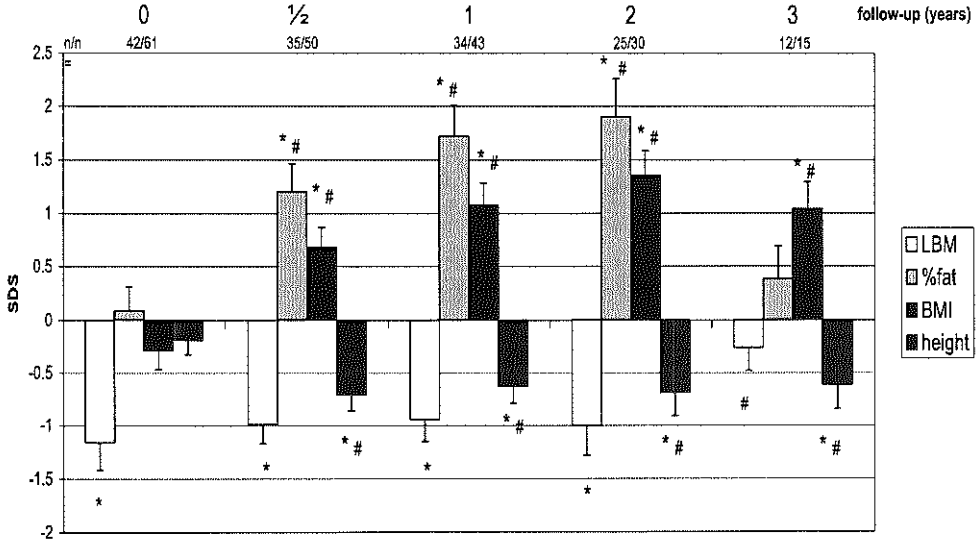

Figure 1. Body composition and height at diagnosis of ALL, during therapy, and after cessation of therapy. [Mean; error bars represent sem].

* Comparison of the mean with zero p< 0.05; # compared to t=0 p<0.05. *LBM* lean body mass; *% fat* percentage body fat; *BMI* body mass index; *SDS* standard deviation score; *n/n* number of LBM and % fat measurements / number of height and BMI measurements.

Lean body mass SDS was significantly reduced at diagnosis and remained low during treatment. Percentage fat was normal at baseline and showed a fast increase in the first 32 weeks.

One year after cessation of therapy, % fat declined ($p<0.001$; t=3 compared to t=2), whereas LBM increased to normal values ($p<0.05$; t=3 compared to t=2).

No differences in height and body composition between the HR and NHR patients were found.

Bone density

Results are shown in Figure 2. At baseline, lumbar spine BMD (mean –0.60; SD=1.55; $p=0.01$) was significantly lower than zero. $BMAD_{LS}$ (mean -0.28; SD=1.51; $p=0.23$) and total body BMD (mean 0.32; SD=1.58; $p=0.20$) did not differ from zero. At baseline, lumbar spine BMD was below –1.5 SDS in 29% of the children. $BMAD_{LS}$ and total body BMD were below –1.5 SDS in 21% and 10% of the patients, respectively.

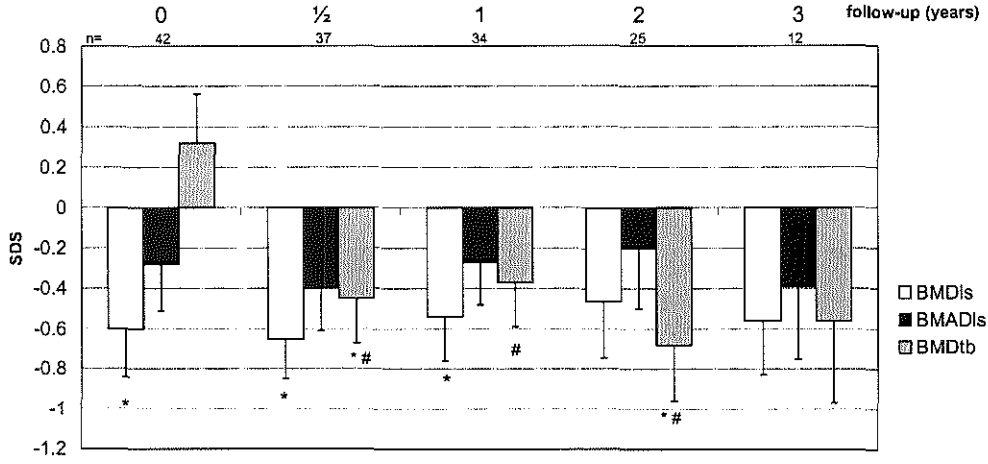

Figure 2. Bone mineral density (BMD) of lumbar spine (LS) and total body (TB) and bone mineral apparent density (BMAD) at diagnosis of ALL, during therapy, and after cessation of therapy. [Mean; error bars represent sem * Comparison of the mean with zero $p< 0.05$; # compared to t=0 $p<0.05$.

During chemotherapy lumbar spine BMD remained below zero at t=½ and t=1. BMAD showed no significant changes during the three years of follow-up. Only BMD of total body showed a significant decrease in the first 32 weeks of treatment, and remained low thereafter. Eighty-four % of the patients showed a decrease in total body BMD SDS between t=0 and t=½, and 50 % showed a decrease in lumbar spine BMD SDS.

After cessation of therapy BMD of lumbar spine and total body showed a tendency to increase, however this did not reach significance. Figure 3 shows the results of BMD_{TB} for 25 patients who had follow-up measurements for at least two years.

When we corrected BMD for bone age instead of chronological age similar patterns were seen. The change in height SDS in the first 32 weeks did not correlate with changes in BMD or BMAD.

At diagnosis, HR patients had significant higher mean lumbar spine BMD SDS compared to the NHR patients ($p=0.04$). This difference disappeared at t=½. No differences in BMD and BMAD

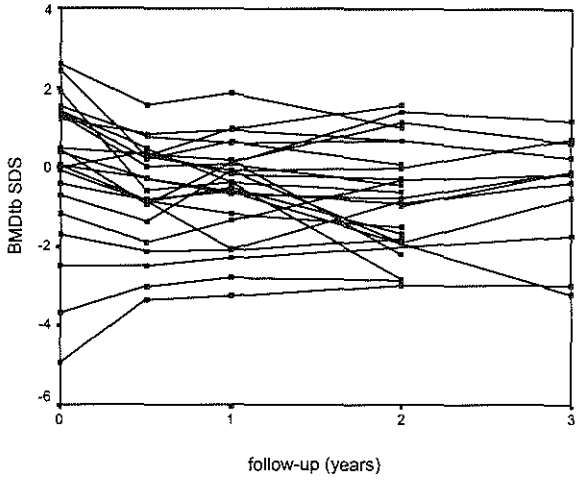

Figure 3. Bone mineral density of total body in ALL patients followed for ≥ two years of therapy.

SDS between boys and girls were found. At diagnosis, age showed a significant positive association with lumbar spine BMD and BMAD SDS ($r=0.38$ and $r=0.41$ respectively; both $p<0.01$). However, in the first 32 weeks of treatment these correlations disappeared. Using multiple regression, with bone density as dependent variable, and age and risk group as independent variables, only age remained a significant factor.

Biochemical markers of bone turnover

Serum markers of bone turnover are presented in Table 2. At diagnosis, serum calcium was below the normal range in 30% of the patients. Mean calcium level increased significantly during treatment. Phosphate SDS was normal at diagnosis and decreased significantly in the first 32 weeks of treatment. The markers of bone formation, ALP and PICP, were significantly lower than zero at diagnosis, and increased significantly during follow-up. ICTP, a marker of bone resorption, was normal at diagnosis and increased during treatment.

Median PTH levels were within the normal range, remaining constant during follow-up. IGF-I SDS was significantly lower than zero at diagnosis, increased during treatment, but remained significantly lower than zero. Mean 1,25-dihydroxyvitamin D levels were within the normal range at baseline and during follow-up, and increased significantly in the first 32 weeks of treatment.

Because the largest changes occurred in the first months of treatment, we calculated the change (Δ) in biochemical and bone density parameters between $t=0$ and $t=½$. The percentage change in PTH was not correlated with changes in bone density. Only, Δ1,25-dihydroxyvitamin D was positively correlated with ΔBMD_{LS} SDS ($r=0.47$; $p=0.02$), $\Delta BMAD_{LS}$ SDS ($r=0.44$; $p=0.03$), and ΔALP SDS ($r=0.54$; $p=0.02$). No correlation between ΔIGF-I SDS and Δ1,25-dihydroxyvitamin D was found. None of the other biochemical parameters were associated with changes in bone density. Δ IGF-I SDS showed no correlation with Δheight, ΔBMD, ΔBMAD or ΔLBM SDS.

Table 2. Serum parameters at diagnosis of ALL, during therapy (Rx), and after cessation of therapy.

Years after start of Rx	**0**	**½**	**1**	**2**	**3**
n	59	49	41	27	15
Calcium (mmol/l)	2.25 (0.20)	2.36 (0.10)#	2.35 (0.13)#	2.34 (0.11)#	2.40 (0.09)#
Phosphate SDS	0.09 (1.14)	-0.35 (1.28)#	-0.42 (1.15)	-0.20 (0.89)	-0.10 (0.63)
ALP SDS	-2.37 (2.38)*	-1.23 (1.22)*	-0.89 (1.48)* #	0.14 (1.57)	0.76 (0.62)*
PICP SDS	-0.83 (0.99)*	1.07 (1.96)* #	0.44 (1.57)#	0.68 (1.21)* #	-0.48 (0.84)#
ICTP SDS	0.21 (2.00)	1.22 (2.39)* #	1.09 (1.35)* #	1.34 (1.24)*	1.09 (1.51)*
1,25 OHvit D_3 (pmol/l)	105.2 (53.7)	127.4(52.0)#	125.3 (42.1)	134.4 (42.6)	159.4 (39.5)
PTH (ng/l)§	19.2 (5-129)	23.3 (6-58)	23.9 (9-80)	21.6 (10-66)	20.3 (6-60)
IGF-I SDS	-2.05 (1.33)*	-0.90 (1.27)* #	-0.86(1.34)* #	-1.44(1.35)*	-0.82 (0.86)*#

* for the comparison of the mean SDS with zero $p<0.05$; # compared to $t=0$ $p<0.05$; § median (range) for PTH, mean (SD) are given for other parameters.

SDS standard deviation score; *ALP* alkaline phosphatase; *PICP* procollagen type I C-terminal propeptide; *ICTP* carboxy terminal telopeptide of type I collagen; *1,25 OHvit D_3* 1,25-dihydroxyvitamin D_3; *IGF-I* insulin-like growth factor I

Calcium intake, physical activities and fractures

At diagnosis, 72 % of the children had sufficient or high calcium intake compared to recommended dietary allowance. This percentage did not change during follow-up. At diagnosis, calcium intake showed a significant positive correlation with BMD of lumbar spine and total body ($r_s=0.34$; $p=0.03$ and $r_s=0.37$; $p=0.02$). No significant associations between calcium intake and bone density were found in the follow-up period. Physical activity was not correlated with lumbar spine or total body BMD SDS or $BMAD_{LS}$.

To date, in total 11 fractures occurred during follow-up. Three patients suffered from one fracture and 2 had two fractures during treatment, 4 fractures occurred in the first year after cessation of treatment, all after trauma. Most fractures were located in the extremities. One boy had a compression fracture of the thoracic vertebrae. The fracture incidence rate ratio was 6.1 (95% confidence interval: [3.0-11.3]; $p<0.001$). Children with fractures did not differ in BMD or BMAD from children without fracture. However, the change in the first 32 weeks in lumbar spine BMD SDS differed in the fracture and non-fracture group ($p=0.04$). Children with fractures showed a decrease in mean lumbar spine BMD SDS, while mean lumbar spine BMD SDS increased in the non-fracture group. No differences in Δheight, ΔBMI, ΔLBM, Δ%fat and change in other parameters of bone turnover were found between the fracture and non-fracture group.

DISCUSSION

We found that children with ALL had reduced BMD of lumbar spine already at diagnosis, remaining low during treatment, suggesting that the disease itself caused osteopenia. Total body BMD was normal at diagnosis, with a fast decrease mainly in the first 32 weeks, in which chemotherapy had the most intensive courses, including high dose dexamethasone and MTX.

The different results for lumbar spine and total body may be explained by differences in bone composition. Lumbar spine mainly consists of trabecular bone, whereas total body consists for

80% of cortical bone[13]. Trabecular bone has a higher metabolic rate, consequently changes in BMD will occur earlier in lumbar spine than in total body BMD[19]. BMD measured by DXA partly depends on bone size and will be underestimated in short stature. To correct for this dependency on bone size we calculated BMAD. Indeed, mean BMAD was reduced in a lesser degree, than lumbar spine BMD. However, Δheight SDS and ΔBMD showed no correlation, so the decrease in BMD is probably not caused by growth retardation.

ALL patients stratified to a high risk protocol had higher lumbar spine BMD SDS compared to the NHR patients, at diagnosis. This difference is mainly explained by the fact that on average the NHR patients were somewhat younger than HR patients, and the younger patients appeared to have lower BMD SDS. The difference in BMD disappeared after 32 weeks of treatment. This may be due to more intensive chemotherapy in the HR group. The difference between HR and NHR protocol mainly consists of almost doubled MTX dose in the HR group, while dexamethasone dose is even somewhat lower than in NHR patients. MTX[20,21] and corticosteroids[22] are both known to impair bone mineralisation. It is not known whether other chemotherapeutic agents might potentiate the side effects of dexamethasone and MTX on bone.

Other putative causes of impaired bone mineralisation are both leukemic infiltration and expansion of the bone marrow that might destruct the spongiosa and various factors secreted by leukemic cells, such as ectopic production of PTH or PTH-related peptide[23], and paracrine secretion of lymphokines[2,24]. Furthermore, decreased physical activity, insufficient calcium intake and lack of sunlight exposure might play an additional role. Approximately 30% of the patients had calcium intake below the recommended allowances. Moreover, the use of glucocorticoids is associated with decreased intestinal absorption of calcium and increased renal calcium excretion[22]. Therefore, higher calcium intake or supplementation might be needed to improve the calcium balance. Future research is necessary to answer the question whether osteoporosis and fractures can be prevented by increasing calcium intake.

Our patients had significant lower lean body mass than healthy controls at baseline, while their fat percentage did not differ. This may reflect the catabolic effect of a malignancy. During therapy, LBM remained low and %fat increased, while both tended to improve after cessation of therapy. Dexamethasone is probably the main cause of increasing fat mass during ALL-treatment[25,26]. The load of muscles on bone is an important stimulator of bone formation[27]. Therefore, a decreased LBM, which consists mainly of muscle mass, will negatively affect bone mineral density.

In accordance with other studies[1,28], we found reduced parameters of bone formation at diagnosis, but normal serum levels of the bone resorption markers. Crofton and colleagues[29] also reported decreased bone formation markers, while ICTP was reduced in a lesser degree. Thus, at diagnosis, bone formation appears to be more impaired than bone resorption. The low bone turnover state at diagnosis is caused by the disease itself and resulted in decreased BMD of lumbar spine. In children, markers of bone turnover reflect both bone modeling (skeletal growth) and bone remodeling. During chemotherapy, bone resorption as well as bone formation markers increased, mainly in the first months. Because height decreased in this period, it must reflect higher bone remodeling rate, resulting in a decrease in BMD.

Fracture risk was 6 times higher in children with ALL compared to our healthy controls. BMD and BMAD did not differ between children with or without fractures. However, the decrease in BMD of lumbar spine was significantly higher than the change in BMD in the non-fracture group, suggesting that the change in BMD plays a role rather than the absolute value of the SD-score.

Additionally, vincristine induced neuropathy may cause clumsiness and less balance skills[30] during ALL therapy, and therefore attributes to a higher fracture risk. However, there are no reports of higher fracture risk in patients treated with high dose vincristine in the absence of dexamethasone and MTX, like for instance nephroblastoma patients. Changes in bone elasticity or micro-architecture, which can not be assessed by DXA, might also play a role.

The effects on bone density varied widely among patients. This suggests that genetic variability might play a role. Polymorphisms of the glucocorticoid receptor may cause differences in glucocorticoid sensitivity, resulting in differences in BMD and BMI[31]. Although we found an association between fractures and a decrease in bone density, we did not find any evidence that children with fractures also had higher susceptibility for other glucocorticoid related side-effects as growth retardation, adiposity, or decreased muscle mass. Other genetic factors associated with bone density and/or fracture risk like polymorphisms of the vitamin D receptor gene and collagen Iα1 gene may also contribute[32,33].

In concordance with our findings, many studies reported a decline in height SDS in children with ALL during intensive chemotherapy, without a significant impairment of height SDS at diagnosis[25,34]. As reported earlier by Crofton et al.[4], we found reduced IGF-I levels at diagnosis with a subsequent increase during therapy. Crofton also reported increased growth hormone (GH) levels at diagnosis, suggesting that ALL itself caused GH resistance. The GH/IGF-I axis plays an important role in longitudinal bone growth, bone density as well as body composition[35]. The low IGF-I serum levels at diagnosis and during therapy might have negatively affected lean body mass and bone mineral density.

In conclusion, children with ALL are prone to develop osteoporosis due to the disease itself and the intensive chemotherapy. After ALL treatment is completed, BMD tends to improve. However, it should be stressed that fracture rate was 6 times higher in ALL children compared to healthy controls, not only during but also shortly after treatment. Interestingly, not the BMD SD-score itself but the change in BMD in the first six months was associated with fracture risk. Future research should be focussed on identifying patients at risk for osteoporosis and on prevention of the very high fracture rate during and shortly after cessation of treatment.

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Chapter 8

BONE DENSITY AND BODY COMPOSITION IN CHRONIC RENAL FAILURE: EFFECTS OF GROWTH HORMONE TREATMENT

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ABSTRACT

Metabolic bone disease and growth retardation are common complications of chronic renal failure (CRF). We evaluated bone mineral density (BMD), bone metabolism, body composition and growth in children with CRF, and the effect of growth hormone treatment (GHRx) on these variables. Thirty-three prepubertal patients with CRF were enrolled including 18 children with growth retardation, who were treated with growth hormone for two years. Every six months, BMD of lumbar spine and total body, and body composition were measured by dual energy X-ray absorptiometry. Biochemical parameters of bone turnover were assessed. Mean BMD of children with CRF did not differ from normal. During GHRx, BMD and bone mineral apparent density of lumbar spine and height SDS increased, whereas BMD of total body did not change. Lean body mass increased in the GH-group. Alkaline phosphatase increased significantly in the GH-group only. The other biochemical parameters of bone turnover increased in both groups, none of them correlated with the changes in BMD. No serious adverse effects of GHRx were reported. In conclusion, BMD of children with CRF did not differ from healthy children. Adequate treatment with alpha-calcidiol or the short duration of renal failure may have attributed to the absence of osteopenia in our patients. BMD of the axial skeleton and growth improved with GHRx.

INTRODUCTION

In patients with chronic renal failure (CRF), metabolic bone disease is a common complication. The term "renal osteodystrophy" is reserved for diseases affecting the control of bone modelling with high or low bone turnover. Secondary hyperparathyroidism plays a central role in the pathogenesis of high-turnover disease, whereas adynamic bone disease and osteomalacia are examples of low-turnover disease[1]. Osteopenia is frequently reported in adult CRF patients[2,3] and after renal transplantation[4,5]. However, reports on bone mineral density and bone metabolism in children with CRF are rather sparse[6,7].

Many patients need a renal transplantation in later life. After renal transplantation, patients are prone to develop osteoporosis due to immunosuppressive treatment with prednisone[8]. Hence, optimising bone acquisition during childhood is very important.

For some years, children with CRF and growth retardation have successfully been treated with growth hormone (GH)[9,10]. In addition to its effects on linear growth, GH also affects bone metabolism, as found in growth hormone deficient patients during GH replacement therapy[11,12]. Growth hormone has a direct effect on bone mass via increasing bone formation and to a lesser extent, bone resorption. Moreover it has an indirect effect on bone via increasing muscle mass[13,14].

The objective of this study was to evaluate BMD, bone metabolism, body composition, and growth in children with CRF. Furthermore the effect of growth hormone treatment on these variables was evaluated. First year results were described previously[15]. We now report the final results after two years of treatment.

PATIENTS AND METHODS

Patients

Thirty-three prepubertal patients with chronic renal failure (25 boys and 8 girls) participated in

the study. The mean age at start was 8.1 years (range 3.4 to 12.6 years). Inclusion criteria were: (1) inulin clearance below 60 ml/min per 1.73 m^2; (2) no clinical evidence of puberty (Tanner stage I); (3) normal thyroid function; (4) no endocrine or metabolic disease (except renal); (5) no treatment with corticosteroids within the preceding six months; and (6) no oxalosis or cystinosis.

Eighteen children had growth retardation, they were treated with growth hormone (GH-group). The other 15 children met all the inclusion criteria, but were not growth retarded and did not receive GH (no-GH group). One growth-retarded boy refused GH treatment and was included in the no-GH group. Growth retardation was defined as either height standard deviation score (SDS) for chronological age less than –1.88[16] and growth velocity below the 50th percentile[17] or height SDS below zero and growth velocity below the 25th percentile.

Both groups had an observation period of 6 months, which was described previously[15]. Thereafter the GH group started with a daily subcutaneous injection of 4 IU/m^2 body surface area recombinant human GH (Norditropin®, Novo Nordisk A/S) over 2 years. Seven children dropped out of the study because of renal transplantation (6 in the GH group). Data from these patients were included in the analyses up to the transplantation. At baseline, three children in the GH group and two in the no-GH group were on dialysis. In the GH group, two children started dialysis during treatment and one in the observation period. All but one patient were treated with 1α-hydroxyvitamin D3 (alpha-calcidiol); none of them received prednisone.

Table 1. Clinical characteristics at baseline (mean and range) and underlying cause of CRF in the growth hormone (GH) group and the no-growth hormone group (no-GH group)

	GH group (n=18)	no-GH group (n=15)
Age	8.2 (3.8-11.7)	8.0 (3.4-12.6)
Bone age	6.3 (2.9-10.3)	7.0 (2.1-13.5)
Height SDS	-2.28 (-3.37 to -1.50)	-098 (-2.08-1.44)
Glomerular filtration rate (ml/min)	25 (6-52)	28 (9-49)
Regimen (number of patients)		
Conservative	15	13
Dialysis	3	2
Underlying disease		
Congenital renal dysplasia	4	3
Reflux nephropathy	4	2
Obstructive nephropathy	3	4
Prune belly syndrome	1	1
Neurogenic bladder	1	0
Polycystic kidney disease	0	2
Chronic glomerulonephritis	1	1
Interstitial nephritis	0	1
Glomerulosclerosis	2	0
Haemolytic uraemic syndrome	1	0
Kidney damage after shock	1	1

The median dose was 0.50 μg/day in the GH group and 0.25 μg/day in the no-GH group at baseline. The dose was adjusted according to levels of PTH, renal function and serum calcium. The median dose did not change in either group during follow-up.
Baseline characteristics, including underlying causes of CRF, are shown in Table 1. With the exception of height SDS, the treated group did not differ from the untreated group.

Anthropometry

Patients were examined every three months. Height was measured with a Harpenden stadiometer and expressed as SDS[16]. Pubertal stage was assessed according to Tanner[17].
Bone age was scored by one investigator using an X-ray of the left hand according to the Tanner-Whitehouse radius-ulna-short bones (RUS) method[18]. Normal range was defined as bone age within 1.3 SD below and above chronological age[19].

Bone mass measurements

BMD (gram/cm^2) of lumbar spine and total body was measured by Dual Energy X-ray Absorptiometry (DXA, Lunar, DPXL/PED, WI, USA). The lumbar spine is mainly composed of trabecular bone, whereas 80% of the total body bone consists of cortical bone[20]. To correct for bone size we calculated apparent BMD of lumbar spine (gram/cm^3) with the model $BMAD_{LS} = BMD_{LS} \times [4/(\pi \times width)]$. This model has been validated by Kröger et al. by in vivo volumetric data obtained from magnetic resonance imaging of lumbar vertebrae[21]. All children were measured by the same apparatus. Quality assurance was performed daily. Body composition was measured by total body DXA. BMD, bone mineral content (BMC), lean tissue mass, and percentage body fat were compared with our own Dutch age- and sex-matched reference values (n=500 , 4-20 years old)[22,23]. The coefficient of variation has been reported to be 1.04% for spine BMD and 0.64% for total body BMD[24].

Laboratory data

Every six months blood samples were obtained for the assessment of calcium, anorganic phosphate, alkaline phosphatase, 25-hydroxyvitamin D, 1,25-dihydroxyvitamin D, PTH, osteocalcin, procollagen type I C-terminal propeptide (PICP) and carboxy terminal telopeptide of type I collagen (ICTP). Osteocalcin and PICP are markers of bone formation and ICTP is a marker of bone resorption. Osteocalcin, 25-hydroxyvitamin D and intact PTH were measured by radioimmunoassay (RIA) (Incstar Corporation, Stillwater, MN,USA) and 1,25-dihydroxyvitamin D by RIA of Immuno Diagnostic System (Boldon, United Kingdom). Radioimmunoassay kits (Orion Diagnostica, Espoo, Finland) were used for measurement of PICP and ICTP. IGF-I and IGFBP-3 were assessed by RIA kits from Med-Genix Diagnostick, Fleurus, Belgium and Diagnostic System Laboratories,(Webster, Texas, USA). IGF-I was expressed as sex- and age-matched SDS[25]. Our own reference values were used for osteocalcin, PICP and ICTP[26]. TSH and free thyroxine (FT4) were assessed in the GH group only. The Amerlite TSH-30 kit (Ortho- Clinical Diagnostics Ltd., Cardiff, UK) and Amerlite MAB FT4 kit were used.

Fasting blood samples were obtained every six months for the assessment of triglycerides (TG), high-density lipoprotein (HDL), low-density lipoprotein (LDL), total cholesterol (TC), apolipoprotein A1 (Apo-A1) and apolipoprotein B (Apo-B). Atherogenic index was calculated as the ratio of TC to HDL. TC, TG, Apo-A1 and Apo-B were measured on a Dupont de Nemours "Dimension" analyser with reagents as provided by the manufacturer[27]. HDL is analysed after apo-

B containing lipoprotein particles has been precipitated from the serum by heparin-$MnCl_2$ solution[28]. LDL is calculated using the Friedewald formula LDL = TC - (HDL + VLDL) where VLDL = TG x 0.45 mmol/l[29]. Dutch age-matched reference values were used for TC and HDL[30] and Finnish reference values for the atherogenic index[31]. For the other lipids our own reference values of 59 healthy children between 2-10 years and available reference data[32] were used.
Acid balance was checked every 3 months to prevent chronic acidosis. In children on conservative treatment glomerular filtration rate was measured yearly by single injection inulin clearance[33].

The study was approved by the medical ethics committee of each participating centre. Written informed consent was obtained from the parents.

STATISTICS

One sample t-tests were performed to compare the mean SDS values to normal. Repeated measurements analysis was used to evaluate changes along time of various variables. With this method each individual has for outcome a linear relationship versus time, with the intercept and slope differing from patient to patient[34]. For PTH, this analysis was done after logarithmic transformation in order to obtain a normal distribution. Wilcoxon signed rank tests were used to test the within patients changes for variables with a non-normal distribution (lipids, GFR, free T4). Pearson's correlation coefficient was calculated to test an association between variables with normal distribution and in the case of a non-normal distribution Spearman's correlation coefficient was used. P-values less than or equal to 0.05 were considered statistically significant.

RESULTS

Height, puberty and bone age

At baseline, mean height SDS was significantly lower than zero in both groups (Table 2). Height SDS of the GH group was significantly lower than in the no-GH group. Height SDS remained stable during the observation period, and increased significantly during GH treatment (mean increase in SDS (ΔSDS) 0.67 /yr, $p<0.001$). Height SDS did not change in the no-GH group (ΔSDS -0.07 /yr, not significant (NS)).

One boy entered puberty during GHRx; in the no-GH group 3 children entered puberty.
At baseline, 55% of the children had delayed bone maturation. No difference in bone age between the GH group and no-GH group was found at baseline. The ratio Δ bone age to Δchronological age in two years follow-up was 1.1 and 1.0 for the GH group and no-GH group, respectively; neither was significantly different from 1.0.

Bone mineral density

Table 2 shows the results of bone mineral density and body composition. At baseline, both groups have normal BMD of lumbar spine($_{LS}$) and total body($_{TB}$). No difference in bone density between children with conservative treatment and children on dialysis was found. During the observation period and in the first year of GH treatment no difference in change in BMD between dialysis and conservative treatment was found. BMD_{LS} increased significantly during GHRx (ΔSDS 0.72 /yr, $p< 0.01$ vs ΔSDS -0.01 /yr, NS in the no-GH group). During follow-up, BMD_{TB} values

Table 2. Mean height, bone mineral density (BMD) and body composition expressed as SD scores (SD), and glomerular filtration rate (GFR in ml/min per 1.73 m^2) in the GH treated and no-GH group

Months	t=0		t=6		t=12		t=18		t=24		t=0-24@
Group (n)	GH (18)	no-GH (15)	GH (18)	no-GH (15)	GH (15)	no-GH (14)	GH (14)	no-GH (14)	GH (12)	no-GH (14)	GH vs no-GH
Height	-2.29 (0.51)*	-0.99 (0.93)*	-1.88 (0.58)*	-1.04 (0.95)*	-1.36 (0.59)*	-0.95 (1.00)*	-0.72 (0.91)*	-1.04 (1.00)*	-0.89 (0.67)*#	-1.05 (1.03)*	P<0.05
BMD_{LS}	0.27 (1.38)	0.40 (1.29)	0.00 (1.28)	0.44 (1.21)	0.72 (1.67)	0.32 (1.34)	1.26 (1.57)*	0.41 (1.19)	1.51 (1.94)*#	0.30 (0.99)	P<0.01
$BMAD_{LS}$	1.18 (1.63)*	0.87 (1.54)	0.92 (1.39)*	1.15 (1.51)*	1.29 (1.84)*	1.11 (1.51)*	1.78 (1.89)*	1.16 (1.30)*	2.14 (2.51)*#	1.11 (1.37)*	NS
BMD_{TB}	0.09 (0.99)	-0.07 (1.85)	-0.05 (0.99)	-0.07 (1.63)	-0.25 (1.19)	-0.06 (1.51)	-0.14 (1.23)	-0.16 (1.68)	0.35 (1.44)	-0.48 (1.02)	NS
BMC	-1.45 (0.83)*	-0.90 (1.20)*	-1.31 (0.83)*	-0.90 (1.20)*	-1.09 (0.85)*	-0.92 (1.08)*	-0.88 (0.80)*	-0.96 (1.07)*	-0.49 (0.89)#	-0.96 (0.89)*	P<0.05
LBM	-1.96 (0.86)*	-1.17 (0.62)*	-1.51 (1.04)*	-1.23 (0.72)*	-1.00 (1.07)*	-1.23 (0.62)*	-1.03 (1.36)*	-1.16 (0.70)*	-0.86 (1.29)*#	-1.24 (0.78)*	P<0.001
% fat	0.00 (1.07)	-0.07 (1.18)	-0.86 (0.93)*	-0.47 (1.15)	-1.04 (0.75)*	-0.47 (1.15)	-1.08 (0.92)*	-0.21 (1.21)	-0.96 (0.90)*#	-0.53 (1.06)#	P=0.05
GFR	25 (19)	28 (14)			38 (32)	34 (14)			41 (34)	27 (14)	NS

* significantly different from zero ($p \leq 0.05$), # significant within patient change during follow-up ($p \leq 0.05$),

@ Comparison between groups of average slope of outcome versus time. *NS* not significant.

LS lumbar spine; *TB* total body; *BMAD* bone mineral apparent density; *BMC* Bone Mineral Content; *LBM* lean body mass; *% fat* percentage body fat

Table 3. Biochemical markers of bone metabolism in the GH and no-GH groups, means (SD). For PTH the median (range) is given

	Reference values	t=0		t=12		t=24		t=0-24
		GH	no-GH	GH	no-GH	GH	no-GH	
Alk Phosph (U/l)	80-225	151.0 (79.8)	191.6 (79.6)	317.9 (201.2)	207.6 (94.8)	353.5 (249.6)*	226.8 (105.1)	##
Calcium (mmol/l)	2.20-2.60	2.50 (0.15)	2.51 (0.21)	2.45 (0.16)	2.39 (0.14)	2.42 (0.12)	2.38 (0.20)*	
Phosphate (mmol/l)	1.0-1.8	1.63 (0.65)	1.52 (0.32)	1.61 (0.30)	1.60 (0.41)	1.69 (0.44)	1.64 (0.51)	
1,25 vit D (pmol/l)	40-140	82.9 (49.1)	98.0 (47.0)	107.9 (59.0)	91.0 (54.6)	91.4 (34.8)	79.5 (33.2)*	
Osteocalcin (μg/l)	4-20	28 (14.7)	28.5 (9.5)	48.4 (24.3)	32.3 (10.7)	59.2 (53.1)*	39.2 (24.7)*	##
PICP (μg/l)	77-626	457.5 (128.8)	419.9 (173.9)	515.0 (163.5)	437.4 (139.5)	600.3 (244.6)*	575.4 (269.5)*	
ICTP (μg/l)	6-19	56.9 (43.1)	50.2 (52.3)	105.0 (82.4)	48.6 (37.8)	99.0 (82.7)*	55.4 (36.4)*	##
PTH (ng/l)	10-55	40.8 (13.2-361.3)	51.2 (8.4-382.5)	77.9 (15.2-1733.8)	63.1 (17.8-256.6)	50.0 (14.1-1671.3)*	52.8 (14.9-792.8)	

* significant change during follow-up ($p \leq 0.05$), ## significant difference in average slope of outcome versus time between the GH and no-GH group ($p \leq 0.05$)

Alk Phosph alkaline phosphatase; *1,25 vit D* 1,25 dihydroxyvitamin D; *PICP* procollagen type I C-terminal propeptide; *ICTP* carboxy terminal telopeptide of type I collagen

were within normal range and remained stable in both groups. $BMAD_{LS}$ SDS was significantly higher than zero at start and increased significantly in the GH group only (ΔSDS 0.53 /yr, $p<0.05$ vs ΔSDS 0.12 /yr in the no-GH group, NS).

The increase in BMD SDS of lumbar spine was significantly higher in the GH group compared to the no-GH group ($p<0.01$). No significant differences in the change in BMD of total body and $BMAD_{LS}$ between the two groups were found.

Body composition

Data are shown in Table 2. At baseline, mean lean body mass (LBM) SDS was significantly lower compared to healthy children in both groups. Percentage fat was normal. During growth hormone treatment, LBM increased (ΔSDS 0.45 /yr, $p<0.001$) and bone mineral content increased as well (ΔSDS 0.67 /yr, $p< 0.05$), whereas percentage fat decreased in both groups (GH group: ΔSDS -0.40 /yr, $p<0.001$, no-GH group: ΔSDS -0.19 /yr, $p<0.05$).The changes in LBM SDS, BMC SDS and percentage fat SDS were significantly higher in the GH group compared to the no-GH group.

Laboratory data

Table 3 shows the results of the laboratory assessments. The mean values of serum calcium and anorganic phosphate remained within normal range. Alkaline phosphatase increased significantly in the GH group only. Osteocalcin, PICP and ICTP increased significantly in both groups.

The increase in osteocalcin and ICTP was significantly higher in the GH group compared to the no-GH group. 1,25-Dihydroxyvitamin D remained stable during GH treatment, but decreased significantly in the no-GH group, though it remained within normal ranges. No significant difference in 1,25-dihydroxyvitamin D between the two groups was found. 25-Hydroxy vitamin D remained stable in both groups. IGF-I SDS increased significantly in the GH group only. IGFBP-3 increased significantly in both groups, with a significantly higher increase in the GH group. Magnesium remained stable. PTH increased significantly in the GH group, and remained stable in the no-GH group.

Table 4. Lipid profile during growth hormone treatment (Means (SD))

months	Reference values	t=0 n=18	t=6 n=18	t=12 n=15	t=18 n=14	t=24 n=12
Cholesterol	3.2-6.0 mmol/l	5.42 (1.73)	5.46 (1.42)	4.96 (1.42)	4.69 (1.07)	4.73 (1.07)
HDL	0.9-1.6 mmol/l	1.37 (0.40)	1.28 (0.38)	1.31 (0.29)	1.28 (0.45)	1.44 (0.58)
LDL	1.3-3.7 mmol/l	3.23 (1.44)	3.03 (1.10)	2.78 (1.37)	2.63 (1.12)	2.51 (0.66)
Triglycerides	0.2-1.7 mmol/l	1.82 (0.89)	1.93 (1.18)	1.93 (1.24)	1.90 (1.30)	1.35 (0.74)
ApoA1	0.8-1.5 g/l	1.63 (0.35)	1.61 (0.52)	1.53 (0.25)	1.54 (0.29)	1.54 (0.50)
ApoB	0.6-1.1 g/l	1.08 (0.37)	1.00 (0.36)	1.00 (0.32)	0.97 (0.21)	0.90 (0.15)
Atherogenic index	< 4.5	4.11 (1.23)	4.55 (1.58)	3.92 (1.19)	3.89 (1.12)	3.52 (0.94)

The mean within patient change from baseline was tested with Wilcoxon signed rank tests; no significant changes were found. *HDL* high-density lipoprotein cholesterol, *LDL* low-density lipoprotein cholesterol, *Apo* apolipoprotein, *Atherogenic index* total cholesterol to HDL ratio.

TSH and free T4 were only assessed in the GH group. Both were within the normal range at baseline. During GH-treatment, free T4 did not change, while TSH decreased significantly.

Table 4 shows the results of lipid profiles at baseline and during GHRx. At baseline, the mean levels of triglycerides and ApoA1 were increased, the other parameters of lipid metabolism were within normal range. During GHRx lipid metabolism did not change significantly. The atherogenic index was and remained normal.

GFR (Table 2) did not change significantly during the observation period and during GH treatment. The change of GFR was not significantly different between the two groups.

Correlations

The percentage change in PTH did not associate with change in BMD SDS or BMAD SDS. The increase in LBM significantly correlated with the increase in BMC in the GH and no-GH group. At baseline, HSDS showed no correlation with BMD_{LS} or BMD_{TB}. No significant correlation was found between the increase in HSDS and the increase in BMD_{LS} or BMD_{TB}. In addition, no correlations were found between ΔHSDS, ΔBMD and the change in parameters of bone turnover, IGF-I and IGFBP-3. BMD and BMAD showed no correlation with the duration of the renal insufficiency.

DISCUSSION

In the present study mean bone mineral density of children with chronic renal failure did not differ from normal. During two years growth hormone treatment the density of the axial skeleton increased, whereas bone density of total body did not change. Height SDS increased only significantly in the GH-treated group. Lean body mass and bone mineral content improved during treatment, while percentage fat decreased.

Unfortunately, we were not able to randomise for GH treatment since GH is registered as a treatment for growth retarded children with CRF. For that reason, we could only compare the GH group with a group of children with chronic renal failure without growth retardation. However, when we analysed all outcomes correcting for the difference in baseline height SDS, all differences found in changes of various parameters remained the same. Therefore, we think that the differences found between the GH-treated and non-treated group are real.

Surprisingly, we found normal baseline values for BMD of lumbar spine and total body, and even increased BMAD of lumbar spine in children with CRF. During two years growth hormone treatment BMD_{LS} and $BMAD_{LS}$ increased, whereas bone density of total body remained stable. Bone turnover in trabecular bone, particularly present in lumbar spine, is higher than in cortical bone, representing 80% of the total skeleton[35]. This may explain the earlier increase in BMD of lumbar spine. Follow-up might have been too short to establish an increase in BMD of total body. Since BMAD of lumbar spine increased, we can conclude that the increase in BMD of lumbar spine is a true increase in bone mass and cannot be explained by an increase in height only.

In the no-GH group the bone density did not change. Bianchi et al.[2] and Gabay et al.[3] however, reported decreased BMD in adult CRF patients on conservative treatment or dialysis. The proximal tubule is the primary site of calcitriol synthesis by the enzyme 1α-hydroxylase. In CRF a significant reduction in calcitriol synthesis occurs. Administration of calcitriol proved to be an effective treatment of secondary hyperparathyroidism[36]. This adequate treatment might have prevented osteopenia in our patients. Furthermore, the shorter duration of CRF in our patients may

explain the discrepancy between our results and the reduced BMD found in adult patients. Nevertheless, we could not find any tendency to reduced bone density in those of our patients with a longer duration of the disease. This might be explained by the relatively short duration of the disease in children compared to adult patients.

The GH group had a higher alpha-calcidiol dose than the no-GH group at baseline and during follow-up. During the six months observation period, in which both groups did not receive GHRx, BMD and BMAD SDS remained stable[15]. Therefore, we may conclude that GHRx and not the higher alpha-calcidiol dose was responsible for the increment of BMD and BMAD.

Recently, a few studies on BMD and the effects of GH, in a similar dose to ours, in children with CRF have been published. Lanes et al.[6] found reduced BMD of cortical and trabecular bone at baseline, while both increased with GH treatment for 1 year. Adjusted for bone age, no difference in BMD was found. All thirteen patients had severe growth retardation. Short stature will underestimate bone density[37] and therefore a correction for bone size should be used. Cochat et al.[38] reported that GH treatment did not affect BMD. Several factors may have contributed to the discrepancy between Cochat's and our results. Firstly, most patients received kidney transplant and were treated with immunosuppressive drugs. Secondly, only 1 year of treatment was reported. Thirdly, a rather small group of 9 patients was studied.

In children with CRF, the results of non-invasive measurements such as DXA are difficult to interpret because of the diversity of skeletal changes. Moreover, DXA is not able to differentiate between osteomalacia and osteoporosis. Reduced cortical thickness, osteitis fibrosa, and osteomalacia combined with the multifactorial influences of uremia could appear as mixed lesions. In uraemia, an increase in woven osteoid is found in which calcium is deposited as amorphous calcium rather than as hydroxyapatite. Bone density measurements could detect normal or even increased mineralisation, without detecting the structural abnormalities[39]. Obviously, only histology can offer information about bone quality and changes during interventions. In future research, one should consider taking bone biopsies. Another alternative could be quantitative computed tomography, the findings of which correlate highly with the results of biopsies[40]. Unfortunately, these methods are costly, invasive or use high radiation doses and thus are less appropriate for use during childhood.

The changes in body composition during GHRx are in agreement with known lipolytic and anabolic effects of GH as reported in adults and children with GHD during GH replacement therapy[11,41]. Feber et al.[42] also found an increase in lean body mass and a decrease in fat mass in children with CRF during 2 years of GH treatment. The increase in lean body mass will have a positive effect on BMD.

A significant improvement in growth rate of children with CRF treated with GH has been well established in various studies[9,43-45]. We used GH therapy with a dose of 4 IU/m^2/day since it induces a better and more prolonged growth response compared to a dose of 2 IU, without evidence of adverse effects[46].

In the present study significant changes in bone markers were found, especially in the GH treated patients, but also in the no-GH group. Osteocalcin and ICTP are small peptides, filtered through the glomerular membrane. In patients with CRF there will be serum retention of both peptides. Due to its large molecular weight, PICP is not filtered by the glomeruli, but is cleared by the liver[47].

In addition to the difficulties caused by the renal clearance of biochemical bone parameters, interpretation of the markers is affected by growth. The parameters cannot differentiate between bone modelling or remodelling. Accelerated growth will increase the bone markers as well. Furthermore, bone markers are influenced by peritoneal dialysis[48]. We therefore conclude that markers of bone turnover are difficult to interpret in children with CRF. This is underscored by the fact that we did not find any correlation between bone markers and the increase in height or BMD.

No serious adverse effects were found during GH treatment. GFR did not change significantly in either group. This finding is in concordance with other studies[9,10,49] which demonstrated no deleterious effect of GH treatment on GFR. Similar to effects during GH replacement therapy in GH deficient children[11], no adverse effects on serum lipids were found. Moreover, thyroid function remained stable.

In the first year of GH treatment, the increase of PTH will subsequently potentially increase renal osteodystrophy (ROD). The increase in ROD might be explained by increased growth rate and bone turnover. Despite alpha-calcidiol treatment, it seems that the bones need time for adaptation to this state of higher bone turnover. Increasing the dose of alpha-calcidol in the first year of treatment might prevent deterioration of secondary hyperparathyroidism and ROD. A review article on GHRx in children with CRF reported no differences in radiographic osteodystrophy scores or PTH levels between the treated and untreated groups[50]. Longitudinal follow-up is required to study the long-term effect of GH on metabolic bone disease.

In conclusion, we have demonstrated that BMD of children with CRF did not differ from healthy children. The absence of osteopenia in our paediatric patients may be related to the short duration of CRF compared to adults. Furthermore, improved treatment with alpha-calcidiol may play a role. Height and BMD of trabecular bone increased significantly during two years of growth hormone treatment. Long-term follow-up is indicated to study whether GH treatment can improve peak bone mass and subsequently decrease the risk for osteoporotic fractures later in life. Since DXA is not able to detect bone quality, bone biopsies or quantitative computed tomography might be more informative in CRF patients. Biochemical parameters of bone turnover were increased in both GH-treated and -untreated patients and did not correlate with the increase in bone mass, indicating that parameters of bone turnover are not very useful in monitoring bone metabolism in children with CRF.

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Chapter 9

LONG-TERM EFFECTS OF GROWTH HORMONE THERAPY ON BONE MINERAL DENSITY, BODY COMPOSITION, AND SERUM LIPID LEVELS IN GROWTH HORMONE DEFICIENT CHILDREN: 6 YEARS FOLLOW-UP

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ABSTRACT

Objective: Growth hormone is essential for normal growth in children, but it also affects bone mineralisation and body composition. We evaluated bone mineral density (BMD), body composition, and serum lipid levels in growth hormone deficient (GHD) children during 6 years of GH treatment (Rx). Patients: 59 GHD children (0.4-16.9 years). *Measurements:* Lumbar spine, total body BMD, and body composition were measured using dual energy x-ray absorptiometry at diagnosis, after six months and yearly thereafter. Volumetric BMD of lumbar spine (bone mineral apparent density, BMAD) was calculated. BMD and body composition were expressed as standard deviation scores using our own normative data. *Results:* We found a significant reduced mean BMD of lumbar spine($_{LS}$) and total body($_{TB}$) at diagnosis. $BMAD_{LS}$ was reduced as well, but in a much lesser degree. During GHRx significant increases in BMD and $BMAD_{LS}$ were found. $BMAD_{LS}$ was normalised after one year of treatment and lumbar spine and total body BMD one year later. At diagnosis, lean body mass (LBM) was reduced and steadily increased during GHRx. %Fat was increased at baseline and normalised within the first six months. Only alkaline phosphatase (ALP) and osteocalcin were decreased at diagnosis. Parameters of bone turnover showed a significant increase mainly in the first six months of GHRx. The change (Δ) in ALP was the only parameter of bone turnover, which correlated with ΔBMD and ΔBMAD. Δ1,25-dihydroxyvitamin D was significantly correlated with $\Delta BMAD_{LS}$ and ΔBMD_{LS}. Bone mineral content highly correlated with LBM. Mean lipid levels were normal at diagnosis and improved during GHRx. *Conclusions:* areal BMD of lumbar spine and total body, and to a lesser extent BMAD, are decreased in GHD children, but normalise within one and two years. The increase in LBM as well as the increase in 1,25-dihydroxyvitamin D might mediate the positive influence of GH on bone mineralisation. Furthermore, GHRx has beneficial effects on body composition and serum lipid levels in GHD children.

INTRODUCTION

Growth hormone (GH) affects longitudinal growth, bone density and body composition. It is well known that adults and children with untreated growth hormone deficiency (GHD) have reduced bone density, increased fat mass and decreased lean body mass[1-3]. The cause of reduced bone mineral density (BMD) in GHD is not fully understood and probably multifactorial. Multiple pituitary deficiencies may play a minor role, because several reports show no difference in BMD between isolated GHD and GHD with multiple deficiencies[4,5]. In addition, the fact that GH replacement therapy can improve bone density[6,7], suggests an important role of GH in determining bone mass.

Reduced bone density is of clinical importance because it is associated with higher fracture risk in adults[8,9] and children[10]. Recently, it has been reported that the prevalence of fractures among a large cohort of adult GHD patients was 2.7 times higher than in the control population[11]. To date, no data regarding fracture risk in GHD children are available, but improving bone mass in childhood onset GHD may help to reduce future problems. Therefore, we evaluated long-term effects of GH replacement therapy in growth hormone deficient children on bone mineral density. We also studied the effects on body composition and lipid metabolism. Two years results of a smaller group of patients have been reported previously[2].

PATIENTS AND METHODS

Patients

Fifty-nine GHD patients (34 boys and 25 girls) participated in the study. The mean age at start was 8.3 years (range 0.4 to 16.9 years). Questionnaires were administered once during GHRx to determine calcium intake.

Twenty-seven children were categorised as classic GHD (17 boys and 10 girls), defined as GH provocation test peaks below 10 IU/l (<5μg/l) and insulin-like growth factor (IGF)-I and IGF-binding protein 3 (IGFBP-3) below the mean of age- and sex-matched controls. Thirty-two patients (17 boys and 15 girls) had non-classic GHD, of whom 24 had partial GHD, defined as GH provocation test peaks between 10 and 20 IU/l and IGF-I and IGFBP-3 below the mean or GH peaks between 20-30 IU/l and IGF-I and IGFBP-3 below -2 SDS.

Eight children formed a special group, three of them had decreased GH peak (<20 IU/l) in the provocation test combined with normal IGF-I or IGFBP-3, two children had normal GH peak with IGF-I or IGFBP-3 below -2.0 SDS, 1 girl with intra-uterine growth retardation without catch-up growth had normal GH peak and IGF-I of –1 SDS and IGFBP-3 of –1.5 SDS and very low height (-4.5 SDS), 2 patients had a GH peak between 20-30 IU/l and IGF-I and IGFBP-3 between 0 to -1.5 SDS.

Forty out of 59 had GHD of known origin: malformation in the central nervous system (13), 12 intracerebral tumor (craniopharyngioma 8, germinoma 1,astrocytoma 2, medulloblastoma 1), syndrome with GHD (Noonan 2, Prader-Willi 1, Robinow 1), 7 children were born small for gestational age. Three children had GHD concomitant with β-thallassaemia, gonadal dysgenesis, or auto-immune polyendocrinopathy. One boy received radiation therapy for a rhabdomyosarcoma of his left ear. Sixteen children (9 boys, 7 girls) had multiple pituitary deficiencies for which they received replacement therapy, 11 of them had acquired GHD of known origin. Patients were treated with a daily GH dosage of 2 IU/m^2 body surface area (~0.02 mg/kg body weight /day) and 5 children were treated with 3 IU/m^2/day. Lipid metabolism was measured in all children and in 15 other GHD children.

Methods

Anthropometry, assessment of biochemical parameters of bone turnover, and BMD and body composition measurements were performed at baseline, after six months, 1 year, and yearly thereafter. Height was assessed using a Harpenden stadiometer. Weight was measured on a standard clinical balance. Body mass index (BMI) was calculated as weight divided by square height (kg/m^2). Height and BMI were compared to Dutch reference values and expressed as standard deviation scores (SDS)[12,13]. Pubertal stage was assessed according to Tanner[14].

Bone age was scored by two trained investigators using an X-ray of the left hand according to the Tanner-Whitehouse Radius-Ulna-Short bones (RUS) method[15].

Bone mineral density of lumbar spine (LS) and total body (TB) was measured by dual energy X-ray absorptiometry (DXA, Lunar, DPXL/PED, WI, USA). The lumbar spine is mainly composed of trabecular bone, whereas 80% of the total body bone consists of cortical bone[16]. To correct for bone size we calculated apparent BMD of lumbar spine with the model $BMAD_{LS} = BMD_{LS} \times [4/(\pi \times \text{width})]$. This model has been validated by Kröger et al. by in vivo volumetric data obtained from magnetic resonance imaging of lumbar vertebrae[17]. All children were measured with the same apparatus. Quality assurance was performed daily. Body composition was measured by total body

DXA. BMD, bone mineral content (BMC), lean tissue mass, and percentage body fat were compared with our own Dutch age- and sex-matched reference values (n=500, 4-20 years old)[18,19]. The coefficient of variation (CV) has been reported to be 1.04% for spine BMD and 0.64% for total body BMD[20]. The CV for the paediatric weight range have been reported 1.0% for LBM, 4.1 % for fat mass, and1.8% for BMC[21] and for the adult weight 1.05% for LBM, 2.2% for fat mass and 0.64 % for BMC[20].

The minimal age for BMD measurements was 4 years, because it is too difficult to lie still for younger children. In 2 out of 4 children under four years DXA was performed after they turned four. At baseline 55 children underwent DXA measurements, after 3 years 52, after 4 years 34, after 5 years 23 and after 6 years 15 children were measured.

Blood samples were obtained in all patients for the assessment of calcium, anorganic phosphate, alkaline phophatase (ALP), 1,25-dihydroxyvitamin D, PTH, osteocalcin, procollagen type I C-terminal propeptide (PICP) and carboxy terminal telopeptide of type I collagen (ICTP). PTH was measured in plasma and sampled on ice. The other parameters were assessed in serum. Intact osteocalcin and intact PTH were measured by immunoradiometric assays (IRMA) (Diasorin, Stillwater, MN, USA) and 1,25-dihydroxyvitamin D by radioimmunoassay (RIA) of Immuno Diagnostic Systems (Boldon, United Kingdom). Radioimmunoassay kits (Orion Diagnostica, Espoo, Finland) were used for measurement of PICP and ICTP. IGF-I and IGFBP-3 were measured by non-extraction IRMA and IRMA respectively (Med-Genix Diagnostics, Fleurus, Belgium and Diagnostic System Laboratories, Webster, Texas, USA). IGF-I was expressed as sex- and age matched SDS[22]. Our own reference values were used for osteocalcin, PICP, ICTP, and 1,25-dihydroxyvitamin D.

In the first morning void of urine the hydroxyproline concentration expressed as mg/g creatinine (OHP/Cr) and the calcium/creatinine ratio were measured. Fasting blood samples were obtained every year for the measurement of triglycerides (TG), high-density lipoprotein (HDL), total cholesterol (TC), apolipoprotein A1 (Apo-A1) and apolipoprotein B (Apo-B). LDL was calculated using the Friedewald formula LDL = TC - (HDL cholesterol + 0.45 x TG)[23]. Atherogenic index was calculated as the ratio of TC to HDL. Cholesterol and triglycerides were determined enzymatically with reagents from Roche Diagnostics (Germany). The HDL-choesterol assay was carried out with the indirect heparine MnCl2 precipitation method[24]. TG, Apo-A1 and Apo-B were measured on Dupont de Nemours "Dimension" analyser with reagents as provided by the manufacturer[25]. Dutch age-matched reference values were used for TC and HDL[26] and Finnish references for the atherogenic index[27]. For the other lipids our own reference values of 59 healthy children between 2-10 years and available reference data were used[28]. Written informed consent was obtained from the parents and from patients older than 12 years of age.

Statistics

Repeated measurements analysis of variance was performed using SAS (Proc Mixed, version 6.12) statistical package. The mean and standard error values given in the tables are based on these analyses. The factors isolated GHD versus multiple deficiencies, classic versus non-classic GHD and pubertal stage were evaluated regarding their impact on the profiles along time of bone density, body composition and height. In this analysis the modifying effect of these factors regarding the response profile was investigated using appropriate interaction terms. To evaluate whether the response profiles levelled out along time, the significance of quadratic terms in

addition to linear terms was determined. PTH and calcium-creatinine ratio showed a non-normal distribution. Therefore these parameters were log-transformed before analysis. Pearson's correlation coefficient was utilised to test the association between two variables with a normal distribution. For the primary outcome variables (bone density and body composition) $p<0.05$ was considered to be the limit of significance. In view of multiplicity of tests the significance level for secondary outcome variables was set at $p=0.01$.

RESULTS

Bone density

Table 1 shows the results of height, bone density and body composition. At start, BMD of lumbar spine and total body, $BMAD_{LS}$ and BMC of total body were significantly lower than zero.

Table 1. Mean height, bone mineral density (BMD) and body composition expressed as SD scores (se) in growth hormone deficient children at baseline and during growth hormone treatment

	baseline	6 months	1 year	2 years	3 years	4 years	5 years	6 years
BMD_{LS}	-1.49(0.18)[a]	-1.14(0.18)[a,1]	-0.79(0.18)[a,1]	-0.32(0.18)[1]	-0.21(0.16)[1]	-0.10(0.18)[1]	-0.20(0.18)[1]	-0.35(0.19)[1]
BMD_{TB}	-0.91(0.17)[a]	-1.18(0.17)[a,3]	-0.88(0.17)[a]	-0.31(0.17)[1]	-0.11(0.17)[1]	0.08(0.19)[1]	-0.16(0.20)[1]	0.08(0.22)[1]
$BMAD_{LS}$	-0.35(0.17)[c]	-0.33(0.16)[c]	-0.19(0.16)	0.11(0.16)[1]	0.03(0.15)[2]	0.29(0.15)[1]	0.19 (0.19)[1]	-0.23(0.28)[1]
BMC	-2.19(0.14)[a]	-1.91(0.16)[a,1]	-1.49(0.17)[a,1]	-0.84(0.19)[a,1]	-0.56(0.18)[b,1]	-0.53(0.24)[c,1]	-0.68(0.23)[b,1]	-0.32(0.21)[1]
LBM	-2.62(0.13)[a]	-1.80(0.12)[a,1]	-1.45(0.13)[a,1]	-1.02(0.15)[a,1]	-0.89(0.15)[a,1]	-0.90(0.16)[a,1]	-0.87(0.21)[a,1]	-1.02(0.20)[a,1]
% fat	1.02(0.26)[a]	-0.07(0.27)[1]	-0.04(0.27)[1]	0.29(0.27)[1]	0.27(0.25)[1]	0.32(0.23)[1]	0.30(0.23)[1]	0.63(0.31)
Height	-3.05(0.13)[a]	-2.41(0.13)[a,1]	-1.98(0.13)[a,1]	-1.50(0.13)[a,1]	-1.25(0.13)[a,1]	-1.10(0.13)[a,1]	-1.07(0.14)[a,1]	-0.78(0.15)[a,1]
BMI	0.54(0.20)[b]	0.40(0.21)[c]	0.41(0.21)[c]	0.73(0.21)[a]	0.70(0.21)[b]	0.84(0.22)[a,3]	0.72(0.22)[b]	0.93(0.24)[a,3]

[a] $p<0.001$ [b] $p<0.01$ [c] $p<0.05$ SDS compared to zero. [1] $p<0.001$ [2] $p<0.01$ [3] $p<0.05$ compared to baseline.

LS lumbar spine; *TB* total body; *BMAD* bone mineral apparent density; *BMC* Bone Mineral Content; *LBM* lean body mass; *% fat* percentage body fat, *BMI* body mass index.

There was a significant increase during follow-up for all these parameters. $BMAD_{LS}$ was normalised after one year of treatment. After 2 years of treatment, BMD of lumbar spine and total body were not different from zero anymore. BMC was normalised after 6 years of treatment. BMD_{LS} increased immediately after start of GHRx and levelled out significantly along time. BMD_{TB} shows an initial decrease and starts to increase after 6 months, with a significant levelling out of the response profile along time.

When we calculated SDS for bone age instead of chronological age mean $BMAD_{LS}$ was not significantly different from zero at any time point of evaluation, while BMD_{LS} was only significantly lower than zero at baseline. Similar to the results of BMD_{TB} SDS for chronological age, we found an initial decrease and a subsequent increase of BMD_{TB} SDS for bone age.

At diagnosis 36 % of the children had BMD_{LS}, and 7% had $BMAD_{LS}$ below –2 SDS. After two years of treatment all patients had BMD_{LS} and $BMAD_{LS}$ above –2. At diagnosis 18% of the children had BMD_{TB} below –2 SDS, after 4 years of GHRx none of the patients had BMD_{TB} below –2 SDS.

Height & Body composition

Although there was a significant increase in height SDS and lean body mass SDS, both remained significantly lower than zero at any time point of evaluation (Table 1). Height and LBM SDS showed a significant levelling out. Mean BMI SDS remained significantly higher than zero. After an initial decrease in the first 6 months, %fat stabilised and did not differ from zero anymore.

Laboratory assessments

Biochemical parameters of bone turnover are presented in Table 2.

Table 2. Biochemical parameters (mean (se))at baseline and during growth hormone treatment in growth hormone deficient children.

	baseline	6 months	1 year	2 years	3 years	4 years	5 years	6 years
Calcium (mmol/l)	2.41 (0.01)	2.42 (0.01)	2.42 (0.01)	2.41 (0.01)	2.40 (0.02)	2.39 (0.02)	2.44 (0.02)	2.64 (0.02)
Phosphate SDS	-0.67(0.15)[a]	0.61(0.16)[a,1]	0.37(0.13)[a,1]	-0.13(0.09)[1]	-0.09(0.11)[1]	0.14 (0.10)[1]	0.19 (0.15)[1]	0.45(0.16)[1]
ALP SDS	-1.30(0.22)[a]	0.49 (0.22)[1]	0.63(0.22)[b,1]	0.20 (0.22)[1]	-0.07(0.22)[1]	0.11 (0.23)[1]	0.57 (0.26)[1]	0.04 (0.30)[1]
Osteocalcin SDS	-1.55(0.19)[a]	0.05 (0.19)[1]	0.11 (0.18)[1]	-0.60 (0.18)[a]	-0.94 (0.19)[a]	-0.92 (0.22)[a]	-0.82(0.25)[b]	-1.34 (0.31)[a]
PICP SDS	-0.20 (0.14)	0.82(0.15)[a,1]	0.65(0.14)[a,1]	0.16 (0.14)	0.12 (0.16)	0.09 (0.18)	0.01(0.20)	-0.48 (0.25)
ICTP SDS	0.14 (0.16)	1.61(0.16)[a,1]	1.81(0.16)[a,1]	1.66(0.16)[a,1]	1.29(0.18)[a,1]	1.30(0.21)[a,1]	1.40(0.24)[a,1]	0.97(0.30)[b,2]
IGF-I SDS	-2.34(0.19)[a]	-0.01(0.21)[1]	0.17 (0.19)[1]	0.22 (0.24)[1]	0.36 (0.20)[1]	0.07 (0.24)[1]	-0.08(0.24)[1]	0.71 (0.32)[1]
IGFBP-3 (mg/l)	1.78 (0.12)	3.11 (0.14)[1]	3.01 (0.14)[1]	3.39 (0.14)[1]	3.68 (0.13)[1]	4.30 (0.20)[1]	4.20 (0.14)[1]	4.04 (0.11)[1]
PTH (ng/l)	20.0 (1.1)	21.4 (1.1)	23.4 (1.1)	22.9 (1.1)	19.5 (1.1)	19.1 (1.1)	17.8 (1.1)	16.2 (1.1)
1,25vitD (pmol/l)	96.5 (3.9)	128.8 (6.2)[1]	140.2 (5.6)[1]	139.1 (5.7)[1]	124.8 (5.6)[1]	123.1 (5.1)[1]	149.0(10.1)[1]	152.7(11.2)[2]
Urine OHP/Cr	115.0 (8.0)	155.4 (7.8)[1]	153.8 (7.6)[1]	124.7 (7.9)	132.8(8.7)	126.9(10.4)	119.0(11.3)	105.8(14.1)
Urine Ca/Cr	0.33 (1.13)	0.30 (1.13)	0.27 (1.13)	0.25 (1.13)	0.29 (1.14)	0.27 (1.17)	0.25 (1.18)	0.27 (1.22)

[a] $p<0.001$, [b] $p<0.01$ SDS compared to zero. [1] $p<0.001$, [2] $p<0.01$ compared to baseline.

ALP alkaline phosphatase; *PICP* procollagen type I C-terminal propeptide; *ICTP* carboxy terminal telopeptide of type I collagen; *IGF-I* insulin-like growth factor I; *PTH* parathyroid hormone; *1,25 vit D* 1,25-dihydroxyvitamin D_3; *OHP/Cr* and *Ca/Cr* hydroxyproline/creatinine (mg/g) and calcium/creatinine ratio

At baseline, osteocalcin, ALP, and IGF-I SDS were significantly lower than zero. ALP, osteocalcin, PICP, ICTP and IGF-I, all expressed as age- and sex matched SDS, showed a significant increase mainly in the first six months of treatment. ALP, ICTP and IGF-I remained significantly higher than baseline, while osteocalcin and PICP showed a steady decrease after 1 year of treatment. IGFBP-3 increased in the first six months and showed a slight (probably age-dependent) increase, thereafter.

Calcium and PTH levels did not change during GHRx, whereas 1,25-dihydroxyvitamin D showed an increase in the first six months and remained stable thereafter.

Urine calcium-creatinine ratio did not change during GHRx. Urine hydroxyproline concentration was the highest at t=0.5 and t=1, and was not different from baseline thereafter.

Lipids

Table 3 shows the results of lipid metabolism before and during GHRx. At baseline and during the 6 years follow-up, the mean levels of all parameters of lipid metabolism were within the normal range.

Table 3. Mean (se) lipid profiles during growth hormone treatment.

	Reference	baseline	1 year	2 years	3 years	4 years	5 years	6 years
Total cholesterol	3.2-6.0 mmol/l	4.48 (0.11)	4.25 (0.12)	4.32 (0.12)	4.26 (0.13)	4.33 (0.13)	4.14 (0.16)	4.20 (0.18)
Triglycerides	0.2-1.7 mmol/l	1.07 (0.06)	1.12 (0.06)	1.10 (0.06)	1.09 (0.06)	1.00 (0.08)	1.11 (0.09)	1.05 (0.11)
LDL	1.3-3.7 mmol/l	2.73 (0.11)	2.56 (0.11)	2.59 (0.11)	2.48 (0.11)[a]	2.54 (0.13)	2.28 (0.15)[a]	2.28 (0.17)[a]
HDL	0.9-1.6 mmol/l	1.21 (0.04)	1.19 (0.04)	1.27 (0.04)	1.31 (0.04)[a]	1.40 (0.05)[a]	1.40 (0.06)[a]	1.47 (0.07)[a]
Free fatty acids	0.2-1.3 mmol/l	0.83 (0.04)	0.73 (0.04)	0.62 (0.04)[a]	0.60 (0.05)[a]	0.52 (0.06)[a]	0.52 (0.07)[a]	0.53 (0.09)[a]
Apo-A1	0.8-1.5 g/l	1.35 (0.03)	1.30 (0.02)	1.37 (0.02)	1.36 (0.03)	1.42 (0.04)	1.37 (0.05)	1.40 (0.08)
Apo-B	0.6-1.1 g/l	0.89 (0.03)	0.85 (0.03)	0.87 (0.03)	0.87 (0.03)	0.87 (0.03)	0.86 (0.04)	0.87 (0.04)
Atherogenic index	<4.5	4.03 (0.19)	3.84 (0.19)	3.66 (0.17)[a]	3.55 (0.18)[a]	3.34 (0.15)[a]	3.21 (0.16)[a]	3.09 (0.16)[a]

Difference with baseline: [a] $p<0.001$ [b] $p<0.01$ *LDL* low density lipoprotein cholesterol; *HDL* high density lipoprotein cholesterol; *Apo* apolipoprotein; *atherogenic index* total cholesterol to HDL ratio.

At baseline, however, 32 % of the patients had an atherogenic index > 4.5, 19 % had FFA levels above and 19 % had HDL levels below the reference range. LDL and total cholesterol were above the reference range in 14 % and 4 % of the patients, respectively. After 3 years GHRx, atherogenic index was above reference range in 11 %, LDL in 2 %, FFA in 4 % of the patients, and HDL was below reference range 9 %. The atherogenic index, FFA, and LDL decreased and HDL increased significantly during GHRx.

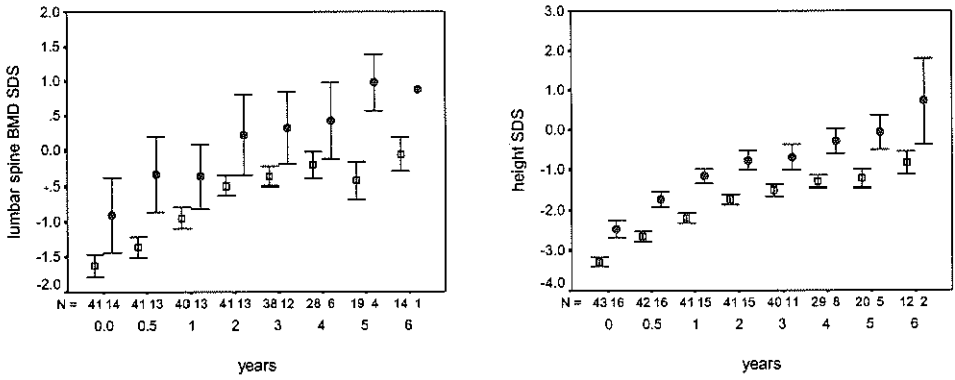

Figure 1. BMD of lumbar spine and height (mean and sem) before and during GHRx for children with isolated GHD (open squares) and children with multiple deficiencies (black dots).

Correlations

In the first six months, the change (Δ) in 1,25-dihydroxyvitamin D significantly correlated with the ΔBMAD and ΔBMD of lumbar spine ($r=0.48$, $p=0.001$ and $r=0.38$, $p=0.01$ respectively) and not with ΔBMD_{TB}. ΔHSDS correlated with ΔBMD_{LS} only in the first 6 months of treatment ($r=0.31$, $p=0.03$), no correlation with $\Delta BMAD_{LS}$ and ΔBMD_{TB} was found.

We correlated the changes of the bone markers expressed as SDS with the changes in BMD, BMAD or height, in the first six months of GHRx. The highest correlations were found between ΔALP and ΔBMD_{LS} or $\Delta BMAD_{LS}$ ($r=0.63$, $p=0.001$ and $r=0.42$, $p=0.03$ respectively).

The change in other markers of bone turnover showed correlation neither with changes in bone density, nor with changes in height. The various biochemical markers of bone turnover were highly correlated, but ΔIGF-I was not correlated with any of the bone markers. ΔIGF-I was not correlated with Δ1,25-dihydroxyvitamin D, Δ%fat, ΔLBM and ΔHSDS after 6 months and after 2 years of treatment.

BMC_{TB} was highly associated with LBM at all time points of evaluation (r varied from 0.81-0.97; $p<0.001$). We also separately analysed all prepubertal children who had a follow-up for at least 3 years. The slope in the regression analyses with BMC as dependent and LBM as independent factor did not change in the follow-up measurements and did not differ form the slope at baseline and follow-up in our reference population. BMD and BMAD were also highly correlated with LBM. Calcium intake was assessed once during follow-up and did not show a significant correlation with BMD of lumbar spine and total body.

Comparison between subgroups of GHD patients

Putative modifiers of lumbar spine BMD, and the effect of GHRx, such as isolated versus multiple deficiencies, classic versus non-classic GHD, and prepubertal versus pubertal during follow-up period, were analysed. Children with multiple deficiencies had 0.7 SDS ($p=0.04$) higher BMD at baseline, than children with isolated GHD. The changes of BMD during GHRx however were similar in both groups (Figure 1). The fact that children had a non-classic or classic GHD and their pubertal stage during treatment did not influence lumbar spine BMD. The same analyses were performed for height SDS (Figure 1). Again, children with multiple deficiencies had 0.9 SDS higher height SDS ($p<0.001$) than children with isolated GHD. However, no difference in the increase in height SDS during GHRx was found. No additional effect of non-classic versus classic GHD or pubertal stage was found.

Children with multiple deficiencies had significantly higher BMI SDS, at baseline. No difference in target height SDS were found between isolated GHD and children with multiple deficiencies.

DISCUSSION

We found a significantly reduced BMD of lumbar spine and total body, $BMAD_{LS}$ and BMC_{TB} in GH deficient children at diagnosis. During GHRx a significant increase was found. $BMAD_{LS}$ was normalised after one year of treatment. One year later, BMD of lumbar spine and total body were normalised as well. The largest changes are seen in the first years of treatment. Total body BMD showed an initial decrease and started to increase after 6 months. Several markers of bone turnover were decreased at diagnosis, and increased during GHRX. In general, serum levels were the highest after six to twelve months of treatment. Alkaline phosphatase appeared to be best predictor of the change in bone density in the first six months of GHRx.

$BMAD_{LS}$ is a calculated volumetric BMD, which corrects for bone size. This bone parameter was decreased in a much lesser degree than the areal BMD of total body and lumbar spine. Areal BMD, measured by DXA, will be underestimated in children with short stature. Therefore, $BMAD_{LS}$ will be a better parameter for assessing bone mineralisation in GHD children. Of course, direct measurement of volumetric BMD, as with quantitative computed tomography (QCT), is preferred, but this technique involves high radiation exposure. However, $BMAD_{LS}$ was decreased before start

of treatment, suggesting that the decrease in BMD was not merely caused by short stature. Since $BMAD_{LS}$ also increased during GHRx and the increase was independent of the change in HSDS, we may conclude that true density improves as well. This is in concordance with the findings of Baroncelli and colleagues[1], who reported that $BMAD_{LS}$ was significantly reduced in GHD children at diagnosis, but to a much lesser degree than areal BMD. Earlier, they have shown increases in BMD during treatment [29] similar to our results.

In our study BMD_{TB} started to increase after an initial decrease in the first six months, while BMC_{TB} and BMD_{LS} increased immediately after start of GHRx. Lumbar spine mainly consists of trabecular bone, which has higher bone turnover than cortical bone[30]. Total body consists for 80 % of cortical bone[16]. As a consequence, changes in bone turnover will occur earlier in lumbar spine. The increase in BMC_{TB} and decrease in BMD_{TB} in first months suggest a faster rate of bone expansion than mineral acquisition in cortical bone.

Surprisingly, we found that children with multiple deficiencies had higher BMD than children with isolated GHD. The response to GHRx, however, was similar. Comparable response profiles were found for height. The severity of the GHD (classic versus non-classic) or pubertal stage of the patients (prepubertal versus pubertal during follow-up) did not play a role in determining bone mass. Several studies showed no difference in BMD between patients with isolated GHD and those with multiple pituitary deficiencies[4,31]. These studies have been performed in somewhat different patient groups of adult men with childhood onset GHD after discontinuation of GHRx. Our patients with multiple pituitary deficiencies were all diagnosed as classic GHD and they received suppletion therapy for their other deficiencies. The latter might explain why GHRx had the same effect in children with isolated GHD and multiple deficiencies, but this cannot really explain why children with multiple deficiencies have even higher BMD. Children with multiple deficiencies also had higher BMI SDS and were taller, this may cause the higher BMD. Taller stature at start could no be attributed to differences in target height SDS. However, almost all children with multiple deficiencies had acquired GHD of known origin, mainly intracerebral tumors, which might result in growth retardation without severe short stature[32].

The pathophysiology of osteoporosis in GHD is complex. GH stimulates IGF-I production in the liver and in skeletal cells. IGF-I enhances bone collagen and matrix synthesis, and stimulates replication of osteoblasts. However, the direct effect of GH on bone is limited. Osteoclasts are indirectly activated via paracrine factors derived from the osteoblasts[33]. Furthermore, renal 1-α-hydroxylase activity is increased by IGF-I[34]. This enzyme is needed to transform 25-hydroxyvitamin D in the active form 1,25-dihydroxyvitamin D, which consequently will increase intestinal calcium absorption and will accomplish other stimulatory effects on bone formation[33]. In addition, GH has an indirect effect by increasing muscle mass and muscle strength, which stimulate bone formation.

According to the mechanostat theory bone mass adapts to strain. The mechanostat acts as a thermostat and is responsible for strain detection. The modelling thresholds of the mechanostat may be modulated by several hormones[35,36]. A large part of the load on bone is generated by the muscles. Indeed, BMC_{TB} was highly associated with LBM, however the association did not change during the follow-up measurements and did not differ from the association in healthy children. This finding suggests that LBM acts as an important predictor of BMC and that the mechanostat or modelling threshold is hardly modulated by GH. Therefore, the increase in BMC might be

explained mainly by an increase in muscle mass with a subsequent 'physiological' adaptation of bone mass, rather than a direct effect of GH on bone.

Lean body mass showed an ongoing increase during GHRx, however it did not normalise in six years. This might be explained by the fact that patients are still significantly shorter than normal after six years or GHRx. The increase in LBM was higher in the first years of treatment. Percentage fat was increased at baseline and showed a fast normalisation in the first 6 months, and stabilises thereafter. Similar changes in body composition are reported in GHD adults and children[6,37,38].

The markers of bone formation and bone resorption were decreased at diagnosis and increased during GHRx. They reflect changes in bone density, with the largest changes in the first six to twelve months. The slight decrease of osteocalcin and PICP after 1 year of treatment, has also been reported in GHD children and adults[39,40]. PICP synthesis has been reported to be down-regulated when bone mineralisation is achieved[41], which occurs during GHRx. In the first six months of GHRx, the change in alkaline phosphatase appeared to be the best predictor of ΔBMD or ΔBMAD. No correlation between changes in BMD or BMAD and the change in ICTP, PICP and osteocalcin was found. The collagen-derived markers such as PICP and ICTP do not only reflect bone turnover, but are also derived from extra-osseous sites. Therefore, they also reflect changes in for example height velocity. Markers of bone turnover have reported to show pronounced variation[42] and they depend on age and sex. We adjusted for age and sex differences by calculating standard deviation scores using our own normative data. Furthermore blood samples were taken under standardised conditions, e.g. early in the morning and the blood was immediately sampled or stored.

In the present study, the mean values of the lipid profiles were normal. Nevertheless, one- third of the patients had an increased atherogenic index and almost one-fifth had increased FFA or decreased HDL. During GHRx, particularly the atherogenic index, HDL, LDL, and FFA improved. Rosen and Bengtsson[43] reported increased vascular morbidity and mortality in adults with hypopituitarism, possibly associated with GHD. The reported effects on lipid levels are inconsistent in adults[44-46], as well as in children[47-49]. The best predictor of coronary heart disease in adults appeared to be the atherogenic index and as a single predictor, HDL[50]. HDL increased and the atherogenic index decreased in our patients, so long-term GHRx seems to have a beneficial effect on lipid profiles in GHD children.

In conclusion, areal bone mineral density of lumbar spine and total body, and to a lesser extent BMAD (volumetric BMD) are decreased in GHD children at diagnosis. BMD and BMAD of lumbar spine increased immediately after start of GH replacement therapy, while BMD of total body increased after an initial decrease. The largest increments are found in the first years of treatment, with a levelling out thereafter. Within one and two years respectively, BMAD and BMD were normalised. In GHD children and children with short stature in general, bone density should be evaluated as volumetric or apparent density, and not as an areal density. Mean lipid levels were normal at start of diagnosis and improved during GHRx. GHRx had lipolytic effects and an anabolic effect on lean body mass. The increase in lean body mass as well as an increase in 1,25-dihydroxyvitamin D might mediate the positive influence of GH on bone density.

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Chapter 10

LONGITUDINAL FOLLOW-UP OF BONE DENSITY AND BODY COMPOSITION IN CHILDREN WITH PRECOCIOUS OR EARLY PUBERTY BEFORE, DURING AND AFTER CESSATION OF GONADOTROPHIN-RELEASING HORMONE AGONIST THERAPY

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ABSTRACT

We studied bone mineral density (BMD), bone metabolism and body composition in 47 children with central precocious puberty (n=36) or early puberty (n=11) before, during and after cessation of gonadotrophin-releasing hormone agonist (GnRH-a). Bone density and body composition were measured with dual energy X-ray absoroptiometry and expressed as standard deviation scores (SDS). Bone age and biochemical parameters of bone turnover were assessed. Measurements were performed at baseline, after six months, and on a yearly basis thereafter.

Mean lumbar spine$_{(LS)}$ BMD SDS for chronological age was significantly higher than zero at baseline, and decreased during treatment. Bone mineral apparent density ($BMAD_{LS}$) and total body$_{(TB)}$ BMD did not differ from normal at baseline and showed no significant changes during treatment. In contrast, BMD SDS for bone age was significantly lower than zero at baseline and at stop of therapy. Two years after therapy BM(A)D SDS for bone age and chronological age did not differ from normal. Markers of bone turnover decreased during treatment, mainly in the first 6 months. Patients had increased % fat and lean body mass at baseline. After an initial increase of % fat during treatment, % fat decreased and normalized within one year after stop of treatment.

Our longitudinal analysis suggests that peak bone mass or body composition will not be impaired in patients with precocious or early puberty after GnRH-a therapy.

INTRODUCTION

Puberty is considered to be a crucial period for bone mass acquisition[1]. Therefore, it is important to know whether children with a disorder in pubertal development will achieve an adequate peak bone mass.

In central precocious puberty (CPP) the hypothalamus-pituitary-gonadal axis is activated before the age of 8 years in girls and before the age of 9 in boys. Treatment is based on administration of gonadotrophin releasing hormone agonist (GnRH-a), which inhibits pituitary gonadotrophin secretion resulting in a decrease of sex steroids levels[2]. Estrogen deprivation, for instance after ovariectomy or natural menopause, is associated with significant bone loss in adult women[3]. A significant decrease in bone density during GnRH-a therapy in women with endometriosis and in men with benign prostatic hyperplasia has been reported[4,5]. Thus, reducing sex steroids levels in CPP could have detrimental effects on bone density and particularly the achievement of peak bone mass could be impaired.

Besides putative negative effects of GnRH-a on bone mass acquisition, concern has been raised that children with CPP are prone to development of adiposity[6,7].

The aim of the present study was to evaluate longitudinally bone mineral density, bone metabolism and body composition in a large group of children with precocious or early puberty before, during and after cessation of GnRH-a treatment. Preliminary results were presented previously[8].

MATERIALS AND METHODS

Forty-seven patients (5 boys and 42 girls) were enrolled in the study. The mean age at start of GnRH-a treatment was 8.3 years (range 2.8-11.4). At diagnosis all patients had a history of increased growth velocity, girls had breast stage $\geq$ 2 and boys had genital stage $\geq$ 2 and testes

volume ≥ 4 ml, bone age was advanced more than 1 year, and a GnRH-stimulated serum LH concentration was greater than 10 IU/l. Thirty-one children had idiopathic central precocious puberty with start of puberty before the age of 9 for boys and before the age of 8 years for girls. Five children had organic central precocious puberty (2 meningomyelocele, 1 hydrocephalus, 1 optic glioma, and 1 craniopharyngeoma). Idiopathic early puberty was found in 11 children, defined as appearance of pubertal signs between 8 and 10 years of age for girls and between 9 and 11 years of age for boys.

All patients were treated with depot leuprolide-acetate 3.75 mg (Lucrin® depot, Abbott) given subcutaneously every two weeks in the first month and every 4 weeks thereafter.

Pubertal suppression was evaluated by clinical evaluation, by repeating GnRH stimulation test after three months, by measuring basal serum levels of luteinizing hormone (LH), follicle stimulating hormone (FSH) and estradiol or testosterone levels every six months of treatment. All children had complete suppression of LH and FSH during the GnRH test after 3 months of treatment (levels <5 IU/l). All patients, except one boy and one girl, had complete suppression during therapy, with prepubertal basal sex steroids concentrations (estradiol < 50 pmol/l and testosterone <1nmol/l). In the boy and girl with incomplete suppression GnRH-a dose was doubled, which resulted in complete suppression.

Anthropometry, assessment of biochemical parameters of bone turnover, and BMD and body composition measurements were performed at baseline, after six months, 1 year and next yearly. All assessments were continued after discontinuation of treatment on a yearly basis. Forty patients completed treatment, of whom 38 agreed to continue participation. Twenty patients (2 boys and 18 girls; 15 CPP and 5 early puberty), had complete follow-up measurements from start of GnRH-a therapy till two years after stop of treatment. This subgroup will be described separately.

Height was measured with a Harpenden stadiometer and expressed as Standard Deviation Score (SDS)[9]. Pubertal stage was assessed according to Tanner[10]. Bone age was scored by one investigator using an X-ray of the left hand according to the Greulich and Pyle method[11].

Bone density and body composition

Bone mineral density of lumbar spine and total body was measured by Dual Energy X-ray Absorptiometry (DXA, Lunar, DPXL/PED, Winconsin, USA). The lumbar spine is mainly composed of trabecular bone, whereas 80% of the total body bone consists of cortical bone[12]. To correct for bone size we calculated apparent BMD of lumbar spine with the model $BMAD_{LS} = BMD_{LS} \times [4/(\pi \times \text{width})]$. This model has been validated by in vivo volumetric data obtained from magnetic resonance imaging of lumbar vertebrae[13]. All children were measured by the same apparatus. Quality assurance was performed daily. The coefficient of variation has been reported to be 1.04% for spine BMD and 0.64% for total body BMD[14]. Body composition was measured by total body DXA. BMD, bone mineral content (BMC), lean body mass, and percentage body fat were compared with our Dutch age- and sex matched reference values (n=500, 4-20 years old)[15,16]. One girl was too young (2.8 years) to compare her DXA results with our normative data. Her data could be used to calculate bone age-adjusted SD-scores. In two children baseline DXA measurement was not performed due to logistic reasons.

Biochemical parameters

Blood samples were obtained for the assessment of calcium, anorganic phosphate, and 1,25-dihydroxyvitamin D. Furthermore, alkaline phophatase and procollagen type I C-terminal propeptide (PICP) were assessed as markers of bone formation, while carboxyterminal telopeptide of type I collagen (ICTP) and urinary hydroxyproline concentration were measured as markers of bone resorption. 1,25-dihydroxyvitamin D was measured by by RIA of Immuno Diagnostic Systems (Boldon, United Kingdom). Radioimmunoassay kits (Orion Diagnostica, Espoo, Finland) were used for measurement of PICP and ICTP. ALP, phosphate, PICP, ICTP were expressed as sex- and age matched SDS using our own reference values. Estradiol and testosterone were assessed by RIA (Orion Diagnositica, Espoo, Finland); LH and FSH by RIA (MedGenix Diagnositics, Fleurus, Belgium). The calcium/creatinine (Ca/Cr) ratio and hydroxyproline concentration expressed as mmol/mol creatinine (OHP/Cr) were assessed in the first morning void of urine. Written informed consent was obtained from the parents of the patients.

Statistical analysis

One sample t-tests were used to compare the mean SDS with the expected zero, which is the mean SDS of age-and sex-matched healthy controls. The within patient change was tested using a paired t-test. To test differences between patients with early puberty and precocious puberty Mann-Whitney tests were used. Pearson's correlation coefficient was utilized to test the association between two variables with a normal distribution and Spearman's correlation in case of a skewed distribution. In view of multiple tests the significance level was set at $p = 0.01$.

RESULTS

Bone density and body composition before treatment

Clinical baseline characteristics are reported in Table 1. The results of bone mineral density and body composition before and during treatment for all patients are shown in Table 2.

Table1. Clinical characteristics at baseline. Mean (range)

	Boys	Girls
Number	5	42
Age (years)	9.0 (4.7 –11.4)	8.2 (2.8 -10.8)
Bone age (years)	11.9 (9.2 -14.5)	10.7 (4.3 -13.3)
Height SDS	0.39 (-1.96 - +2.09)	0.80 (-1.77 - + 4.71)
BMI SDS	0.63 (-0.47 - +2.79)	1.17 (-0.76 – +2.80)

SDS standard deviation score; *BMI* body mass index

At baseline, mean BMD SDS of lumbar spine was significantly higher than zero. $BMAD_{LS}$ SDS and BMD SDS of total body were above normal but this did not reach significance. However, after correction for bone age instead of chronological age, BMD_{LS} and BMD_{TB} SDS corrected for bone age were significantly lower than zero. $BMAD_{LS}$ SDS corrected for bone age was normal.

Height, bone mineral content (BMC), percentage fat, lean body mass and BMI SDS (all SDS for chronological age) were significantly increased at baseline.

Forty patients completed treatment, their mean treatment period was 2.7 years (range: 1.4 - 5.4 years). After an initial increase, BMD_{LS} and BMD_{TB} corrected for chronological age tended to decrease (Table 2). BMAD showed the same trend, but these changes were not significant. However, when adjusted for bone age, BMD_{TB} SDS for bone age increased significantly during treatment. As for chronological age, no significant changes in $BMAD_{LS}$ corrected for bone age were found during treatment. BMD_{LS} SDS for bone age did also not change during treatment.

% Fat SDS increased and LBM SDS decreased significantly (Table 2). Height SDS decreased, after one year of treatment height SDS did not differ significantly from zero anymore. BMI SDS increased significantly during treatment.

Table 2. Mean height, bone mineral density (BMD) and body composition expressed as SD scores (sem) in children with precocious puberty at baseline and during GnRH-a treatment

Time	baseline	0.5 yr	1 yr	2 yrs	3 yrs
n	44	45	45	39	24
SDS for chronological age					
BMD_{LS}	0.67 (0.18)[b]	0.83 (0.16)[b 1]	0.65 (0.17)[b]	0.48 (0.18)	0.39 (0.25)
$BMAD_{LS}$	0.36 (0.18)	0.50 (0.16)[a]	0.41 (0.17)	0.38 (0.18)	0.05 (0.22)
BMD_{TB}	0.19 (0.19)	0.39 (0.19)	0.50 (0.17)[a 1]	0.38 (0.19)	0.31 (0.27)
SDS for bone age					
BMD_{LS}	-0.54 (0.15)[b]	-	-0.57 (0.14)[b]	-0.36 (0.18)	-0.29 (0.22)
$BMAD_{LS}$	-0.15 (0.18)	-	-0.20 (0.17)	-0.06 (0.19)	-0.29 (0.25)
BMD_{TB}	-0.85 (0.15)[b]	-	-0.64 (0.14)[b 2]	-0.40 (0.17)[2]	-0.39 (0.24)[1]
SDS for chronological age					
LBM	0.92 (0.21)[b]	0.79 (0.20)[b 1]	0.71 (0.20)[b 1]	0.49 (0.25)[2]	0.31(0.33)[1]
% fat	0.47 (0.18)[a]	0.98 (0.18)[b 2]	1.28 (0.18)[b 2]	1.30 (0.21)[b 2]	1.10 (0.26)[b 2]
Height	0.75 (0.20)[b]	0.65 (0.22)[a]	0.55 (0.22)[1]	0.53 (0.23)[2]	0.18 (0.37)[2]
BMI	1.11 (0.13)[b]	1.24 (0.13)[b]	1.46 (0.13)[b 2]	1.47 (0.17)[b 2]	1.22 (0.21)[b]

[a] $p<0.01$ [b] $p<0.001$ for the comparison of the mean SDS with zero

[1] $p<0.01$ [2] $p<0.001$ compared to baseline

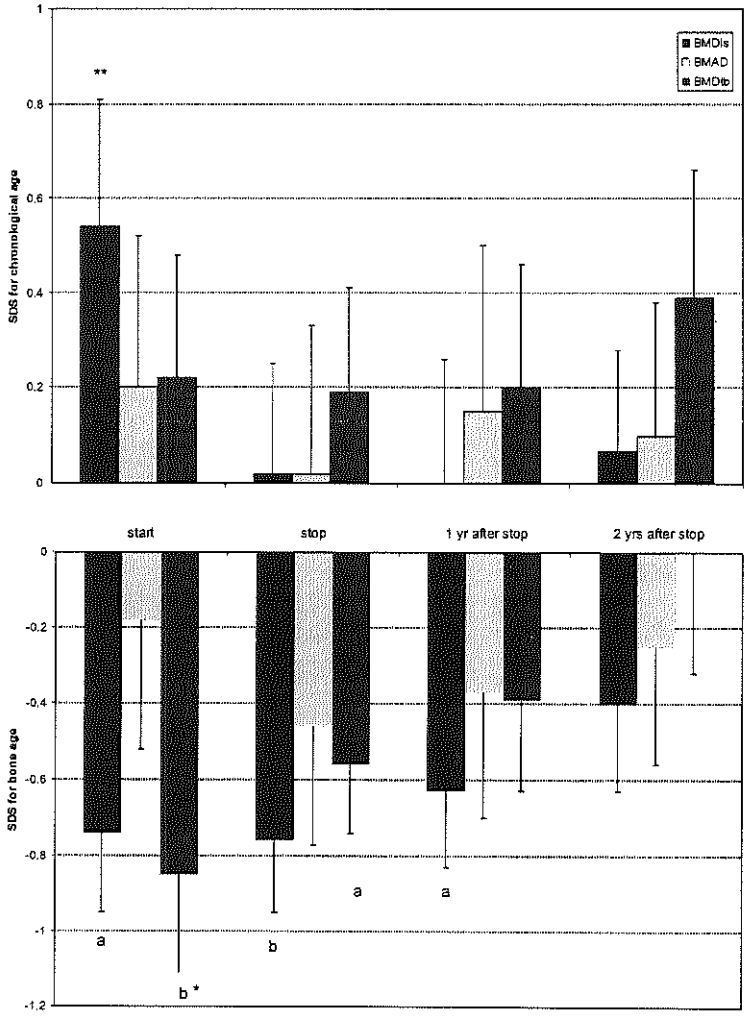
LS lumbar spine; *TB* total body; *BMAD* bone mineral apparent density; *LBM* lean body mass; *% fat* percentage body fat, *BMI* body mass index.

Results of 20 patients followed-up from start to 2 years after cessation of therapy

Figure 1 shows bone density and body composition before, at cessation of GnRH-a treatment, and two years after cessation of treatment in 20 patients, who were followed-up for this entire period. For this particular subgroup, mean (sd) age and bone age at start was 8.7 (1.1) and 11.3 (1.24) years respectively. Mean (sd) age at cessation of therapy was 11.3 (0.8) years. Mean (sd) bone age was 12.4 (0.7) years at stop of therapy, and 14.6 (1.0) years two years after stop of therapy. Mean treatment period for this subgroup was 2.6 years (range 1.4 - 4.1 years).

At baseline, BMD_{LS} SDS adjusted for chronological age was 0.54 (sd=1.15; p=0.06). Lumbar spine BMD SDS for chronological age decreased significantly during treatment, while total body

BMD and $BMAD_{LS}$ SDS showed no significant change. After cessation of therapy, no significant changes in bone density were found (Figure 1, upper panel).



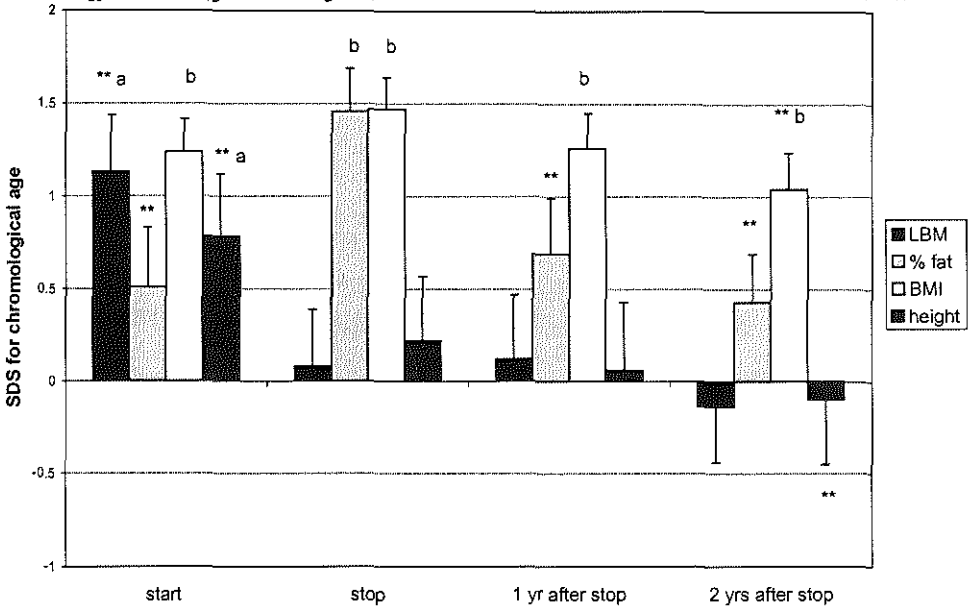
[a] $p<0.01$ [b] $p<0.001$ for the comparison of the mean SDS with zero;
* $p<0.01$ ** $p<0.001$ compared to stop

Figure 1. Bone mineral density adjusted for chronological age (upper panel) and adjusted for bone age (lower panel) before start, at cessation, and after cessation of GnRH-a therapy in children with precocious and early puberty (mean (sem)).
BMD bone mineral density; *BMAD* bone mineral apparent density; *ls* lumbar spine; *tb* total body

After correction for bone age (Figure 1, lower panel), only BMD_{TB} showed a significant increase, while $BMAD_{LS}$ and BMD_{LS} did not change during treatment. BMD_{LS} and BMD_{TB} remained significantly lower than zero at stop of treatment. After cessation of GnRH-a therapy all three bone

density parameters slightly increased compared to stop. After two years none of bone density parameters differed from zero anymore.

The mean absolute change between start and cessation of GnRH-a therapy was 0.09 gram/cm^2 for BMD_{LS} and BMD_{TB} and 0.01 gram/cm^3 for BMAD. None of the children had an absolute



[a] p<0.01 [b] p<0.001 for the comparison of the mean SDS with zero; * p<0.01 **p<0.001 compared to stop

Figure 2. Body composition and height adjusted for chronological age (mean (sem)) at start, at cessation, and after cessation of GnRH-a therapy in children with precocious puberty. *LBM* lean body mass; *% fat* percentage body fat; *BMI* body mass index.

decrease in BMD during therapy. Most children showed an absolute increase in BMAD as well, but in three children a decease in BMAD (0.01 to 0.02 gram/cm^3) was found. In the two years after cessation of therapy, BMD and BMAD showed an absolute increase in all children.

Lean body mass decreased significantly during treatment, and % fat increased (Figure 2). After cessation of treatment LBM and height showed a further decrease, while % fat decreased to pre-treatment values. Although BMI decreased significantly after stop of treatment, it remained significantly higher than zero after treatment. Two years after cessation of therapy, LBM, % fat, and height SDS did not differ significantly from zero.

Biochemical parameters

Table 3 shows the results of biochemical parameters at baseline, during treatment and after stop of treatment. Only baseline and six months data are given, because the substantial changes were found in this period. During GnRH-a treatment, serum calcium was normal and remained stable. At baseline, ICTP SDS was significantly higher than zero. PICP SDS was also increased, but in a lesser degree (p=0.03). Mean phosphate SDS, alkaline phosphatase (ALP) SDS, 1.25-

dihydroxyvitamin D, and Ca/creatinine ratio were normal. Urine OHP concentration was in the high reference range.

Table 3. Biochemical parameters at baseline and after cessation of GnRH agonist treatment in children with precocious puberty (mean (sem)).

	Before and during GnRH-a		After cessation of GnRH-a		
	baseline	6 months	stop	1 year	2 years
n=	45	44	33	29	18
Calcium (mmol/l)	2.42 (0.02)	2.42 (0.02)	2.38 (0.02)	2.37 (0.02)	2.39 (0.03)
Phosphate SDS	-0.07 (0.11)	-0.23 (0.12)	-0.23 (0.08)**	-0.13 (0.15)	-0.33 (0.17)
ALP SDS	0.10 (0.19)	-1.05 (0.15)[b]**	-1.18 (0.24)[b]**	-0.56 (0.26)[1]	-1.48 (0.44)[a]
PICP SDS	0.57 (0.25)	-0.74 (0.21)[a]**	-1.23 (0.19)[b]**	-0.45 (0.29)	-1.11 (0.26)[b]
ICTP SDS	1.78 (0.14)[b]	0.26 (0.14)**	-0.03 (0.32)**	0.68 (0.23)[a]	-0.48 (0.33)
1,25 vit D (pmol/l)	132.1 (7.0)	125.1 (7.1)	117.5 (5.7)	138.4 (6.4)[1]	129.6 (7.8)
Urine Ca/Cr	0.20 (0.02)	0.40 (0.04)*	0.33 (0.03)*	0.24 (0.05)	0.28 (0.06)
Urine OHP/Cr (mmol/mol)	132 (9)	104 (16)**	77 (4)**	86 (9)	52 (5)[1]

[a] $p<0.01$ [b] $p<0.001$ for the comparison of the mean SDS with zero; * $p<0.01$ ** $p<0.001$ compared to baseline; [1] $p<0.01$ [2] $p<0.001$ compared to stop

SDS standard deviation score; *ALP* alkaline phophatase; *1,25 vit D* 1,25-dihydroxyvitamin D; *PICP* procollagen type I C-terminal propeptide; *ICTP* carboxy terminal telopeptide of type I collagen, *OHP/Cr* and *Ca/Cr* hydroxyproline/creatinine and calcium/creatinine ratio

ALP SDS, ICTP SDS, PICP SDS and urine Ca/Cr ratio decreased significantly during treatment, mainly in the first six months, and stabilized thereafter. Phosphate SDS showed a slight decrease. Urinary hydroxyproline concentration decreased significantly during treatment.

At cessation of therapy, all serum markers, except calcium and 1,25-dihydroxyvitamin D, were decreased compared to baseline. Phosphate, PICP, and ALP SDS were significantly lower than zero at stop of treatment.

After cessation of therapy ALP, PICP, and ICTP increased with a subsequent decrease. Two years after treatment ALP and PICP SDS for chronological age were significantly lower than zero. After correction for bone age, however, normal SD-scores were found. Urine OHP concentration showed an ongoing decrease, while urine Ca/Cr ratio did not change.

Precocious puberty versus early puberty

The mean treatment period was 2.8 years in the CPP patients and 2.2 years in patient with early puberty. Patients with CPP had higher height SDS and LBM SDS than children with early puberty, at baseline and at stop of treatment. No differences in BMI and % fat were found. At baseline BMD and BMAD for chronological age and bone age did not differ. Total body and lumbar spine BMD for chronological age were significantly higher in the CPP group, at cessation of therapy. BMAD and BMD for bone age did not differ at stop of treatment.

Correlations

The changes (Δ) between values at cessation of treatment and at start of treatment were calculated. ΔHeight SDS and ΔLBM SDS were positively correlated with ΔBMD_{LS} ($r= 0.46$

$p=0.007$; $r=0.61$ $p<0.001$ respectively), but not with $BMAD_{LS}$ or BMD_{TB}. ΔBMI SDS was not significantly correlated with ΔBMD or ΔBMAD SDS. Changes in parameters of bone turnover showed correlation with neither Δheight SDS, nor with changes in BMD or BMAD SDS.

Discussion

The present study showed increased lumbar spine BMD for chronological age in children with precocious or early puberty. During GnRH agonist treatment lumbar spine BM(A)D and total body BMD for chronological age decreased, after an initial increase, to the normal range. However, after correction for bone age, lumbar spine and total body BMD were reduced. Using $BMAD_{LS}$ (volumetric or apparent BMD) corrected for bone age normal bone density before, during and after cessation of treatment was found.

Sex steroids, and especially estrogens, are very important in the acquisition of bone mass. This became more clear when two new syndromes were described, each representing a human model in which estrogen was lacking. A female with aromatase deficiency[17] and man with estrogen receptor defect[18] had severe undermineralization of the skeleton and no epiphyseal closure. So, androgens do not cause normal epiphyseal closure and bone mineralization in the absence of estrogens. In GnRH-a treated precocious puberty, a decrease in sex steroids by GnRH-a may explain the decrease in bone density. The initial increase in BMD in our patients may be explained by incomplete suppression of puberty during the first months.

In children with growth disorders, BMAD may be a more appropriate parameter to evaluate bone mineralisation than BMD. As children with precocious puberty have tall stature, BMD measured by DXA, will be overestimated. By calculating BMAD, a correction for bone size is made. Indeed, BMAD was increased to a much lesser extent than areal BMD of lumbar spine. Together, these findings suggest that increased BMD_{LS} and increased bone turnover at baseline mainly reflect increased growth, rather than increased bone mineralisation.

In adults with GnRH-a therapy an absolute decrease in BMD has been reported[4,5]. In contrast, absolute BMD increased during GnRH-a therapy in children, albeit at lower rates than before start of therapy, since their SDS decreased. Adults reach their peak bone mass and will not further increase their bone mass in physiological conditions. Absolute BMD also increases in healthy prepubertal children who are not exposed to high levels of sex steroids. This might explain that during gonadal suppression in our CPP patients BMD continued to increase.

Heger et al.[19] studied patients after cessation of GnRH-a therapy at final height. These young women had normal bone density of lumbar spine and femoral neck. Most studies found a BMD high for chronological age, but appropriate for bone age[19-21], before start of therapy. The described effects of GnRH-a on bone are still controversial. No change during 2 years of treatment have been found[20], as well as a decrease in trabecular bone density during GnRH-a therapy, whereas cortical bone of the radius did not change[22]. Saggese et al.[23] reported a reduction in BMD in cortical bone of the radius within 6 months. Most studies are performed in rather small patients groups varying from 10 to 13 children. Additionally, differences in reference values and differences in site, which has been measured, and differences in timing of start of treatment, may have contributed to some of the discrepancy with our results. Furthermore, our study also included children with early puberty. Children with CPP were taller, but did not have higher BMD than children with EP, at baseline. Despite a somewhat longer treatment in CPP, BMD was even

somewhat higher in CPP at cessation of therapy. This difference disappeared after correction for bone size, by calculating BMAD. Bertelloni et al.[24] stated that peak bone mass (PBM) was not impaired in girls treated with GnRH-a. Since PBM of lumbar spine was measured at the chronological age of 13.4 years, this might have been too early. Bone mineral accrual continues in the postpubertal years after linear growth has ceased. Furthermore the age that PBM is reached appeared to be site-dependent[25]. Our patients had mean age of 13.4 and bone age of 14.7 years at their last visit. Theoretically, children with precocious puberty might attain their PBM at an earlier chronological age, because of their advanced bone maturation. However, all patients showed an ongoing increase in lumbar spine BMD after cessation of therapy. So, we cannot draw any final conclusions yet regarding PBM. Nonetheless, our findings, e.g. normal bone mass and bone turnover 2 years after cessation of therapy, do not suggest that PBM will be impaired in children with a history of GnRH-a therapy.

We found that, the ICTP was significantly higher and PICP was slightly higher than normal before start of treatment. During treatment, bone turnover decreased mainly in the first six months of treatment and stabilized thereafter. Other authors found similar results. Antoniazzi et al.[22] showed that patients with CPP had pubertal osteocalcin levels that decreased during treatment. Hertel et al.[26] reported that girls with CPP had normal PICP levels that decreased within 2 months after initiation of GnRH-a and remained below baseline values. So, markers of bone resorption as well as markers of bone formation decrease during GnRH-a therapy. However, biochemical markers are not specific for bone modeling or remodeling. Additionally, PICP, ICTP and ALP are not bone-specific. Thus, changes in biochemical markers may reflect changes in growth as well as changes in bone mineralization. Two years after cessation of therapy, the markers of bone turnover were in the normal range for bone age.

Obesity is a common problem in children with precocious puberty[6,27,28]. Indeed, in the present study, % fat was increased at baseline. At cessation of therapy, % fat was significantly higher than normal, and normalized thereafter. Thus, after an initial aggravation of adiposity, no prolonged negative effects on % body fat were found. In concordance with our study, two other studies showed increased BMI at start, but no change in BMI during treatment[6,19]. BMI cannot distinguish between lean body mass and fat mass, BMI will not change when LBM decreases and fat mass increases as occurred in our study. Therefore, DXA is preferred to evaluate body composition.

The decreased GH and IGF-I levels that have been reported during GnRH-a therapy[27,29,30] might play a role in the increment of fat mass and decrease in lean body mass. Kamp et al.[27] showed that GH levels in GnRH-a treated children with CPP were inversely related to BMI. Growth hormone deficient patients have decreased bone density, increased fat mass and decreased LBM, which improved during GH replacement therapy[31]. Besides a decrease in estrogens and GH levels, the decrease in muscle mass during GnRH-a therapy may also affect bone mineralization.

In conclusion, children with precocious and early puberty had normal total body BMD, and increased lumbar spine BMD for chronological age. Only lumbar spine BMD decreased significantly during GnRH-a therapy. In contrast, BMD corrected for bone age was significantly lower than normal. Using $BMAD_{LS}$ (volumetric or apparent BMD) corrected for chronological age or bone age, normal bone density before, during and after cessation of treatment was found. Two years after cessation of therapy, bone density for bone age and chronological age did not differ from normal and markers of bone turnover adjusted for bone age were normal. Thus, this study suggests that PBM will not be impaired in children with a history of GnRH-a therapy. However, our

patients did not reach their PBM yet, so further follow-up is still needed. After an initial aggravation of adiposity during treatment, their % fat decreased to normal values after stop of treatment.

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Chapter 11

GENERAL DISCUSSION AND FUTURE RESEARCH

In this chapter we will discuss the most important findings of this thesis, and put them into perspective of current knowledge. This discussion will finish with suggestions for future research.

DETERMINANTS OF BONE MASS

Genetic determinants

Peak bone mass is considered to be under strong genetic control[1-3]. Identification of the genes mediating effects on bone mass may lead to better understanding of the pathogenesis of osteoporosis, and might help us to identify subjects at risk. Twin and family studies showed, that up to 80% of the variation in bone mass is determined by (poly)genetic factors[2-5]. So far, many candidate genes have been suggested. The choice of a candidate gene is based on a selection of genes coding for proteins involved in bone matrix and for factors that regulate bone turnover. Meta-analyses showed that VDR polymorphisms[6,7] and COLIA1 Sp1 polymorphism[8] are genetic factors affecting at least a part of the variation in bone mineral density (BMD) in adult populations.

In order to study genetic effects on BMD in children, we investigated polymorphisms in the VDR gene and the COLIA1 gene in healthy children and young adults. We found that the T-allele of the G to T substitution in the Sp1 binding site of the COLIA1 gene was associated with lower bone mineral content (BMC) as well as lower bone mineral density (BMD). These associations were demonstrated in two bone density measurements with an interval of approximately 4 years. An obvious frame size effect was observed: the association was most apparent for BMC, less for BMD of total body and lumbar spine, while no significant association between BMAD and genotype was found. In these subsequent steps, BMC (gram) is corrected for bone area (BMD, gram/cm^2), and for bone volume (BMAD, gram/cm^3). In line with this, the T-allele also tends to be associated with shorter stature and smaller bone size.

Furthermore, the VDR genotype showed a weak association with bone mineral apparent density (BMAD), in which BMD is corrected for bone size. VDR haplotype '3' was associated with taller stature and bigger bone size. Most studies that used areal BMD as an end-point to study did not find an association[9,10], while Sainz et al.[11] and our study used volumetric density and found an association. Thus, due to differences in frame size areal BMD might be a less suitable end-point to study the effects of VDR polymorphisms on bone mass.

Interestingly, COLIA1 and VDR polymorphism both affect frame size parameters, such as height and vertebral body width. In the COLIA1 gene the T-allele appeared to be a risk allele for short stature and small bone size, whereas non-haplotype '3' alleles carriers appeared to be risk alleles among the VDR haplotype alleles. By combining the risk genotypes of VDR ([1,1],[1,2],[1,4]or [2,2]) and collagen IA1 genotype ([GT] or [TT]), an additive genotype effect on height (Figure 1) and vertebral body width was found.

The major drawback of our study in healthy children and adolescents was the limited sample size. Furthermore, the wide age range including various pubertal stages might theoretically have weakened some of the associations. Differences between different genotypes might be more difficult to demonstrate during puberty, when large increases in skeletal size and bone mass occur. Finally, putative gender differences might be better detectable in larger groups.

In conclusion, we found polymorphisms in the VDR and COLIA1 gene to be associated with various bone characteristics, but especially with height and bone size. Bone density is polygenetically determined, and large studies are needed to study other candidate genes, and

their possible interaction, and find epidemiological support to clarify the interaction between lifetyle factors and genetic predisposition.

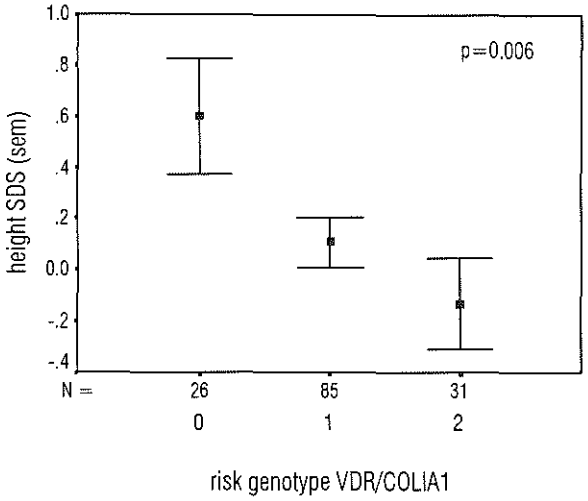

Figure 1. Interaction of effects of VDR genotype and COLIA1 genotype on height.
The combined genotypes were analysed as a risk score: '0' those not carrying a risk genotype of VDR nor COLIA1, '1' those carrying a risk genotype of either VDR or COLIA1, and '2' those carrying a risk genotype of VDR as well as COLIA1.P value of the regression analysis is given.

Environmental and clinical determinants

Bone mass acquisition in childhood is also associated with weight, height, hormonal status, and lifestyle factors such as physical activity and calcium intake[12-15]. Some of these determinants might be under genetic control as well[13].

In the previous study in 500 healthy children and young adolescents, the major determinant of BMD during childhood appeared to be weight in boys and pubertal development in girls[12]. Multiple regression analysis with weight, height, Tanner stage, calcium intake, and physical activity as determinants with adjustment for age with BM(A)D as the dependent variable resulted in the models presented in Table 1.

Table 1. Determinants of bone density in childhood[12]

	Boys		Girls	
	Model	R^2	Model	R^2
BMD_{LS}	Weight & height	85%	Weight & Tanner stage	80%
BMD_{TB}	Weight & calcium intake	88%	Weight & Tanner stage	85%
$BMAD_{LS}$	Weight	46%	Tanner stage	57%

A combination of clinical and genetic determinants of BMD of lumbar spine and femoral neck were analysed in a somewhat older group of approximately 700 Canadian women (18-35 years)[13]. The multiple regression analysis was performed resulting in a model with weight, height, physical activity (as an adolescent and currently), age, family history of osteoporosis, and VDR genotype (*BsmI*, *ApaI* and *TaqI*) for femoral neck BMD. This model explained about 22% of the variation in femoral neck BMD. The addition of VDR RFLPs in the final model explained less than 1% of the variation in femoral neck BMD. The genetic effect of VDR was not evident in the lumbar spine BMD. Consequently, the model for BMD of lumbar spine included weight, physical activity, age, and a family history of osteoporosis (R^2=17%).

Thus, unmeasured environmental variables may account for a part of the variance of peak bone mass, however, as yet the bulk of the variance is likely a result of undetected genetic factors.

THE USE OF SERUM MARKERS OF BONE TURNOVER

Biochemical parameters of bone turnover in childhood are difficult to interpret as they reflect growth (bone modelling) and bone remodelling. First of all, reference data are needed to interpret data found in pathological conditions. Chapter 4 presents reference data for biochemical markers of bone turnover. In concordance with other studies[16-19], most parameters peaked during puberty, in girls approximately 2.5 years earlier than in boys. The markers of bone formation and resorption were highly correlated, which could be expected because bone resorption and formation are strongly coupled. Many markers of bone turnover showed large variation. Correction for age and gender might be helpful and necessary for long-term follow-up. However, one single measurement will not be informative for monitoring bone mass. Indeed, no correlation between markers of bone turnover and BMD was found in our cross-sectional study. Other studies showed only weak associations between cross-sectional markers of bone turnover and bone gain in the following year in children[19], while no association in young adults was found[20]. In adults, however, markers of bone turnover have been shown to be independent predictors of fracture risk[21-23] and are especially valuable in predicting BMD response to various therapies[24-26].

What can be the use of serum markers in children? In our longitudinal studies, we found decreased alkaline phophatase (ALP) and osteocalcin levels before start of growth hormonde (GH) treatment in growth hormone deficient (GHD) children (*Chapter 9*). Parameters of bone turnover increased significantly mainly in the first six months of treatment. The change in ALP was the only parameter which correlated with changes in BM(A)D. In children with precocious or early puberty (*Chapter 10*), parameters of bone resorption (ICTP, urine OHP/Cr) as well as bone formation PICP and ALP decreased, mainly the first six months of GnRH-a treatment. After cessation of therapy no substantial changes in bone markers were found. The changes in bone turnover reflected the changes in BMD and in growth velocity.

In newly diagnosed children with ALL (*Chapter 7*) bone formation markers (ALP and PICP) were significantly reduced at diagnosis. During treatment, both formation and as well as resorption markers increased, the net effect however was a decrease in BMD and in height. Moreover, interpretation of various bone markers is complicated because osteocalcin and ICTP are filtered through the glomerular membrane and PICP is cleared by the liver. As a consequence, changes in bone marker levels might reflect impaired renal and liver function due to chemotherapy.

Finally, in children with chronic renal failure (CRF) with or without GH therapy osteocalcin, ICTP and PICP increased in both groups, while ALP increased only in the GH treated children (*Chapter 8*). Interpretation of the parameters of bone turnover in CRF is complicated by peritoneal dialysis[27] and impaired renal clearance.

Bone markers may play a role in improving the care of children with growth disorders, for instance in monitoring or even predicting the effect of growth hormone treatment. Recently, Schönau et al.[28] presented a new prediction model for growth response to GH treatment in GHD children. The best model included a marker of bone resorption, e.g. urinary levels of deoxypyridinoline after 1 month of therapy. The second best model included ALP, PICP and urinary deoxypyridinoline. On the other hand, markers of bone turnover may provide rapid assessment of the side effects on growth and bone. For example, PICP has been shown to decrease shortly after start of glucocorticoid treatment in children[29].

In conclusion, biochemical markers of bone turnover might be valuable tools to evaluate the efficacy of treatment of osteoporosis[24,30-32]. Furthermore, they may provide insight into the pathophysiology of bone disorders. However, no single marker fulfils all the criteria for an ideal marker. Various limitations should be kept in mind, such as the tissue specificity of the marker and sources of variability (stability of the parameter, diurnal variation[33], day-to-day[34] and circannual variation[35]). Most importantly, the markers of bone turnover reflect growth (skeletal modelling) and remodelling in children. Therefore, the markers of bone turnover are difficult to interpret especially in children.

BONE DENSITY IN VARIOUS DISEASES

Children with the following diseases were evaluated:

- long-term survivors of ALL
- acute lymphoblastic leukemia (ALL)
- chronic renal failure
- growth hormone deficiency
- precocious and early puberty

All studies had a longitudinal design, with the exception of the study in long-term survivors of ALL. The results of our studies in ALL patients are summarised in Figure 2. We found normal bone mineral density in long-term survivors of ALL ten years after cessation of ALL treatment according to the ALL6 protocol. However, 39% of the children reported one or more fractures, this is almost twice as much as in our healthy reference group. Most fractures occurred during or immediately after cessation of ALL therapy. Although the sample size was rather small, the strength of this study was that all children were treated with the same (ALL6, dexamethasone-based) protocol. Whereas, most studies comprised long-term survivors of childhood cancer, which include various malignancies and treatment schedules[36-38].

The currently used ALL9 treatment protocol is very similar to the ALL6 protocol. Longitudinal analysis of BMD before, during and 1 year after cessation of ALL therapy revealed substantial changes in BMD. Fracture rate was six times higher compared to healthy controls. Not BMD SD-

score itself but a decrease in lumbar spine BMD in the first six month was associated with higher fracture rate. Observations in adults showed that reduced BMD is only one of the risk factors of fractures[39]. BMD accounts for 75-85% of the capability of bone to resist strain[40], consequently 15-25% may be explained by other skeletal or extraskeletal factors. Bone quality, reflected in bone resorption rate, bone architecture and bone matrix components[22,23,41,42], and the risk of falling or weight[39,43] may play an additional role. Thus, other factors besides BMD may account for the increased fracture risk that we found in children with ALL.

In conclusion, children with ALL have a temporary decrease in BMD and increased fracture rate, predominantly during and shortly after cessation of therapy. The absence of cranial irradiation in the ALL6 and ALL9 protocol might have prevented side effects on bone density on the long term. Because of the substantial increase in fracture rate, preventive measures are recommended in an early stage.

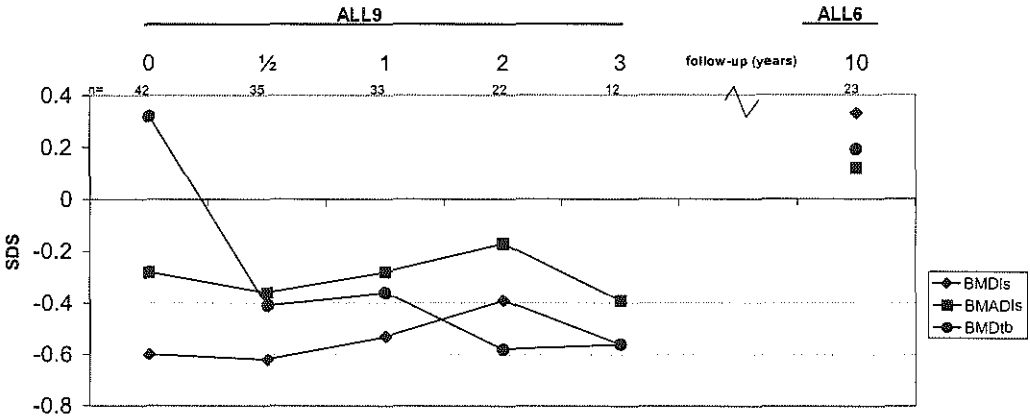

Figure 2. Bone mineral density in ALL

Interestingly, BMD measured with DXA was normal in our patients with CRF. Suggesting that adequate treatment with alphacalcidiol may have prevented detrimental effects on bone. During GH treatment height, BMD and BMAD of lumbar spine increased. In the no-GH group BMD and BMAD remained stable.

Interpretation of non-invasive bone analysis, such as DXA, is especially difficult in children with CRF, because the skeletal changes vary widely. Bone biopsies may show osteomalacia, osteitis fibrosa, and cortical abnormalities. The skeletal changes in CRF are due to hyperparathyroidism and uremia. In CRF bone biopsies show increased woven osteoid instead of lamellar osteoid. Woven osteoid can be mineralised, but the calcium may be deposited as amorphous calcium instead of as hydroxyapatite[44]. As a result, X-ray and DXA will show normal or increased bone density, however, structural changes cannot be excluded using these techniques. Therefore, to assess the real effects of GH treatment in CRF on bone mineralisation, bone biopsies

are needed. Unfortunately, bone biopsies are rather invasive, which makes this method less appropriate for children.

Our findings in GHD children show that patients have decreased BMD before start of GH replacement therapy. Of course these children have short stature, and therefore BMD will be underestimated in DXA measurements. Indeed, BMAD was reduced to a lesser extent. During GH treatment, BMD and BMAD normalised within one to two years (Figure 3).

On the other hand, in children with precocious or early puberty concern has been raised about the detrimental effects on bone mass acquisition during GnRH-a treatment. Our results show that although lumbar spine BMD for chronological age decreased during GnRH-a treatment, it still remained within the normal range. In contrast, BMD corrected for bone age was significantly lower than normal at start and increased during therapy. At cessation of therapy BM(A)D for bone age and chronological age were in the normal range, two years after cessation of therapy all patients showed an ongoing increase in BMD and normal bone turnover.

In conclusion, these two studies suggest that peak bone mass will not be impaired in children with early or precocious puberty with a history of GnRH-a therapy and in GHD children with GH replacement therapy. Furthermore, both studies showed that BMAD, in which a correction for bone size is made, was less affected than BMD. Therefore, both studies underscore the fact that BMAD is a more appropriate parameter than BMD in children with tall or short stature.

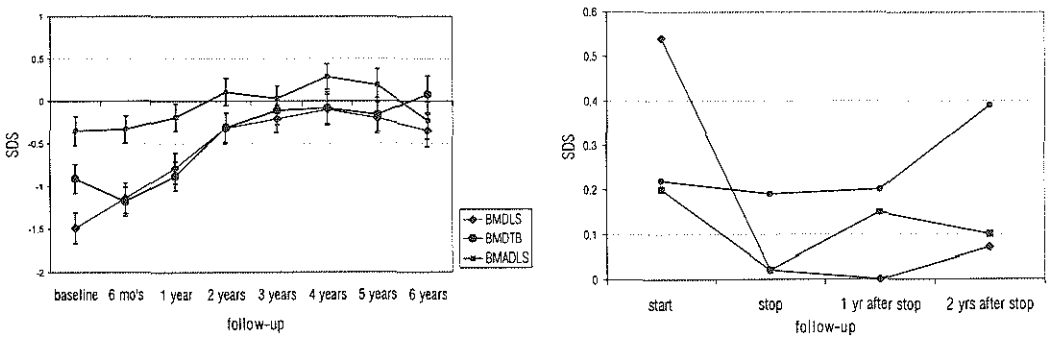

Figure 3. BMD and BMAD in growth hormone deficiency (left panel) and precocious puberty (right panel).

BONE DENSITOMETRY TECHNIQUES

Dual energy X-ray absorptiometry

To date, dual energy X-ray absorptiometry (DXA) is the method of choice to measure BMD. Low radiation dose, great precision, accuracy and a short scanning period make DXA also suitable for children[45]. An important shortcoming of DXA is that it measures an areal density (g/cm^2), as a consequence BMD might be underestimated in short stature and overestimated in tall stature. Mathematical models are used to correct for bone size and calculate bone mineral apparent density (BMAD) or volumetric density. In the model that we used, the lumbar vertebra is assumed to have a cylindrical shape. The volume of a cylinder is $\pi r^2 h = \pi(\text{width}/2)^2(\text{area}/\text{width})$. Thus, BMAD = BMC/volume= BMD x $[4/(\pi \times \text{width})]$[46]. Such corrections are especially required in children with short or tall stature, but remain estimates of the 'real' volumetric density.

An inaccuracy of DXA measurements has been reported. A change in the fat content of the body may affect DXA results[47-49]. In a phantom study, BMC increased with an increase of body weight whereas the derivative BMD decreased with an increase of body weight[49]. In follow-up studies, this might affect BMD measurements in children with weight changes and with changes in body composition.

Computed tomography

In contrast to DXA, computed tomography provides a three-dimensional image, which enables to measure true volumetric density. Furthermore a spatial separation of trabecular from cortical bone is possible. Thus far, only Gilsanz et al.[50,51] have reported the use of QCT of the lumbar spine in larger paediatric study groups. Usually the lumbar spine is measured, a disadvantage of this method is the relatively high radiation dose (70-400 μSv). To avoid this high radiation exposure peripheral quantitative computed tomography (pQCT) can be used. In this technique radiation exposure is reduced to less than 2 μSv[44]. Peripheral QCT may also provide an estimation of bone strength strain index (SSI)[52] and is becoming more widely available nowadays.

Quantitative ultrasound

A rather new method to assess bone density is quantitative ultrasound (QUS). Most commercially available devices measure the speed of sound (SOS) and the loss of amplitude of a sound wave (the broad-band ultrasound attenuation, BUA). QUS measurements can be performed at various sites, mostly used sites are the calcaneus[53] and the phalanges[54], but the cortex of the tibia could also be measured[55]. The SOS is dependent on the density, microstructure, elasticity and the macrostructure of the bone[56]. However, it is until now still not evident what exactly is measured. Advantages of QUS are low costs, ease of use and the absence of radiation. However, during childhood the macrostructure changes constantly, and regions of interest may therefore change during follow-up. To overcome this problem imaging QUS has been suggested. This method places a region of interest (ROI) at the site of the lowest intensity. However, Van den Berg et al.[53], who used this technique in a large group of healthy Dutch children, found only small changes of SOS with increasing age. Therefore, they suggested that calcaneal SOS might not be an appropriate measurement in children.

Radiographic absorptiometry

In radiographic absorptiometry a radiograph of the left hand is taken. An aluminium reference wedge is placed on the film prior to exposure. The optical density of the skeleton is compared to that aluminium wedge[57]. This makes it possible to express the BMD as gram equivalent Al/cm^2. At the department of Radiology of the Erasmus University Rotterdam, this technique was further developed. Two perpendicular views of the second phalanx of the index finger of the left hand are obtained, making it possible to obtain a 'true' volumetric BMD expressed in gram equivalent Al/cm^3. Recently, normative data for Caucasian children have been published[58]. It is a relatively easy and cheap technique, however only the peripheral skeleton is measured.

In general, it is required to have normative data, preferably collected locally, for all types of bone mass measurements. When reference values from other centres are used, one should use

reference data collected with similar software from the same manufacturer. Age-and sex-adjusted Z-scores should be calculated using ethnic–specific data where possible. Besides correction for age and gender, results should be adjusted for height, pubertal stage, or bone age in children with impaired growth or maturation. One should keep in mind that all techniques have their own limitations and (dis)advantages, which should be taken into consideration when interpreting the results.

CLINICAL IMPLICATIONS

Patients at risk

In children, diagnosing osteoporosis in the absence of fractures is not easy as the result of both bone densitometry and biochemical markers need to be interpreted in the light of age, growth and stage of pubertal development. Table 1 in the General introduction shows causes of primary and secondary osteoporosis, which might help to decide if a DXA measurement is indicated. This list, however, is not complete. On the other hand, we do not perform bone densitometry any longer in newly diagnosed GHD children, since our study showed that BMD is reduced at diagnosis, but will normalise during GH replacement therapy. However, it might be necessary to evaluate bone density again in the years after stop of GH therapy when adult height is reached. In patients, who remain GH deficient after final height is reached, the restart of GH therapy might be necessary to maintain bone mass.

In newly diagnosed children with early or precocious puberty no DXAs are performed anymore, because our study suggests that in general GnRH-a therapy will not negatively affect their bone mass acquisition in the long-term.

A large group of patients, who need special attention, are children with diseases that require long-term corticosteroid therapy. Polymorphisms in the glucocorticoid receptor may cause differences in glucocorticoid sensitivity, which resulted in differences in BMD and BMI among the genotypes in healthy adults[59]. In addition to the (chronic) disease which may affect bone density itself, differences in receptor sensitivity may explain the variation in side-effects. For example in children with ALL, we found substantial differences in BMD among patients. In our view, every child with severe inflammatory bowel disease or rheumatoid disease, even without glucocorticoid therapy, should undergo DXA measurements, preferably starting before glucocorticoid treatment is initiated.

Bone densitometry

Although bone densitometry has been performed for decades, it still seems to be a problem interpreting the results. This issue was addressed at the Paediatric Endocrinology Meeting (Montreal 2001)[60]. Thirty-one DEXA scans from children 5-17 years of age who were referred for childhood osteoporosis were reviewed. Eighty-seven % of the scans had at least one misinterpretation. The most frequent error was the use of T-scores (67%) instead of Z-scores. Other errors included neglecting gender and ethnical differences, short stature or delayed puberty. Reinterpretation of the scans revealed that more than fifty percent of the children had normal bone mass. This presentation underscores the need to train paediatricians how to interpret bone densitometry results.

Therapy in general

Although, therapeutic options for osteoporosis in childhood are limited, because of lack of randomised controlled studies, simple measures as adequate calcium and vitamin D intake and stimulating physical activity are applicable for all children in health and disease.

What recommendations can be given to provide adequate calcium intake in children? The recommended dietary allowance for calcium in healthy children depends on age (see Table 2 General introduction). Basic dietary calcium intake without dairy consumption is 200-400 mg. One litre of milk or yoghurt contains 1200 mg calcium, whereas 100 gram cheese contains 600-1000 mg calcium. As one standard dairy consumption is defined as 150 ml of milk or yoghurt (≈180 gram calcium) or 20 mg of cheese (=1 sandwich, ≈120-200 mg calcium), consequently, a calcium intake of 1000 mg/day can be reached by approximately four standard dairy consumptions in addition to the basic dietary calcium intake.

As far as primary osteoporosis is concerned, spontaneous recovery is often seen in idiopathic juvenile osteoporosis[61]. In severe osteogenesis imperfecta cyclic administration of intravenous bisphosphonates reduces bone pain, and fracture rate and increases BMD[62-64].

In secondary osteoporosis, bone mass acquisition may be optimised by treating the underlying disease. For instance, treatment of Cushing's disease[65,66], hyperthyroidism[67,68] and GHD resulted in a substantial increase or even normalisation of BMD.

Children with glucorticoid treatment, for example children with inflammatory bowel disease or rheumatic diseases are at major risk to develop osteoporosis. In these conditions bone density is decreased due to a combination of inflammatory activity, malabsorption, and decreased physical activity. When glucocorticoid therapy is inevitable, general measures are recommended, such as using the minimal effective dose, prescribing inhaled or topical glucocorticoids where possible or using alternate day therapy when systemic therapy is needed. Since corticosteroids reduce intestinal calcium absorption and increase renal calcium excretion[69], an adequate calcium and vitamin D intake should be ensured. It might be necessary to give dietary advice or to prescribe supplementation (500 mg calcium and 400 IU vitamin D/day). Furthermore, physical activity should be encouraged.

Calcium intake and vitamin D

The mean calcium intake in the Netherlands is relatively high compared to recommended dietary allowances[12]. Patients with diseases involving total or partial withdrawal from dairy products for a prolonged period, as in lactose intolerance and cow's milk allergy, are at risk to develop osteoporosis[70]. Several calcium supplementation trials performed in children show that bone mineral density increased after supplementation with calcium-enriched foods[71], calcium salts[72,73], or dairy products[74]. Unfortunately, follow-up studies indicate that after discontinuation of calcium supplements the benefits of supplementation disappear[75,76]. Longer-term trials are needed to determine whether sustained calcium supplementation improves peak bone mass.

Vitamin D is produced in the skin during exposure to sunlight and is present in our diet for example in (fortified) margarine and butter. Firstly, vitamin D is hydroxylated in the liver to 25-hydroxyvitamin D_3, secondly 1,25-dihydroxyvitamin D_3 is formed after an additional hydroxylation step in the kidney. As a consequence, vitamin D deficiency can occur due to malabsorption, malnutrition, lack of sun exposure, or as a result of liver or renal disease. Moreover, Negroid children are at risk for vitamin D deficiency, especially during winter time, because increased skin

pigment reduces vitamin D synthesis in the skin[77]. In these cases vitamin D supplementation should be prescribed.

Physical activity

Physical activity is an important determinant of bone density in children and is therefore recommended for enhancing peak bone mass. Especially high load, weight-bearing activities cause mechanical stress that stimulates new bone formation[13,15,78-82]. As a consequence, disuse osteoporosis can occur in congenital or acquired paraplegia, and cerebral palsy[83,84]. According to Wolff's law, bone adapts to strain applied to it. Muscles cause large loads on bones, and these strains help to control the biological mechanisms that determine whole-bone strength. Therefore, the strength of children's load-bearing bones depend strongly on growing muscle strength and subsequent bone response. In addition some agents, as growth hormone and sex steroids, long thought to exert bone effects solely by acting directly on bone cells, affect muscle strength too. In that way they could affect bone strength indirectly. Thus, muscle and bone are an operational unit, the so-called 'muscle-bone unit'[85,86].

Growth hormone

Growth hormone is essential for normal growth and bone density in children. Children with growth hormone deficiency may have low bone density, but this normalises within one or two years after initiation of GH replacement therapy. The action of GH on bone metabolism is two-fold: it stimulates both bone formation and bone resorption. GH increases bone formation in two ways: via direct stimulation of GH receptors on osteoblasts and via an induction of IGF-I. It is not known how much of the GH effect is mediated by IGF-I, but the direct effects of GH on bone are likely to be limited. Osteoclasts are indirectly activated via paracrine factors derived from the osteoblasts. It is also still unknown, whether osteoclasts express GH receptors. In addition, IGF-I has an anabolic effect on muscles and increase 1α-hydroxylase activity in the kidney, thereby increasing 1,25-dihydroxyvitmin D levels[87,88]. In GHD adults, a biphasic effect of GH treatment on bone turnover has been noticed: initially GH increases bone resorption resulting in bone loss, subsequently bone formation increases. After approximately 6 month, formation is stimulated more than resorption, and a net increase will be found[87,89].

The anabolic effects of GH might also have beneficial effects in glucocorticoid induced osteoporosis or osteoporosis due to chronic diseases. Preliminary results indicate that growth velocity increased during GH treatment in children with juvenile chronic arthritis[90], but the reported effects on bone density are ambiguous[91,92]. One year might be too short a time period to conclude that GH does not increase BMD. Currently, two studies on the effects of GH therapy in children with inflammatory bowel disease or rheumatic diseases with growth retardation and osteopenia, are performed in our department. Preliminary results show promising effects with respect to growth in the growth-retarded children, although BMD did not (yet) show substantial changes. GH treatment for osteoporosis is expensive, but may have additional benefits especially in children who are also growth retarded.

Bisphosphonates

Bisphosphonates inhibit bone turnover by decreasing bone resorption. They do this both directly, by inhibiting the recruitment and function of osteoclasts and by stimulating osteoblasts

to produce an inhibitor of osteoclast formation. They also shorten the life span of osteoblasts[93]. Bisphosphonate therapy is effective in patients with postmenopausal osteoporosis[94] and glucocorticoid induced osteoporosis in adults[95,96]. In severe primary osteoporosis in childhood bisphoshonates are also used.

Short-term studies of bisphosphonates administration in children with connective tissue disease and chronic juvenile arthritis have been published with promising results[97,98]. However, sclerotic epi- and metaphyseal bands have been reported following bisphosphonates therapy in children. Sclerosis was most prominent in loci were growth activity is the largest, but appears to be reversible after closure of the growth plate. Linear growth progressed normally during bisphosphonates therapy and bone biopsies of the iliac crest showed normal mineralisation[99,100]. Nevertheless, long-term controlled studies are needed to assess the risks and benefits of bisphosphonates in children with glucocorticoid induced osteoporosis.

PTH

Parathyroid hormone (PTH) stimulates bone formation and resorption, depending on the mode of administration. Continuous infusion leads to greater bone resorption, than do daily subcutaneous injections. PTH also stimulates 1α-hydroxylase activity in the kidney, thereby increasing 1,25-dihydroxyvitamin D_3 levels[101]. Recently, in a trial involving approximately 1600 postmenopausal women with osteoporosis, a daily subcutaneous injection with human recombinant PTH(1-34) showed an increase in BMD and a decrease in vertebral fracture risk. The PTH injections were well-tolerated[102]. Although osteosarcomas have been reported during long-term PTH administration in young rats, these tumours were not found in the postmenopausal women[102]. Long-term follow-up is needed to study especially the safety of PTH administration. In the future, it might be used in children as well, however caution is recommended especially in the growing child.

FUTURE RESEARCH

Definitions and consensus

Osteoporosis is defined as reduced bone mineral density, deterioration of the micro-architecture of bone tissue, and increased fracture risk[103]. Guidelines have been proposed by the World Health Organisation (WHO)[104] for the interpretation of bone mass measurements in Caucasian women. In children, age- and sex-adjusted Z-scores or standard deviation scores are used to describe bone mass measurements. No consensus on cut-off points for intervention in children is reached yet. Thus, before we can establish guidelines for treatment or prevention, firstly consensus should be reached on the interpretation of measurements in childhood.

Optimising bone mass acquisition during childhood may not only be positive in the short-term, but may also reduce the incidence of osteoporosis and fractures in later life. It seems logical to assume that lower peak bone mass results in a higher risk of osteoporosis later. In fact, only longitudinal studies conducted over 50 to 60 years, are able to test the hypothesis that high peak bone mass protects for osteoporosis in later life.

Genetic studies

To increase statistical power of our study on genetic determinants of bone mass larger study groups are necessary. The group of healthy children and young adults will be enlarged to study various candidate genes, such as polymorphisms in the VDR gene, estrogen receptor gene and COLIA1 gene. Of special interest would be the young adults, who have reached their peak bone mass. In this group the genetic determinants of peak bone mass can be studied. Furthermore, a large study population enables evaluation whether there is any differences in genotype effect between different pubertal stages.

The COLIA1 Sp1 polymorphism is a functional polymorphism. The COLIA1 gene and the COLIA2 gene together code for the two polypeptides that in a 2:1 ratio constitute the collagen type I fibril, which is an important protein of the bone matrix. A disturbance in the synthesis ratio of COLIA1/COLIA2 occurs in T-allele carriers, resulting in a different structure of the collagen type I. In contrast, the *BsmI-ApaI-TaqI* RFLPs of the VDR gene are not functional and merely act as markers through linkage disequilibrium for truly functional sequence variation elsewhere in the gene. Finding functional sequence variants and studying the pathways of these genes is needed in future research.

In the ALL9 study, a large interindividual variability in risk of osteoporosis has been noticed. The major part of the variance in bone mass in healthy people can be explained by genetic factors, for example by polymorphisms in the COLIA1 and VDR gene. We also know that the individual sensitivity to glucocorticoids is variable, due to for instance polymorphisms in the glucocorticoid receptor. These polymorphisms may help us to identify patients at risk for osteoporosis. This will also be one of the aims in the follow-up study in children with ALL.

Densitometry techniques

Conventional X-rays show a reduced radiation transparency in case of osteoporosis. These changes, however, can only be detected after a 20 to 40% reduction in bone mass[105]. Radiogrammetry has been known since the early sixties as a technique that manually measured bone dimensions in a radiograph[106]. Using an X-ray of the hand, the total width and medullary witdh of the metacarpals were measured at the midpoint of the metacarpals. The correlation between these measurements and ashed bone ($r=0.8$), as well as the correlation with bone density at other skeletal site measured with photon absortiometry are quite good[57]. Recently, Pronosco® introduced a fully computerised approach that estimates BMD of the metacarpals II-IV. This method is still developing and even bone age assessment can be performed fully automatically. This relatively inexpensive method would be really suitable for children. Especially since an additional hand X-ray is usually needed, when other bone densitometry techniques are used, to adjust the results for bone age.

Peripheral quantitative computed tomography (pQCT) is also a promising technique. It provides a three-dimensional image, which enables the measurement of true volumetric density. Furthermore, trabecular and cortical bone can be evaluated separately, additionally muscle mass can be measured. A disadvantage of this method is that it only measures peripheral bone density. But the low radiation exposure and short scanning time make this method suitable for children. In the future, good normative data and comparison with other bone densitometry techniques are needed to establish the use of these new methods.

Therapy

In further research the major goal should be to establish the efficacy and safety of therapeutic agents, such as calcium and vitamin D supplements, bisphosphonates, PTH, and growth hormone for prevention and treatment of osteoporosis in childhood. Two ongoing studies are conducted to evaluate the effects of GH therapy on growth and BMD in children with rheumatic diseases and inflammatory bowel disease in the Sophia Children's Hospital in collaboration with other academic centres in the Netherlands. Although preliminary results show less effect on bone density, the time period might have been too short to detect these changes. These two studies will be continued over the next years.

Moreover, the effects of physical training programmes should be evaluated. In the Sophia Children's Hospital an intervention study in children with ALL will start in the near future. Intervention will be based on physical activity programme in combination with calcium and vitamin D supplementation. The programme contains extra short-burst activities with high load, because not duration but short periods of unexpected and irregular high load will stimulate bone formation[78,79,82,107,108].

In general, future research should focus on early detection of young people at risk for osteoporosis allowing early application of adequate preventive or therapeutic measures which may decrease morbidity and health costs both at young and old age. It will be a challenge for paediatricians to address these issues in the near future.

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Chapter 12

SUMMARY

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NEDERLANDSE SAMENVATTING VOOR DE NIET-MEDICUS

SUMMARY

This thesis contains several studies on bone mineral density and body composition in healthy children, and in children with various diseases. Diseases and/or their treatment may affect bone metabolism already at a young age. Consequently, osteoporosis can also occur in childhood and is not solely a disease of the elderly. Reduced bone mineral density (BMD) has been shown to be associated with increased fracture risk in adults as well as in children. As BMD in later life depends largely on peak bone mass achieved in adolescence or young adulthood and on the subsequent bone loss, a high peak bone mass may provide a larger reserve later in life. Thus, impaired bone mass acquisition in childhood may cause osteoporosis and increased fracture risk both at young and at old age.

After a general introduction (*Chapter 1*) the thesis continues with two articles about genetic determinants of anthropometric and bone characteristics, such as height, bone size and bone density. We studied a polymorphism in the collagen Iα1 (COLIA1) gene (*Chapter 2*) and polymorphisms in the vitamin D receptor (VDR) gene (*Chapter 3*).

The COLIA1 Sp1 polymorphism is a functional polymorphism. The COLIA1 gene and the COLIA2 gene together code for the two polypeptides that in a 2:1 ratio constitute the collagen type I fibril, which is an important protein of the bone matrix. A disturbance in the synthesis ratio of COLIA1/COLIA2 occurs in T-allele carriers, resulting in a different structure of the collagen type I. In contrast, the studied polymorphisms in the VDR gene are anonymous and merely act as markers through linkage disequilibrium for truly functional sequence variation elsewhere in the gene. Polymorphisms in the VDR gene and COLIA1 gene both have been shown to affect bone density, fracture risk and bone metabolism in elderly women. These polymorphisms might play a role in bone mass acquisition during childhood, as well. Therefore, we studied the G to T substitution in the Sp1 binding site of the COLIA1 gene in relation to bone mass in 148 Caucasian children and young adults (*Chapter 2*). All but six participated previously in our study on the determinants of bone mass and body composition, approximately 4 years ago. Bone density was measured by dual energy X-ray absorptiometry (DXA), while tibial ultrasound was performed once at follow-up. The genotype distribution was 70% GG, 27% GT and 3% TT, similar to other Caucasian populations. Carriers of the T-allele had significantly lower bone mineral content (BMC) and bone mineral density (BMD). An obvious frame size effect was observed: the association was most apparent for total body BMC, less for BMD of total body and lumbar spine, while no significant association with apparent BMD (BMAD) was found. In these subsequent steps, BMC (gram) is corrected for bone area (BMD, gram/cm^2), and for bone volume (BMAD, gram/cm^3). Moreover, T-allele carriers had shorter stature and smaller bones, as measured by vertebral body width of the lumbar spine. The T-allele was also associated with lower 'Speed of Sound' as assessed by tibial ultrasound. The change in BMD and BMC between the first and second measurement did not differ between the GG and GT&TT group. In conclusion, the COLIA1 polymorphism in children and young adults is associated with several bone characteristics. However, at least a part of the COLIA1 effect on bone mass may be related to differences in frame size and bone quality.

We also studied the association between anthropometric and bone characteristics and vitamin D receptor (VDR) genotype using the *BsmI*, *ApaI*, *TaqI* restriction fragment length polymorphisms (RFLPs, *Chapter 3*). Informative alleles can be combined in multi-allelic markers to monitor three

clustered RFLPs at the VDR gene locus simultaneously. This study was performed in the same Caucasian subjects of the COLIA1 study. We found increased height and increased width of the lumbar vertebral body in the haplotype '3' (=*bAT*) allele carriers. The direct haplotyping procedure showed a trend towards an increased bone mineral apparent density (BMAD) in haplotype '2' allele carriers (=*BAt*) and a decreased BMAD in haplotype '3' allele carriers (=*bAT*). No differences in bone gain between the VDR genotypes were demonstrated. Taller stature and increased bone size in the haplotype '3' allele carriers neutralised the genotype effect on BMD, while a weak association with BMAD, in which BMD is corrected for bone size, was observed. Interestingly, VDR genotype and COLIA1 genotype were found to have an additive effect on height and bone size.

Chapter 4 reports a cross-sectional study on biochemical parameters of bone turnover and vitamin D metabolites in children and young adults. The objective of this study was to gain reference data for calcium, inorganic phosphate, 25-hydroxyvitamin D_3, and 1,25-dihydroxy vitamin D_3, and markers of bone turnover. Blood samples were taken in 176 healthy Dutch children and young adults (7.6-25.3 years) to assess serum calcium, alkaline phosphatase (ALP), inorganic phosphate, osteocalcin, collagen type I cross-linked N-telopeptide (NTx), N-terminal propeptide of type I procollagen (PINP), 25-hydroxyvitamin D_3, and 1,25-dihydroxyvitamin D_3 levels. Cross-linked telopeptide of type I collagen (ICTP) and carboxyterminal propeptide of type I procollagen (PICP) were assessed in 286 subjects (1.4-25.3 years). ALP, osteocaclin, PICP, and PINP are markers of bone formation, while ICTP and NTx are used as markers of bone resorption. Calcium and vitamin D levels were independent of age. The peak concentrations for NTx, ICTP, PICP, PINP, ALP and osteocalcin were found during puberty, in girls approximately 2.5 years earlier than in boys. Strong correlations were found between the markers of bone turnover, while no correlation was found between the markers of bone turnover and bone mineral density measured by dual energy X-ray absorptiometry. We concluded that single measurement of bone markers cannot predict bone density. Reference data according to age, gender and Tanner stage are given, which allow the calculation of standard deviation scores adjusted for age and gender.

Chapter 5 presents new reference data for bone density and body composition as assessed by DXA. Previously, 500 healthy Dutch children 4 to 20 years of age (444 Caucasians) were studied to gain reference values. 198 Caucasian children were willing to participate in the follow-up study. The mean follow-up time was approximately 4 years. The results of the first and second study were combined to expand the reference data for bone mineral density and body composition in children and young adults. Bone density and lean body mass increased with age, with a maximal increase around the age of 13 years in girls and approximately two years later in boys. Most of the skeletal mass in lumbar spine and total body was reached before the end of the second decade, with a slight increase thereafter. Reference data according to gender, age and Tanner stage are given.

Since childhood leukemia has increasing numbers of survivors more emphasis is being placed on side effects. In Chapter 6 bone mineral density, body composition and height were evaluated in 23 children and young adults, who were diagnosed with acute lymphoblastic leukemia (ALL) approximately ten years ago and were all in first remission. The ALL-6 protocol of the Dutch

Childhood Leukemia Study Group involved high dose dexamethasone and methotrexate, and no cranial irradiation. BMD and body composition were measured by DXA. We found that mean BMD and BMAD were normal. None of the subjects had BMD below –2 SDS, only one subject had a BMAD below –2 SDS, which was within the normal range 1.5 years thereafter. Mean SDS for lean body mass, percentage fat and height were not significantly different from zero. However, nine subjects reported traumatic fractures (8 during or shortly after therapy). Thus, ten years after ALL-6 treatment no long-term side effects on height, bone density and body composition were found, despite high dose dexamethasone and methotrexate. This study suggests that ALL treatment without cranial irradiation may not be associated with long-term side effects on growth and BMD. The ALL-6 protocol was a moderately intensive treatment protocol without cranial irradiation and proved to be very successful with respect to survival rate. Therefore, the protocol was reintroduced in the Netherlands in 1997 as the currently used ALL-9 protocol.

We also evaluated 61 newly diagnosed children with ALL, who were treated according to the ALL-9 protocol (*Chapter 7*). BMD and body composition were measured by DXA and blood samples were taken to assess parameters of bone turnover. All measurements were performed at diagnosis, after 32 weeks, one year, 2 years (cessation of therapy) and one year after cessation of therapy. Lumbar spine BMD was significantly reduced at diagnosis, and remained low during therapy. Total body BMD was normal at diagnosis, with a fast decrease mainly in the first 32 weeks, in which chemotherapy was relatively intensive. BMAD was reduced as well, but this did not reach significance at diagnosis and during follow-up. Bone formation markers were reduced at diagnosis, and formation as well as resorption markers increased during treatment. Fracture rate was 6 times higher in ALL patients compared to healthy controls. Most fractures were located in the extremities, but one boy had a compression fracture of the thoracic vertebrae. Not the BMD SD-score itself, but a decrease in lumbar spine BMD in the first six months was associated with higher fracture risk. Substantial changes in body composition were observed. Lean body mass was already decreased at baseline. % Body fat increased significantly during therapy. After ALL treatment was completed, BMD, body composition and height tended to improve.

Thus, children with ALL are at risk for osteoporosis due to the disease itself and the intensive chemotherapy. It should be stressed that fracture rate was 6 times higher in ALL children compared to healthy controls, not only during but also shortly after treatment. Future research should be focussed on identifying patients at risk for osteoporosis and prevention of the very high fracture rate during and shortly after cessation of treatment.

Metabolic bone disease and growth retardation are common complications of chronic renal failure (CRF). In *Chapter 8* the effects of growth hormone treatment on bone density, body composition and growth are discussed. Thirty-three prepubertal patients with CRF were enrolled. Eighteen children had growth retardation, they were treated with growth hormone (GH group). The other 15 children met all the inclusion criteria, but were not growth retarded and did not receive GH (no-GH group). Every six months, BMD and body composition were measured by DXA and biochemical parameters of bone turnover were assessed. At baseline, mean BMD of children with CRF did not differ from normal. During GH therapy, BMD and BMAD of lumbar spine and height SDS increased, whereas BMD of total body did not change. Lean body mass increased in the GH group. Alkaline phosphatase increased significantly in the GH group only, while the other

biochemical parameters of bone turnover increased in both groups. None of the biochemical parameters correlated with the changes in BMD. Fortunately, no serious adverse effects of GH therapy were reported. Thus, BMD of children with CRF did not differ from healthy children. Adequate treatment with alpha-calcidiol or the short duration of renal failure may have attributed to the absence of osteopenia in our patients. BMD of the axial skeleton and height increased with GH therapy.

Growth hormone is essential for normal growth in children, but it also affects bone mineralisation and body composition. *Chapter 9* reports BMD, body composition, and serum lipid levels in 59 growth hormone deficient (GHD) children during 6 years of GH treatment. BMD and body composition were measured using DXA at diagnosis, after six months and yearly thereafter. We found a significant reduced mean BMD of lumbar spine and total body at diagnosis. BMAD was reduced as well, but in a much lesser degree. BMD and BMAD increased significantly during GH therapy. BMAD was normalised after one year of treatment and lumbar spine and total body BMD one year later. At diagnosis, lean body mass was reduced and steadily increased during GH treatment. %Fat was increased at baseline and normalised within the first six months. Only alkaline phosphatase and osteocalcin were decreased at diagnosis. Parameters of bone turnover showed a significant increase mainly in the first six months of GH therapy. The change in alkaline phosphatase was the only parameter of bone turnover, which correlated with the change in BMD and BMAD in the first six months. The change in 1,25-dihydroxyvitamin D_3 was significantly correlated with the change in BMAD and BMD of lumbar spine. Bone mineral content highly correlated with lean body mass. Mean lipid levels were normal at diagnosis. During GH therapy, HDL increased, whereas the atherogenic index and LDL decreased. In conclusion, areal BMD of lumbar spine and total body, and to a lesser extent BMAD, are decreased in GHD children, but normalise within one and two years. The increase in lean body mass as well as the increase in 1,25-dihydroxyvitamin D_3 might mediate the positive influence of GH on bone mineralisation. Furthermore, GH therapy has beneficial effects on body composition and serum lipid levels in GHD children.

Chapter 10 reports BMD, bone metabolism and body composition in 47 children with central precocious puberty (n=36) or early puberty (n=11) before, during and after cessation of gonadotrophin-releasing hormone agonist (GnRH-a) therapy. GnRH-a treatment inhibits pituitary gonadotrophin secretion resulting in a decrease of sex steroids levels and suppression of pubertal development. The decrease in sex steroids may impair bone mass acquisition. Measurements were performed at baseline, after six months, and on a yearly basis thereafter. Mean lumbar spine BMD corrected for chronological age was significantly higher than zero at baseline, and decreased during treatment. BMAD of lumbar spine and total body BMD did not differ from normal at baseline and showed no significant changes during treatment. In contrast, BMD corrected for bone age was significantly lower than zero at baseline and at stop of therapy. Two years after therapy BMD and BMAD corrected for bone age and chronological age did not differ from normal. Markers of bone turnover decreased during treatment, mainly in the first 6 months. Patients had increased %fat and lean body mass at baseline. After an initial increase of %fat during treatment, %fat decreased and normalised within one year after stop of treatment. Thus, our

longitudinal analysis suggests that peak bone mass or body composition will not be impaired in patients with precocious or early puberty after GnRH-a therapy.

Finally, *Chapter 11* discusses the results of our studies, and puts them into perspective of current knowledge. In addition, clinical implications are discussed and suggestions for future research are given.

SAMENVATTING VOOR DE NIET-MEDICUS

Dit proefschrift bestaat uit diverse onderzoeken naar botdichtheid en lichaamssamenstelling bij gezonde kinderen, maar ook bij kinderen met diverse ziekten. Met botdichtheid wordt de kalkhoudendheid van het skelet bedoeld. De botdichtheid werd in dit onderzoek gemeten met "dual energy X-ray absorptiometry" (DXA-scan). Dit röntgenologisch onderzoek duurt 20 tot 30 minuten. De botdichtheid van de lendenwervels en het hele lichaam werd gemeten. Een bijkomend voordeel van de meting van het hele lichaam is, dat behalve de botdichtheid ook de lichaamssamenstelling wordt gemeten. Er is dus direct bekend hoeveel vet en vetvrije massa (dit bestaat met name uit spiermassa) het kind heeft.

De DXA-scan geeft diverse resultaten van wat wij de 'botdichtheid' noemen. Allereerst de 'bone mineral content' (BMC), dit is de totale hoeveelheid mineraal wat in het bot zit en wordt uitgedrukt in gram. Hoe ouder en dus groter een kind is, hoe hoger de BMC zal zijn. Door de BMC te delen door het totale botoppervlak (cm^2) dat is gemeten, krijgen we de 'botminerale dichtheid' (BMD) in $gram/cm^2$. Bij de BMD wordt dus al gedeeltelijk gecorrigeerd voor de grootte van het kind, maar het is nog geen dichtheid! Dichtheid impliceert een driedimensionale meting uitgedrukt in $gram/cm^3$. Indien bij twee kinderen de echte botdichtheid constant wordt gehouden zal de BMD bij een klein kind onderschat en bij een groot kind overschat worden. Dit levert met name problemen op bij kinderen met groeistoornissen. Dit probleem kan grotendeels worden opgelost door met behulp van een formule de 'bone mineral apparent density' (BMAD, in $gram/cm^3$) te berekenen. In deze berekening wordt de grootte van het bot meegenomen en kan gecorrigeerd worden voor de botgrootte/lengte van de kinderen. Deze methode wordt binnen de kindergeneeskunde veel gebruikt en is gevalideerd met driedimensionale metingen verricht met een MRI-scan.

Diverse ziekten en de behandeling van ziekten kunnen de botstofwisseling reeds op jonge leeftijd beïnvloeden. Daardoor kan osteoporose (botontkalking) ook op jonge leeftijd voorkomen en is het niet alleen een ouderdomsziekte. Studies hebben aangetoond dat een lage botdichtheid de kans op botbreuken verhoogt bij volwassenen maar ook bij kinderen. Tijdens de kinderleeftijd neemt de botmassa toe totdat de piekbotmassa wordt bereikt op de jongvolwassen leeftijd. De botdichtheid op oudere leeftijd is voor een groot deel afhankelijk van deze piekbotmassa en het botverlies dat daar op volgt. Een slechte botopbouw tijdens de kinderjaren kan dus osteoporose en een verhoogd risico op botbreuken veroorzaken op jonge en oudere leeftijd.

Genetische factoren

Botdichtheid wordt voor een belangrijk deel bepaald door genetische factoren. Collageen type I is een eiwit dat veel voorkomt in bot en vitamine D speelt een belangrijke rol in de botstofwisseling. Daarom bestudeerden wij polymorfismen in twee genen, het collageen Iα1 (COLIA1) gen en het vitamine D receptor (VDR) gen, die een rol kunnen spelen in de botstofwisseling. Een polymorfisme is een genetische variatie die bij meer dan 1% van de bevolking voorkomt. Het COLIA1 Sp1 polymorfisme is een functioneel polymorfisme, dat wil zeggen dat een aanwezige verandering in het gen resulteert in een verandering van de structuur van de collageen vezels in het bot. Op dit moment is de functionele betekenis van het VDR polymorfisme nog onbekend.

Het is aangetoond dat polymorfismen in het VDR gen en het COLIA1 gen geassocieerd zijn met verschillen in botdichtheid, fractuurrisico en botmetabolisme bij ouderen. Deze polymorfismen zouden echter ook een rol kunnen spelen tijden de botopbouw gedurende de kinderjaren. Om dit te bestuderen werden bij 148 gezonde kinderen botdichtheidsmetingen verricht met DXA en echo van de tibia (scheenbeen), en werd bloed afgenomen voor de genetische bepalingen. Indien het COLIA1 polymorfisme aanwezig is, is in het gen een guanine vervangen door een thymine base (de G naar T substitutie). In iedere cel zijn twee kopieën aanwezig, namelijk gelegen op de twee chromosomen afkomstig van de vader en van de moeder. Een enkele kopie van het gen noemen we een 'allel' en de set van twee allelen noemen we een 'genotype'. Omdat een genotype wordt bepaald door twee allelen, zijn er 3 verschillende COLIA1 genotypen, t.w. GG, GT en TT. In onze studie (*Hoofdstuk 2*) was de verdeling van de genotypen 70% GG, 27% GT en 3% TT. Dit is vergelijkbaar met andere blanke populaties. Dragers van het T-allel (kinderen met een GT of TT genotype) hadden een lagere BMC en BMD, het verschil in BMAD was echter niet meer significant. Opvallend was hoe meer er gecorrigeerd werd voor botgrootte hoe kleiner de verschillen tussen de GG en de gecombineerde GT&TT groep werden. Bovendien bleken dragers van een T-allel een kortere lengte en kleinere botten te hebben. De botgrootte werd bepaald door de breedte van de lendenwervels te meten. Middels echogafisch onderzoek van de tibia (scheenbeen) werd gemeten hoe snel een geluidsgolf door het bot gaat. Hoe sneller een geluidsgolf gaat, hoe hoger de botdichtheid. Daarnaast kan de echo mogelijk ook informatie verschaffen over de kwaliteit van het bot. Het T-allel was geassocieerd met lagere snelheid van de geluidsgolf. Wij concludeerden dat het COLIA1 polymorfisme bij kinderen en jongvolwassenen geassocieerd is met diverse botkarakteristieken. Echter een deel van het COLIA1 effect op bot is gerelateerd aan verschillen in botgrootte en de kwaliteit van het bot.

In *hoofdstuk 3* bestudeerden wij bij dezelfde groep kinderen ook de associatie tussen lengte, botgrootte, en botdichtheid en VDR polymorfismen. In het VDR gen werden drie nabij gelegen polymorfismen onderzocht, nl. de *BsmI, ApaI* en *Taq-I*-RFLPs. Combinaties van allelen op die drie plaatsen noemen we een 'haplotype'. Twee haplotypen combineren ook weer tot een genotype. Kinderen met een bepaalde haplotype (haplotype '3') in het VDR gen bleken langer te zijn en bredere wervels te hebben. Er werden geen verschillen in BMC en BMD geconstateerd, de BMAD was echter wel enigszins verlaagd in haplotype '3'. Doordat kinderen met haplotype '3' langer zijn en grotere botten hebben werd het effect op BMD geneutraliseerd, terwijl er wel een lagere BMAD in haplotype 3 carriers werd gevonden, waarin gecorrigeerd wordt voor de botgrootte. Omdat de drie VDR polymorfismen voor zover bekend niet functioneel zijn, wordt wel aangenomen dat ze als marker dienen voor een daadwerkelijk functioneel polymorfisme dat elders in het VDR gen moet liggen. Een interessante bevinding was dat wanneer de COLIA1 en VDR polymorfismen gecombineerd werden, er een additief effect op lengte en botgrootte werd gevonden.

Botmetabolisme en botdichtheid bij gezonde kinderen

Voor veel onderzoek geldt dat voordat er conclusies ten aanzien van patiëntengroepen mogelijk zijn, eerst bekend moet zijn hoe de resultaten zouden zijn bij gezonde personen. In *hoofdstuk 4* worden referentiewaarden gepresenteerd voor diverse markers van botombouw en voor vitamine D metabolieten, die in het bloed gemeten kunnen worden. Markers van de botombouw zijn te verdelen in markers van de botopbouw en markers van de botafbraak. Er werden normaalwaarden verzameld voor calcium, anorganisch fosfaat, 25-hydroxyvitamine D, en 1,25-dihydroxyvitamine

D. Als markers van de botopbouw werden alkalisch fosfatase, osteocalcine, PICP, en PINP bepaald. Ntx, en ICTP werden bepaald als markers van de botafbraak. De vitamine D- en calciumspiegels waren onafhankelijk van de leeftijd. Er werd een duidelijke piekconcentratie tijdens de puberteit gezien voor Ntx, ICTP, PICP, PINP, osteocalcine en alkalisch fosfatase, bij meisjes ongeveer 2.5 jaar eerder dan bij jongens. Alhoewel er een sterke correlatie tussen de markers van botombouw werd gevonden, correleerden de botmarkers niet met de botdichtheid gemeten met DXA. Wij concludeerden dat één meting van de botmarkers de botdichtheid niet kan voorspellen. In dit hoofdstuk worden de referentiewaarden naar puberteitsstadium gerapporteerd en is het mogelijke om leeftijds- en geslachtsgecorrigeerde standaarddeviatiescores te berekenen.

In *hoofdstuk 5* worden nieuwe referentiewaarden voor botdichtheid en lichaamssamenstelling gemeten met DXA gepresenteerd. In 1994-1995 werden bij 404 blanke kinderen van 4 tot 20 jaar DXA scans verricht om referentiewaarden te verkrijgen. Ongeveer 4 jaar later werden alle kinderen opnieuw gevraagd of ze met een vervolgstudie mee wilden doen, 198 kinderen wilden nog een keer participeren. De data van de eerste en tweede studie werden gecombineerd om de referentiewaarden voor leeftijd, geslacht en puberteitsstadium uit te breiden. Er werd een leeftijdsafhankelijke toename van botdichtheid en spiermassa gevonden, met een maximale toename rond de 13 jaar bij meisjes en bij jongens ongeveer 2 jaar later. Het grootste gedeelte van de botmassa van het hele lichaam en van de lendenwervels is opgebouwd voor het twintigste levensjaar, vervolgens wordt nog slechts een lichte toename gevonden.

Leukemie

Tegenwoordig is het overlevingspercentage van acute lymfatische leukemie op de kinderleeftijd ongeveer 80%. De behandeling probeert men door voortdurend onderzoek nog steeds te verbeteren. Door de goede overleving wordt onderzoek naar de negatieve effecten van de behandeling op korte en lange termijn steeds belangrijker. Eén van de bijwerkingen van de behandeling is osteoporose, maar ook door de ziekte zelf kunnen kinderen osteoporose ontwikkelen. Verder kunnen de kinderen door de behandeling slechter gaan groeien en te dik worden. In *hoofdstuk 6* worden de resultaten besproken. Ongeveer 10 jaar geleden werden kinderen in Nederland behandeld volgens het 'ALL6'-protocol. Dit protocol was zeer succesvol en werd daarom geherintroduceerd in 1997 (het ALL9-protocol). De behandeling duurt twee jaar en bestaat uit een combinatie van chemotherapeutica. Er wordt tegenwoordig geen schedelbestraling meer gebruikt. Wij evalueerden de botdichtheid, lichaamssamenstelling en lengte van de kinderen en jongvolwassenen die in het verleden leukemie hebben gehad en destijds allen volgens het ALL6-protocol zijn behandeld. De gemiddelde botdichtheid van de groep was normaal. Slechts 1 van de 23 kinderen had een te lage botdichtheid. Het vetpercentage, de spiermassa, en de lengte van de kinderen waren ook normaal. Echter 9 kinderen hadden één of twee botbreuken gehad, tijdens of vlak na het staken van de behandeling. Dit is ongeveer 2x zoveel als wij zouden verwachten bij gezonde kinderen. Wij concludeerden dat er op lange termijn geen verhoogd risico is op botontkalking, kleine lichaamslengte of overgewicht. Gezien het hoge aantal botbreuken is er zeer waarschijnlijk wel een passagère botdichtheidsverlaging geweest.

Dit werd bevestigd in een tweede onderzoek bij kinderen met ALL die nieuw gediagnosticeerd zijn (*Hoofdstuk 7*). Dit maakte het mogelijk de patiënten voor, tijdens en na hun behandeling te vervolgen. De botdichtheid van de lendenwervels was reeds verlaagd bij diagnose en bleef laag

tijdens de behandeling. De gemiddelde botdichtheid van het hele lichaam was normaal bij diagnose, maar liet een sterke daling zien in de eerste 32 weken van de behandeling. In deze periode is de chemotherapie zeer intensief. De botstofwisseling was verlaagd bij diagnose. Het aantal botbreuken was 6 keer hoger dan bij onze gezonde vrijwilligers. De meeste botbreuken waren gelokaliseerd in de ledematen, één jongen had echter een ingezakte wervel. Grote veranderingen in lichaamssamenstelling werden gevonden. De spiermassa was afgenomen bij diagnose en bleef verlaagd, terwijl het vetpercentage sterk toenam tijdens de behandeling. Eén jaar na het stoppen van de therapie leek de botdichtheid en lichaamssamenstelling weer enigszins te verbeteren.

Uit de twee onderzoeken bij kinderen met ALL werd geconcludeerd dat deze patiënten een verhoogd risico op osteoporose hebben door de ziekte zelf en de chemotherapie. Bovendien was het aantal botbreuken tijdens of kort na stoppen van de behandeling sterk verhoogd. Verder onderzoek moet zich dan ook vooral richten op het identificeren van kinderen met een verhoogd risico en de preventie van botbreuken tijdens en kort na het stoppen van de behandeling.

Chronische nierinsufficiëntie

Metabole botziekten en groeiretardatie zijn veel voorkomende complicaties bij kinderen met chronische nierinsufficiëntie. In *hoofdstuk 8* worden de effecten van groeihormoonbehandeling op botdichtheid, lichaamssamenstelling en groei besproken. Aan dit onderzoek deden 33 kinderen mee. Na een half jaar werd gekeken welke kinderen een ernstige groeivertraging hadden en zij werden behandeld met groeihormoon (18 kinderen), de resterende 15 kinderen fungeerden als controle groep. Om de zes maanden werd er een DXA-scan verricht en werd bloed afgenomen voor onderzoek. Bij start van het onderzoek bleek de gemiddelde botdichtheid bij beide groepen normaal te zijn. Tijdens groeihormoonbehandeling nam de lengte en de botdichtheid van de lendenwervels toe, terwijl de botdichtheid van het hele lichaam niet veranderde. Ook de spiermassa nam toe tijdens groeihormoonbehandeling. Alkalische fosfatase nam alleen toe in de groep met groeihormoonbehandeling, de andere biochemische botmarkers stegen in beide groepen, geen van de markers correleerde met een verandering in botdichtheid. Door o.a. de verminderde nierfunctie zijn de biochemische botmarkers echter moeilijk te interpreteren bij kinderen met chronisch nierinsufficiëntie. Tegenwoordig worden kinderen behandeld met vitamine D om metabole botziekte te voorkomen. Deze vitamine D behandeling en het relatief kort bestaan van de nierinsufficiëntie bij kinderen kunnen ertoe hebben bijgedragen dat er, in tegenstelling tot wat vaak beschreven wordt bij volwassen nierpatiënten, een normale botdichtheid bij onze patiënten werd gevonden.

Groeihormoondeficiëntie

Groeihormoon is essentieel voor normale groei van kinderen, maar het heeft ook effect op botmineralisatie en lichaamssamenstelling. Bij kinderen met een tekort aan groeihormoon (groeihormoondeficiëntie) kan dit dus vele effecten hebben. In *hoofdstuk 9* wordt de botdichtheid en lichaamssamenstelling beschreven bij 59 kinderen met groeihormoondeficiëntie voor en tijdens groeihormoonbehandeling. Metingen werden verricht bij diagnose, na een half jaar groeihormoonbehandeling en vervolgens jaarlijks. De botdichtheid (BMD) was significant verlaagd bij diagnose, de BMAD was ook verlaagd echter in mindere mate. BMAD en BMD namen toe tijdens groeihormoonbehandeling en normaliseerden respectievelijk binnen 1 à 2 jaar. De

spiermassa was verlaagd bij diagnose en nam toe tijdens behandeling. Het vetpercentage was verhoogd bij diagnose, maar dit normaliseerde binnen 6 maanden. De verandering in alkalisch fosfatase was de enige biochemische botmarker die correleerde met de verandering in botdichtheid. Daarnaast was er een associatie tussen de verandering in vitamine D spiegels en de verandering in botdichtheid. De gemiddelde lipiden(vet)-spiegels in het bloed waren normaal bij diagnose en de profielen verbeterden tijdens groeihormoonbehandeling. Wij concludeerden dat BMD en in mindere mate BMAD zijn verlaagd bij kinderen met groeihormoondeficiëntie, maar dat dit normaliseert na 1 à 2 jaar groeihormoonbehandeling. Zowel de toename in spiermassa als de toename in vitamine D spiegels hebben een positief effect op de botmineralisatie. Bovendien heeft groeihormoontherapie een positief effect op de lichaamssamenstelling en lipidenspiegels.

Pubertas praecox

Hoofdstuk 10 beschrijft de effecten van de behandeling van centrale pubertas praecox (vroege puberteit) op botdichtheid, botmetabolisme en lichaamssamenstelling. De behandeling van pubertas praecox bestaat uit gonadotrofine releasing hormoon agonisten (GnRH-a). GnRH-a behandeling remt de afgifte van gonadotrofinen (de hormonen LH en FSH) door de hypofyse wat resulteert in een verlaging van de geslachtshormonen en onderdrukking van de puberteitsontwikkeling. De daling van geslachtshormonen zou ook de botopbouw negatief kunnen beïnvloeden. Om dit te onderzoeken werden bij 47 kinderen met pubertas praecox DXA-scans verricht en biochemische botparameters bepaald bij start van de behandeling, na een half jaar en vervolgens jaarlijks. Na het stoppen van de behandeling werden de metingen gecontinueerd. De botdichtheid van de lendenwervels was verhoogd bij starten van de behandeling, en nam af tijdens GnRH-a behandeling. De BMAD en botdichtheid van het hele lichaam waren niet hoger dan normaal, en toonden geen significante veranderingen tijdens de behandeling. Twee jaar na stoppen van de behandeling was de BMD en BMAD normaal. Tijdens de behandeling, vooral in de eerste 6 maanden, nam de botombouw af. Bij start van de behandeling was het vetpercentage verhoogd. Na een toename tijdens de behandeling, nam het vetpercentage weer af na stoppen van de behandeling en normaliseerde het binnen 1 jaar. Dit longitudinale onderzoek suggereert dat de piekbotmassa en de lichaamssamenstelling op de lange termijn niet aangedaan zullen zijn bij kinderen die een behandeling ondergaan voor pubertas praecox.

Tenslotte worden in *hoofdstuk 11* de resultaten van de verschillende studies besproken en worden er aanbevelingen gedaan voor verder onderzoek.

ABBREVIATIONS

ALL	acute lymphoblastic leukemia
ALP	alkaline phosphatase
Apo-A1	apolipoprotein A1
Apo-B	apolipoprotein B
BMAD	bone mineral apparent density
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
Ca/Cr	ratio of calcium and creatinine in urine
COLIA1	collagen Iα1
CPP	central precocious puberty
CRF	chronic renal failure
DXA	dual energy x-ray absorptiometry
FFA	free fatty acids
FSH	follicle stimulating hormone
GH	growth hormone
GHD	growth hormone deficiency
GHRx	growth hormone treatment
GnRH-a	gonodotrophin-releasing hormone agonist
HDL	high-density lipoprotein
ICTP	cross-linked telopeptide of type I collagen
IGF-I	insulin-like growth factor 1
IGFBP-3	insulin-like growth factor binding protein 3
IRMA	immuno radiometric assay
IU	international units
LBM	lean body mass
LDL	low-density lipoprotein
LH	luteinizing hormone
LS	lumbar spine
MTX	methotrexate
NTx	collagen type I cross-linked N-telopeptide
OHP/Cr	hydroxyproline concentration expressed as mmol/mol creatinine
PICP	carboxyterminal propeptide of type I procollagen
PINP	amino-terminal propeptide of type I procollagen
PTH	parathyroid hormone
RFLP	restriction fragment length polymorphism
RIA	radio immuno assay
Rx	treatment
SD	standard deviation
SDS	standard deviation score
SE	standard error (SEM: standard error of the mean)
SOS	speed of sound

TB	total body
TC	total cholesterol
TG	triglycerides
TH	target height
VDR	vitamin D receptor
VLDL	very low-density lipoprotein
1,25 diOH vit D	1,25-dihydroxyvitamin D_3
25 OH vit D	25-hydroxyvitamin D_3
% fat	percentage body fat

CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 15 juni 1970 te Kampen. Vanaf 1982 bezocht zij het Stedelijk Lyceum in Zutphen en behaalde in 1988 haar VWO-diploma. In 1988 begon zij enthousiast aan de studie Gezondheidswetenschappen aan de Katholieke Universiteit Nijmegen, maar al snel werd duidelijk dat de studie Geneeskunde haar voorkeur had. In 1989 startte zij aan dezelfde universiteit haar studie Geneeskunde, 4 jaar later werd het doctoraalexamen (cum laude) behaald. Tijdens haar studie verrichtte zij onderzoek bij de afdelingen kindergeneeskunde en dermatologie. Na het arts-examen (cum laude) in 1996 was zij één jaar werkzaam als arts-assistent kindergeneeskunde in het Merwedeziekenhuis (Albert Schweitzer Ziekenhuis) te Dordrecht. In juni 1997 begon zij als assistent-in-opleiding (AIO) aan haar promotieonderzoek onder begeleiding van Dr. S.M.P.F. de Muinck Keizer-Schrama bij de afdeling Kinderendocrinologie van het Sophia Kinderziekenhuis Rotterdam (hoofd: Prof.dr. S.L.S. Drop) in samenwerking met de afdeling Radiologie van het Academisch Ziekenhuis Rotterdam (hoofd: Prof.dr. G.P. Krestin). Zij is bestuurslid van de DRCO (Dutch Registrar's Club in Osteoporosis) en zij is lid van de landelijke botcommissie van de Stichting Nederlandse Werkgroep Leukemie bij Kinderen (SNWLK), die onderzoek verricht naar botdichtheid en avasculaire botnecrose bij kinderen met leukemie. Haar onderzoek naar botdichtheid en lichaamssamenstelling bij kinderen met acute lymfatische leukemie werd in 2001 bekroond met de 'Dr de Vaanprijs'. Sinds 1 oktober 2001 is zij werkzaam als arts-assistent kindergeneeskunde in het Sophia Kinderziekenhuis te Rotterdam (hoofd: Prof.dr. H.A. Büller).

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Appendix

REFERENCE VALUES FOR BONE MINERAL DENSITY AND BODY COMPOSITION

Mean bone mineral density values of lumbar spine (BMD_{LS}mean g/cm^2), bone mineral apparent density ($BMAD_{LS}$mean g/cm^3) and bone mineral density of total body (BMD_{TB}mean g/cm^2) and standard deviations (SD) in boys and girls.

Boys						
Age (years)	$BMAD_{LS}$mean	SD	BMD_{LS}mean	SD	BMD_{TB}mean	SD
4-4.9	0.250	0.036	0.592	0.062	0.799	0.029
5-5.9	0.262	0.036	0.631	0.067	0.819	0.034
6-6.9	0.269	0.036	0.665	0.073	0.839	0.038
7-7.9	0.273	0.036	0.694	0.078	0.859	0.043
8-8.9	0.276	0.036	0.719	0.084	0.880	0.048
9-9.9	0.278	0.036	0.742	0.089	0.900	0.053
10-10.9	0.280	0.036	0.764	0.095	0.920	0.057
11-11.9	0.282	0.036	0.791	0.100	0.942	0.062
12-12.9	0.285	0.036	0.828	0.106	0.967	0.067
13-13.9	0.290	0.036	0.886	0.111	1.000	0.072
14-14.9	0.300	0.036	0.968	0.117	1.045	0.076
15-15.9	0.315	0.036	1.064	0.123	1.103	0.081
16-16.9	0.332	0.036	1.152	0.128	1.158	0.086
17-17.9	0.349	0.036	1.214	0.134	1.200	0.091
18-18.9	0.360	0.036	1.251	0.139	1.229	0.096
19-19.9	0.367	0.036	1.271	0.145	1.251	0.100
20-20.9	0.370	0.036	1.281	0.150	1.270	0.105
21-21.9	0.372	0.036	1.286	0.156	1.287	0.110
22-22.9	0.373	0.036	1.289	0.162	1.305	0.115

Girls						
Age (years)	$BMAD_{LS}$mean	SD	BMD_{LS}mean	SD	BMD_{TB}mean	SD
4-4.9	0.280	0.023	0.631	0.055	0.790	0.048
5-5.9	0.284	0.025	0.660	0.063	0.809	0.051
6-6.9	0.288	0.027	0.689	0.070	0.827	0.053
7-7.9	0.293	0.029	0.718	0.078	0.845	0.056
8-8.9	0.297	0.031	0.747	0.086	0.864	0.058
9-9.9	0.302	0.032	0.779	0.094	0.886	0.061
10-10.9	0.309	0.034	0.819	0.102	0.913	0.063
11-11.9	0.319	0.036	0.876	0.109	0.947	0.066
12-12.9	0.335	0.038	0.957	0.117	0.990	0.068
13-13.9	0.355	0.040	1.049	0.125	1.036	0.071
14-14.9	0.372	0.042	1.128	0.133	1.079	0.073
15-15.9	0.383	0.043	1.181	0.141	1.114	0.076
16-16.9	0.391	0.045	1.214	0.148	1.139	0.078
17-17.9	0.396	0.047	1.236	0.156	1.156	0.081
18-18.9	0.401	0.049	1.252	0.164	1.168	0.083
19-19.9	0.406	0.051	1.265	0.172	1.177	0.086
20-20.9	0.410	0.052	1.277	0.180	1.184	0.088
21-21.9	0.415	0.054	1.287	0.187	1.190	0.091
22-22.9	0.419	0.056	1.297	0.196	1.196	0.093

Mean lean body mass values (LBMmean gram), bone mineral content of total body (BMC_{TB}mean, gram), and percentage body fat (%fat), width of the lumbar vertebral body (cm) and standard deviations (SD) in boys and girls.

Boys							width	
Age (years)	LBMmean	SD	BMCmean	SD	ln(%fatmean)*	Ln(%fatsd)*	mean	SD
4-4.9	15885	2351	708	69	2.35	0.53	2.97	0.26
5-5.9	18092	2636	839	99	2.35	0.53	3.10	0.26
6-6.9	20467	2921	965	129	2.35	0.53	3.19	0.26
7-7.9	22985	3207	1084	159	2.35	0.53	3.27	0.27
8-8.9	25616	3492	1197	190	2.35	0.53	3.33	0.27
9-9.9	28324	3777	1310	220	2.35	0.53	3.39	0.27
10-10.9	31065	4062	1438	250	2.35	0.53	3.47	0.28
11-11.9	33802	4347	1599	280	2.35	0.53	3.59	0.28
12-12.9	36586	4633	1813	310	2.35	0.53	3.75	0.28
13-13.9	40687	4918	2087	340	2.35	0.53	3.94	0.29
14-14.9	49492	5203	2406	370	2.35	0.53	4.12	0.29
15-15.9	55472	5488	2725	400	2.35	0.53	4.27	0.29
16-16.9	57983	5773	2997	430	2.35	0.53	4.36	0.29
17-17.9	59943	6059	3200	460	2.35	0.53	4.41	0.30
18-18.9	61690	6344	3336	490	2.35	0.53	4.44	0.30
19-19.9	63249	6629	3419	521	2.35	0.53	4.45	0.30
20-20.9	64628	6914	3469	551	2.35	0.53	4.46	0.31
21-21.9	65836	7199	3498	581	2.35	0.53	4.47	0.31
22-22.9	66937	7499	3515	612	2.35	0.53	4.47	0.31

Girls							width	
Age (years)	LBMmean	SD	BMCmean	SD	ln(%fatmean)*	ln(%fatsd)*	mean	SD
4-4.9	15468	2317	714	112	3.22	0.24	2.83	0.20
5-5.9	17434	2499	832	134	3.24	0.24	3.01	0.20
6-6.9	19452	2682	939	156	3.26	0.24	3.10	0.21
7-7.9	21535	2865	1039	179	3.28	0.24	3.15	0.21
8-8.9	23762	3047	1147	201	3.29	0.24	3.20	0.22
9-9.9	26297	3230	1275	223	3.31	0.24	3.27	0.22
10-10.9	29323	3412	1438	246	3.33	0.24	3.38	0.22
11-11.9	32759	3595	1640	268	3.35	0.24	3.51	0.23
12-12.9	36070	3778	1871	290	3.37	0.24	3.65	0.23
13-13.9	38692	3960	2104	313	3.39	0.24	3.77	0.23
14-14.9	40512	4143	2313	335	3.41	0.24	3.86	0.24
15-15.9	41736	4325	2477	357	3.42	0.24	3.92	0.24
16-16.9	42588	4508	2595	379	3.44	0.24	3.95	0.25
17-17.9	43213	4690	2673	402	3.46	0.24	3.96	0.25
18-18.9	43692	4873	2723	424	3.48	0.24	3.97	0.25
19-19.9	44069	5056	2753	446	3.50	0.24	3.98	0.26
20-20.9	44370	5238	2772	469	3.52	0.24	3.98	0.26
21-21.9	44611	5421	2783	491	3.53	0.24	3.98	0.26
22-22.9	44815	5612	2790	514	3.55	0.24	3.98	0.27

* Because of a skewed distribution, a logarithmic transformation for percentage body fat (%fat) was performed. We showed natural logarithm (ln) of the data. Calculating standard deviation scores(SDS) for boys %fatsds = (ln(%fat)-ln(%fatmean))/ln(%fatsd). For girls %fatsds = (ln(%fat+10)-ln(%fatmean))/ln(%fatsd). For the other parameters SDS=(measured value-mean)/SD.