DEFINING ADEQUATE VITAMIN D INTAKE –
CROSS-SECTIONAL AND INTERVENTION STUDIES

ACADEMIC DISSERTATION

To be publicly discussed, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, in Auditorium XII of the University Main Building, on May 23rd, 2008, at 12 noon

Helsinki 2008
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To Onni and Helmi
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TIIVISTELMÄ, Finnish summary


ABSTRACT

Vitamin D is required for normal bone growth and maintenance of the skeleton throughout life. In Finland, like in many other Western countries, the population suffers from inadequate or deficient vitamin D status, especially during winter, which is thought to increase the risk of osteoporosis. New strategies to prevent osteoporosis are actively being sought. The main objective of this thesis was to determine whether vitamin D is feasible in the primary prevention of osteoporosis; does it affect bone mineral accrual during the growth period? A second goal was to ascertain whether seasonal variation in calcitropic hormones affects bone remodelling, and to elucidate the vitamin D intake needed to overcome this variation in different age groups.

The subjects were healthy, free-living representatives of their respected age groups: 11- to 12-year-old girls (N=228), 21- to 49-year-old men (N=54) and 65- to 85-year-old women (N=52). Subjects participated in an intervention trial in which they were randomly assigned to a group receiving 0, 5, 10 or 20 µg of vitamin D as a supplement. All studies were performed double-blinded. Fasting blood and urine samples were collected together with data about, for instance, dietary intake of calcium and vitamin D and physical activity. In two studies bone mineral density (BMD) of subjects was measured at enrolment and at the end of the study. Laboratory analysis consisted of measurements of serum 25-hydroxyvitamin D (S-25-OHD), parathyroid hormone (S-PTH) and bone remodelling markers.

Differences among months were observed in calcitropic hormones, bone formation marker, and BMD of the femur and vertebra in a cross-sectional study of early and mid pubertal girls, thus predicting seasonal variation. Vitamin D supplementation increased bone mineral accrual dose-dependently both in the femur and the vertebra of adolescent girls. Bone mineral accrual was 17.2% higher in the femur and 12.5% higher in the vertebra with 10 µg of vitamin D than with placebo, but the effect depended on compliance. The effect of vitamin D on bone was mediated through decreased resorption. Based on the results, a total intake of 15 µg/d appears to be sufficient for adolescent girls.

The effect of vitamin D supplementation (5-20 µg/d) on S-25-OHD concentration of elderly women reached a plateau within six weeks. A concentration of 80 nmol/l, which is considered optimal, was not achieved with these dosages. We estimated that a concentration of 60 nmol/l, which is typically seen during summer in Finland, requires a total intake of 24 µg/d of vitamin D in elderly women.
Seasonal variation in calcitropic hormones and bone resorption marker, but not in volumetric BMD of the radius or bone formation marker, was noted in healthy men in a prospective study. Vitamin D supplementation increased S-25-OHD, inhibited winter elevation of PTH and decreased bone formation marker, but did not affect BMD during this 6 month study. Adequate intake to avoid the season-related changes was calculated to be 17 µg/d.

In summary, vitamin D intake remains inadequate among the target groups of this thesis, as reflected by seasonal variation in calcitropic hormones and bone metabolism. Dietary intake of vitamin D should be increased to achieve at least an adequate vitamin D status (S-25-OHD>50 nmol/l) and possibly an optimal vitamin D status (S-25-OHD>80 nmol/l) throughout the year. This could be accomplished by introducing new vitamin D-fortified foods to the market.
ABBREVIATIONS

1,25(OH)\(_2\)D 1,25-dihydroxyvitamin D, calcitriol
24,25(OH)\(_2\)D 24,25-dihydroxyvitamin D
25-OHD 25-hydroxyvitamin D, calcidiol
AI adequate intake
ALTM all laboratory trimmed mean
ANCOVA analysis of covariance
ANOVA analysis of variance
BA bone area
BALP bone specific alkaline phosphatase
BAP bone specific alkaline phosphatase activity
BMC bone mineral content
BMD bone mineral density
BMI bone mass index
CaBP calcium binding protein
CB compliance based
CBA competitive protein binding assay
CSA cross-sectional area
CT computed tomography
CV\% coefficient for variation percentage
D\(_2\) ergocalciferol
D\(_3\) cholecalciferol
DBP vitamin D binding protein
DEQAS vitamin D external quality assessment scheme
DPyr deoxypyridinoline
DR dose-response
DXA dual-energy x-ray absorptiometry
EIA enzymeimmunoassay
FFQ food frequency questionnaire
HPLC high-performance liquid chromatography
HSD honestly significant difference
IGF-1 insulin-like growth factor 1
IT intention to treat
IU international unit
LSD least significant difference
NOAEL no observed adverse effect level
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>NTX</td>
<td>cross-linked N-telopeptides of type I collagen</td>
</tr>
<tr>
<td>OC</td>
<td>osteocalcin</td>
</tr>
<tr>
<td>PICP</td>
<td>carboxy-terminal propeptide of type I procollagen</td>
</tr>
<tr>
<td>PINP</td>
<td>amino-terminal propeptide of type I procollagen</td>
</tr>
<tr>
<td>PBM</td>
<td>peak bone mass</td>
</tr>
<tr>
<td>pQCT</td>
<td>peripheral quantitative computed tomography</td>
</tr>
<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>Pyr</td>
<td>pyridinoline</td>
</tr>
<tr>
<td>RCT</td>
<td>randomized controlled trial</td>
</tr>
<tr>
<td>RIA</td>
<td>radioimmunoassay</td>
</tr>
<tr>
<td>S-</td>
<td>serum</td>
</tr>
<tr>
<td>TRACP</td>
<td>tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>U-</td>
<td>urinary</td>
</tr>
<tr>
<td>UL</td>
<td>upper limit</td>
</tr>
<tr>
<td>vBMD</td>
<td>volumetric bone mineral density</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
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</tbody>
</table>
List of original publications

This thesis is based on the following publications, which are referred to in the text by their Roman numerals (I-IV):


1. INTRODUCTION

Although 60-80% of bone mass is determined by genes, lifestyle factors, such as nutrition and physical activity, and possibly their interaction play a crucial role in bone mass accrual during growth, maintaining stable bone turnover in adulthood and preventing bone loss with age. With the exception of lung cancer, osteoporosis causes more disability than any other chronic disease (Johnell & Kanis 2006). Within the next 30 years, the incidence of osteoporosis is estimated to increase threefold due to ageing of the population; an inactive lifestyle and unhealthy food patterns contribute to this increase (Kannus et al. 1999).

The primary focus of this thesis is on supporting bone health with adequate dietary intake of vitamin D. The relationship between vitamin D and bone was described in 18th century when ultraviolet light and sunlight were discovered as cures for rickets in both England and Leiden. Since the 18th century cod liver oil has been used as a medicine for rickets, although the antirachitic factor in cod liver oil was not recognized until 1920 (Mellanby 1919). In Finland, the prevalence of rickets among newborn and small children decreased dramatically from 1950s with systematic vitamin D supplementation (Hallman et al. 1964). In present studies the final outcome variables are not as distinct and obvious as were the clinical symptoms of rickets, but they describe bone health in a modern way.

The dietary guidelines of vitamin D for children aged under 3 years have changed over the years, from 60-100 µg in 1940 to 10 µg in 1996 (Ala-Houhala 2002). Initially, recommended doses were too high, causing toxic effects as hypercalcaemia and kidney stones. The currently recommended intake of 7.5-10 µg is safe for all age groups. The safety aspect was carefully considered by the Scientific Committee on Food in 2002 before announcing NOAEL (no observed adverse effect level) and UL (upper limit) for vitamin D intake. Long-term vitamin D intake in children aged less than 11 years should not exceed 25 µg and in adults 50 µg No other nutrient has as narrow limit for adequate and safe intakes (Scientific Committee on Food 2002) although they are challenged now and then (e.g Vieth et al. 2001, Heaney et al. 2003a).

Vitamin D is required for normal skeletal growth. In addition, it is agreed that vitamin D insufficiency enhances the development of osteoporosis. Nowadays, the focus on the prevention of
osteoporosis by maximizing bone mass accrual during the growth phase (primary prevention),
which might begin as early as in the uterus. Vitamin D therapy is also feasible and cost-effective at
other stage of life (secondary and tertiary prevention). These aspects are discussed in this work and
novel evidence is provided to support the old hypothesis once again – vitamin D is supporting bone
health in all age groups.
2. REVIEW OF THE LITERATURE

2.1 Vitamin D

2.1.1 Sources of vitamin D
Globally, the most important vitamin D source is sunlight UVB (290-315 nm) radiation, which induces photosynthesis of vitamin D in the skin (Webb et al. 1989). The energy of the radiation converts the provitamin 7-dehydrocholesterol to previtamin D. The amount of previtamin D is regulated by transforming it to photoisomers tachysterol and lumisterol, which are abundant when the sunlight exposure is prolonged (MacLaughlin et al. 1982). After sun exposure, previtamin D is converted to cholecalciferol (D$_3$), which is catalysed by skin temperature. Vitamin D$_3$ is then translocated from epidermal to dermal circulation. If isomers are not reconverted to previtamin D, they are sloughed off during natural skin turnover.

Skin regulates vitamin D$_3$ production by tanning. After an erytremal dose, which expresses time to reach equilibrium in previtamin D$_3$ production, takes 20 min in white skin. Pigmented skin does not absorb all UVB radiation, and the time required for an erytremal dose is 3-6 longer (Lo et al. 1986). An equivalent dose to 10 $\mu$g is achieved by exposing 5% of skin area to sunlight for 10-15 min (Davie et al. 1982). By comparing oral dosing of vitamin D with sun exposure, a full body exposure to sunlight for 10-15 min is estimated to enhance vitamin D synthesis up to 218-250 $\mu$g (Vieth 1999). Sun protection filter tells how much longer a person can stay in the sun compared with unprotected skin. Sun screen with sun protection filter and clothing decreases vitamin D$_3$ production. In addition, 7-dehydrochlesterol concentration in the skin decreases during normal ageing (MacLaughlin & Holick 1985).

Although the sun is globally the most important vitamin D source, food is also relevant in countries located north of 60$^\circ$. In these areas, sunlight exposure is limited during winter, and virtually no UVB radiation exists for 6 months, or the angle of the radiation is insufficient to produce vitamin D in the skin (Webb et al. 1989).

Traditionally, fish, liver and egg yolk are considered as major food sources of natural vitamin D$_3$. Minor concentration of vitamin D$_3$ can also be isolated from meat and milk, but the content depends
on the feed and sun exposure of the animals providing these products. Fish in dark-watered lakes and oceans contain less vitamin D$_3$ than fish in clear waters, probably due to more generous vitamin D synthesis in clear waters (Mattila et al. 1995). In addition, farmed fish have lower vitamin D$_3$ content than wild fish (Lu et al. 2007). Plant-origin vitamin D is called ergocalciferol (D$_2$). Some wild-grown mushrooms (Outila et al. 1999) and algae contain D$_2$. In industry, vitamin D$_2$ is formed by the photolysis of specific algae, while D$_3$ is extracted from fish liver oils or as a by-product of lamb wool (Trang et al. 1998).

In the human body both forms of vitamin D are metabolized, but vitamin D$_2$ is thought to have lower bioavailability than vitamin D$_3$ (Armas et al. 2004). Vitamin D$_2$ increases S-25-OHD concentration less than D$_3$ (Trang et al. 1998, Armas et al. 2004). In the United States, the vitamin D added to foods, is mainly D$_2$ which is the major form found in supplements as well. On the other hand, in Europe, D$_3$ is more abundantly used in industry and in fortified foods. D$_3$ is claimed to be more photo- and temperature-stable than D$_2$ (Holick 1998, Trang et al. 1998). In this text, vitamin D is used as synonym for vitamin D$_3$.

Because of relatively few vitamin D sources in the diet and the growing awareness of the negative impact of low vitamin D status in the general population, the number of vitamin D-fortified products or foods on the market has increased. Good fortification practice requires evaluating different possible carriers for vitamin D. In Europe and the US, milk products, juices, spreads, cereals, oils and bread are favoured fortification targets (Lu et al. 2007).

In Finland, margarines and spreads have been fortified since 1940 with vitamin D (7.5 µg/100 ml). In addition, the vitamin D content of low-fat and fat-free milk was recovered to the natural level found in unprocessed milk (0.08 µg/100 ml) in the early 1980s. Lamberg-Allardt et al. 2001 observed that the dietary intakes of vitamin D were insufficient, as roughly 30% of the healthy adult population suffered from vitamin D deficiency (S-25-OHD $\leq$ 25 nmol/l) during wintertime and over half of the population had inadequate vitamin D status (S-25-OHD $\leq$ 40 nmol/l). Based on the simulated calculations suggested by Lamberg-Allardt et al. (2003), the Ministry of Social Affairs and Health expanded the fortification of liquid milk products to 0.5 µg/100 g including milk, sour milk and yoghurt, excluding organic products and increased the fortification of margarines and spreads to 10 µg/100 g. Although the fortification is voluntary, most dairy companies began supplementing milk and sour milk products, but fortification of yoghurts has proceeded slowly.
### 2.1.2 Vitamin D metabolism

Dietary vitamin D is absorbed nearly 100% in the ileum. It is transported with other fats in chylomicrons via lymphatic vessels that empty the contents to the thoracic duct. In the liver, vitamin D undergoes its first hydroxylation to 25-carbon and forms 25-hydroxyvitamin D (25-OHD), which is the most abundant vitamin D metabolite in the body (Fig. 1). Part of the ingested vitamin D$_2$ is also transformed to D$_3$, but 25-hydroxylase is capable of hydroxylating D$_2$ as well. The activity of liver 25-hydroxylase is not regulated (Brown et al. 1999) when the body stores of 25-OHD are within the normal range (20-120 nmol/l), but certainly vitamin D, 25-OHD and 1,25(OH)$_2$D can control its activity by negative feedback, at least in in vitro studies.

The major metabolic site of vitamin D is the liver. The activity of the circulating 25-OHD molecule depends mainly on the amount of vitamin D binding protein (DBP) also known as Gc globulin, a specific transporter of vitamin D in the circulation. The normal serum concentration of DBP varies from 4 to 6 µmol/l depending on the phenotype of Gc (Lauridsen et al. 2005). Vitamin D originating from the skin is bound to DBP when entering the circulation. Excess vitamin D is stored in fat and muscle tissue, and small amounts in the liver. The stores are mobilized when the concentration of circulating 25-OHD decreases. DBP has a higher affinity for 25-OHD than for 1,25(OH)$_2$D or vitamin D, and this affinity favours the release of the active metabolite into the blood. The half-life of 25-OHD varies between 20 and 30 days, differing among Gc phenotypes (Lauridsen et al. 2005).

To become metabolically active, vitamin D needs to be 1-hydroxylated, which systemically occurs in the kidney. The concentration of the active metabolite, 1,25(OH)$_2$D, is strictly regulated by negative feedback of 1,25(OH)$_2$D concentration, parathyroid hormone (PTH), growth hormone, estrogens and serum phosphate and the lifespan of calcitriol is extremely short (from 4 to 6 hours) (Clements et al. 1992). Yet most tissues contain 1-α-hydroxylases, enabling the local production of calcitriol (e.g. Brown 1999), which may partly explain why calcidiol concentration correlates with PTH (Vieth et al. 2003) and bone mineral density (BMD) (Outila et al. 2001, Välimäki et al. 2004, Bischoff-Ferrari et al. 2004a).

Free 1,25(OH)$_2$D enters all cells due to its fat solubility. The active metabolite binds to its specific receptor, VDR, in the nucleus and activates genes alone as a homodimer or in heterodimer form with retinol receptor superfamily members. There are alternative pathways in vitamin D metabolism that are related to further hydroxylation of 23-, 24- or 26-carbons (Brown 1999). Although some
metabolites have their own specific activity, like 24,25(OH)$_2$D in the fibrous tissue, the hydroxylation steps mainly increase the water solubility of the molecule and induce the metabolic inactivation and secretion of the body. Calcitronic acid is the final form of vitamin D, which is secreted in urine.

Figure 1. Simplified schematic of vitamin D metabolites.

2.1.3 Functions in the body
The 25-OHD-DBP complex of enters cells via a receptor-mediated endocytic pathway (Willnow & Nukjaer 2005). This pathway includes the release of 25-OHD from the complex as well as the recycling of the transmembrane proteins cubilin and megalin (Willnow & Nukjaer 2005). After the conversion of 25-OHD to 1,25(OH)$_2$D, this binds to VDR and affects the production of proteins. The main function of vitamin D in cells is to increase calcium binding proteins (CaBP) in the cytoplasma. CaBP can modulate calcium fluxes in the cell, thus regulating cell proliferation and differentiation. Because VDRs are found in most cells of the body, the biological responses to vitamin D vary according to cell.

One of the main functions of vitamin D is calcium absorption from the intestine. In a repleted vitamin D status, the amount of CaBP in mucosal cells is increased and active calcium transport is optimally working (DeLuca 1979). The threshold for maximum calcium absorption capability has been suggested to be 80 nmol/l (Heaney et al. 2003b). In a vitamin D depleted state, calcium absorption occurs only with passive diffusion, which is hardly adequate to meet the needs of the body.

Vitamin D acts in the bone by increasing the production of osteocalcin, a matrix protein. In addition, it regulates bone resorption, and thus, maintains bone turnover. Over the years, additional
target organs have been recognized, including muscles (Ritz et al. 1980, Bischoff-Ferrari et al. 2004b). Epidemiological studies have revealed a relationship between deficient vitamin D status and different chronic diseases such as type I diabetes, multiple sclerosis, osteoarthritis, prostate and colon cancer, vascular calcification and coronary heart disease (Zittermann 2003, Bischoff-Ferrari et al. 2006). Moreover, some evidence suggests that immune response (Cannell et al. 2006), depression (Berk et al. 2007) and infertility (Panda et al. 2001) are also related to vitamin D insufficiency.

2.1.4 Vitamin D status

The most abundant vitamin D metabolite in the body, serum 25-OHD, is generally used to reflect the vitamin D status of the body. Other markers of calcium metabolism, such as S-Ca, U-Ca, S-PTH and S-1,25(OH)\textsubscript{2}D, are used alongside S-25-OHD, as they are tightly involved in the clinical status of vitamin D (Need et al. 2000). While no international consensus has been reached regarding reference ranges to describe each status, some common guidelines are recognized (Table 1).

<table>
<thead>
<tr>
<th>S-25-OHD, nmol/l</th>
<th>Status</th>
<th>S-iPTH</th>
<th>1,25(OH)\textsubscript{2}D</th>
<th>BMD</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;25</td>
<td>Deficiency</td>
<td>+ 15 %</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>25-49.9</td>
<td>Inadequate/Insufficient</td>
<td>+ 15 %</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>50-79.9</td>
<td>Adequate/Sufficient</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>≥ 80</td>
<td>Optimal</td>
<td>0</td>
<td>0</td>
<td>+</td>
</tr>
</tbody>
</table>

+ increase, 0 no effect, - decrease

In vitamin D deficiency, the S-25-OHD concentration is generally considered to be below 20-25 nmol/l (Need et al. 2000, Lips 2004). Vitamin D deficiency is related to rickets and osteomalacia among children and adults, respectively, both of which feature low mineralization of bones. Subclinical vitamin D deficiency or vitamin D insufficiency, also called vitamin D inadequacy, is characterized by serum levels of 25-40 nmol/l (Need et al. 2000) or 25-50 nmol/l (Lips 2004). In vitamin D insufficiency, the vitamin D stores of the body are depleted, the concentration of PTH is increased by at least 15% and 1,25(OH)\textsubscript{2}D is also elevated (Lips 2004).
Adequate vitamin D status is characterized by S-25-OHD concentration above 40 nmol/l (Need et al. 2000) or above 50 nmol/l (Lips 2004, Rizzoli et al. 2008), and normal concentrations of PTH and 1,25(OH)\_2D. For general bone health (Dawson-Hughes et al. 2005) as well as for the prevention of certain chronic diseases (Zittermann 2003, Bischoff-Ferrari et al. 2006), the optimal 25-OHD concentration has been speculated to be around 80 nmol/l, although some consider this to be an overestimate, more suitable for risk groups than for the normal population (Rizzoli et al. 2008).

Changes in vitamin D status are mediated through PTH, which is the main regulator of calcium metabolism (Maver and Davis 2001) (Fig. 2). In inadequate vitamin D status, the decreased absorption of calcium from the small intestine affects serum calcium intermittently, stimulating the parathyroid glands to secrete PTH. PTH activates 1-α-hydroxylases in the kidney to produce more calcitriol and further accelerates vitamin D metabolism. Serum calcium concentration is corrected with increased absorption from the intestine. During the next step the amount of 25-OHD is insufficient to form calcitriol; this state is known as vitamin D deficiency. Because calcium absorption can no longer increase, calcium is mobilized from the skeleton by resorption. The clinical features of vitamin D deficiency are osteomalacia and rickets.
Figure 2 illustrates the development of vitamin D deficiency as described by Maver & Davies (2001).

2.1.4.1 Methods for assessing S-25-OHD
At least six different methods are used worldwide to measure s-25-OHD concentration. These include high-performance liquid chromatography (HPLC), radioimmunoassay (RIA), enzymeimmunoassay (EIA), competitive protein binding assay (CBA) and chemiluminescence assay. Although the HPLC method is considered the gold standard for measuring 25-OHD, the number of laboratories using it is declining (Carter et al. 2004). This could be due to its complexity, the pre-treatment of samples necessary and the poor recovery. Its advantages comprise separation of 25-OHD$_2$ and 25-OHD$_3$ and a rather small bias compared with other analytical methods. The most frequently used method is RIA, in which $^{125}$I isotope is preferred (Carter et al. 2007). Advantages of RIA are its good precision and ease of use. On the other hand, handling of radioactive isotopes requires its own places in the laboratory and produces hazardous waste which is disadvantageous. Sometimes RIA has difficulties in recognizing 25-OHD$_2$, which is commonly used in dietary...
supplements in the US (Carter et al. 2004). Increasing numbers of laboratories have chosen EIA because of its ease and rapid results. Its performance is not the best, as it tends to overestimate, especially higher concentrations, and the results are likely affected by the sample matrix (Carter et al. 2004).

Despite the good precision of the method the results are not necessarily true. Interlaboratory comparisons are justified to ensure validity of the measurement. The International Vitamin D External Quality Assessment Scheme (DEQAS) was created for this purpose. DEQAS delivers serum samples to each participating laboratory and sums up the results. This way all laboratory trimmed mean (ALTM) values are provided to facilitate comparisons of different methods. ALTM is the mean value for all results within three standard deviations of the mean. Recently, Dawson-Hughes et al. (2005) proposed that standardized values for S-25-OHD should be reported in scientific articles.

2.1.5 Dietary guidelines and current intakes in Finland

In 2004, the National Nutrition Council (2005) increased the recommendation for vitamin D intake from 5 to 7.5 µg a day for individuals aged 3-60 years of age. For children under 3 years and persons over 60 years including pregnant and lactating women as well as other risk groups the recommended intake is 10 µg. The dietary guidelines in Finland are based on the Nordic Nutrition Recommendations (Nordic Council of Ministers 2004). In the United States, the Institute of Medicine has set only an adequate intake (AI) for vitamin D, as scarce evidence exists for a recommended daily allowance. The AI for groups 0-50 years, 51-70 years and >71 years is 5 µg, 10 µg and 15 µg, respectively (Institute of Medicine 1997). Recommended nutrient intakes published by the World Health Organization are in accord with the guidelines of the Institute of Medicine.

In the Findiet 2002 Study, the mean dietary intake of vitamin D among 25- to 64-year-old men and women was 5.8 µg/d and 3.8 µg/d, respectively (National Public Health Institute 2003). The mean energy-standardized daily intake of vitamin D was 0.6 µg/MJ. As mentioned earlier, vitamin D fortification of food stuff expanded in 2003. The current recommended intake (National Nutrition Council 2005) is achieved if the diet contains three glasses of fortified milk (0.5 µg /100 ml), fortified spreads (10 µg /100 g) and at least two portions of fish dishes a week (Table 2). Vitamin D intakes were examined in a follow-up study on vitamin D status in the Finnish population (Lamberg-Allard et al. 2006). The dietary intake of vitamin D was found to increase on average 2.3
µg/d between 2002 and 2004, of which 1.8 µg originated from fortified milk products and 0.3 µg from spreads (Lamberg-Allardt et al. 2006). The use of vitamin D-containing supplements was beneficial, but despite the supplements the total intakes were still inadequate among 4- to 6-year-old girls, 14- to 17-year-old girls and 27- to 35-year-old men and women.

**Table 2. Vitamin D content of selected food product**

<table>
<thead>
<tr>
<th>Food product</th>
<th>µg of vitamin D per 100 g of product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmon</td>
<td>9.8</td>
</tr>
<tr>
<td>Tuna</td>
<td>1.8</td>
</tr>
<tr>
<td>Whitefish</td>
<td>22.0</td>
</tr>
<tr>
<td>Herring</td>
<td>18.0</td>
</tr>
<tr>
<td>Coalfish</td>
<td>1.5</td>
</tr>
<tr>
<td>Cod</td>
<td>7.5</td>
</tr>
<tr>
<td>Milk</td>
<td>0.5</td>
</tr>
<tr>
<td>Margarine</td>
<td>9.2</td>
</tr>
<tr>
<td>Egg</td>
<td>2.2</td>
</tr>
<tr>
<td>Liver</td>
<td>0.8</td>
</tr>
<tr>
<td>Poultry</td>
<td>0.7-1.0</td>
</tr>
<tr>
<td>Meat</td>
<td>0.2-0.5</td>
</tr>
<tr>
<td><em>Cantharellus cibarius</em></td>
<td>12.8</td>
</tr>
<tr>
<td><em>Boletus edulis</em></td>
<td>2.9</td>
</tr>
</tbody>
</table>

1 Source: National Food Database Fineli®, National Public Health Institute
2.2 Bone

2.2.1 Bone growth at puberty

Typically, growth slows down during late childhood and a clear acceleration occurs at puberty. Pubertal growth consists of three phases: slow acceleration at prepuberty, growth spurt for two years at midpuberty and normalization and termination of growth at late puberty. Genetic, hormonal and nutritional factors modify the tempo and timing of growth. Improved health and nutritional status over the decades has advanced sexual maturation in industrialized countries. Body fat content is one of the most common modulators of pubertal development (Marshall & Tanner 1969, Aloia et al. 1995).

Pubertal growth starts earlier in girls than in boys. The evaluation of pubertal development is based on external characteristics of height increment, pubic hair and breast/penis (Table 3). The growth spurt, in which the height increment exceeds 6 cm per year, peaks at the age of 12 years in girls and at 14 years in boys (Boot et al. 1997, Bailey et al. 2000). In both genders, peak height velocity is observed at pubertal stage 3 (Mølgaard et al. 1999). The greater height increment and longer duration of the growth period are thought to be the reasons why boys reach a larger skeletal size than girls (Bailey et al. 2000, Heaney et al. 2000).

In late childhood and prepuberty, the height of long bones, i.e the femur, tibia and forearm, increases dramatically (Boot et al. 1997). Mineralization occurs in the growth plate cartilage at the epiphyses of long bones, thus increasing the length of the bone. Fig. 3 illustrates the modelling of the radius involving both bone formation and bone resorption. Typically, during puberty the size of the trunk increases, comprising vertebral growth and widening of the pelvis (Bass et al. 1999).

During growth the size and the shape of the skeleton changes constantly. Heaney et al. (2000) stated that the size of the bone increases first, producing collagen-rich bone. Hydroxyapatite crystals are later added. The lag between collagen-rich bone and mineralization among girls is longer than among boys mainly because the increase of bone size goes together with an increase of lean body mass. This mechanostat theory links the bone development to the strain caused by muscle contraction (Frost et al. 1998, Fricke & Schoenau 2007). In boys, with greater lean body mass accrual (Mølgaard et al. 1999), the muscles surround the bones and apply pressure to unmineralized bone more extensively during growth, leading to quicker mineral accrual. Among girls, this lag between increased bone size and mineralization is from 3 to 6 months. Thus, bone strength decreases...
transiently, and this could create a problem. An increasing rate of fractures occurs during this highly intensive growth period that is typically known for the peak height velocity (Bailey et al. 1989).

Table 3. Puberty development in girls based on Tanner stage (modified from Marshall & Tanner 1969).

<table>
<thead>
<tr>
<th>Tanner stage</th>
<th>Description</th>
</tr>
</thead>
</table>
| 1            | Height: At basal rate  
              | Breast: Papilla elevation  
              | Pubic hair: Unpigmented, villus hair only |
| 2            | Height: Increased rate, over 5 cm annually  
              | Breast: Buds palpable and areolae enlarge  
              | Pubic hair: Some coarse, pigmented hair, mainly on labia |
| 3            | Height: Increases at peak rate, 8 cm per year  
              | Breast: Elevation of breast contour, areolae enlarge  
              | Pubic hair: Dark, coarse, curly hair spreads over mons pubis  
              | Axillary hair develops |
| 4            | Height: Increased rate  
              | Breast: Areolae forms secondary mound on the breast  
              | Pubic hair: Adult-like hair, but not spread  
              | First menstrual bleeding, menarche |
| 5            | Height: Increases modestly  
              | Breast: Adult breast contour  
              | Pubic hair: Adult distribution of hair  
              | Menses quite regular |

Figure 3. Bone growth and modelling occur at the epiphyses of long bones. Here the situation in the radius over an 8- to 11-month period is presented (adapted from Rauch et al. 2001a).
Bone mineral accrual continues after the cessation of axial (longitudinal) growth (Bailey et al. 2000). In late adolescence, 80-90% of the adult peak bone mass (PBM) is accrued (Matkovic et al. 1994, Bonjour et al. 1994). PBM is reached before the age of 16 years in girls and around 18 years in boys in most skeletal sites except of the cortices of long bones (Bonjour et al. 1991, Theintz et al. 1992, Lorentzon et al. 2005), although individual and racial differences exist. Many of the discrepancies related to the achievement of PBM are derived from different measurements (Wren et al. 2007). Axial growth ceases as the growth plates in long bones are closed by oestrogens in both genders.

2.2.2 Ageing of bone

After the achievement of PBM, the skeleton continues to change (Heaney et al. 2000). To optimize its strength and minimize its weight, the bone architecture is reorganized in a process called bone mineral apposition (Schoenau et al. 2001). Bone strength depends on both material and geometric properties (Heaney 2000, van der Meulen 2001); generally, the bigger the bone, the stronger the bone. To maximize its strength, the bone material is placed as far from its mean axis as possible (Seeman 2003a), in nature reflected as mineral apposition from the endochondral envelope to the outer surface of the bone. During growth this mineral apposition increases both the outer diameter of the bone and the wall thickness (Seeman 2003a, 2004).

Periosteal expansion is facilitated by androgens. In addition, growth hormone, IGF-1 and mechanical loading are thought to be the primary modulators of periosteal apposition. On the other hand, oestrogens are responsible for the opposite mineral apposition called endocortical apposition (subtraction) (Schoenau et al. 2001). In endocortical subtraction, the mineral is placed on the endochondral envelope, which maintains the outer diameter but decreases the inner diameter of the bone. Here, the mineral plays no real role in the mechanical strength of the bone, but it serves as a mineral reservoir that could easily be mobilized to meet the needs of the body in for example, pregnancy or lactation (Schiessl et al. 1998, Schoenau et al. 2001).

During ageing, the mineral apposition varies from the inner cortex to the outer cortex, increasing the outer diameter of bone and thus maintaining the width of the bony wall (Seeman 2003, 2004). The main goal is to make the bones as strong as possible using as little material as possible (Schoenau et al. 2001). Figure 4 is a schematic of longitudinal growth and bone mass reorganization.
Figure 4. During growth cross-sectional area and bone length increase (A). The shape of bone might also alter. Periosteal apposition increases whole bone diameter and wall thickness (B). Endochondral subtraction; whole bone diameter is maintained, but wall thickness is increased (C).

Skeletal integrity and bone mass are relatively stable between the ages of 20 and 40 years. However, lifestyle factors, such as nutrition, smoking, alcohol abuse, physical activity and disease (malabsorption, anorexia, hypogynadism, etc.), and certain medicines contribute to changes in BMD, which varies 1-2% annually (Bergstralh et al. 1990). Bone loss is a normal physiological process; it occurs in all humans due to hormonal changes and declines in physical activity and muscle mass. After menopause, bone loss accelerates in women, as the anabolic effect of oestrogens ceases. By the age of 80 years women are estimated to lose 30-50% of their bone mass (Väänänen 1996) and men from 25-30% (Fig. 5).

Lower PBM and earlier and greater bone loss are the main reasons why women are more likely to suffer from osteoporosis than men. Of population aged over 50 years, an estimated 20% of women and 14% of men will suffer from osteoporotic fracture during their life time. The incidence of osteoporosis was speculated to increase three fold by 2030, based on the incidence of hip fracture from 1960 to 1994 (Kannus et al. 1999). At this point, this seems unlikely since only 8000 hip
fractures occurred in 2006, which is 2000 less than predicted by Kannus et al. (1999). However, a newer publication by Kannus et al. (2006) showed a decreasing trend since 1994 for hip fractures in women but a slight increase in men. Hip fractures have been considered the final outcome variable for osteoporosis, but whether hip fractures actually reflect the general well-being of the elderly or the standards of care (Jensen et al. 2002) rather than osteoporosis itself is arguable. Typically, hip fractures occur in elderly persons after a fall. There are a variety of risk factors for falls, including number of used medicines, environmental factors, personal motor functioning and vision. Low-energy fractures of the vertebra or lower arm are proposed to predict the incidence of osteoporosis more clearly than the current strategy (Johnell & Kanis 2006).

![Lifetime bone mass accrual and loss among men and women. Error bars represent SD. Data from http://courses.washington.edu/bonephys/index.html](image)

**Figure 5.** Lifetime bone mass accrual and loss among men and women. Error bars represent SD. Data from [http://courses.washington.edu/bonephys/index.html](http://courses.washington.edu/bonephys/index.html)

### 2.2.3 Bone modelling and remodelling

Bone is living material that constantly renews in a process called remodelling; osteoclasts remove old bone by resorption and osteoblasts form new bone (Parfitt 1988) (Fig. 6). In addition, the basic multicellular unit in which the bone remodelling occurs contains lining cells and osteocytes that also participate in the process in ways as yet uncharacterized. During growth there is less
remodelling but more modelling, which means that new osteons are formed and more bone is formed than resorbed i.e. the balance is placed more on the osteoblastic site (Parfitt 1988). Modelling occurs predominantly at the epiphyses of long bones, altering the shape and size of bone (Rauch et al. 2001a).

In healthy adults, a balance exists between osteoclasts and osteoblasts to maintain skeletal function and integrity. A negative calcium balance initiates the remodelling cycle at the edge of the bone. Both lowered and accelerated bone turnover is harmful to bone health, as they affect the homeostasis between osteoclasts and osteoblasts, and bone turnover becomes uncoupled (Eastell et al. 1993, Parfitt 2003). Bone turnover accelerates and becomes uncoupled after menopause, reflected as an increase in the number of resorption cavities and undermineralized osteons. In lowered bone turnover by contrast, microcracks tend to accumulate and bone strength decreases (Seeman 2004). Microcracks are recognized by osteocytes that initiate the remodelling cycle in the inner structures of bone (Parfitt 1988). Lowered bone turnover is seen more commonly among men, in African Americans and after antiresorptive therapy in osteopenic patients (Seeman 2004).

Figure 6. Remodelling cycle in bone starts with the resting phase (1), where lining cells cover the edge of bone. In the resorption phase (2), multinuclear osteoclasts secrete proteolytic enzymes to break down the old bone matrix. A resorption cavity is formed. During the formation phase (3), osteoblasts refill the cavity with collagen. During the maturation phase (4), the collagenous matrix is mineralized. Based on Parfitt (1988).
Remodelling rates differ between cortical and trabecular bone. Trabecular bone, located mainly in the epiphyses of long bones and the vertebra, is metabolically more active. It contains more cells and nutrients are more abundant than in the cortex; its remodelling cycle is therefore shorter than in the cortex. The remodelling cycle in trabecular bone takes about three months, with resorption lasting for two weeks, whereas the cycle in cortical bone is four months (Dempster 1995). All bones are covered with a variable layer of cortical bone, but most cortical bone is situated in the diaphysis of long bones. Only 3% of cortical bone is renewed annually, while the proportion is 25% in trabecular bone (Dempster 1995). A number of hormones, including PTH, sex hormones, growth hormone, thyroid hormones and glucocorticoids, affect bone turnover. In addition, such local factors as growth factors, cytokines and prostaglandins contribute to local changes.

2.2.3.1 Markers of bone remodelling
Bone remodelling markers are specific biomarkers of bone turnover that are utilized in preclinical and clinical trials (Watts 1999). The biomarkers of bone are divided into subgroups describing either bone formation or resorption. Although some markers are specifically linked to certain phase of the remodelling cycle, (for example TRACP), others describe the general turnover rate (for example BALP) (Seibel 2003).

Bone formation markers are biproducts formed during the production of new bone. The main function of osteoblasts is to produce collagen type I matrix. Procollagen peptides or their fractions, like carboxy-terminal propeptide of type I procollagen (PICP) or amino-terminal propeptide of type I procollagen (PINP) are formed during collagen matrix synthesis (Fig. 6). These can be detected in serum or urine with highly specific methods that reflect bone formation rate (Tähtelä et al. 1997). Another well-known formation marker is bone-specific alkaline phosphatase (BALP), which describes osteoblastic function. BALP is located in the plasma membrane of osteoblasts and the measured activity reflects the function of the osteoblasts. Another marker, osteocalcin (OC), is non-collagenous protein that mature osteoblasts secrete when bone matrix has been mineralized. The amount of OC can be detected in the serum and is highly bone-specific. New methods are currently being invented to detect different fragments of OC to further elucidate the turnover process (Ivaska et al. 2004).
Bone-resorbing cells, osteoclasts, secrete proteolytic enzymes, cathepsins, which in a normal situation start the degradation of the bone matrix, but other enzymes, such as metalloproteinase, accompany them. However, alternative collagen cleavage sites predominate in a pathological resorption process (e.g. Nishi et al. 1999), and possibly also in different sites of bones (Everts et al. 2006). Deoxypyridinoline (Dpyr), pyridinolines (Pyr) or peptides from the amino- or carboxyterminal telopeptides are released when bone-specific type I collagen is broken down. These pyridinoline compounds and crosslinks are released into the circulation, and as they are not recycled in the body, they could serve as biomarkers. The latest marker on the market is serum tartrate-resistant acid phosphatase (TRACP), which describes either osteoclastic activity or number according to different isoforms (Halleen et al. 2006).

There are a number of reasons why resorption markers are more frequently used than formation markers in prospective studies. Resorption is thought to be more easily affected than formation, but in fact the bone remodelling cycle starts with the resorption of old bone, and in many bone diseases, accelerated bone resorption is the underlying reason (Borderie et al. 2001). In general, within three months, an effect of the therapy is noticed in remodelling markers, but still when interpreting the results individual, diurnal and seasonal variation should be taken into consideration as well as the menstrual cycle in females (Dempster 1995). In addition, remodelling markers are utilized in the follow-up of bone disease or metastasis. While remodelling markers are not related to the diagnosis of osteoporosis, they could be utilized to predict fractures.

Some researchers (Van Coeverden et al. 2002, Fares et al. 2003) have observed that bone remodelling markers tend to vary during puberty. The concentration of bone remodelling markers increases gradually as puberty proceeds, peaking at mid puberty. After menarche, bone remodelling markers decrease again, and they reach adult levels around 16-20 years, a bit earlier in girls than boys (Van Coeverden et al. 2002, Fares et al. 2003). This growth-related variation is seen in both remodelling markers, but to greater extent in formation markers.

### 2.2.4 Assessments of bone mass and structure

The current primary diagnostic technique for BMD is dual-energy x-ray absorptiometry (DXA) but other x-ray and ultrasound techniques have been widely used for the same purpose. Although ultrasound was abandoned in calcaneus BMD measurements, it has been reintroduced to characterize human trabecular bone microstructure (Hakulinen et al. 2006). Mineral properties are
commonly accepted as being responsible for bone strength, but increasingly more emphasis is being placed on the microarchitecture of bone (Parfitt 1988). Bone microarchitecture alters in osteoporosis (Chappard et al. 2005). In addition, microarchitecture may explain gender differences (Seeman 2004) and is related to fracture risk assessment (Legrand et al. 2007). Parameters derived by microcomputed tomography, microCT, describe the histomorphometry of bone, especially trabecular bone. For preclinical use, the microCT application is suitable for *ex vivo* and *in vivo* samples. Human applications require bone biopsy from transiliac bone, which is unethical in studies involving healthy human beings. In future, images of CT will provide us with further understanding of the structure and properties of bone during growth.

**2.2.4.1 Dual-energy x-ray absorptiometry (DXA)**

DXA is a non-invasive method to quantify bone mass. The method for measuring bone is calibrated against a hydroxyapatite phantom. The radiation dose is highly localized, and the effective dose is around 2.1 microSiever (µSv) which is equal to a 1-to 2-days radiation dose from the environment.

DXA gives a planar image of bone, allowing the bone mineral content of the whole body or from specific sites, such as lumbar spine, femoral neck (Fig. 7) or forearm, to be calculated. It is crucial to understand that the size and shape of the bone affects the bone mineral content (BMC) (Heaney 2003). A typically parameter used, the bone mineral density (BMD), which is calculated by dividing the BMC with corresponding bone area (BA) (mg/mm²), predicts apparent rather than real density. DXA-derived BMD is used in the diagnosis of osteoporosis (Compston et al. 1995, Table 4), and it is thought to predict fractures most reliably, especially in the femoral neck (Nevitt et al. 1994). A change of one standard deviation in the BMD increases fracture risk 1.5-3.0 times (Compston et al. 1995). The use of BMD in growth studies is questionable, as it is size-related (Heaney 2003). However, no consensus exists about BMC, BMD or BA to describe the size of the bone among growing children, but are recommended to use all three to characterize the situation most clearly.

The precision of DXA is fairly good, around 1%. Errors are related to the movement of the body and placement of subjects during measurement. Reference values are used to categorize subjects’ results. The summary of T and Z scores is presented in Table 5. Although areal BMD reflects bone fracture risk quite well, it reveals virtually nothing about bones’ inner structure, geometry or mass distribution, which are important determinants of bone strength in general (Seeman 2004).
Figure 7. DXA scans of the lumbar spine (L1-L4) and left hip.

Table 4. Standardized reference values in evaluating areal BMD results (Compston et al. 1995).

<table>
<thead>
<tr>
<th>Value</th>
<th>T-score</th>
<th>Z-score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Description</td>
<td>a young adult of the same gender with peak bone mass</td>
<td>other people in same age group and of same size and gender e.g. children</td>
</tr>
<tr>
<td>&gt; -1</td>
<td>Normal BMD</td>
<td>Normal BMD</td>
</tr>
<tr>
<td>-1- (-2.5)</td>
<td>Osteopenia</td>
<td>Normal BMD</td>
</tr>
<tr>
<td>&lt;-2.5</td>
<td>Osteoporosis</td>
<td>Normal BMD</td>
</tr>
</tbody>
</table>

2.2.4.2 Peripheral quantitative computed tomography (pQCT)

Peripheral quantitative computed tomography (pQCT) elucidates bone structure and geometry (Fig. 8 and 9). With the pQCT scanner, the radiation source rotates around the measured particle and creates an image of the cross-section of the bone. Typically measured sites are the radius and tibia from different positions. Because of its cross-sectional nature, pQCT gives the bone composition or cortical to trabecular bone ratio. In addition, the actual bone volumetric density (vBMD) is calculated. The effective dose is higher than in DXA, 0.22 μSv (Meditechnish, Stratec), but this is more localized than in DXA. Fig. 6 presents simple parameters that could be obtained from pQCT measurements. In addition, there are a number of parameters that can be calculated to estimate mechanical strength of long bone or its resistance to torsion or bending stress (Schoenau et al. 2001). One of basic ones is section modulus, which indicates resistance of diaphyseal bone to stress. Another much used parameter is polar moment of inertia, which is inversely related to shear stress (Specker & Schoenau 2005). Reference values are currently available for adult vBMD only.
Figure 8. Trabecular or cancellous bone is marked with spotted texture. R represents radii and inner R endocortical or trabecular R. Cortical bone is the cortex surrounding the trabecular bone. Outer R is also called the total or periosteal radius. Circular ring model is assumed when calculating these parameters.

Figure 9. An image of distal radius at 4% position with pQCT.
2.2.5 Modulators of bone mass

Although 60-80% of PBM is determined by genetic factors (Slemenda et al. 1991), environmental factors, such as physical activity and nutrition, can also modulate bone mass accretion during growth (Welten et al. 1994, Bonjour et al. 1997) and maintain it during adulthood. In addition, nutrients and genes interact with each other (e.g. Ferrari et al. 1998). Low bone mass is a risk factor for the development of osteoporosis. In primary prevention, the goal is to optimize bone acquisition during growth, which is justified as tracking of bone mass occurs (Cooper et al. 2001, Matkovic et al. 2004).

Weight-bearing exercise is crucial for bone mass accrual, which is supported by the mechanostat theory (Frost et al. 1998, Heaney et al. 2000). Physical activity started in early childhood has been speculated to be a one-time opportunity to gain long-lasting positive effects on bone (Heaney et al. 2000) since activity in childhood is associated with adult BMD (Slemenda et al. 1991, Pesonen et al. 2005). The effect of long-term physical activity on bone health becomes apparent with age (Uusi-Rasi et al. 2002), especially at weight bearing sites. Adults with high-impact, weight-bearing activities have enlarged bone cortices and thus better bone strength than counterparts without this type of activity (Kontulainen et al. 2006). Recreational exercise has been shown to have a positive effect on bone health, possibly induced via muscular performance (Uusi-Rasi et al. 2006). Because the skeleton adapts to exercise, varying types of activity are recommended to maintain bone mass and integrity during adulthood (Vainionpää et al. 2007).

There are a number of nutrients that play a role in bone health (e.g. Heaney et al. 2000), one of them being calcium. Calcium is typically considered the limiting factor for bone mineral accrual (Bailey et al. 2000). Calcium intake of 1300 mg is required to fulfill the retention rates that are observed in puberty (Bailey et al. 2000). Positive effects of calcium on bone mineral accrual have been noticed among children (Specker & Binkley 2003) and adolescent (Bonjour et al. 1997, Rozen et al. 2003). Calcium supplementation is claimed to extend the growth period or add some extra mineral to bones (Rozen et al. 2003, Bonjour 2005). How long these positive effects are maintained is another issue. In adults, the positive effects are not maintained after cessation of supplementation, if the habitual intake of calcium becomes clearly lower than during the intervention (Prentice 2004). However, the changes that occur before or during early puberty are thought to be maintained after supplement cessation (Bonjour et al. 2001, Bonjour 2005), although not all studies agree (e.g. Zhu et al. 2006).
The body apparently can adapt to different levels of calcium intake: thus the absorption from the gut and the retention to bone vary (Bailey et al. 2000). This adaptation illustrates efficient function of the vitamin D-PTH –axis in maintaining stable serum calcium concentration (Fig.10). The adaptation predominates until menopause, but thereafter calcium balance of the body tends to become negative more easily than before (Heaney et al. 2003b). Diurnal rhythm of PTH It is suggested to support the maintenance of trabecular bone, while chronically elevated PTH levels are related to enhanced bone loss (Gabet et al. 2006). Age-related changes in calcium metabolism may stimulate chronic PTH secretion (North American Menopause Society 2006), but renal function may also play a role (Vieth et al. 2003).

**Figure 10.** The regulation of calcium metabolism. Low S-Ca stimulates PTH secretion, which affects bone and the kidney. In kidney, PTH increases the reabsorption of calcium from urine and activates 1-alpha-hydroxylase to form calcitriol. Both PTH and calcitriol mobilize calcium from bone. In addition, calcitriol increases calcium absorption from intestine. Calcitriol regulates its own concentration by negative feedback.
2.3 Vitamin D and bone

2.3.1 Association between 25-OHD and bone health

Vitamin D is considered a threshold nutrient for bone health (Heaney et al. 2005), which means that at a lower concentration than the threshold for serum 25-OH the association with BMD is linear, but beyond the threshold there are no further benefits (Fig. 10). Recently, an expert board suggested the vitamin D threshold for optimal bone health (Dawson-Hughes et al. 2005) should be over 80 nmol/l. This conclusion is based on different studies worldwide in which a concentration of 80 nmol/l is shown to benefit calcium absorption (Heaney et al. 2003b), prevent bone loss among the elderly (Dawson-Hughes et al. 1995), decrease fracture risk among the elderly (Dawson-Hughes et al. 1997), be associated with higher BMD for both genders and different races (Bischoff-Ferrari et al. 2004a) and suppress PTH secretion (Vieth et al. 2003). In addition, for subjects over 70 years, an additional benefit for bone health is achieved if a vitamin D status above 100 nmol/l is maintained (Vieth et al. 2003). This is based on findings that elderly subjects require higher doses of vitamin D to suppress PTH secretion, possibly because of lowered kidney function.

Figure 11. Association between BMD and serum 25-OHD concentration (based on Heaney et al. 2005).
2.3.2 Effect of seasonal variation on bone remodelling

During winter there is limited amount of UVB irradiation to synthesis vitamin D on the skin. Together with an inadequate vitamin D supply from the diet, this leads to deprivation of vitamin D stores. To maintain adequate serum calcium concentration, PTH secretion increases and further activates vitamin D metabolism. This seasonal variation increases bone resorption and decreases formation during the winter and possibly has opposite effect during the summer (e.g. Rosen et al. 1994). A positive association between vitamin D status and formative markers has been described (Woitge et al. 1998), and in follow-up studies resorptive markers increase during winter (Storm et al. 1998, Woitge et al. 2000, Rapuri et al. 2002) due to increased PTH secretion.

Seasonal variation in serum 25-OHD, PTH and possibly 1,25(OH)₂D is believed to determine the seasonal variation in bone mass (Krall et al. 1989, Dawson-Hughes & Harris 1993). In healthy adults, the seasonal variation in bone mass is around 1-2% (Bergstralh et al. 1990, Meier et al. 2004), whereas in the elderly it comprises a 6-8% change, at least, according to cross-sectional studies (Rapuri et al. 2002, Bhattoa et al. 2004). Furthermore, a prospective community-based study demonstrated that the rates of bone fractures and falls follow this seasonal periodicity (Jacobsen et al. 1991, Pasco et al. 2004). These observations were made in Australia and the United States, taking into account the geographical levels as well as the influence of winter weather conditions. The peak in fracture incidence during the colder season is most obvious in warm locations without icy and slippery weather conditions (Bischoff-Ferrari & Dawson-Hughes 2007).

2.3.3 Effect of vitamin D on bone metabolism in children and adolescents

Vitamin D deficiency in childhood causes rickets, which is characterized by disturbed bone formation. Clinical symptoms include swollen growth plates in long bones and deformed legs and pelvis; in severe cases the spine is affected as well. In rickets, mineralization of growth plate cartilage is stunted and bone formation rate is accelerated; new osteoids are formed, but they do not mature because of undermineralization (Parfitt 2003). Poor mineralization of bone deteriorates bone strength, which is seen as bending of long bones. Especially weight-bearing bones are affected (Bishop 1999). When diagnosed early, rickets can be cured with extensive vitamin D therapy, but if deformities are severe and growth plates start to mature in puberty, no cure exists (DeLuca 1979). Studies have shown that vitamin D therapy stabilizes bone turnover in rickets (Scariano et al. 1995). Osteomalacia was the main cause of death among childbearing women in 19th century England.

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(Wharton & Bishop 2003). Underdeveloped or malformed pelvis hindered the delivery and caused the death of the mother or both mother and baby (Ala-Houhala 2002).

In the early 1900s, the incidence of rickets was 44-80% in Finland (Ylppö 1925, Leppo 1940). Supplementing the diets of newborns and small children with vitamin D started in the 1940s in Finland. Initially the doses were 2400-4000 IU (60-100 µg) for weaning and small children and 4000-6000 IU (100-150 µg) for older children. However, these doses were soon deemed too extensive, as they caused hypercalcaemia and kidney stones (Hallman et al. 1964). In 1960, the recommendation was diminished to 2000 IU (50 µg), and a decade later to 1000 IU (25 µg). Since 1970, the incidence of rickets has been less than 0.6% according to child welfare clinics (Ala-Houhala et al. 1995). In 1992, the guidelines for weaning and small children declined to 400 IU (10 µg); in daily use, this was considered safe and adequate intake for children aged under 3 years (Ala-Houhala et al. 1995). Cases of rickets are very rare today in Finland, but those present are virtually always related to insufficient of vitamin D supplementation.

Some vitamin D supplementation studies have been carried out in healthy children and adolescents (Docio et al. 1998, Guillemant et al. 2001, Lehtonen-Veromaa et al. 2002, Moyer-Mileur et al. 2003, Cheng et al. 2006, El-Hajj Fuleihan et al. 2006), but only four of these (Lehtonen-Veromaa et al. 2002, Moyer-Mileur et al. 2003, Cheng et al. 2006, El-Hajj Fuleihan et al. 2006) had BMD as the outcome variable. In studies of Guillemant et al. (2001) and El-Hajj Fuleihan et al. (2006), dosages were notably higher than considered safe for continuous longitudinal use by children under 11 years of age (Scientific Committee on Food, 2002). Moreover, Guillemant et al. (2001) reported that three oral doses of 2.5 mg of vitamin D$_3$ at 2-month intervals for 13-to 17–year-old boys maintained the post-summer 25-OHD concentration at 55 nmol/l throughout the winter. In the study of El-Hajj Fuleihan et al. (2006), adolescent girls reached a S-25-OHD of 95 nmol/l (measured with competitive protein binding assay) with a daily dose of 50 µg.

A prospective study from Switzerland showed that children given vitamin D supplementation for 1-2 years during early childhood had higher BMD 13 years later than control subjects who had received no supplementation (Zamora et al. 1999). Lehtonen-Veromaa et al. (2002) reported that supplementation benefited more girls with low initial vitamin D status than those that initially had adequate vitamin D status; BMAD increased more in subgroup with low vitamin D status than in subgroup with adequate vitamin D status, although their results concerning 25-OHD were suspicious because either S-25-OHD or the assessment method did not respond to D$_2$
supplementation. Adolescent girls from Libanon responded to vitamin D supplementation by increasing total hip BMC as compared with placebo (El-Hajj Fuleihan et al. 2006), but Cheng et al. (2005) did not report any beneficial effects in their vitamin D intervention study with 10-to 11-year-old Finnish girls. However, they did find a positive association between higher 25-OHD together with low PTH and higher BMD in a cross-sectional study (Cheng et al. 2003), which is in line with the observation of Outila et al. (2001). In addition, combination therapy of calcium and vitamin D improved trabecular bone BMD in 12-year-old girls compared with the unsupplemented placebo-receiving group in a one-year study (Moyer-Mileur et al. 2003).

2.3.4 Effect of vitamin D on bone metabolism in adults and the elderly

Vitamin D deficiency induces osteomalacia in adults, in which the body’s calcium balance becomes negative and calcium is mobilized from bone. Bone formation rate is lowered, which contributes to increased osteoid thickness and osteoid volume per unit of bone volume, as observed in histological experiments (Parfitt 2003). In DXA, this is seen as decreased BMD, but osteomalacia could not be distinguished from osteoporosis. From the bone biopsy of the ilium, trabecular structure of the bone is maintained in osteomalacia, but under the cortex an undermineralized area is present (Parfitt 2003). Muscle weakness and pain, also called myopathy, are typically associated with osteomalacia. Osteoporosis, in turn is characterized by lowered BMD due to accelerated bone turnover, in which more bone is resorbed than formed. The inner structure of bone is affected, which is noted as a decrease in bony bridges in bone biopsy.

There is general agreement that insufficient vitamin D status promotes the development of osteoporosis (e.g. Zitterman 2003). Vitamin D intervention studies have been done mainly in the elderly to prevent bone loss (Dawson-Hughes et al. 1995, Ooms et al. 1995, Chapuy et al. 2002, Trivedi et al. 2003). Intervention trials in which vitamin D supplementation was beneficial to BMD or decreased fracture risk are few (Dawson-Hughes et al. 1995, Chapuy et al. 2002), while others have detected no change (Hunter et al. 2000, Patel et al. 2001, Grant et al. 2005). The effective dose of vitamin D varies between subjects with weight and initial vitamin D status having an impact on response (Heaney et al. 2003a). In many studies where positive effects were seen, calcium supplementation was included as well (Chapuy et al. 1992, Dawson-Hughes et al. 1997); thus, the calcium intake is thought to modulate the vitamin D requirement (Lips 2004). A recent meta-analysis of vitamin D (Bischoff-Ferrari & Dawson-Hughes 2007) supports the beneficial effect of vitamin D on bone health maintenance, but also states that the dose required to reach a serum 25-
OHD of at least 75 nmol/l is 17.5-20 µg (700-800 IU), which is in line with the report of Vieth et al. (2007).

2.3.5 Mechanisms of action of vitamin D on bone

The main effect of vitamin D on bone is proposed to be mediated through calcium balance, as vitamin D-deficient persons with adequate calcium intake benefit from vitamin D supplementation (Lips 2004, Heaney 2005). When positive effects on bone health have been seen, the calcium balance was improved (Heaney. 2005). Vitamin D intake increases calcium absorption from the intestine and promotes mineralization of the skeleton. Recently, an alternative mechanism by which vitamin D might affect bone via muscles or a Gc-globulin-related mechanism has been described (Lauridsen et al. 2004). These routes do not exclude each other. Although the systemic effect of calcitriol focuses on the body calcium balance, the other mechanisms make sense in this context as well.

In osteoblast cells, as in many other cells, 1-α-hydroxylases are present, which enables the cells to form calcitriol locally. Calcitriol upregulates matrix protein synthesis in osteoblasts (Xue et al. 2006), thus reinforcing bone formation independently of PTH. In addition, vitamin D has a role in muscle performance and muscle strength maintenance via specific nuclear receptor (e.g. Bischoff-Ferrari et al. 2004b, Wicherts et al. 2007), which is observed especially among the elderly, but possibly is also present among children. However, in both rickets and osteomalacia, clinical symptoms include myopathy (Parfitt 2003). Because muscles surround bones and induce mechanostatic loading to bone (Heaney et al. 2000), this could be considered a mechanism affecting bone as well. Finally, 25-OHD may have an independent and direct effect on bone since Gc-globulin transporting 25-OHD in the circulation forms a complex with transmembrane proteins of osteoclasts. The genotype with the lowest Gc concentration had less fractures that other genotypes in a study of 600 pre-menopausal women (Lauridsen et al. 2005). However, the association between Gc genotype and bone health requires further investigations.
3. AIMS OF THE STUDY

The objectives of this thesis were to characterize the effects of vitamin D supplementation on different outcome variables such as S-25-OHD, S-iPTH and BMD. A deeper goal was to elucidate adequate vitamin D intake to preserve bone health for each target group (adolescent girls, men and the elderly).

Specific research questions as follows:

Study I: Does vitamin D increase bone mineral accretion dose-dependently among adolescent girls with high habitual intake of calcium in a randomized, placebo-controlled one-year intervention?

Study II: Do S-25-OHD, S-iPTH, BMD or bone remodelling markers vary between different months in early and mid-pubertal Caucasian girls in a cross-sectional setting?

Study III: Does vitamin D increase S-25-OHD concentration dose-dependently in ambulatory elderly women? Which dose increases vitamin D status above the insufficient level (S-25-OHD≥50 nmol/l) and decreases S-iPTH concentration within 12 weeks?

Study IV: Is there seasonal variation in bone remodelling markers and radius BMD in a 6-month study? Which vitamin D dose prevents these changes among healthy 21-to 49-year-old men?
4. SUBJECTS AND METHODS

4.1 Subject recruitment and study designs

4.1.1 Intervention trial in adolescent girls (I)

A total of 228 adolescent girls, aged 11-12 years were studied in the capital region of Helsinki (60°N), in southern Finland. Recruitment was conducted in primary schools. The subjects were healthy, used no medicines known to affect calcium metabolism and were of Caucasian origin.

The subjects first presented between September 2001 and March 2002 and were later examined every 6th month. The study lasted one year. During each visit height, weight and pubertal development were measured. Background data on diet, physical activity and serum and second void urinary samples were collected. Bone mineral density (BMD) was measured twice with a Hologic DXA (Fig. 11).

A stratified randomization process was performed three times. The stratification factor was pubertal development, Tanner stage. At each Tanner stage, an equal number of subjects was randomly assigned to three treatment groups receiving 0 μg (placebo), 5 μg, or 10 μg of vitamin D₃ as a supplement (Scanpharm Ltd., Denmark). The vitamin D₃ content of the tablets was confirmed by an analysis performed at the Danish Institute for Food and Veterinary Research, Söborg, Denmark. The subjects were instructed to take one tablet daily for 28 days during each month of the one-year study. The blister packs were returned to researchers during each visit, and compliance was confirmed by pill counts. The study was double-blinded.

4.1.2 Cross-sectional study of adolescent girls (II)

Girls entering the longitudinal intervention study and scoring Tanner stage 2 (=early puberty) or 3 (=mid-puberty) were selected for the cross-sectional study (N=196). Data were collected during the first visit and included fasting blood samples, second void urinary samples, DXA-measured BMD and background information.
4.1.3 Short-term intervention in elderly women (III)

Women aged 65-85 years were recruited from the Helsinki area by an announcement in a local paper in Helsinki. Fifty-two women participated in the 12-week intervention trial from the beginning of January to April 2002, when UVB radiation is scarce in Finland (Fig. 12). Of the 52 enrolled subjects, three dropped out due to the strict schedule of the study; thus, 49 completed the study. According to the protocol, travelling to sunny places was not allowed during the study. Subjects were randomly assigned to four groups receiving placebo, 5 µg, 10 µg or 20 µg of vitamin D$_3$ a day. Scanpharm Ltd. (Birkerød, Denmark) supplied the vitamin D$_3$ tablet for groups receiving placebo, 5 µg or 10 µg of supplementation per day. Ferrosan Ltd. (Espoo, Finland) supplied the vitamin D$_3$ drops used in the 20-µg group.

Figure 12. Flow chart of Study I.
Recruitment of 65-to 85-year-old women via a local paper in December 2001
- Exclusion criteria: liver or kidney disease

Randomized into four groups, N=52
- Background data and FFQ

Placebo
N = 13

5 µg
N = 13

10 µg
N = 13

20 µg
N = 13

Study lasted 12 weeks from January to early April 2002
- sampling every other week (7 times)
- 24-hour urine collected 3 times
- FFQ was filled out again

**Figure 13.** Design of Study III.

### 4.1.4 Intervention trial in healthy adult men (IV)

Men of Caucasian origin, aged 21-49 years, were recruited from the Helsinki area by an announcement in a campus area of the University of Helsinki. This was a randomized, double-blind, placebo-controlled, 6-month vitamin D intervention study in which 54 men participated (Fig. 13). Participants were allocated to three groups and assigned four tablets consisting of 20 µg (800 IU), 10 µg (400 IU), or placebo with morning meals daily. All tablets were provided by Verman Oy, Minisun, and were similar in size and taste. Subjects were asked to take tablets until trial closure and to return any unused tablets during the study to researchers for pill counting. In addition, participants were advised to record the days they forgot to take pills in a follow-up diary. Compliance was confirmed with pill counts and the diary. A total of 49 men completed the study. According to the protocol, travelling to sunny places or using tanning salons was not allowed during the study.

Randomization was performed by minimizing the variation of age, weight, initial S-25-OHD status and dietary intake of vitamin D in three groups.
The trial lasted from the beginning of November until the end of April, altogether 26 weeks. Fasting blood samplings were collected at 5-to 6-week intervals, altogether 6 times at the same time-points during the day between 7:30 and 9:30 am. Body weight, height and distal and proximal radius BMD were measured by peripheral QCT at enrolment and after 6 months.

**Figure 14.** Flow chart of Study IV.

### 4.1.5 Ethical consideration

For each study protocol, ethical approval was obtained from the Ethics Committee of the Helsinki and Uusimaa Hospital District. In addition, the protocol of Study I was disclosed to the National Institute of Medicine. The subjects and parents of minors gave informed written consent in accord with the Helsinki Declaration before entering the study. Subjects could withdraw their participation without a reason at any time. Subjects were provided copies of their personal results for dietary intakes of calcium and vitamin D, S-25-OHD concentration and BMD. The overseeing doctor discussed low BMD values or other unhealthy findings with subjects.
4.2 Methods

4.2.1 Background characteristics (I-IV)
All subjects completed a form containing questions on their medical history, use of vitamin D and calcium supplements, time spent outdoors, sunny holidays and physical activity. Weight and height of subjects were measured at enrolment and at the end of the study.

The physical activity of subjects consisted of school or work trips, guided leisure time activity and leisure time activity on their own (Outila et al. 2001). The activity was calculated in minutes per day.

In Studies I and II, the pubertal stage of the subject was assessed *ad modum* Tanner (Marshall & Tanner 1969). A self-assessment protocol concerning the evaluation of breast development and timing of menarche was completed during an interview.

Dietary intakes of vitamin D and calcium were assessed with a food frequency questionnaire (FFQ), a validated semi-quantitative questionnaire covering over 70 foods (Outila et al. 2001). The nutrient contents of the foods were calculated using the Finnish National Food Composition Database, Fineli®; version 2001, which is maintained by Nutrition Unit of the National Public Health Institute of Finland.

All forms were checked by the researchers, and if needed, additional information was requested.
Table 6 summarizes background and nutritional data of subjects in each study presented with means (SDs).

**Table 6.** Background data in Studies I-IV presented with mean (SD) and median.

<table>
<thead>
<tr>
<th>Study</th>
<th>N, gender</th>
<th>Age, years</th>
<th>Height, cm</th>
<th>Weight, kg</th>
<th>Physical activity, min/d</th>
<th>Vitamin D intake, µg/d</th>
<th>Calcium intake, mg/d</th>
<th>Supplement users, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>228, F</td>
<td>11.4</td>
<td>149.2</td>
<td>42.4</td>
<td>169</td>
<td>5.6 (4.0)</td>
<td>1260</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.4)</td>
<td>(7.5)</td>
<td>(9.8)</td>
<td>(92)</td>
<td></td>
<td>(550)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>196, F</td>
<td>11.4</td>
<td>149.0</td>
<td>41.7</td>
<td>169</td>
<td>5.8 (4.3)</td>
<td>1230</td>
<td>18.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(0.4)</td>
<td>(7.2)</td>
<td>(9.0)</td>
<td>(90)</td>
<td></td>
<td>(530)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>52, F</td>
<td>71.7</td>
<td>162.1</td>
<td>68.7</td>
<td>-</td>
<td>10.2 (5.7)</td>
<td>1050</td>
<td>33.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3.9)</td>
<td>(6.4)</td>
<td>(12.1)</td>
<td></td>
<td></td>
<td>(420)</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>54, M</td>
<td>28.7</td>
<td>178.6</td>
<td>79.2</td>
<td>42</td>
<td>7.7 (5.2)</td>
<td>1340</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(6.7)</td>
<td>(7.8)</td>
<td>(14.2)</td>
<td>(32)</td>
<td></td>
<td>(870)</td>
<td></td>
</tr>
</tbody>
</table>

- not defined
4.2.2 **Laboratory measurements (I-IV)**

Overnight fasting blood samples were collected in the morning between 7:30-9:30 to avoid the diurnal rhythm of PTH to confound the results. Venoject gel tubes were used to obtain clear sera. Blood samples were processed within 3 h and centrifuged at 3000 rpm for 15 min. Serum was stored in aliquots at -70°C until analysis.

From serum samples, 25-OHD concentration (S-25-OHD$_2$ and S-25-OHD$_3$) was measured with HPLC analysis at the Danish Institute for Food and Veterinary Research, Söborg, Denmark (I-III). In Study IV, S-25-OHD was measured with an OCTEIA® enzyme immunoassay (IDS, Boldon, UK). The accuracy of both methods was monitored by participation in the Vitamin D External Quality Assessment Scheme (DEQAS, Charing Cross Hospital, London, UK). Standardized concentrations of 25-OHD were provided for Study IV. Other parameters measured from serum samples are summarized in Table 7.

Second void urine samples were collected in Studies I, II and IV. In Study III, 24-hour urine collections were performed. Portions of urine were stored at -20°C until analysis. Calcium and phosphate secretion together with bone resorption markers deoxypyridinoline and pyridinoline were measured from urine samples (Table 7) with specific methods.
### Table 7. Summary of laboratory measurements in Studies I-IV.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Biomarker</th>
<th>Method</th>
<th>Study of analysis</th>
<th>Location of analysis</th>
<th>CV% intra- and interassays</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vitamin D status</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcidiol</td>
<td>S-25-OHD</td>
<td>HPLC</td>
<td>I-III</td>
<td>DFVR</td>
<td>4.3, 6.3</td>
</tr>
<tr>
<td></td>
<td>S-25-OHD</td>
<td>EIA</td>
<td>IV</td>
<td>HU</td>
<td>2.0, 7.9</td>
</tr>
<tr>
<td>Parathyroid hormone</td>
<td>S-iPTH</td>
<td>EIA</td>
<td>I-IV</td>
<td>HU</td>
<td>2.3, 4.0</td>
</tr>
<tr>
<td><strong>Calcium metabolism</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum calcium</td>
<td>S-Ca</td>
<td>Spectrophotometric</td>
<td>I-IV</td>
<td>HU</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Urinary calcium</td>
<td>U-Ca</td>
<td>Spectrophotometric</td>
<td>I-IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum phosphate</td>
<td>S-Pi</td>
<td>Spectrophotometric</td>
<td>I-IV</td>
<td>HU</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Urinary phosphate</td>
<td>U-Pi</td>
<td>Spectrophotometric</td>
<td>I-IV</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Bone formation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteocalcin</td>
<td>S-OC</td>
<td>ELISA</td>
<td>I, II</td>
<td>UCC</td>
<td>&lt;11</td>
</tr>
<tr>
<td>Bone-specific alkaline phosphatase activity</td>
<td>S-BALP</td>
<td>EIA</td>
<td>IV</td>
<td>HU</td>
<td>2.9, 5.0</td>
</tr>
<tr>
<td><strong>Bone resorption</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Deoxypyridinoline</td>
<td>U-DPyr</td>
<td>HPLC</td>
<td>I, II</td>
<td>UCC</td>
<td>3</td>
</tr>
<tr>
<td>Pyridinoline</td>
<td>U-Pyr</td>
<td>HPLC</td>
<td>I, II</td>
<td>UCC</td>
<td>5</td>
</tr>
<tr>
<td>Tartrate resistant acid phosphatase</td>
<td>S-TRACP</td>
<td>EIA</td>
<td>IV</td>
<td>HU</td>
<td>4.0, 4.0</td>
</tr>
</tbody>
</table>

HPLC high performance liquid chromatography  
EIA enzyme immunoassay  
ELISA enzyme linked immunoassay  
DFVR, Danish Institute for Food and Veterinary Research, Søborg, Denmark  
HU, Department of Applied Chemistry and Microbiology at the University of Helsinki  
UCC, Department of Food and Nutritional Sciences and Biosciences Institute, University College Cork, Ireland

#### 4.2.3 Measurements of bone mineral density

**4.2.3.1 DXA (I, II)**

The bone area (BA), bone mineral content (BMC) and areal bone mineral density (aBMD) were measured at the beginning and end of the study with a dual-energy x-ray absorptiometry (DXA) model A (QDR 4500, Hologic, Waltham, MA, USA) from the lumbar spine L1-L4 vertebrae and left femur region. The femur region includes the femoral neck, trochanter, Ward’s triangle and the inter-area, which is analysed as a single area of interest. Calibration of the measurement was performed using a spine phantom; the interassay CV for the phantom was 0.31%. Intra-assay CVs were determined with duplicate measurements of 10 subjects. CVs for BMD in the left femur and lumbar vertebrae were 0.67% and 1.39%, respectively.
The site-specific bone mineral augmentation was determined following changes in BMC, as suggested for growth studies (Heaney 2003). The change in BA confounds the interpretation of BMC since the correlation between the change in BMC and BA was strong ($r =0.8, P<0.001$); we adjusted BMC with BA (Prentice et al. 1994, Carter et al. 2001, Lehtonen-Veromaa et al. 2002, Mølgaard et al. 2004). In addition, BMC values were adjusted for change in weight and Tanner stage.

4.2.3.2 pQCT (IV)
Peripheral quantitative computed tomography (pQCT) to acquire peripheral BMD from the non-dominant radius was used in Study IV. Two 2-mm thick slices (voxel size of 0.50 mm) were scanned in the proximal direction from the distal radius (XCT-2000, Stratec, Pforzheim, Germany). Sites were 4% and 66% of the axial length, referred to as the distal and proximal radii in this text. A 30-mm planar scout view was used to locate a standard anatomical site for the radius reference line at the distal end. Length of the forearm was defined as the distance between the olecranon and the processus styloideus, forming the basis for the location of distal and proximal slices. At 6 months measurements were performed at the same sites, which were recognized by comparing the scout views.

The data were analysed using version 5.4 of the manufacturer’s software package in which the outer contour of bone was defined with a threshold of 280 mg/cm$^3$ (Rauch et al. 2001b). The trabecular (Trab) BMD was determined as a mean density of 45% of the total bone (TB) cross-sectional area (CSA) at 4% site. At 66% site an additional threshold of 770 mg/cm$^3$ was introduced to define cortical bone (Cort) BMD. This autoanalyse was performed on all measurements.

Short-term precision (CV%) was determined with duplicate measurements of seven subjects. CVs for the BMD and CSA in TB, Cort and Trab bone were 2.15, 1.99, 0.71, 0.88 and 1.32, 1.99, respectively. Phantom scans were executed daily, to maintain quality assurance. The long term CV% for the phantom BMD and CSA were 1.9, 1.1, 2.7, 0.79 and 0.50, 0.78 in the TB, Cort and Trab bone, respectively.
4.2.4 Statistical analyses

Statistical analyses were performed with SPSS version 12.0 for Windows (SPSS Inc., Chicago, IL, USA, 2000). The normal distribution of variables was tested with the Kolmogorov-Smirnov test. If normality was not present, logarithmic transformations were performed. The relationship between variables was shown with Pearson or Spearman correlations, depending on the distribution of the variables.

Repeated measure analysis of variance (ANOVA) was used to detect the effect of supplementation on S-25-OHD and S-iPTH in Studies I, III and IV. Comparison between groups was performed with contrasts. The baseline value of the corresponding variable was used as a covariate in each of these studies. Baseline characteristics and the total change in variable (Δvariable = final value - baseline value) were tested with one-way ANOVA. The post-hoc tests were performed with Tukey’s honestly significant difference (HSD), Dunnett’s test or least significant difference (LSD) depending on the feature of the tested variable.

BMD, BMC, bone remodelling markers and corresponding Δvariable were tested with both ANOVA and analysis of covariance (ANCOVA) to illustrate the effect of confounding factors. Comparison of groups in ANCOVA was performed with contrasts, and for each analysis, covariates are shown in footnotes. Covariates were selected if their correlation with the outcome variable was highly significant or if it was known to affect the outcome according to the literature. For example, with regard to BMC, typical covariates are weight, calcium intake and physical activity (Carter et al. 2001, Lehtonen-Veromaa et al. 2002).

Evidence from randomized controlled trial (RCT) is novel and allows causality to be determined (Uhari & Nieminen 2001). Regression models in longitudinal studies can be constructed to define and clarify causality as well. In Study I, analyses were performed with both an intention-to-threat (IT) and a compliance-based (CB) method.

Results were considered statistically significant when p < 0.05. P values between 0.05 and 0.1 were considered trends. In tables, the standard deviation (SD) is presented in parenthesis after the mean value. In figures, the standard error of the mean (SEM) is shown.
5. RESULTS

5.1 Dietary intakes of vitamin D and calcium and their corresponding serum 25-OHD and PTH concentrations (I, III, IV)

Three studies (I-III) were performed before the vitamin D fortification of liquid milk products began in March 2003 (Fig. 15). In 2004, the Nordic Council of Ministers established new nutrient recommendations, which are applied in this thesis when evaluating adequacy of dietary intake of vitamin D.

Figure 15. Timing of studies, fortification and renewed recommendations.

Baseline total intakes of vitamin D and calcium included intake from supplements together with corresponding 25-OHD and PTH concentrations are presented in Table 8.

Table 8. Baseline values for the intakes of vitamin D and calcium, 25-OHD and PTH presented with mean (SD).

<table>
<thead>
<tr>
<th>Study</th>
<th>N, gender</th>
<th>Age, years</th>
<th>Total vitamin D intake, µg/d, median</th>
<th>Calcium intake, mg/d</th>
<th>S-25-OHD, nmol/l</th>
<th>S-iPTH, pmol/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>228, F</td>
<td>11.4 (0.4)</td>
<td>5.6 (4.0) <strong>4.5a</strong></td>
<td>1260 (550) b</td>
<td>47.6 (17.2) c</td>
<td>3.35 (1.07)</td>
</tr>
<tr>
<td>III</td>
<td>52, F</td>
<td>71.7 (3.9)</td>
<td>10.2 (5.7) <strong>9.2</strong></td>
<td>1050 (420) d</td>
<td>47.2 (14.7) d</td>
<td>3.91 (1.18)</td>
</tr>
<tr>
<td>IV</td>
<td>54, M</td>
<td>28.7 (6.7)</td>
<td>7.7 (5.2) <strong>6.6</strong></td>
<td>1340 (870) e</td>
<td>67.2 (16.9) e</td>
<td>2.56 (0.74)</td>
</tr>
</tbody>
</table>

a N=223  
b January 2002  
c September 2001 to March 2002  
d N=223  
e October 2004
The use of supplements was expedient (Fig. 16), as with supplements the mean total vitamin D intake among girls was about 1.2 µg higher than the mean dietary intake of vitamin D. However, using the median value instead of the mean is advisable because the distribution of the intakes was not Gaussian. In this case, the difference between total and dietary intake narrowed to 0.5 µg among girls (Fig. 16), concluding that both intakes were lower than recommended (= 7.5 µg).

Similarly 50% of those studied in each age group had an intake below the recommended level. Note that Study IV was performed in 2004, when fortification of fluid milk products was ongoing.

**Figure 16.** Distribution of vitamin D intakes in Study I among adolescent girls without A) and with B) supplements. Five of the 228 girls did not fill in the FFQ.

Baseline S-25-OHD concentration illustrates more seasonal differences than differences related to dietary intakes among groups. Vitamin D status in winter best reflects the dietary intake of vitamin D and its adequacy (Table 9).

**Table 9.** The distribution of vitamin D status (based on Dawson-Hughes et al. 2005) during winter months in Studies II-IV.

<table>
<thead>
<tr>
<th>S-25-OHD, nmol/l</th>
<th>Vitamin D status</th>
<th>Study II (%)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Study III (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Study IV (%)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 25</td>
<td>Deficient</td>
<td>15 (19.2)</td>
<td>3 (6.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>25-49.9</td>
<td>Inadequate</td>
<td>54 (69.2)</td>
<td>25 (51)</td>
<td>9 (56.3)</td>
</tr>
<tr>
<td>50-79.9</td>
<td>Adequate</td>
<td>8 (10.3)</td>
<td>20 (40.8)</td>
<td>7 (43.7)</td>
</tr>
<tr>
<td>≥ 80</td>
<td>Optimal</td>
<td>1 (1.3)</td>
<td>1 (2.1)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>78 (100)</td>
<td>49 (100)</td>
<td>16 (100)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Included girls that enrolled the study during February and March 2002

<sup>b</sup> All subjects included during January 2002

<sup>c</sup> Included men in the placebo group during March 2005
Wintertime dietary intake of vitamin D and the concentration of 25-OHD correlated positively among girls and elderly women (Fig. 17) \( (r = 0.357, p = 0.002 \) and \( r = 0.415, p = 0.003 \), respectively). In Study IV, this was not tested, because baseline data was collected in October when results are confounded by solar exposure.

**Figure 17.** Correlation between S-25-OHD and dietary intake of vitamin D among elderly woman in Study III \( (r = 0.415, p = 0.003) \).

An inverse correlation was found between 25-OHD and PTH in girls and men (Fig. 18 A and B, respectively), but not in elderly women. In winter, elevated PTH \(( \geq 4.1 \text{ nmol/l})\), reflecting secondary hyperparathyroidism, was observed in 17 (23.9 %) among girls, 17 (34.7 %) among elderly and 3 (18.8 %) in men. Elevated PTH values were present although subjects were healthy and their calcium intake was adequate.

**Figure 18.** Significant negative correlations between S-25-OHD and S-iPTH were observed among A) girls \((N=228)\) and B) men \((N=52)\).
5.2 Seasonal variation in serum 25-OHD, PTH, bone remodelling markers and bone mineral density (II, IV)

In Study II the mean serum 25-OHD concentration differed between months (ANOVA; p<0.001); the highest mean concentration was measured in September-October and the lowest in February. The opposite was observed in S-iPTH concentration, but the difference among months was not significant in the whole group, (p=0.627), but among early puberty girls (= Tanner stage 2) a tendency of variation was observed, p=0.069. The monthly mean value for serum 25-OHD and PTH are shown in Table 10.

Table 10. The monthly mean value for serum 25-OHD and S-iPTH are shown with mean (SD).

<table>
<thead>
<tr>
<th>Month</th>
<th>N</th>
<th>S-25-OHD (nmol/l)</th>
<th>PTH (pmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>September</td>
<td>35</td>
<td>59.5 (13.4)</td>
<td>3.18 (1.16)</td>
</tr>
<tr>
<td>October</td>
<td>57</td>
<td>54.7 (13.4)</td>
<td>3.30 (0.91)</td>
</tr>
<tr>
<td>November</td>
<td>35</td>
<td>41.9 (16.4)</td>
<td>3.35 (1.30)</td>
</tr>
<tr>
<td>February</td>
<td>29</td>
<td>37.3 (15.5)</td>
<td>3.41 (0.85)</td>
</tr>
<tr>
<td>March</td>
<td>40</td>
<td>38.2 (12.7)</td>
<td>3.56 (1.11)</td>
</tr>
</tbody>
</table>

*a* p<0.001 for whole group, p<0.001 for early (=Tanner stage 2, N= 121) p=0.027 for mid puberty girls (=Tanner Stage 3, N= 75)

In the whole group of early and midpuberty girls S-OC and U-DPyr concentrations varied among months after adjustment for age, puberty development, weight, height, physical activity and dietary intake of calcium (ANCOVA; p=0.062 and p=0.035, respectively) , whereas U-Pyr did not, p=0.635. After stratification to pubertal stage, only S-OC was found to differed among months (ANCOVA; p<0.001) among early puberty girls (see original publication fig. 4).

Similarly, the seasonality was seen only among early puberty girls in the BMD of the femur and lumbar vertebrae. The femoral BMD differed among months (ANCOVA; p=0.047) among early puberty girls. The BMD in October was 7.6% higher than in March (p<0.008, by contrast). Neither the femoral BMC nor BA followed the seasonal rhythm (ANCOVA; p=0.229 and p=0.314, respectively). In the lumbar vertebrae BMD, a trend for seasonal variation was seen (p=0.057), although the difference between October and March was 7.0 % significant, (contrast; p=0.007). No seasonality was noted in the lumbar vertebrae BMC or BA (ANCOVA; p=0.168 and p=0.619, respectively) (see original publication fig. 2 and fig. 3).
In a longitudinal study of men (IV), a seasonal variation in S-25-OHD and PTH concentration was observed in the placebo group consisting of 16 subjects (repeated measures ANOVA; p<0.001 and p=0.011, respectively) (Fig. 19 A).

Bone formation marker, S-BALP was maintained in the placebo group of study IV during six month interval. But for the same men, resorption marker S-TRACP decreased significantly through winter (repeated measures ANOVA; p<0.05) (Fig. 19B). The difference between November and April was 7.8%.

In study IV, vBMD was measured from the distal and proximal parts of the radius with pQCT. No seasonal variation was noted.

**Figure 19.** Seasonal variation in a follow-up study of men (IV) in A) S-25-OHD and PTH and B) bone remodelling markers S-TRACP and S-BALP (N=16).

**5.3 Effect of vitamin D supplementation on serum 25-OHD and PTH concentration (I, III, IV)**

In supplementation studies (I, III and IV), vitamin D supplementation increased S-25-OHD concentration dose-dependently (Fig. 20); the higher the dose, the higher the increment in S-25-OHD concentration. Fig. 21 presents the response of PTH to supplementation.
Figure 20. Response of S-25-OHD concentration to vitamin D supplementation A) adolescent girls, B) elderly woman and C) men, tested with repeated measures ANOVA; *p*<0.001 for each study. Error bars represent SEM. (■) placebo, (▲) 5µg, (●) 10 µg and (○) 20 µg.

Figure 21. Response of S-iPTH concentration to vitamin D supplementation A) adolescent girls, B) elderly woman and C) men. Only for men, *p*<0.001. Error bars represent SEM. (■) placebo, (▲) 5µg, (●) 10 µg and (○) 20 µg.
The dose-response was calculated by dividing the increment in S-25-OHD concentration with a given dose. Dose-responses were independent of the dosage used. Table 11 summarizes the mean dose-response (DR) of each study.

**Table 11. Dose-responses in Studies I, III and IV presented with means (SDs).**

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Baseline 25-OHD, nmol/l</th>
<th>DR, nmol/l/ȝg median</th>
<th>95% CI for mean DR</th>
<th>Correlation between DR and baseline 25-OHD</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Adolescent girls</td>
<td>47.6 (17.2)</td>
<td>1.24 (2.51) <strong>1.15</strong></td>
<td>0.81-1.68</td>
<td>-0.606**</td>
</tr>
<tr>
<td>III</td>
<td>Elderly women</td>
<td>47.2 (14.7)</td>
<td>1.57 (1.18) <strong>1.32</strong></td>
<td>1.17-1.97</td>
<td>-0.467**</td>
</tr>
<tr>
<td>IV</td>
<td>Men</td>
<td>67.2 (16.9)</td>
<td>1.54 (1.24) <strong>1.48</strong></td>
<td>1.08-2.00</td>
<td>-0.552**</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td></td>
<td>**1.36 (2.17) <strong>1.23</strong></td>
<td><strong>1.05-1.56</strong></td>
<td></td>
</tr>
</tbody>
</table>

** p<0.001

We observed that S-25-OHD increased more in subjects with a lower initial 25-OHD concentration. This finding was repeated in each study. In Study III, a plateau in 25-OHD concentration was reached within 6 weeks with each given dose; dose-responses did not differ between the 6- and 12-week time-points. In summary, mean dose-responses did not differ between studies, reflecting that the effect of supplementation over a short period is apparent and that the concentrations reached are similar to those in longer studies.

Although supplementation did not affect PTH in the whole group of Study I (Fig. 21A), there was a positive trend present in the autumn cohort (Fig. 22). With 10 ȝg of vitamin D, a stable PTH was maintained throughout the year in adolescent girls. However, PTH was not affected in the short-term study of elderly women. Positive effects were seen among men; 10 ȝg inhibited the seasonal variation in PTH concentration. For men, total damping down of PTH was obtained with 20 ȝg of vitamin D (Fig. 21C).

The determinants of dose-response were investigated with the linear regression model (Fig. 23). The model included possible determinants such as age, weight, height, pubertal development stage, hormone replacement therapy, activity time, intakes of calcium and vitamin D and initial 25-OHD concentration. Of these, the significant determinants were chosen by a stepwise backward method. In each study, the final significant determinant was baseline 25-OHD concentration; the ß intercept was: -0.101 (p<0.001), -0.037 (p=0.030) and 0.058 (p<0.001) for Studies I, III and IV, respectively.
Figure 22. Response of S-iPTH to supplementation in the autumn cohort of Study I (N=102). The overall effect was not significant (repeated measures ANOVA; p=0.301), but a trend of difference was observed at the 6-month time-point (March-April) (p=0.056). Comparison with contrast indicated that with 10 µg PTH was lower by 0.723 (0.324) pmol/l (p=0.024) and with 5 µg lower by 0.576 (0.312) pmol/l (p=0.067) than with placebo.

Figure 23. Pooled data from Studies I, III and IV (N=197). The association between S-25-OHD and dose-response was linear as follows $y = 4.9 - 0.07 \times x$. According to this equation, the dose-response is zero at 70 nmol/l.

5.3.1 Estimation of adequate vitamin D intake (I, III, IV)

Adequate vitamin D intake was estimated in Studies I, III and IV either with the help of dose-response or based on total vitamin D intakes in subjects with a target 25-OHD concentration. The goal was to quantify the vitamin D intake that maintains stable 25-OHD throughout the year (Whiting & Calvo 2005). The target concentration was 60-62 nmol/l, and extrapolations for 80 nmol/l were also performed. In the calculations, the mean dose response was assumed to be 1.4 nmol/l/µg, which is somewhat higher than earlier reports (0.6-1.0 nmol/l/µg) (Heaney et al. 2005).
In Study I, the mean decrease from baseline in the placebo autumn cohort was 23.0 (12.3) nmol/l, and according to dose-response an required intake of 16 µg/d would maintain this concentration. However, the mean total intake among girls in the 10-µg group was 14.5 µg, thus, in practice, this was adequate. To achieve 80 nmol/l, girls would have needed to increase the 25-OHD concentration by 32.5 nmol/l; this could have been achieved with roughly 23 µg/d of vitamin D.

Among the elderly in Study III, the mean decrease in the placebo group was 4.5 (13.7) nmol/l, which would be avoided with an additional intake of 3.2 µg/d, thus increasing the total intake to 13 µg/d. However, the basal mean 25-OHD concentration was 47 nmol/l, hence increasing the S-25-OHD by 15 nmol/l to 62 nmol/l is achieved with an additional intake of 11 µg/d (total 21 µg/d). We observed that the total intake of vitamin D among subjects with S-25-OHD concentration around 60 nmol/l was 24 µg/d.

In Study IV, baseline S-25-OHD concentration was 62.4 (14.6) nmol/l. The 25-OHD concentration decreased 16.8 (11.7) nmol/l in the placebo group until March. According to dose-response, this would be avoided with an additional intake of vitamin D by 12 µg/d; thus, with basal intake this sums to 19 µg/d. Similarly, mean total intake among subjects who had maintained stable 25-OHD (±2) throughout the study was 17 µg/d. Total vitamin D intake among subjects achieving an optimal vitamin D status (78-82 nmol/l) was 26 µg/d.

5.4 Effect of vitamin D supplementation on bone remodelling markers (I, IV)
In Study I, the resorptive marker, U-DPyr was further decreased by vitamin D supplementation among adolescent girls (ANOVA; p=0.042) (Fig. 24). The formation marker S-OC was unaffected.

In Study IV, S-BALP decreased in vitamin D supplemented groups whereas it was maintained in the placebo (ANCOVA; p=0.044) (Fig. 25A). Resorption marker, S-TRACP decreased during the 6-month study, but the change was similar in each group. The ratio of TRACP to BALP was calculated at baseline and again at 6 months (Fig. 25B) and adjusted for physical activity and calcium intake. The ratio acted similarly in the groups (P=0.303), but it was determined by changes in PTH and 25-OHD. In a simple regression model, Δs-iPTH explained 8.8% of the variation in the TRACP to BALP ratio (P = 0.04), and the standardized β was -2.108. Likewise, Δs-25-OHD explained 6.3% of this variation, (P=0.09) and the standardized β was 0.251.
Figure 24. Bone remodelling marker changes in adolescent girls presented with bars with SEMs. The change in U-Dpyr differed among study groups (ANCOVA; p=0.042). The change was larger in the group receiving 5 µg of vitamin D compared with the placebo group (p=0.013) (marked with *), but not with the 10-µg group (p=0.304). No difference between the highest group and the placebo group was noted (p=0.130). The change in OC did not differ among groups (ANCOVA; p=0.239).

The coupled bone turnover is described with a positive correlation. At baseline, the bone remodelling markers were positively associated with each other (r = 0.309, p = 0.031) in Study IV. However, after 6 months, the bone remodelling markers, S-BALP and S-TRACP correlated positively in the 10 µg- and 20-µg groups, (r = 0.521, P = 0.038 and r = 0.502, p = 0.046, respectively), but not in the placebo group.

Figure 25. Bone remodelling markers in men presented with bars and SEMs. Panel A presents the change in serum formation marker BALP (µg/ml), which differed among groups (ANCOVA; p=0.044). The effect of vitamin D on BALP was dose-dependent (*p=0.05 for the comparison of placebo and 10 µg and ** p=0.019 for comparison of placebo and 20 µg). The change in serum TRACP (U/l) did not vary between groups. Panel B presents the ratio of TRACP to BALP at baseline and at 6 months. The ratio did not change between groups (p=0.303).
5.5 Effect of vitamin D supplementation on BMD (I, IV)

With regard to Study I the main results based on IT analysis indicate no effect of vitamin D supplementation on bone mineral accrual in the femur or the lumbar vertebrae (ANCOVA; p = 0.256 and p = 0.144, respectively) of the adolescent girls. On the other hand, according to CB results, vitamin D increased dose-dependently bone mineral augmentation both in the femur and the lumbar spine (Fig. 26) in Study I.

Since BMC augmentation depends on pubertal stage, we tested whether the effect of supplementation differed between early puberty (Tanner 1 or 2) and mid-puberty (Tanner 3-5) subgroups. The response to supplementation was similar between subgroups in the femur, but it differed in the lumbar vertebrae. Among mid-pubertal girls, BMC augmentation in the lumbar vertebrae was dose-dependent and significant (ANCOVA; p = 0.012), but this was not the case among early pubertal girls (see original publication I).

The effect of wintertime vitamin D supplementation on vBMD was studied among men (IV) with pQCT. The change in BMD in TB, Trab and Cort was analysed with ANCOVA, in which the corresponding $\Delta$CSA was used as a covariate. No difference was observed in the $\Delta$TB BMD (p=0.126) or in $\Delta$Trab BMD among groups in the distal radius (Fig. 27). Similarly, no change was present in $\Delta$Cort BMD among groups (p=0.175) in the proximal radius.

**Figure 26.** Site-specific bone mineral accrual in adolescent girls presented with bars and SEMs. Panel A indicates the change in femoral BMC in the study groups (ANCOVA; p=0.028). Bone mineral accrual was 14.3% higher with 5 µg and 17.2% higher with 10 µg than with placebo. Panel B shows the results of the lumbar vertebrae; note the dose-dependent effect. A significant change was observed only with the highest dose (ANCOVA; p=0.039), which increased BMC by 12.5% compared with placebo. P< 0.05 is indicated with * and p<0.01 with **.
Figure 27. Changes in BMD in total (A), trabecular (B) and cortical (C) bone observed in the study groups presented with bars and SEMs.
6. DISCUSSION

6.1 Methodology

6.1.1 Study design and selection bias
Studies I, III and IV were RCTs, which enabled causality to be assessed. A strict follow-up may interfere with the behaviour of subjects; this is the main bias related to experimental science. However, this type of study design is thought to give the most reliable evidence of certain causality.

Intention-to-treat (IT) analysis/approach of RCT is preferred in medical sciences. The results of IT and compliance based (CB) studies are known to differ. For example, both in a secondary prevention study, RECORD, (Grant et al. 2005) and Women Health Initiative (WHI) study (Jackson et al. 2006) the effect of vitamin D supplementation together with calcium on fracture prevention was poor and hardly detectable due to poor compliance, which has been strongly criticized. (Sambrook 2005, Boonen et al. 2007). Likewise, the results of Study I varied according to whether or not the adolescent girls had taken the supplements; positive effect was more pronounced in subjects following the protocol than in the whole group. Subjects with poor compliance weakened the effect of the supplementation on S-25-OHD and BMC augmentation, which justifies emphasizing the CB results. Furthermore, our primary aim was to define the effect of vitamin D on bone mineral accrual is Study I. Another issue was to determine the best method to increase the vitamin D intake in this target group. In addition, the groups were not distorted in basic characteristics when CB analysis was used.

A cross-sectional study is a type of descriptive research in which subjects and phenomena are more intact and discrete than in experimental research. Although the results of follow-up and cross-sectional studies may differ (e.g. Bailey et al. 2000), cross-sectional studies offer some advantages such as low drop-out rates, less affected behaviour of subjects, and especially in studies exploiting densitometry measurements, minimal exposure to radiation. However, careful planning of a cross-sectional study is needed to address the research question.

Selection bias means that participants differ from the typical population; generally volunteers are considered to be more health-orientated, healthier and better educated than non-participants of the
target group, which affects generalizability of results. This was the case particularly in studies of elderly women (III) and men (IV). The use of vitamin D-containing supplements was quite popular among elderly women in Study III, with one-third taking them. Another marker of health awareness was the use of hormone replacement therapy; roughly half of the elderly used it. Based on these assumptions, vitamin D status is likely more impaired among elderly women in general than seen in Study III, supporting the results of Andersen et al. (2005). This means that the calculated dose-response in Study III might have been higher due to lower vitamin D status, i.e. typical elderly women might have responded more prominently to supplementation than seen in our study. Similarly, men in Study IV had an academic background and were probably more health-aware than their non-participating peers. In addition, a strict study schedule was thought to be the reason for drop-outs, as men appeared to be very active during their leisure-time. However, dietary intake of vitamin D and vitamin D status were in line with the study of Lamberg-Allardt et al. (2006), suggesting that the men were good representatives of the target group and that the results could be generalized for healthy men in Finland.

In Study I, schools of adolescent girls were randomly chosen. The girls together with their parents decided whether they would participate. Over 90% of girls were willing to participate and their vitamin D status was similar to that of other teenagers in Finland (Lehtonen-Veromaa et al. 1999, Cheng et al. 2003), implying no selection bias. The results are therefore applicable to this age group at least in Finland.

6.1.2 Sample size
The number of recruited subjects in each study was based on power calculations assuming 90% power; however, in the majority of studies, 80% power is adequate to detect an effect (Scheinin 2000). The power and sample size calculations are dependent on the main outcome variable of the study. Both too small and large sample sizes are unethical. This suggests that pilot studies are unethical, which is debatable. Small-scale studies are needed to become familiar with a phenomenon and to learn new methods. Drop-out rates are difficult to predict, although researchers should anticipate them. In the study of adolescent girls (I) the sample size was based on BMD, although BMC is considered a better marker of bone mineral accrual (Heaney 2003). In Studies III and IV, the main outcome variable was 25-OHD, although other variables were measured as well. Only vBMD results in Study IV lacked the intended power, and thus, these results are somewhat disputable.
6.1.3 Methods

An overview of selected methods, including the food frequency questionnaire (FFQ), an analytical method for detecting serum 25-OHD concentration and methods for detecting BMD, is provided here. Other methods are discussed elsewhere.

From the nutritional point of view, the estimation of dietary intake of vitamin D is challenging because natural sources of vitamin D are scarce and the frequency of the consumption of these sources as well as the portion size vary between populations. Dietary history has been used for the calculation of vitamin D intake (Willet 1998). The FFQ was validated against food diaries (for a total of 25 days over a one-year period) of 45 healthy adults. During winter, the dietary intake of vitamin D by the current FFQ correlates strongly with the corresponding S-25-OHD concentrations (Studies II-IV) and determines one-fourth of the wintertime 25-OHD (Lamberg-Allardt et al. 2006), confirming the validity of the FFQ.

The FFQ includes portions of foods that are either rich sources of vitamin D, like salmon, or sources in frequently use, such as milk. The questionnaire is retrospective, exploring the previous month. Volumes are generally thought to be more easily remembered by subject than weights (Willet 1998). Thus, portion sizes could be memorized efficiently with the help of the pictures included in the FFQ. In addition, more variety exists in frequencies of consumed foods, than in portion sizes between individuals.

The FFQ tends to overestimate dietary intake of vitamin D compared with a 3- to 4-day food record at the individual level, but at a group level the difference is less obvious (less than 0.5 µg) (Lamberg-Allardt et al. 2006). The sources of vitamin D vary among the age groups; since 2003 fortified milk products have been the major source for children and young adults, but fish for older age groups (Lamberg-Allardt et al. 2006). The 4-day food record may thus be more useful for the calculation of vitamin D intake among children and young adults, whereas the FFQ may be more useful for older persons with a more varied diet including such sources as fish, which are not eaten daily.

According to the literature review, at least six different methods are available to assess S-25-OHD. Large differences are present between methods and laboratories (Carter et al. 2004). Comparing literature with results should be done carefully, taking into consideration the methods used and applying DEQAS standardized 25-OHD values when possible (Dawson-Hughes et al. 2005). In the
near future, automated analysis will become more common, increasing the repeatability of results. Issues related to precision will, however, remain. Reference ranges for vitamin D status have changed over the years, with different cut-off values used in the past, complicating the comparability of results. The current reference ranges are justified, as they are associated with better bone health throughout life, as well as with other health outcomes as described elsewhere (Bischoff-Ferrari et al. 2006). However, only a fraction of the Finnish population reached an optimal vitamin D status in 2004 (Lamberg-Allardt et al. 2006).

Finally, DXA-derived BMD is size-dependent and reflects bone mass rather than bone density. However, in growth studies BMC might characterise bone mass accrual better than BMD (Heaney 2003), but it is not utilized as much as it could be because it strongly depends on bone size as well. Among adolescent girls, we were intrigued by the BMC increasing due to an increase in bone size as bone grew, as well as to enhanced mineralization as a result of vitamin D supplementation. To investigate bone mineral accrual, we needed to adjust the increase in BMC by the increase in bone size, assuming that the growth was similar in groups, which it pretty much was, but the anabolic effect of vitamin D was neglected. Vitamin D-supplemented girls gained more weight during the study, which could be due to higher mineral accrual or to extra lean mass, probably muscle mass.

In Study I, we could not conclude, whether vitamin D induced mineralization in Trab or Cort compartment; here pQCT is more suitable than DXA. However, the limitation of pQCT is that only peripheral sites can be measured (Specker & Schoenau 2005), and in peripheral sites, changes are believed to occur over a longer period than in central sites (Szulc et al. 2003). DXA and pQCT measurements are highly biased by the user, by positioning of the subject and by performing the analysis similarly, etc. Fairly small changes become undetectable if these confounding factors are not taken into account. Duplicate measurements of a small group of subject will clarify the repeatability issue of results.

6.2 Vitamin D status and intake among target groups
Mean baseline 25-OHD concentration among adolescent girls in Study I was 47.6 (17.2) nmol/l over a 6-month period. This concentration could be regarded as illustrating the average vitamin D status among 11- to 12-year-old Finnish girls in 2001-2002. However, this semiannual mean value includes a seasonal variation. Between November and March the mean 25-OHD was lower than 50 nmol/l, which is considered the cut-off value for inadequate vitamin D status (Dawson-Hughes et al.
Before November, the total vitamin D intake from the diet and sun exposure was adequate for these adolescent girls. Most studies that examine vitamin D status are performed in the winter. When comparing results of the lowest is reasonable, but the vitamin D status in the summer completes the picture.

Among adolescent girls, the mean 25-OHD during winter was 36.0 (13.7) nmol/l, which agrees with other studies for this age group (Lehtonen-Veromaa et al. 1999, Outila et al. 2001). We observed that 19% of adolescent girls studied during February and March were vitamin D-deficient (S-25-OHD<25 nmol/l) and 69% had an inadequate vitamin D status, both of these conditions being characterized by increased PTH concentration, which is harmful for the skeleton in the long run. A higher (32-37%) prevalence of vitamin D deficiency has been reported among Finnish adolescent girls (Cheng et al. 2003, Andersen et al. 2005), as has a lower (13%) prevalence (Lehtonen-Veromaa et al. 1999, Outila et al. 2001). Of these studies, those dated 2003 or earlier have used Incstar Corporation Stillwater RIA for assessing 25-OHD, as opposed to HPLC, which would have overestimated 25-OHD about 5% compared with the RIA, thus explaining part of the differences. The vitamin D status of adolescent girls has been quite similar across Europe (Poland, Ireland, Denmark, Finland) (Andersen et al. 2005) as well as in North America (Gordon et al. 2004, Harkness et al. 2005). The median dietary intake of vitamin D calculated with FFQ was 4.5 (4.0) µg among adolescent girls, which agrees with the FFQ-assessed vitamin D intake among Finnish 9- to 15-year-old and 12- to 13-year-old girls (Lehtonen-Veromaa et al. 1999, Outila et al. 2001, Andersen et al. 2005). Lower intakes (3.0-3.7 µg) have been observed in the same age group with a 4-day food record (Cheng et al. 2003).

The ambulatory elderly women in Study III had a mean 25-OHD concentration of 47.2 (14.7) nmol/l during winter, reflecting substantially better vitamin D status than in adolescent girls. In fact, only 6% of elderly women were vitamin D-deficient, in line with another study (Nurmi et al. 2005) (based on RIA method). The low prevalence of vitamin D deficiency partly could be due to bias related to the selection of subjects, as discussed above, as Andersen et al. (2005) reported the prevalence of vitamin D deficiency to be 17% among elderly Finnish women. The elderly in nursing homes and in long-term care commonly suffer from vitamin D deficiency (Lamberg-Allardt 1984, Suominen et al. 2004), but the ambulatory elderly are aware of the risk and include supplements in their diet accordingly. Total vitamin D intake among the elderly women was 9.2 (5.7) µg/day, which nevertheless is a somewhat lower than recommended for this age group (Nordic
Council of Ministry 2004), but similar to figures reported in other studies (Lamberg-Allardt et al. 2006).

At enrolment (in October 2004), the mean 25-OHD concentration was 67.2 (16.9) nmol/l among healthy men in Study IV. In January 2005, the mean 25-OHD concentration dropped below 50 nmol/l. No cases of vitamin D deficiency existed, but 56% of unsupplemented men had inadequate vitamin D status in winter. This differs from the results of Välimäki et al. (2007), who reported vitamin D deficiency (S-25-OHD below 20 nmol/l) in 29% and S-25-OHD between 20 and 37.5 nmol/l in 44% of young men. In their study, both the RIA and HPLC methods for analysing 25-OHD were used, but there was no standardization of results (variation in result -6% to +7%) and the cut-offs used have been replaced with newer ones. Our results do, however, agree with those of Lamberg-Allardt et al. (2006), who showed that the median 25-OHD concentration among 27- to 35-year-old men was 44 nmol/l and among 36- to 60-year-old men 52 nmol/l. The dietary intake among men in study IV was 6.6 (5.2) µg a day, which is in line with Lamberg-Allardt et al. 2006.

In general, the use of vitamin D-containing supplements was beneficial in all target groups. Without supplement users, the median and mean values for vitamin D intake were even lower (see Study I). Thus, vitamin D supplements are recommended, when an individual does not consume fortified milk over 500 ml a day, consume spreads on bread and in cooking or eat fish at least twice a week.

In summary, vitamin D intake and vitamin D status among adolescent girls were both inadequate. The situation was better among elderly women and among men, as reflected by their intake and vitamin D status. In 2003, the fortification of liquid milk started, and this improved vitamin D intake and status in the Finnish population (Lamberg-Allardt et al. 2006). The currently recommended adequate intake for vitamin D is 7.5 µg, which is an improvement but likely still too low for these age groups.

6.3 Effect of seasonal variation in vitamin D status on bone (re)modelling and BMD
Seasonal variation was illustrated in 25-OHD and PTH concentration both in the cross-sectional study of adolescent girls and in the longitudinal study of men, is consistent with earlier studies (Lamberg-Allardt 1984, Rozen et al. 1994, Woitge et al. 2001, Carnevale et al. 2001, Meier et al. 2004). Typically, circannual variation is observed in S-25-OHD, but less often in PTH or calcitriol (Woitge et al. 2000) due to sampling because PTH has a diurnal rhythm and calcitriol changes only
in rare cases such as in severe vitamin D deficiency or menopause (e.g. Heikkinen et al. 1997). In addition, the biological half-life of calcitriol is relatively short (12-18 h), and it has a quick response to PTH (Brown et al. 1999).

The impact of seasonal variation on bone is studied by following bone remodelling markers (Rozen et al. 1994, Woitge et al. 2000, Lehtonen-Veromaa et al. 2002) and BMD (Bergstralh et al. 1990, Dawson-Hughes & Harris 1993, Rozen et al. 1994, Meier et al. 2004). Error sources related to cross-sectional studies might overestimate the effect on bone or bone turnover (Woitge et al. 1998, Seibel et al. 2004). However, in Study II on adolescent girls, the group was homogenous with regard to age, weight, height and sexual maturation, the main potential sources of bias related to cross-sectional studies. Longitudinal follow-up studies have utilized DXA in BMD measurement, but to our knowledge, pQCT, which was exploited in Study IV, has not been used for this purpose before.

Previously bone resorption was suggested to increase and bone formation to decrease during winter, and vice versa during summer (Rozen et al. 1994). Currently, bone turnover is shown to accelerate during winter, meaning that more bone is resorbed than formed in winter, however, the bone turnover seems to recover to the initial level in summer (Meier et al. 2004). This explains why BMD decreases during winter, but does not reach to the initial value during the following summer (Meier et al. 2004, Seibel et al. 2004). In the cross-sectional study of adolescent girls (II), the bone formation marker osteocalcin was lower in winter, but the resorption markers were similar over months. Bone resorption markers are thought to be more sensitive to dietary manipulation than formation markers (Borderie et al. 2001), but among these rapidly growing early and mid-pubertal girls the balance in bone remodelling/modelling is placed on the formation site, which might explain our observations. In addition, a positive association between vitamin D status and formative markers has been described by others (Woitge et al. 2000, Lehtonen-Veromaa et al. 2002). In regard to resorption markers, the individual variation in bone remodelling marker levels has been demonstrated to be very high at puberty, and one-time specimen is thus not informative in a cross-sectional study (Watts 1999, Borderie et al. 2001).

In the prospective study of men (IV), the effect of season was observed in the placebo group; the resorption marker S-TRACP decreased significantly, on average 7.8% from November to April, while the S-BALP concentration was maintained. Whether the change in TRACP is clinically relevant is unclear. Firstly, the intra-CV% for this assay was 4%. However, the ratio or an index
applying both remodelling markers might be more informative than an individual marker (Eastell et al. 1993). The ratio of TRACP to BALP decreased somewhat during the 6-month period, which shows that the bone turnover rate slows down when vitamin D status is impaired. Previous studies (e.g. Woitge et al. 2000, Meier et al. 2004) have proposed that no seasonal variation exists in bone turnover among men. In addition, bone remodelling in general is more stable among men than women (Seeman 2003b). Remodelling is vital for normal bone renewal (Parfitt 2003). Vitamin D may act as a coupling factor in the remodelling process (Eastell et al. 1993, Gurlek et al. 2002), affecting both formation and resorption, which is supported by our results.

The results concerning the BMD in the cross-sectional study (II) suggest, that season contributed to 7.5-8.2% of the change in the BMD of the lumbar spine and femur, respectively, in early pubertal girls. The change is higher than reported in prospective studies of healthy adults (1-2%) (Bergstralh et al. 1990, Meier et al. 2004), but similar to that in the cross-sectional studies of the elderly (Rapuri et al. 2002, Bhattoa et al. 2004). Interestingly, the early pubertal girls (II) seemed to be more sensitive to seasonal rhythm than their mid-pubertal peers possibly due to sex hormones that acted on the vitamin D-PTH axis. In fact, there is increasing evidence that oestrogens influence bone mineral accrual and maintenance (e.g. Rizzoli et al. 2001), and this could explain why the bones of early pubertal girls (before menarche) and postmenopausal women are more susceptible to changes of vitamin D status.

Men with higher vitamin D intake from the diet and with more stable bone turnover are proposed not to be exposed to seasonal variation like women (Carnevale et al. 2001, Seibel et al. 2004). In fact, previous studies that have reported seasonal variation in areal BMD (Bergstralh et al. 1990, Dawson-Hughes et al. 1991, Rozen et al. 1994, Meier et al. 2004) included mostly female subjects. The volumetric BMD from the non-dominant radius, considered a clinically relevant site in the study of men (IV) did not indicate any seasonal variation. However, the distribution of volumetric BMD from a certain site is much narrower than the distribution of areal BMD from the same site (Riggs et al. 2004). Inadequate vitamin D status affects bone mass; with DXA, this is marked as decreased BMC and BMD, whereas with pQCT it is expected to be observed firstly and predominantly in Trab BMD (e.g. Rüegsegger et al. 1995) and after a time, in the Cort BMD, as the chronically elevated PTH increases Cort resorption, especially at the outer surface of bone (Adami et al. 1998, Bilezikian 2003). During impaired vitamin D status, the mineralization of bone decreases, but how soon does the bone compensate for this by rearranging and replacing the remaining minerals - thus increasing its diameter to aid in maintaining bone strength (van der
Meulen 2001). Longer prospective studies are needed to fill in the gaps in our knowledge of seasonal variation and bone health.

The discrepancies between the data on BMD and remodelling markers are likely explained by bone remodelling markers reflecting the situation in the central skeleton (Parfitt 2003, Szulc et al. 2003), whereas the vBMD was measured from a peripheral site. It is believed that seasonal variation of BMD should be avoided as it could complicate bone mass accrual during puberty (Lehtonen-Veromaa et al. 2002). In addition to icy and snowy weather conditions, lowered vitamin D status during winter might explain why Scandinavian countries have a higher incidence of osteoporotic fractures than other countries in Europe (Epos 2002, Bischoff-Ferrari & Dawson-Hughes 2007). One approach to estimate adequate vitamin D intake for a target group is to find the dose that maintains stable vitamin D status and PTH concentration throughout winter (Heaney et al. 2003a, Whiting & Calvo 2005).

### 6.4 Effect of vitamin D supplementation on serum 25-OHD and PTH

Vitamin D supplementation increased serum 25-OHD concentrations dose-dependently in Studies I, III and IV. Calculated dose-responses (nmol/l/µg) enable us to compare results from different studies and to extrapolate the dose needed to reach a certain 25-OHD concentration. The mean dose-response did not differ among studies, indicating that the short-term study was long sufficiently to attain the effect described in longer studies. The vitamin D pool in the body has been shown to turn over every 42 days (Heaney et al. 2003a); our results are consistent with this, as a plateau in serum 25-OHD concentration was reached in 6 weeks in elderly women. However, this should not be confused with the biological half-life of the 25-OHD molecule, which could vary between 1 and 3 months depends on the circulating levels of PTH and factors regulating PTH secretion, calcium and phosphorus intake and other hormones (Brown et al. 1999).

The mean dose-response was 1.36 (2.17) nmol/l/µg depending on the initial 25-OHD concentration. In the pooled data, these showed a strong linear inverse association, which has been reported by others as well (e.g. Heaney 2005). Thus, when the baseline 25-OHD concentration exceeds 70 nmol/l, supplementation of 5-20 µg does not increase it any further. This might indicate that the initial hydroxylation step of vitamin D (25-hydroxylation) that takes place in the liver occurs predominantly without control when the initial 25-OHD concentration is low, but is down-regulated when the initial 25-OHD is higher. Some studies have shown that 1,25(OH)₂-vitamin D could
down-regulate liver 25-hydroxylases (e.g. Bell et al. 1985), at least intermittently. However, we suggest that increasing 25-OHD could activate other possible routes of the metabolism, as also proposed by Willnow & Nukjaer (2005), although other explanations, including degradation of 25-OHD have been tendered. Weight has been reported to modify the dose-response in a few studies (Heaney et al. 2003a), but no association was noted with dose-response in linear regression models in Studies I, III or IV when data were analysed separately or pooled.

A plateau in the 25-OHD concentration noted in the study of elderly women (III) illustrates that vitamin D metabolism reaches a balance at certain dosages. Intake and utilization are thus balanced, or beyond a certain threshold, consumed vitamin D is stored rather than circulated in the blood. However, continuous long-term use of supplements is not recommended, as this could lead to accumulation of vitamin D in the body (Vieth 1999), seen as a further increase in circulating 25-OHD concentration after the first plateau. To avoid this, regular monitoring of 25-OHD concentration is advisable if large dosages are used. The upper limits for intake of 25 µg for children under 11 years and 50 µg for individuals older than 11 years (Scientific committee on Food 2002) thus seem reasonable.

In the study of adolescent girls (I), 25-OHD increased with supplementation of 10 µg to 60 nmol/l, which is lower than defined as optimal (Dawson-Hughes et al. 2005); this nevertheless had a significant effect on bone mineral accrual and maintained stable PTH throughout the year. However, with the HPLC method, the bias is 5% (Carter et al. 2004). The concentration is believed to be underestimated, the standardized 25-OHD concentration would thus be 63 nmol/l. Moreover, the vitamin D requirement may be modified by calcium intake (Lips 2004). In addition, the review of Dawson-Hughes and colleagues (2005) did include only one study concerning adolescents (Lehtonen-Veromaa et al. 2002), which was not successfully performed. In their study, 13- to 15-year-old girls were supplemented with vitamin D$_2$ and they reached a 25-OHD concentration of 41 nmol/l (analysed by RIA, which has a poor sensitivity for 25-OHD$_2$) (Lehtonen-Veromaa et al. 2002). In light of these data, we suggest that S-25-OHD concentration of 60 nmol/l is beneficial for bone in conditions such as those in Finland with a high habitual intake of calcium. The total intake among adolescents in the highest supplemented group was on average 14.5 µg (10 µg from supplements and 4.5 µg from the diet), and this seems adequate for this age group.

In the study of the elderly (III), the total intakes in each 25-OHD category (10-nmol/l categories) were calculated. A concentration of 40-49.9 nmol/l, which has previously been considered
sufficient by McKenna & Freaney (1998) and Need et al. (2000), is maintained with 17 µg, but a concentration above 60 nmol/l, which is typical during summer in Finland, is achieved with 24 µg.

In the study of healthy men (IV), optimal 25-OHD concentration was achieved with supplementation doses of both 10 µg and 20 µg a day, but the latter dose further suppressed the PTH from baseline, which was not an objective of the study. A stable PTH was maintained with 10 µg of vitamin D. Thus, the total intake of vitamin D required for healthy men is about 17 µg (10 µg+6.6 µg).

6.5 Effect of vitamin D supplementation on bone
Vitamin D intervention studies have not been carried out in adolescent girls in a RCT setting with a clinically relevant BMD as an outcome variable. Intervention trials with vitamin D alone on the growing and adult skeleton are required.

The results of the study of adolescent girls (I), especially those of BMD and change in BMC, were highly depended on compliance. Although the main result of the RCT, with all subjects included, showed no effect of vitamin D on bone mineral accrual either in the femur or in the lumbar spine, we performed the CB analysis to focus on the compliant subjects. There were two logical reasons why the CB was based on pill counts in Study I. Girls were advised to take 28 pills each month for 12 months. The total amount of pills consumed was thus 336. Those following the protocol had consumed at least 80% of the pills (269 pills). With this method, we excluded those who had not taken pills for over 2.4 months. In fact, there is a correlation between taken pills and the s-25-OHD in both supplemented groups ($r=0.212$, $p=0.015$). In addition, the half-life of s-25-OHD molecule is 2-3 months, and unequal distribution of oral vitamin D could be seen in lowered S-25-OHD concentrations. The study was double-blinded, meaning that subjects with poor adherence in the placebo group were excluded as well. However, the exclusion seemed to affect more the mean values of BMD and BMC in the placebo group than those in the vitamin D-supplemented groups probably because more subjects were excluded, and the excluded girls seemed to be somewhat (but not significantly) taller and heavier in the placebo group than in the other groups. These may partly explain the observed decrease in the BMC and BMD values. However, the exclusion of the subjects did not distort the baseline characteristics in the groups.
Finally, the changes in BMC were tested with ANCOVA using as covariates changes in BA (Prentice et al. 1994, Carter et al. 2001, Lehtonen-Veromaa et al. 2002, Mølgaard et al. 2004), weight and Tanner stage, as these may confound the interpretation. For example, an increase in BMC may be neutralised by an increase in BA, although vitamin D is expected to increase BMC by enhancing mineralization. All girls were growing and their bone size increasing, so using ΔBA as a covariate adjusted the growth and allowed focusing on the increase in BMC. Similarly, increases both in weight and the Tanner stage induces BMC gain, which justifies using these in the model. Choosing different covariates in the model may influence the result. It is recommended to use baseline values as covariates in such a model (Vickers & Altman 2001). Our model did not initially include baseline values. Further analyses showed that they only had a minor effect on the results (data not shown).

CB results show that vitamin D supplementation increased BMC augmentation dose-dependently both in the femur and in the lumbar spine in adolescent girls (I). Retention in the femur region was 14.3% and 17.2% higher in groups receiving either 5 μg or 10 μg of vitamin D, respectively, than in the placebo group. Similar results were demonstrated in the lumbar vertebra, albeit only the highest dose increased BMC augmentation significantly in the whole group. Our results agree with previous studies in which vitamin D promotes bone mineral accretion in adolescent girls (Lehtonen-Veromaa et al. 2002, Moyer-Mileur et al. 2003). In addition, our results suggest that the effect of threshold nutrients on bone mineral augmentation during growth is vaster than during other phases of life (Heaney et al. 2000). A year is a relatively short period of time when BMD or BMC is the main outcome; adult bone turnover varies between 1 and 2% annually, which is the same variance as in the repeatability of DXA. The least-significant change in DXA studies is about 3 %. However, during puberty (before menarche) and after menopause, bone turnover is accelerated (accrual or loss) and the impact of vitamin D is more distinct. Puberty is an extremely sensitive growth period during which retention occurs maximally, and any nutrient deficiency, such as calcium or vitamin D deficiency, could hinder the PBM accumulation (Bailey et al. 2000). Eating disorders and unhealthy food habits during puberty should be handled extra carefully as their impact on bone is greater than during any other time of life (Specker & Schoenau 2005).

Spine growth accelerates at puberty (Bass et al. 1999). This could be the reason why mid-pubertal girls in Study I responded more efficiently to vitamin D supplementation than early pubertal girls when considering the results of lumbar vertebra. In fact, among early pubertal girls, BMC accrual at the lumbar vertebra did not differ among study groups. Tempo of pubertal growth might be
interesting factor to consider as teens with a high growth speed tend to achieve lower PBM than
teens with a longer growth spurt (Bass et al. 1999). Nutrients and genes are the main determinants
of growth tempo. Among undernourished or severely obese children, the tempo of growth changes
growth influence the adult stature by decreasing the predicted adult height (Dunkel 2000).

Vitamin D intervention studies have been carried out mainly in the elderly (Ooms et al. 1995,
Dawson-Hughes et al. 1997, Trivedi et al. 2003), and the focus has been on the prevention of bone
loss and adjacent bone fractures, in other words, the focus is on secondary and tertiary prevention of
osteoporosis. Sometimes the results of interventions in the elderly have been discordant (Lips et al.
where positive effects on bone health were marked, calcium supplementation was also included
(Dawson-Hughes et al. 1995, Chapuy et al. 2002), reinforcing the traditional concept that the effect
of vitamin D on bone is mediated through calcium balance, as vitamin D-deficient persons with
adequate calcium intake will benefit from vitamin D monotherapy. A meta-analysis by Tang et al.
(2007) concluded that the best therapeutic effect for persons over 60 years is attained with at least
1200 mg of calcium and 20 µg of vitamin D for decreasing fracture risk and BMD changes.
However, the effect of vitamin D might act via influence through muscle tissues (e.g. Bishoff-
Ferrari et al. 2004c), as vitamin D supplementation have been shown to improve the maintenance
and performance of muscles. Myopathy is frequently related to osteomalacia (Parfitt 2003). In
addition, immobilized elderly persons have a higher risk for vitamin D deficiency than more
ambulatory seniors. According to this muscle theory, vitamin D deficiency might have caused the
immobilization rather than vice versa.

In Study IV with healthy men, vBMD measurements were repeated after 6 months, which may have
been a too short follow-up. However, we considered this justified since if seasonal variation is
present in the peripheral skeleton, it should be detectable within 6 months when the number of
subjects is adequate. We calculated retrospectively the power analysis with 80 %, that a sample size
of 37 in each group would have been required to detect a clinically relevant 2 % change in Cort
BMD. Our initial sample size in Study IV based on 25-OHD was unfortunately not large enough for
vBMD. The effect of vitamin D on radius vBMD in men remains unsolved. The study of Moyer-
Mileur et al. (2003) with preadolescent girls indicated that calcium and vitamin D therapy increased
Trab vBMD in the distal tibia, but to our knowledge, there are no other studies that have used pQCT
for this purpose. In some studies with male subjects (Szulc et al. 2003, Välimäki et al. 2004), a
positive association between 25-OHD and areal BMD was reported, and a similar association was observed among young females (Outila et al. 2001).

Interestingly, vitamin D supplementation had no effect on the bone formation marker osteocalcin; this concentration decreased slightly among adolescent girls during the Study I, as is expected to during normal puberty (Bass et al. 1999, Fares et al. 2003). The U-Pyr concentration, which reflects bone resorption, decreased more in the vitamin D-supplemented groups than in the placebo group, but the effect was not dose-dependent. This implies that supplemental vitamin D has antiresorptive properties that spare bone mineral. We assume that these functions are due to improved calcium balance (Heaney et al. 2000, Prentice 2004), as calcium has been shown to decrease bone resorption (Wastney et al. 2000). Our results conflict with those of Schou and coworkers (2003), who reported that vitamin D supplementation, had no effect on bone remodelling markers in 20 children aged 6-14 years. However, the number of subjects in the study of Schou et al. (2003) was small and the results were not controlled for individual variation, e.g. age and pubertal development, which could have obscured the effect. Adequate vitamin D status has been associated with decreased bone resorption markers in other studies (Lehtonen-Veromaa et al. 2002, Cheng et al. 2003), and in vitamin D deficiency all biochemical markers of bone turnover are known to be elevated (Scariano et al. 1995).

Bone remodelling markers might act differently in men than in women due to different bone turnover rates (Woitge et al. 2000). Resorption and formation are strongly coupled in bone remodelling (Parfitt 1989, Woitge et al. 2000), which we noted at baseline in Study IV. However, vitamin D supplementation of 10 µg and 20 µg significantly decreased S-BALP concentration by 9.1% and 11.3%, respectively. BALP is generally thought to indicate the viability of osteoblasts, but it could also be considered a marker of bone turnover (Avbersek-Luznik et al. 2007). The generalization of bone matrix parallels the maturation of osteoblasts, thus decreasing BALP (Parfitt 2003). Among vitamin D-deficient children, the BALP concentration is typically elevated; the bone formation rate is high (Wharton & Bishop 2003), and the concentration of BALP decreases as mineralization occurs (Scariano et al. 1995, Ros et al. 2005). Another possible explanation is that the total bone turnover rate decelerated, as PTH was lower in vitamin D-supplemented groups than in the placebo. Nevertheless, PTH within a normal range is required for normal bone turnover (e.g. Parfitt 1989, Gabet et al. 2006). With 10 µg of vitamin D, a stable, normal PTH concentration was maintained throughout the study.
The concentration of TRACP decreased in all groups in a similar way. The results of TRACP are discordant with earlier studies in which bone resorption increased during winter (Woitge et al. 2000, Meier et al. 2004). However, the basal intake of men was nearly 7 µg, and men in the placebo group did not become vitamin D-deficient, only vitamin D-inadequate. Thus the drop in 25-OHD was not high sufficiently to induce bone resorption among men, who generally have more stable bone turnover than women (Seeman 2003b). Furthermore, the ratio or an index applying both remodelling markers might be more informative than individual markers (Eastell et al. 1993). Our results support this, as the ratio of TRACP to BALP was shown to vary according to PTH and 25-OHD. The ratio was kept stable with 20 µg, while it decreased somewhat in other groups during the 6-months of winter. In addition, a strong correlation was observed between TRACP and BALP at the beginning of the study, which also illustrates coupling. In the vitamin D-supplemented groups, bone turnover remained coupled at the end of the study, supporting the coupling effect of vitamin D (Gurlek et al. 2002).
7. SUMMARY AND CONCLUSIONS

Novel findings in this thesis were related to vitamin D and bone metabolism. Firstly, a seasonal variation of calcitropic hormones was indicated to affect bone metabolism in healthy men, and in a cross-sectional study of adolescent girls, BMD was observed to differ among months, reflecting seasonal variation. Secondly, in a randomized placebo-controlled trial, vitamin D was shown to enhance bone mineral accretion in adolescent girls, which seemed to occur dose-dependently and by decreasing bone resorption, but these results were dependent on compliance. Finally, dose-responses obtained in each study enable to calculate vitamin D intakes required to achieve a certain S-25-OHD concentration.

Specific findings in each study were:

§ The main analysis did not support that vitamin D supplementation had a beneficial effect on bone mineral accumulation in the adolescent girls. However, confining the analyses only to those subjects that had at least a 80% compliance rate, the results showed that 10 µg of vitamin D increased BMC by 17.5% in the femur, and by 12.5% in the lumbar spine compared to the placebo. This effect was mediated by a decrease in bone resorption, possibly through an improved calcium balance. The positive results depended on the compliance of the subjects, indicating that supplementation may not be the optimal means to increase vitamin D intake among adolescent girls. However, increased vitamin D intake had clinically relevant effect on bone mineral accrual in adolescent girls with habitually high dietary intake of calcium. Changes that occur during growth period in bone mineralization are possibly maintained after the cessation of the supplementation, suggesting that puberty is optimal timing for enhancing bone mineral accrual.

§ In a cross-sectional study of adolescent girls, calcitropic hormones, BMD and bone formation marker differed among months reflecting seasonal variation. Early pubertal girls were more pronounced to this variation than mid-pubertal girls, proposing that estrogens may modify the effect of calcitropic hormones on bone metabolism. Although no causality could be pointed out in cross-sectional study, seasonal variation is speculated to complicate PBM attainment during
growth, and thus, increase the risk of osteoporosis later in life. With vitamin D intake of 15 µg, this variation could be avoided in adolescent girls.

In the elderly study, a plateau in S-25-OHD concentration was achieved in six weeks. Calculated mean dose-response was 1.36 nmol/l/µg, which enables one to estimate the effect of supplementation on S-25-OHD. Based on this, we estimated that ambulatory elderly require on average 18 µg a day to maintain adequate vitamin D status (above 50 nmol/l) during winter. To reach 60 nmol/l, which is a typical 25-OHD concentration in summer, the total intake should be approximately 24 µg. Three months study was not long enough to affect PTH among the elderly. Previously, the elderly were shown to require higher vitamin D dosages to suppress PTH; however, cases with primary hyperparathyroidism were not ruled out.

Vitamin D supplementation over a period of six months did not reveal (statistically) significant differences in radial volumetric BMD in healthy men, possibly due to the low number of subjects. However, the calcitropic hormones and the bone remodelling markers responded to supplementation. Bone turnover seemed to become uncoupled in the placebo group, which is considered harmful in the long run. With 18 µg of vitamin D, most of the changes were reversed, but more observations are required to confirm the effects of vitamin D supplementation on bone health in men.

The main objective of this thesis was to define an adequate vitamin D intake for healthy adolescent girls, men and elderly women. An adequate intake of vitamin D maintains stable S-25-OHD and PTH concentrations, and thus, avoids seasonal changes in bone turnover, which is considered beneficial for bone health. Based on these studies, adequate vitamin D intake varies between 15 µg and 24 µg per day for the target groups. A question, arising from the results, is how these adequate vitamin D intakes are achieved? Daily dietary intake of 10 µg is achieved by following current dietary guidelines, but to increase the intake beyond that either supplements or new tailor made products are needed to keep the diet as balanced as possible.
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