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Vitamin D deficiency and supplementation

Studies from infancy to young adulthood

Elisa Holmlund-Suila

ACADEMIC DISSERTATION

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Painosalama Oy

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To my family

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Abstract

Vitamin D deficiency in infancy and childhood impairs normal bone development and growth: defective bone mineralization leads to rickets. In adults, vitamin D deficiency causes osteomalacia, softening of the bones. For many decades in Finland, vitamin D supplementation in infants and small children has been successful in preventing rickets. However, along with increasing knowledge of non-skeletal vitamin D actions, optimal vitamin D status, defined by serum 25-hydroxyvitamin D (25OHD) concentration, has been under debate. Moreover, vitamin D deficiency has been prevalent in many populations and age groups. The need for vitamin D is affected by individual variation in vitamin D metabolism, state of health and many extrinsic factors. The optimal vitamin D status and the dose of supplemental vitamin D in different populations may differ considerably.

This doctoral thesis aimed to define the prevalence of vitamin D deficiency (serum 25OHD <50 nmol/l) in Finnish children, focusing on individuals with high risk of vitamin D deficiency, and studying in chronically ill children the factors that further increase the prevalence of low serum 25OHD concentration. Vitamin D interventions in infants and young adults enabled examination of the effect and safety of higher than currently recommended vitamin D supplementation, and exploring vitamin D and mineral metabolism in more detail.

The study populations comprised 113 healthy term newborns and 42 young adults who participated in vitamin D intervention, and 1,335 children followed at Children's Hospital Helsinki between 2007 and 2010 for a chronic illness. The vitamin D interventions were double-blinded controlled randomized trials. The newborns received either 10, 30 or 40 µg of vitamin D_3 daily from 2 weeks to 3 months, and blood samples were obtained at baseline and at 3 months. The young adults were either normal weight (n=24), or suffered from severe childhood-onset obesity (n=18). Both obese and normal-weight individuals received either placebo or 50 µg of vitamin D_3 daily for 12 weeks. Blood samples were taken during follow-up visits at baseline, 6 and 12 weeks. Data on chronically ill children were collected in a retrospective manner from the hospital laboratory database. Serum 25OHD, parathyroid hormone, and other parameters of calcium and phosphate metabolism were available for analyses.

Vitamin D deficiency was common, as more than 40% of the overall study population presented a serum 25OHD concentration <50 nmol/l. More than half of the adolescents with a chronic disease and of the obese young adults were vitamin D deficient. Obesity correlated inversely with 25OHD concentrations. Seasonal variation was evident in school-age children with a chronic disease, with the lowest prevalence of vitamin D deficiency in summer, and the highest prevalence in winter and spring. In younger children, on the other hand, vitamin D deficiency was less prevalent, and seasonal variation was lacking. Serum 25OHD concentration in chronically ill children was higher in 2010 compared with 2007–2009. Daily vitamin D_3 supplementation with 30 to 40 µg in infants, and 50 µg in young adults was safe in short-term follow-up. When adherence to intervention was good, both 30

and 40 µg dosing increased infant 25OHD concentration to >80 nmol/l. The vitamin D_3 intervention did not affect serum fibroblast growth factor 23 (FGF23) concentrations, but a distinct sex difference in active FGF23 concentration was observed, girls having higher concentration than boys at three months of age. The response to vitamin D supplementation was lower in obese than in normal-weight young adults. Obese individuals receiving 50 µg vitamin D_3 daily achieved similar 25OHD concentrations as normal-weight subjects who received placebo.

In conclusion, the prevalence of vitamin D deficiency exceeded 40% in the study cohorts: especially adolescents and obese individuals were at risk for low 25OHD concentration. Seasonal variation in 25OHD was evident, the concentrations being lowest in winter and spring. High-dose vitamin D supplementation in infants proved to be safe and effective in short-term follow-up. Long-term effects require further studies. Obesity associated with inferior response to vitamin D_3 supplementation. Chronically ill children and obese subjects need individualized vitamin D supplementation and follow-up.

Tiivistelmä

D-vitamiinin puute lapsuus- ja nuoruusiässä aiheuttaa riisitaudin, johon liittyy luuston ja kokonaiskasvun häiriintyminen. Riisitauti on seurausta luuston puutteellisesta mineraalistumisesta. Aikuisilla D-vitamiinin puute johtaa luiden pehmenemiseen eli osteomalasiaan. Suomessa on jo usean vuosikymmenen ajan ehkäisty riisitautia antamalla vastasyntyneille ja pienille lapsille säännöllistä D-vitamiinilisää. Elimistön D-vitamiinitilannetta kuvaa seerumin 25-hydroksi-D-vitamiinipitoisuus (25OHD). Samalla kun tieto luuston ulkopuolisista D-vitamiinivaikutuksista on lisääntynyt, keskustelu parhaasta mahdollisesta veren D-vitamiinipitoisuudesta on kiihtynyt. D-vitamiinin puute on ollut yleistä monessa maassa ja useissa eri ikäryhmissä. D-vitamiinin tarpeeseen voi vaikuttaa yksilöllinen aineenvaihdunta, terveydentila ja monet ulkoiset tekijät. Elimistön kannalta ihanteellinen 25OHD-pitoisuus ja tarvittava D-vitamiinilisän suuruus voivat vaihdella huomattavasti eri populaatioiden ja yksilöiden välillä.

Tässä vaitöskirjatutkimuksessa selvitettiin D-vitamiinin puutoksen yleisyyttä lapsilla ja nuorilla, joilla on suuri riski kärsiä D-vitamiinin puutoksesta. Seerumin 25OHD-pitoisuus <50 nmol/l määriteltiin D-vitamiinin puutteeksi. Tutkimuksessa pyrittiin selvittämään matalalle 25OHD-pitoisuudelle altistavia taustatekijöitä. Nykyistä suositusta suuremman D-vitamiinilisän tehoa ja turvallisuutta sekä vaikutuksia luuston aineenvaihduntaan tutkittiin vastasyntyneillä ja nuorilla aikuisilla.

Helsingin Lastenklinikalla vuosien 2007 ja 2010 välisenä aikana poliklinikkaseurannassa olleiden pitkäaikaissairaiden lasten 25OHD-määritykset poimittiin sairaalan laboratoriotietokannasta. Mukaan otettiin myös samanaikaisesti määritetyt kalsium- ja fosfaattiaineenvaihduntaa kuvaavat laboratorioarvot. Tämä poikittaistutkimus koostui 1335 lapsen tiedoista. Vastasyntyneiden D-vitamiinitutkimukseen osallistui 113 täysiaikaisena syntynyttä imeväistä, jotka saivat sokkoutetusti joko 10, 30 tai 40 µg D_3-vitamiinia päivässä 2 viikon iästä 3 kuukauden ikään saakka. Verinäyte laboratoriotutkimuksia varten saatiin napasuonesta syntymän jälkeen ja 3 kuukauden seurantakäynnillä ihopistonäytteenä. Nuorten aikuisten D-vitamiinitutkimukseen osallistui 18 nuorta aikuista, jotka olivat kärsineet vaikeasta lapsuusiällä alkaneesta lihavuudesta, ja 24 heille valittua samanikäistä ja normaalipainoista verrokkia. Molemmat ryhmät arvottiin saamaan sokkoutetusti joko lumevalmistetta tai 50 µg D_3-vitamiinia päivässä 12 viikon ajan. Seurantakäyntien yhteydessä (lähtötilanne, 6 ja 12 viikkoa) otettiin verinäytteet.

Yli 40 % kaikista 25OHD-pitoisuuksista oli alle 50 nmol/l, ja jopa yli puolella yli 10-vuotiaista pitkäaikaissairaista ja lihavista nuorista todettiin D-vitamiinin puutos. Kouluikäisillä pitkäaikaissairailla lapsilla havaittiin merkittävää vuodenaikaisvaihtelua 25OHD-pitoisuudessa. D-vitamiinin puutos oli yleisintä talvella ja keväällä ja harvinaisinta kesällä. Alle kouluikäisillä vuodenaikaisvaihtelua ei havaittu, ja heillä D-vitamiinin puutos oli muita harvinaisempaa. Vuoden 2010 aikana pitkäaikaissairaiden lasten 25OHD-pitoisuudet olivat korkeammat kuin vuosina 2007–2009. Lihavuuteen liittyi matalampi veren 25OHD-pitoisuus normaalipainoisiin verrattuna. Nykysuositusta korkeampi

päivittäinen D_3-vitamiinilisä osoittautui turvalliseksi sekä aikuisilla että vastasyntyneillä. Vastasyntyneillä 30 ja 40 µg:n vuorokausiannos D_3-vitamiinia nosti 25OHD-pitoisuuden >80 nmol/l. D-vitamiinilisä ei vaikuttanut fosfaatin aineenvaihduntaan osallistuvan seerumin fibroblastikasvutekijä 23:n (FGF23) pitoisuuteen, mutta aktiivisen FGF23:n pitoisuuksissa oli selvä ero sukupuolten välillä: kolmen kuukauden iässä pitoisuus oli tytöillä korkeampi kuin pojilla. Lihavuuteen liittyi huonompi vaste D_3-vitamiinille: 50 µg:n vuorokausiannos nosti lihavien aikuisten 25OHD-pitoisuuden samalle tasolle kuin lumevalmistetta saaneilla normaalipainoisilla.

Yhteenvetona voidaan todeta, että väitöskirjan tutkimusryhmissä D-vitamiinin puutos oli yleistä. Etenkin nuorilla ja ylipainoisilla riski D-vitamiinin puutokseen on kohonnut. Vuodenaikaisvaihtelu 25OHD-pitoisuudessa on huomattavaa, puutosta esiintyy eniten talvi- ja kevätkuukausina. Jopa 40 µg:n lyhytaikainen päivittäinen D-vitamiinilisän käyttö imeväisellä on turvallista, mutta pitkäaikaisvaikutusten arviointiin tarvitaan jatkotutkimuksia. Pitkäaikaissairaat lapset ja nuoret sekä ylipainoiset henkilöt tarvitsevat yksilöllistä ohjausta D-vitamiinilisän käytöstä ja seurannasta.

List of original publications

This thesis is based on the following publications:

I Holmlund-Suila E*, Koskivirta P*, Metso T, Andersson S, Mäkitie O, Viljakainen HT. Vitamin D deficiency in children with a chronic illness - seasonal and age-related variations in serum 25-hydroxy vitamin D concentrations. PLoS One. 2013 Apr 9;8(4):e60856. doi: 10.1371/journal.pone.0060856. Print 2013.

II Holmlund-Suila E, Viljakainen H, Hytinantti T, Lamberg-Allardt C, Andersson S, Mäkitie O. High-dose vitamin D intervention in infants - effects on vitamin D status, calcium homeostasis, and bone strength. J Clin Endocrinol Metab. 2012 Nov;97(11):4139-47. doi: 10.1210/jc.2012-1575. Epub 2012 Aug 29.

III Holmlund-Suila E, Viljakainen H, Ljunggren Ö, Hytinantti T, Andersson S, Mäkitie O. Fibroblast growth factor 23 concentrations reflect sex differences in mineral metabolism and growth in early infancy. Horm Res Paediatr. 2016;85(4):232-41. doi: 10.1159/000443988. Epub 2016 Mar 5.

IV Holmlund-Suila E, Pekkinen M, Ivaska K, Andersson S, Mäkitie O, Viljakainen H. Obese young adults exhibit lower total and lower free serum 25-hydroxycholecalciferol in a randomized vitamin D intervention. Clin Endocrinol (Oxf). 2016 May 5. doi: 10.1111/cen.13093. [Epub ahead of print]

*equal contribution

The publications are referred to in the text by their Roman numerals. These articles were reprinted with the permission of the copyright holders. Some previously unpublished data are also presented.

Abbreviations

AI	adequate intake
ALTM	all laboratory trimmed mean
ANOVA	analysis of variance
ANCOVA	analysis of covariance
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
D_2	vitamin D_2, ergocalciferol
D_3	vitamin D_3, cholecalciferol
DBP	vitamin D-binding protein
DEQAS	vitamin D external quality assessment scheme
DXA	dual-energy X-ray absorptiometry
$1,25(OH)_2D$	1,25-dihydroxyvitamin D, calcitriol
$1,24,25(OH)_3D$	1,24,25-trihydroxyvitamin D
$24,25(OH)_2D$	24,25-dihydroxyvitamin D
25OHD	25-hydroxyvitamin D, calcidiol
FGF23	fibroblast growth factor 23
iFGF23	intact, i.e., active fibroblast growth factor 23
cFGF23	C-terminal, i.e., inactive fibroblast growth factor 23
IOM	Institute of Medicine
NIST	reference measurement, the national institute of standards and technology
pQCT	peripheral quantitative computed tomography
PTH	parathyroid hormone
RCT	randomized controlled trial
RDA	recommended dietary allowance
RI	recommended intake
OC	osteocalcin
U-Ca/Cr	urine calcium/creatinine ratio
UL	tolerable upper intake level
VDR	vitamin D receptor
VDSP	vitamin D standardization program

1 Introduction

Vitamin D, a fat-soluble vitamin, is vital to humans, and is by definition a hormone. In addition to dietary intake (vitamin D_2 and D_3), the skin is able to produce vitamin D_3. After hydroxylation in the liver and kidneys vitamin D is eventually secreted to the circulation in its active form: 1,25-dihydroxyvitamin D ($1,25(OH)_2D$). In the target tissues, $1,25(OH)_2D$ binds to the intranuclear vitamin D receptor (VDR), and affects directly or indirectly gene expression in numerous cells.

Vitamin D has profound effects on mineral metabolism regulating the concentrations of calcium and phosphate. Vitamin D deficiency results in impaired bone mineralization leading to rickets in children and osteomalacia in adults. On the other hand, vitamin D excess leads to hypercalcemia and vitamin D intoxication. In addition to its impact on bone development and metabolism, vitamin D participates in several non-skeletal physiological processes. Vitamin D deficiency associates with several pathological conditions, such as infections, autoimmune diseases, cancers, and cardiovascular morbidities, even though a causal connection is still unconfirmed in many cases. Abundant vitamin D research has increased the understanding of the role of vitamin D in mineral metabolism, bone development, and in the development of various diseases.

Serum 25-hydroxyvitamin D (25OHD) concentration reflects vitamin D status. An optimal 25OHD concentration has not been confirmed. However, 25OHD <50 nmol/l is suggested to reflect vitamin D deficiency, and some consider 25OHD >75 nmol/l as a target concentration. In order to overcome the insufficient cutaneous synthesis of vitamin D, common in many countries, regular vitamin D supplementation or vitamin D fortification of foods, or both, is often necessary. In Finland, vitamin D supplementation has a long tradition in preventing rickets. However, the recommendations of supplemental vitamin D dose and the fortification of foods have changed over time. In fact, the currently recommended dose of supplemental vitamin D in infants has decreased from 100 µg in the 1940s to 10 µg in 1992. Dietary intake of vitamin D, on the other hand, has improved due to increased vitamin D fortification of foods. Thus, vitamin D status has likely changed with time, and needs intermittent evaluation.

2 Review of the literature

2.1 Vitamin D metabolism and action

Ultraviolet B (UVB) radiation converts 7-dehydrocholesterol in the epidermis to pre-vitamin D, which is further transformed in a temperature-dependent process to vitamin D_3 (Holick et al. 1980). In addition, some foods contain naturally ergocalciferol (vitamin D_2) (e.g. mushrooms), and cholecalciferol (vitamin D_3) (e.g. fish, egg yolk), while several spreads, bread, breakfast cereal, and juice are currently fortified with vitamin D_3. Moreover, fortification of some foods, e.g. milk and yoghourt, expands the diversity of vitamin D sources. Mother's breast milk does not provide sufficient vitamin D for the breast-fed infant (Vieth Streym et al. 2015). Maternal supplementation with vitamin D, however, may increase the vitamin D content in breast milk (Wall et al. 2015). In general, vitamin D supplements are an important source of vitamin D in some groups, such as children, especially infants, pregnant and lactating women and the elderly.

From the skin and the intestine vitamin D passes to the circulation, where it is mainly bound to vitamin D-binding protein (DBP).

2.1.1 Structure and metabolism

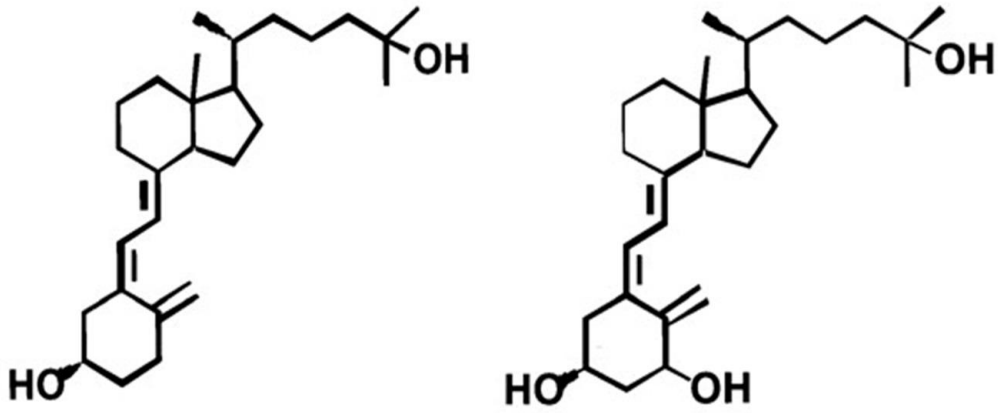

Figure 1 *Structure of 25-hydroxyvitamin D (left) and 1,25-dihydroxyvitamin D (right).*

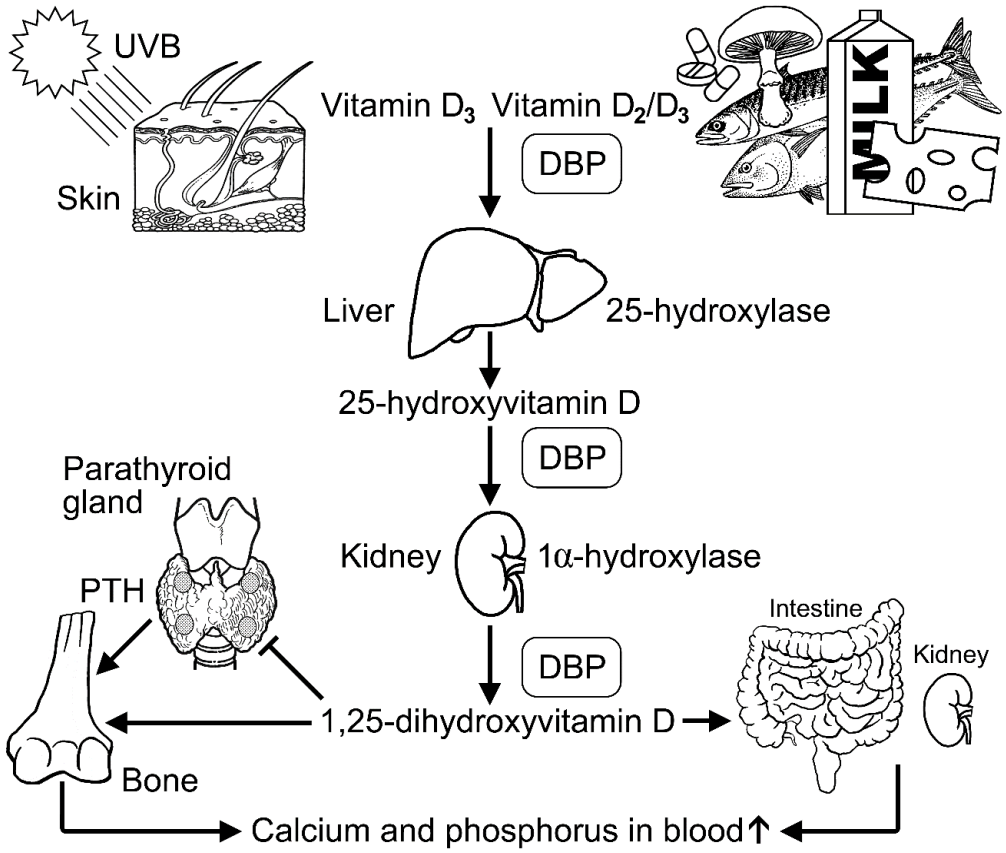

Figure 2 *Vitamin D metabolism and classical actions on mineral metabolism. Modified from Holick 2006. UVB, ultraviolet B radiation; DBP, vitamin D-binding protein; PTH, parathyroid hormone.*

25-hydroxyvitamin D

DBP-bound vitamin D is transported to the liver where cytochrome P450-linked 25-hydroxylases (CYP2R1) convert it to the main circulating form of vitamin D, 25-hydroxyvitamin D (25OHD) (DeLuca 2004). This hydroxylation is substrate-dependent, without tight regulation. In the circulation, DBP binds up to 90% of the 25OHD, and albumin approximately 10%, stabilizing 25OHD concentrations. Less than 1% of the total 25OHD is circulating free (Bikle et al. 1985). Such free 25OHD may enter cells in several tissues without carrier-protein interaction. DBP-bound 25OHD enters proximal tubular cells in the kidneys through receptor-mediated endocytosis (Nykjaer et al. 1999). Megalin, a transmembrane protein, serves as a cell-surface receptor for DBP-25OHD complex (Moestrup and Verroust 2001). Binding to megalin leads to endocytosis of 25OHD.

1,25-dihydroxyvitamin D

1,25-dihydroxyvitamin D ($1,25(OH)_2D$) is the biologically active form of vitamin D. Despite local production in several tissues, the circulating $1,25(OH)_2D$ results from renal hydroxylation, which is under regulation of plasma parathyroid hormone (PTH), fibroblast growth factor 23 (FGF23), and serum calcium and phosphate concentrations (DeLuca 2004, Shimada et al. 2004b). In the proximal tubular cells mitochondrial cytochrome P450 enzyme (1α-hydroxylase, CYP27B1) converts 25OHD to $1,25(OH)_2D$ (Zehnder et al. 2001) which is secreted to the circulation, and bound to DBP. In target cells $1,25(OH)_2D$ binds to the VDR. This complex then binds to specific sequences in the genome (vitamin D response elements, VDREs), in conjunction with retinoid X receptor (RXR), and regulates gene expression (Pike and Meyer 2014).

Intracrine activation of 25OHD to $1,25(OH)_2D$, without a tight regulation, occurs in several cells that may also express VDR. Such cells include e.g. macrophages, enterocytes, osteoblasts, osteoclasts and parathyroid cells (Adams et al. 2014). Regulation of local production of $1,25(OH)_2D$ differs from the renal activation. For example, cytokines such as interferon-gamma (INFγ), but not calciotropic hormones, regulate the activity of macrophages to produce $1,25(OH)_2D$, and $1,25(OH)_2D$ may escape the macrophage and act as a paracrine factor (Adams et al. 2014). In addition, $1,25(OH)_2D$ produced by osteocytes may regulate bone remodeling independent of circulating renal $1,25(OH)_2D$ (Turner et al. 2014).

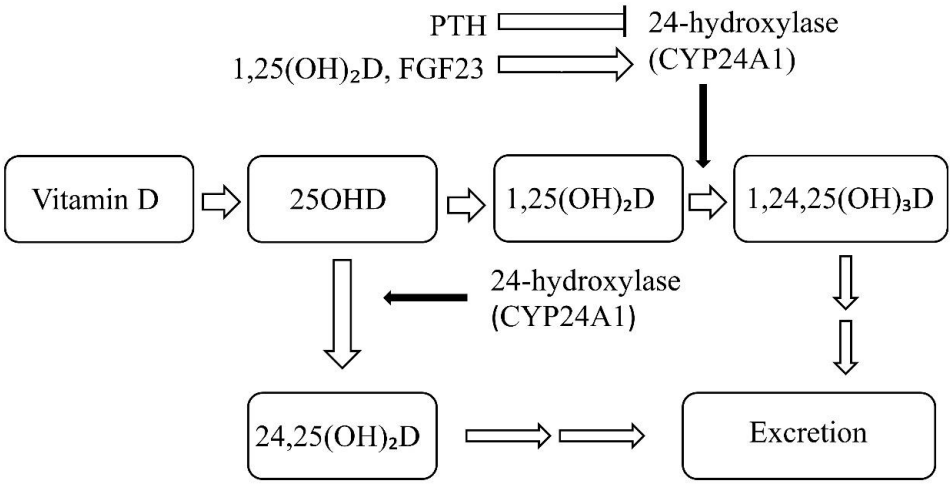

Figure 3 *Catabolism of vitamin D by 24-hydroxylase (CYP24A1). 25OHD, 25-hydroxyvitamin D; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; $1,24,25(OH)_3D$, 1,24,25-trihydroxyvitamin D; $24,25(OH)_2D$, 24,25-dihydroxyvitamin D; PTH, parathyroid hormone; FGF23, fibroblast growth factor 23.*

CYP24A1 enzyme (24-hydroxylase) inactivates both $1,25(OH)_2D$ and 25OHD in target tissues to water-soluble $1,24,25(OH)_3D$ and $24,25(OH)_2D$, respectively (Makin et al. 1989). The main regulators of 24-hydroxylase activity are $1,25(OH)_2D$, PTH, and FGF23 (Schlingmann et al. 2011, Petkovich and Jones 2011). Figure 3 illustrates the catabolism of vitamin D.

Vitamin D-binding protein

Vitamin D-binding protein (DBP), originally named as group-specific component of serum (Gc-protein), is a polymorphic protein with several functions: in addition to binding vitamin D, it also modulates immune and inflammatory responses and regulates bone development (White and Cooke 2000, Gomme and Bertolini 2004). DBP, a glycoprotein with a molecular weight of 52-59 kDa, is synthetized in the liver. Abundant genetic variation in *DBP* results in versatile physiological characteristics, the three most common alleles being *Gc1F*, *Gc1S* and *Gc2* (Bhan 2014). Although DBP binds all main vitamin D metabolites, it has greatest affinity to 25OHD, enabling regulation of vitamin D bioavailability (White and Cooke 2000, Gomme and Bertolini 2004). Binding to DBP stabilizes concentrations of 25OHD and $1,25(OH)_2D$, especially in situations with restricted vitamin D intake (Chun et al. 2014). Moreover, the binding affinity and DBP concentration vary between different genotypes, and their prevalence is in turn affected by ethnicity (Taes et al. 2006, Chun et al. 2012).

2.1.2 Biological actions

The best-characterized actions of vitamin D are the regulation of calcium and phosphate homeostasis in the body. In the intestine, vitamin D induces calcium and phosphorus absorption (Rizzoli et al. 1977, Suda et al. 2015). Intestinal calcium absorption occurs mainly via a $1,25(OH)_2D$-dependent process: $1,25(OH)_2D$ increases intestinal calcium channels (Van Cromphaut et al. 2001, Christakos et al. 2014). However, when calcium intake is high, as in infancy, calcium can also diffuse passively in an $1,25(OH)_2D$-independent manner (Masuyama et al. 2003, Kovacs 2014). The role of $1,25(OH)_2D$ in the intestinal phosphate absorption, on the other hand, is minor compared with the passive, concentration-dependent absorption (Kovacs 2014). In the intestine, $1,25(OH)_2D$ likely increases phosphate absorption by acting on sodium-dependent phosphate cotransporters (Christakos et al. 2014).

Despite passive reabsorption of minerals at the proximal tubules, the role of $1,25(OH)_2D$ is fundamental in promoting renal reabsorption of calcium and phosphate (Kurnik and Hruska 1985, Hoenderop et al. 2001, Blaine et al. 2015). The renal action of $1,25(OH)_2D$ is similar to its action in the intestine, resulting in increased transcellular transport of calcium in the distal tubulus and increased reabsorption of phosphate in the proximal tubulus (Suda et al. 2015). In addition to $1,25(OH)_2D$, the regulation of phosphate reabsorption in the kidneys involves FGF23, Klotho, PTH, and calcitonin (Urakawa et al. 2006, Blaine et al. 2015).

Relatively well-established renal and intestinal actions of $1,25(OH)_2D$ increase essential minerals available for bone mineralization. Instead, direct $1,25(OH)_2D$ action on bone is still poorly understood. In mice, $1,25(OH)_2D$ stimulates bone resorption by inducing osteoclastogenesis and activation of osteoclasts, the cells that are responsible for bone resorption (Galli et al. 2008). However, one of the principal actions of $1,25(OH)_2D$, tightly connected to mineral metabolism, is the inhibition of synthesis and secretion of PTH in the parathyroid gland (Jones et al. 1998), leading to reduced bone resorption (Suda et al. 2003).

In addition to its role in mineral metabolism, vitamin D regulates immune responses (Chesney 2010) and the cell cycle, thus exerting antineoplastic activity (Christakos et al. 2016). In macrophages, locally activated $1,25(OH)_2D$ enhances innate immunity, for example by increasing secretion of the antimicrobial peptide cathelicidin (Liu et al. 2006, Adams et al. 2007a). Vitamin D may reduce the risk of cancer via its actions on the cell cycle, enhancement of apoptosis and cell differentiation, and reduction of cell proliferation (Davis and Milner 2011, Moukayed and Grant 2013).

The connection between vitamin D and cardiovascular health is complex. Vitamin D deficiency associates with several risk factors for cardiovascular disease, and vitamin D is accepted as being important for cardiovascular health (Reddy Vanga et al. 2010, Joergensen et al. 2010, Vacek et al. 2012). Despite observational evidence supporting an association between vitamin D and cardiovascular morbidity, causality is still unconfirmed (Pittas et al. 2010, Christakos et al. 2016).

2.1.3 Toxicity

The risk for vitamin D toxicity is relevant due to a growing interest in increased vitamin D supplementation and free availability of high-dose supplements. Moreover, the attitude to supplementation in Finland is relatively liberal today.

In the 1950s, fortification of dairy products and foods with vitamin D was widely banned in Europe due to hypercalcemia in infants in Great Britain, which was presumed, but not confirmed, to result from vitamin D (Chesney 1989). Vitamin D toxicity has not been an issue in Finland. In 1963 a cohort of 160 healthy newborns in Helsinki received either 100 µg or 50 µg vitamin D daily for 3 to 5 months without any signs of excess vitamin D effect (Hallman et al. 1964). Reports on vitamin D toxicity occur intermittently with excessively high doses of vitamin D (6,650 to 100,000 µg/day) (Barrueto et al. 2005, Joshi 2009, Kara et al. 2014). Notably, the response to vitamin D supplementation, and hence the risk for toxicity, varies greatly between individuals (Vanstone et al. 2012, Rajakumar et al. 2013).

Vitamin D intoxication is caused by the increase in total 25OHD in the circulation, which leads to increased local production of $1,25(OH)_2D$ in extra-renal tissues and displacement of $1,25(OH)_2D$ from the vitamin D-binding protein (DBP) (Vieth 1990, Pettifor et al. 1995). At the same time, the degradation and excretion of vitamin D metabolites may be diminished. When measuring vitamin D excess in the body, hypercalcemia and

hypercalciuria are the commonly used markers. In the intestine, $1,25(OH)_2D$ increases the absorption of calcium (Heaney et al. 1997), leading to hypercalcemia and increased excretion of calcium to the urine. Increased $1,25(OH)_2D$ also leads to bone resorption and consequent increase in serum calcium (Suda et al. 2003). Hypercalcemia causes the typical symptoms of vitamin D intoxication: nausea, vomiting, constipation, anorexia, polyuria, dehydration, muscle weakness, nonspecific pains etc. (Barrueto et al. 2005, Chambellan-Tison et al. 2007, Ozkan et al. 2012), and may, if prolonged, lead to nephrocalcinosis (Joshi 2009).

Serum 25OHD concentration can rise above 200 nmol/l in adults with abundant sunlight exposure without signs of vitamin D excess (Barger-Lux and Heaney 2002). Such an exposure would be equivalent to oral daily dose of 250 µg of vitamin D (Stamp 1975, Holick 1995). In vitamin D intoxication, the lowest serum 25OHD concentration resulting in hypercalcemia was 220 nmol/l, and in adults a daily dose higher than 250 µg increases the risk for vitamin D intoxication (Vieth 1999). The aforementioned numbers cannot as such be adopted to children, and unfortunately data on safety in children are limited. Most toxicity data come from single patient reports, and only a few randomized controlled trials have studied both the effect and safety of vitamin D supplementation. A recent review on current literature by the Drugs and Therapeutics Committee of the Pediatric Endocrine Society emphasizes that serum 25OHD concentration needs to be monitored when using long-term vitamin D supplementation with doses at or above the recommended upper intake level (Table 2), and serum calcium concentration should be monitored if serum 25OHD concentration is above 375 nmol/l (Vogiatzi et al. 2014).

2.1.4 Nutritional rickets

Rickets was first defined in a paper by F. Glisson in 1650 (Dunn 1998). Rickets was an endemic disease in industrialized cities in northern parts of Europe. In the beginning of the 1900s, the prevalence in the UK was 25% (Paterson and Darby 1926). In Finland, rickets was extremely common in the 1920s. Archiater A. Ylppö noted in 1925 that 35% of 3- to 6-month-old infants and 50 to 70% of 1- to 2-year-old children visiting an out-patient clinic in Helsinki had signs of rickets (Ylppö 1925). Between 1920 and 1950, the overall prevalence of rickets among children in Finland was as high as 80% (Uuspää 1950).

Vitamin D deficiency, often accompanied by low intake of calcium, causes nutritional rickets. The risk for rickets is greatest during infancy and adolescence, the periods of rapid growth (Saggese et al. 2015). Rickets of prematurity, on the other hand, is primarily due to insufficient intake of phosphate, not vitamin D (Backström et al. 1996). Characteristic features in rickets are growth retardation, skeletal deformities, bone pain, and muscle weakness with delayed gross motor development (Elder and Bishop 2014, Munns et al. 2016). Hypocalcemia may result in convulsions and tetany, in addition to dilated cardiomyopathy, and failure to thrive. Radiological findings include abnormal mineralization, bowing of the long bones, metaphyseal widening at long-bone ends, and rachitic rosary (expansion of the anterior rib ends at the costochondral junctions). Other

skeletal deformities include frontal bossing, craniotabes, and delayed closure of the fontanelle.

In rickets (Figure 4), the differentiation of chondrocytes is abnormal, and deficiency of phosphate results in failure to mineralize newly formed bone (osteoid). Impaired endochondral mineralization at the growth plate leads to deformity of the long bones and impaired longitudinal growth (Munns et al. 2016). Due to hypocalcemia, as a result of vitamin D deficiency, PTH secretion increases, and this leads to increased loss of phosphate in the urine, and hence hypophosphatemia. Similarly, hypophosphatemia resulting from a variety of inherited disorders, or hypophosphatasia, may also cause rickets (Elder and Bishop 2014).

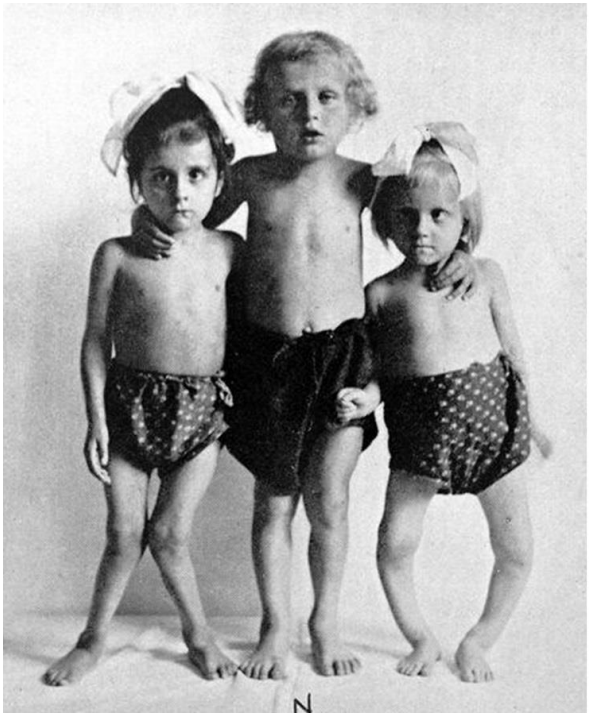

Figure 4 *Children with rickets in the beginning of the 1900s. (Wellcome Library, London)*

2.1.5 Vitamin D supplementation

History

The understanding of the treatment and prevention of rickets improved in the late 1910s and early 1920s, following the discovery of vitamin D. As early as 1889, lion cubs with rickets

were effectively treated with cod-liver oil (Bland-Sutton 1889). McCollum discovered Vitamin D as a nutrient in the 1920s (McCollum et al. 1922). In the beginning of the 1920s, sun light and cod-liver oil proved successful in preventing and treating rickets among infants (Chick 1976). The structure of vitamin D was identified in the 1930s (Wolf 2004), and the chemist Windaus received the Nobel Prize in chemistry in 1928 "for his studies on the constitution of the sterols and their connection with vitamins".

Vitamin D supplementation has been commonly used in Finland in the form of cod-liver oil since the 1930s, in addition to fortification of dairy products. From the 1940s to the 1960s, the recommended daily vitamin D dose was 5,000 to 4,000 IU (125-100 µg) (Hallman et al. 1964). As a result of the potential risks of vitamin D intoxication, reports on hypercalcemia in infants in Great Britain, and the observations that rickets can be avoided with smaller doses, Hallman et al. proposed in 1964 that the recommended dose be decreased to 2,000 IU (50 µg) /day. Since the prevalence of rickets in Finnish children decreased from 7% in 1962 to 0.6% in 1972 the National Board of Health decreased the recommended daily dose to 25 µg (Ala-Houhala et al. 1995). According to a national questionnaire survey, between 1980 and 1990 only 335 children in Finland were diagnosed as having rickets, and the most common predisposing factor was poor adherence to regular vitamin D supplementation (Ala-Houhala et al. 1995). In 1992 the recommendation was further decreased to the current daily dose of 10 µg (Sosiaali- ja terveyshallitus 1992).

The first Nordic Nutrition Recommendation was published in 1980, and the latest recommendation is from 2013 (http://www.norden.org/nnr). Finland has modified national recommendations on the basis of the common Nordic recommendations. The first recommendation from the National Nutrition Council was published 1987, and the most recent update in 2014 (http://www.ravitsemusneuvottelukunta.fi/portal/fi/julkaisut). These guidelines include recommendations for daily vitamin D intake from food and supplements.

Randomized trials on vitamin D supplementation

A teaspoon of cod-liver oil contains approximately 10 µg of vitamin D_3, which is the most commonly used supplemental dose of vitamin D (Holick 2007a). Despite increased understanding of the physiological actions of vitamin D, there are relatively few randomized controlled trials (RCT) investigating the effects of vitamin D in children. For example, the Cochrane database (http://www.cochrane.org/) review on the effects of vitamin D supplementation on bone mineral density (BMD) in children comprised only six RCTs (Winzenberg et al. 2010), although vitamin D action on mineral metabolism and bone development is well established. The aforementioned meta-analysis concluded that children with low 25OHD concentration (<50 nmol/l) may benefit from vitamin D supplementation in terms of BMD. Several studies in adults exist, and vitamin D has proven useful in fracture prevention (Bischoff-Ferrari et al. 2012).

Table 1. *Recent randomized trials on vitamin D effect and safety in healthy children.*

Study	**Year**	**N**	**Mean age**	**Intervention**	**Duration**	**BL 25OHD**	**25OHD**
Maalouf	2008	340	13 years	D3 350 µg/wk	1 year	36 (19)	91 (50)
				D3 35 µg/wk		38 (20)	46 (16)
Gordon	2008	40	10 months	D2 50 µg/d	6 weeks	43 (9) (N=40)	90 (N=40)
				D2 1,250 µg/wk			
				D3 50 µg/d			
Dong	2010	49	16 years	D3 10 µg/d	16 weeks	34 (11)	60 (18)
				D3 50 µg/d		33 (9)	86 (30)
Abrams	2013	64	7 years	D3 25 µg/d	8 weeks	69 (19)	90 (26)
				Placebo		69 (18)	75 (31)
Gallo	2013	132	34 days	D3 40 µg/d	11 months	57	>75 (100%)
				D3 30 µg/d			>75 (92%)
				D3 20 µg/d			>75 (81%)
				D3 10 µg/d			>75 (55%)
Putman	2013	54	16 years	D3 25 µg/d	11 weeks	73 (18)	75 (17)
				D3 5 µg/d		70 (16)	72 (18)
Lewis	2013	323	11 years	D3 100 µg/d	12 weeks	70 (19)	146
				D3 50 µg/d			103
				D3 25 µg/d			91
				D3 10 µg/d			76
				Placebo			61
Rajakumar	2015	157	11 years	D3 25 µg/d	6 months	50 (19)	67 (19)
				Placebo		47 (17)	56 (18)

BL, baseline; 25OHD, 25-hydroxyvitamin D, mean (SD) (nmol/l); D3, vitamin D_3; D2, vitamin D_2; wk, week; d, day

Table 1 summarizes pediatric trials from the last decade assessing the effect and safety of vitamin D supplementation in healthy children. The studies were heterogeneous in terms of race, age of subjects, given dose, duration, and baseline 25OHD concentration. Some studies include only black individuals (Maalouf et al. 2008, Dong et al. 2010), whereas others include ≥40% of Caucasian origin (Gordon et al. 2008b, Abrams et al. 2013, Gallo et al. 2013, Putman et al. 2013, Lewis et al. 2013, Rajakumar et al. 2015). None of these studies showed any adverse effects. In an intervention study on healthy Canadian infants, however, the highest vitamin D dose of 40 µg/day was discontinued at three months due to high serum 25OHD concentration (Gallo et al. 2013). In this group the mean concentration of 25OHD was 180 nmol/l. The researchers also examined bone mineral content (BMC) and BMD by dual-energy X-ray absorptiometry (DXA); these did not differ between intervention groups.

Table 2. *Current recommendations of vitamin D intake and tolerable upper intake level for children.*

Age	**PES**	**IOM AAP†**	**EFSA ESPGHAN**	**NNR**
0–6 mo	10[1]	10*	10[3]	10[3]
6–12 mo	10[1]	10*	10[3]	10[3]
1–3 y	15[1]	15[2]	-	10[2]
4–8 y	15[1]	15[2]	-	10[2]
9–10 y	15[1]	15[2]	-	10[2]
11–18 y	15[1]	15[2]	-	10[2]
Tolerable upper intake level (µg/d)				
0–6 mo	50	25	25	25
6–12 mo	50	38	25	25
1–3 y	100	63	50	50
4–8 y	100	75	50	50
9–10 y	100	100	50	50
11–18 y	100	100	100	100

PES, Pediatric Endocrine Society; IOM Institute of Medicine; AAP, American Academy of Pediatrics; EFSA, European Food Safety Authority; ESPGHAN, European Society of Pediatric Gastroenterology, Hepatology, and Nutrition; NNR, Nordic Nutrition Recommendations

Recommendations given as follows: [1]daily requirement, [2]dietary allowance, [3]daily supplementation

†AAP has agreed on IOM statements

*Adequate daily intake, i.e., the level that is assumed to meet the daily requirements, with limited scientific evidence

Present recommendations

Current recommendations of vitamin D intake vary according to different authorities; they provide recommendations for daily requirement, dietary allowance (RDA) or daily supplementation of vitamin D (Ross et al. 2011, Holick et al. 2011, Agostoni et al. 2012, Fogelholm 2013, Braegger et al. 2013, Golden et al. 2014). The Institute of Medicine (IOM) states that adequate daily intake (AI) of vitamin D for infants before age 1 year is 10 µg, and RDA for children over 1 year and for adolescents is 15 µg (Ross et al. 2011). AI is reported when scientific evidence to develop an RDA is insufficient. The IOM also states that tolerable upper intake level (UL) for infants 0 to 6 months and 6 to 12 months is 25 µg and 37.5 µg/day, respectively. RDA or recommended daily intake (RDI) estimates an average daily intake (from food and supplements) to meet the nutrient requirement of 97.5% of the population. The requirement is defined by target concentration: serum 25OHD above 50 nmol/l (Ross et al. 2011). UL defines the highest daily intake above which the risk of adverse events increases. Table 2 summarizes the current pediatric recommendations of vitamin D intake. Moreover, a recent consensus highlights the means to prevent and treat nutritional rickets (Munns et al. 2016).

In Finland the current recommendation for daily supplemental vitamin D in children, adolescents, and pregnant and lactating women throughout the year is:

1. children 0 to 2 years: 10 µg
2. children 2 to 17 years: 7.5 µg (recommended daily intake 10 µg)
3. pregnant and lactating women: 10 µg.

2.2 Vitamin D and bone

2.2.1 Bone growth

Bone is derived from mesenchymal cells which differentiate to either chondrocytes or osteoblasts (Berendsen and Olsen 2015). By the 8th week of gestation chondrocytes form a model of the bone, which is then replaced by mineralized bone in axial and appendicular skeleton, and parts of the skull in a process called endochondral bone formation (Kovacs 2014). On the other hand, osteoblasts form bone directly in the skull and in parts of the clavicles (intramembranous bone formation).

Most of the mineralization of the fetal bone occurs during the third trimester. Active placental transport of calcium, phosphorus, and magnesium to the fetus ensures sufficient concentrations of minerals for bone formation. The bone formation is regulated by PTH, and especially PTH-related protein (PTHrP) (Kovacs 2014). Postnatally normal bone homeostasis requires adequate intake of minerals, and several hormones regulate bone

metabolism (e.g. PTH, $1,25(OH)_2D$, FGF23, calcitonin, sex steroids). Bone growth and mineral accretion is a continuous process from childhood to young adulthood (Molgaard et al. 1999), and most of the peak bone mass is achieved by the end of puberty (Bailey et al. 1999).

Longitudinal growth after birth occurs mainly at growth plates, at the metaphyseal ends of the long bones. Similarly as in fetal bone development, chondrocytes undergo tightly regulated maturation and differentiation into hypertrophic chondrocytes, and eventually form the growth plate (Adams et al. 2007b, Wang et al. 2011). The cartilage model of the bone is then replaced by bone. In this process hypertrophic chondrocytes may transform into osteoblasts, and further into osteocytes, and matrix mineralization starts (Yang et al. 2014, Tsang et al. 2015). As mentioned in section 1.4 Nutritional rickets, inadequate supply of minerals may lead to poor mineralization, deformity of the growth plate, various skeletal defects, and growth retardation.

2.2.2 Vitamin D actions on bone

Fetal bone development is independent of vitamin D (Miller et al. 1983, Brommage and DeLuca 1984). Physiologic hypocalcemia of the newborn results from cessation of active placental calcium transport (Stulc et al. 1994). Hypocalcemia induces PTH production and subsequent activation of 25OHD into $1,25(OH)_2D$ (Kovacs 2014). During the early postnatal period intestinal absorption of minerals is mostly passive, but gradually $1,25(OH)_2D$-dependent mineral absorption from the intestine becomes essential for normal bone growth and development (Kovacs 2012).

VDR and 1α-hydroxylase activity are present in all major bone cell types: osteoblasts, osteocytes, and osteoclasts (van Driel et al. 2006, Morris and Anderson 2010). Autocrine action of locally activated $1,25(OH)_2D$ in bone cells is still poorly understood. Renal $1,25(OH)_2D$, however, regulates FGF23 production of osteocytes, and thereby phosphate metabolism and bone mineralization (Lanske et al. 2014). In osteoblasts $1,25(OH)_2D$ regulates proliferation, differentiation and mineralization (van Driel and van Leeuwen 2014). Interestingly, $1,25(OH)_2D$ has a dual action on bone metabolism, as it also stimulates bone resorption by inducing osteoclastogenesis (Takahashi et al. 2014). Thus, $1,25(OH)_2D$ is one of the regulators of bone remodeling, the continuous process of removal of mature bone tissue and formation of new bone tissue (Ormsby et al. 2014). It is unknown if $1,25(OH)_2D$ has a direct effect on mineralization. However, by enhancing mineral absorption in the intestine, $1,25(OH)_2D$ stimulates bone mineralization indirectly (Tanaka and Seino 2004). The regulatory role of vitamin D on the growth plate is also poorly understood, but vitamin D metabolites (e.g. $24,25(OH)_2D$) may have an impact on the differentiation of chondrocytes (Nilsson et al. 2005, Boyan et al. 2010, Tsang et al. 2015).

2.2.3 Bone as an endocrine organ

Bone is an active tissue with continuous remodeling, and production of at least two hormones: FGF23 and osteocalcin (OC) (Fukumoto and Martin 2009). FGF23 is a phosphaturic hormone while OC is suggested to participate in energy metabolism.

Fibroblast growth factor 23

FGF23 is a hormone that regulates phosphate homeostasis (Quarles 2008). Mineralized osteocytes produce FGF23 (Yoshiko et al. 2007). Hyperphosphatemia and 1,25$(OH)_2$D enhance FGF23 production, but the overall regulation of FGF23 still remains insufficiently characterized (Saito et al. 2005, Smith et al. 2014). Moreover, PTH is able to increase production of FGF23 (Burnett-Bowie et al. 2009, Lavi-Moshayoff et al. 2010). Full-length, i.e., intact FGF23 (iFGF23) is the biologically active form. The main target of iFGF23 is in the proximal tubules in the kidneys, where it inhibits renal phosphate reabsorption by reducing the expression of type 2a and 2b sodium-phosphate cotransporters (Shimada et al. 2004a, Miyamoto et al. 2007). iFGF23 requires the co-receptor Klotho to exert its effects in the kidneys (Urakawa et al. 2006). iFGF23 also reduces the expression of renal 1α-hydroxylase, which converts 25OHD to 1,25$(OH)_2$D, and thereby indirectly reduces the intestinal absorption of phosphorus (Shimada et al. 2004a). The net result is decreased concentration of phosphate in the circulation. FGF23 may also affect bone mineralization in an autocrine or paracrine fashion by regulating the secretion of osteopontin, a protein that inhibits mineralization (Murali et al. 2015).

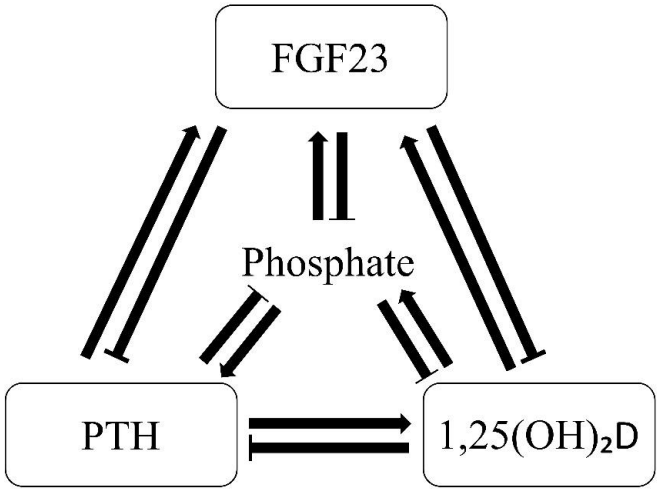

Figure 5 *Interaction between the main regulators of phosphate metabolism, adapted from Fukumoto, 2014. FGF23, fibroblast growth factor 23; PTH, parathyroid hormone; 1,25$(OH)_2$D, 1,25-dihydroxyvitamin D.*

After phosphorylation, iFGF23 is proteolytically processed into N- and C-terminal fragments (Tagliabracci et al. 2014). These fragments do not regulate phosphate metabolism directly, but the C-terminal FGF23 (cFGF23) may have a regulatory role (Goetz et al. 2010). The C-terminal fragment may adhere to Klotho and thus compete with iFGF23.

Increased production or decreased degradation of FGF23 results in several diseases involving the mineral homeostasis and the skeleton. Excess FGF23 due to impaired degradation is evident in autosomal dominant hypophosphatemic rickets (ADHR) (ADHR Consortium 2000) and X-linked hypophosphatemic rickets (XLH) (Yamazaki et al. 2002), whereas in tumor-induced osteomalacia (TIO) increased FGF23 production by the tumor results in renal phosphate loss (Shimada et al. 2001). On the other hand, diseases with hyperphosphatemia due to FGF23 deficiency also exist but they are rare (Folsom and Imel 2015).

Osteocalcin

Osteoblasts produce OC, a bone-derived hormone that regulates energy metabolism (Lee et al. 2007). In the circulation two forms of OC are present: carboxylated and undercarboxylated OC. The latter is able to increase β-cell proliferation, insulin secretion and sensitivity (Ferron et al. 2010). Moreover, osteoblasts express insulin receptors, and insulin can regulate osteoblast development, and further increase OC activity (Fulzele et al. 2010). Hence, bone and pancreas form an endocrine loop, regulating each other. As osteoblasts produce OC, it can be used as a bone formation marker (Fukumoto and Martin 2009). In obese individuals lower OC has been observed (Viljakainen et al. 2014). However, the overall role of OC in energy homeostasis and its potential other functions remain inadequately understood.

2.2.4 Bone assessment

Radiography

Conventional radiography is widely used as a primary tool to examine bone morphology and fractures and to assess bone maturation, i.e., bone age. Radiological findings seen in rickets are well-defined (Figure 6). However, radiographs do not provide detailed and accurate information on BMC, BMD, bone quality, bone architecture, or body composition, and have therefore limited value in the assessment of skeletal characteristics.

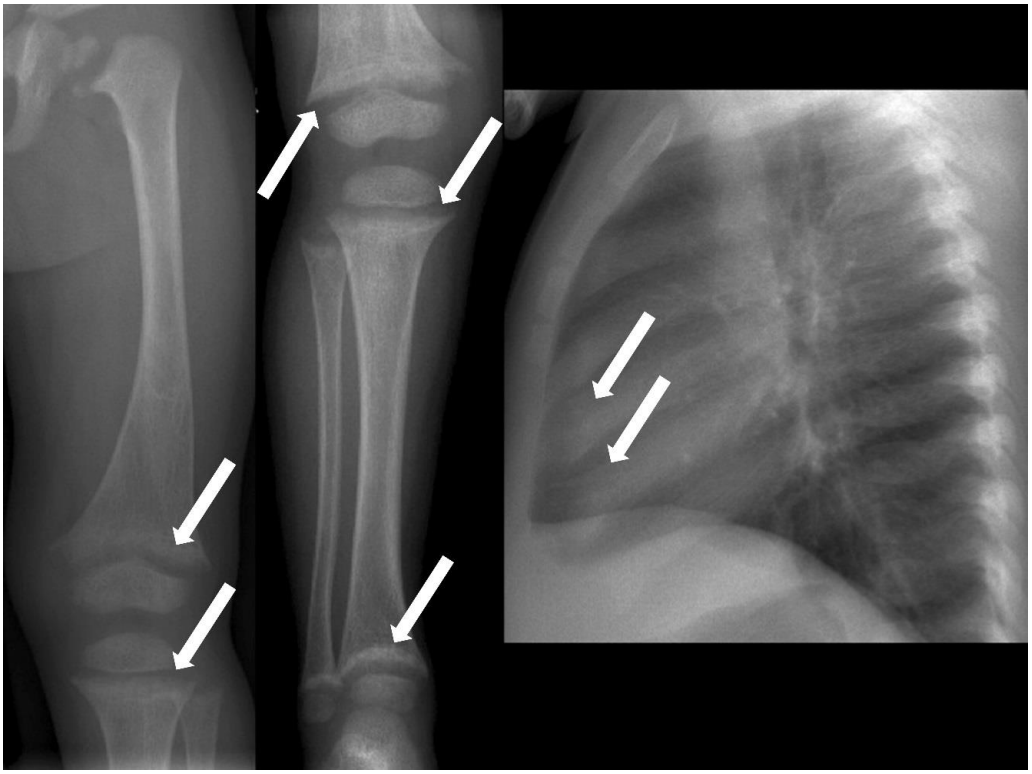

Figure 6 *Radiographic findings of rickets: widening and cupping of the metaphysis and enlarged costochondral junctions.*

Dual-energy X-ray absorptiometry

DXA enables rapid and non-invasive examination of bone area, BMC, BMD, in addition to body composition, with a relatively low radiation dose. It is an established method in clinical use to diagnose osteoporosis and to predict fracture risk in adults (Cummings et al. 2002). Hip and lumbar spine are the most common sites for BMD measurement in adults while in pediatric use lumbar spine (Figure 7) and whole body are the recommended sites (Crabtree et al. 2014). As DXA does not provide three-dimensional data, it is biased by different size and shape of the target and cannot provide data on volumetric BMD.

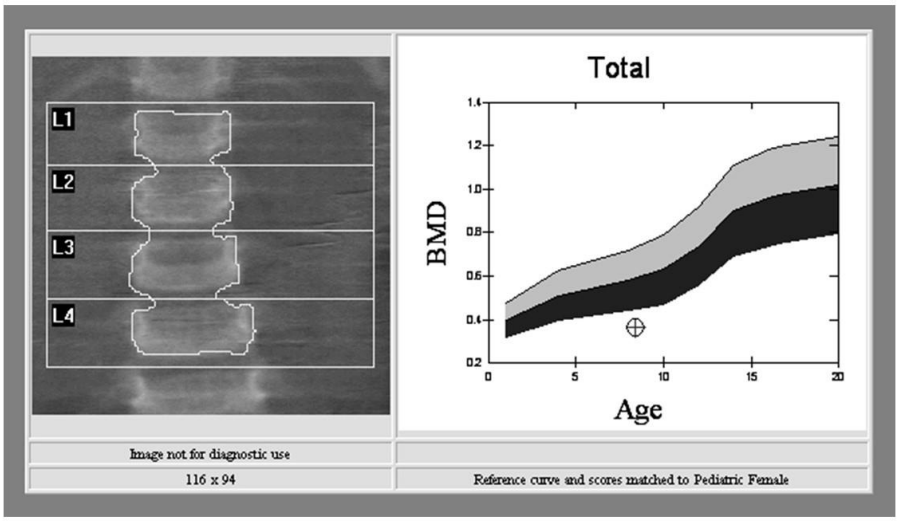

Figure 7 *DXA scan at lumbar spine in a child. The area between the curves (right) indicates normal BMD for age (Z-score between -2.0 and +2.0).*

Peripheral quantitative computed tomography

Peripheral quantitative computed tomography (pQCT) is a tool for imaging bone and surrounding soft tissues, fat and muscle (Figure 8). This method is not in clinical use but it is useful for research purposes, as it provides data on total, trabecular, and cortical true volumetric BMD and bone area, as well as an estimate of bone strength (stress and strain index, SSI) (Zemel et al. 2008). This leads to several important advantages in pQCT measurement compared with DXA: lower radiation dose, the ability to differentiate between cortical and trabecular bone, and to quantify volumetric BMD without confounding effects of size, and the ability to examine relationships between bone and soft tissues locally (Schoenau et al. 2002, Binkley et al. 2008). On the other hand, the measurements sites are peripheral (e.g. tibia and radius), while proximal or central skeleton (e.g. hip and spine) needs to be examined with DXA. In fact, different techniques complete each other by providing additive information (Amstrup et al. 2015, Daneff et al. 2015). Every method has its challenges, and in pQCT movement artifacts and difficulty in positioning the site of interest may cause bias (Blew et al. 2014). In addition, researchers need to deal with selection of analysis mode, resolution, and thresholding (Ashe et al. 2006).

Bone metabolism markers

Type 1 collagen is the main form of collagen in bone. During the continuous formation and resorption of bone tissue type 1 collagen is synthetized and degraded (Szulc et al. 2000).

During the synthesis of type 1 collagen N-terminal propeptide (PINP) is released. Thus, in addition to bone alkaline phosphatase and OC, which reflect osteoblast activity, PINP serves as a marker of bone formation (Yang and Grey 2006). On the other hand, during bone tissue resorption, as a result of osteoclast activity, N- and C-terminal fragments of collagen are released into the circulation. Measurement of cross-linked telopeptides of N- and C-terminal fragments reflects the rate of bone resorption (Yang and Grey 2006).

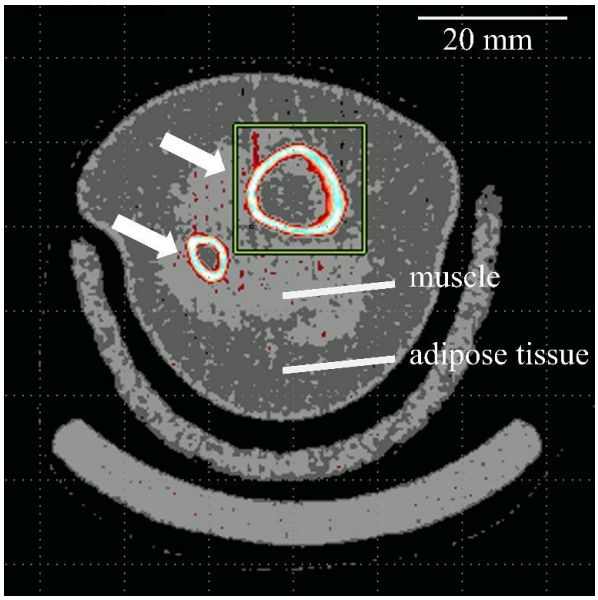

Figure 8 *Example of pQCT scan of a 1-year-old child's leg. Upper arrow points at tibia (right) and lower arrow at fibula (left).*

2.3 Vitamin D status

Research communities widely agree, based on scientific evidence, that the concentration of the most abundant vitamin D metabolite, circulating 25OHD, reflects the vitamin D status of the body. Still, a consensus on definition of vitamin D deficiency, insufficiency and sufficiency is under debate. The Pediatric Endocrine Society defined in 2008 that 25OHD <37.5 nmol/l indicates deficient, 37.5 to 50 nmol/l insufficient, and 50 to 250 nmol/l sufficient vitamin D status (Misra et al. 2008). In line with this, the American Academy of Pediatrics and ESPGHAN Committee on Nutrition stated that 25OHD concentration in infants and children should be ≥50 nmol/l (Wagner et al. 2008, Braegger et al. 2013). This is the same target concentration that the IOM definition of vitamin D RDA is based on (Ross et al. 2011). On the other hand, the Endocrine Society defined in 2011 vitamin D deficiency as 25OHD <50 nmol/l, insufficiency as 25OHD 50 to 75 nmol/l, and sufficiency as 25OHD 75 to 250 nmol/l (Holick et al. 2011). These thresholds are similar to those recommended

for adolescents for whom the optimal concentration was defined as 75 to 125 nmol/l (Society for Adolescent Health and Medicine 2013).

2.3.1 Assessing vitamin D status

Methods for assessing 25-hydroxyvitamin D

The concentration of the biologically active form of vitamin D, $1,25(OH)_2D$, does not consistently correlate with signs of vitamin D deficiency or excess. Hence, in a widely accepted manner, measurement of serum 25OHD concentration defines vitamin D status (Vieth 2007, Holick 2007b). The use of several different 25OHD assessing methods in vitamin D studies complicates interpretation of the results and comparison of individual studies (Barake et al. 2012, Sarafin et al. 2015). The estimated difference between the results in various 25OHD assays may exceed 20 nmol/l (Sempos et al. 2015). In order to deal with this challenge in verifying the accuracy and specificity of the 25OHD results in different studies, the International Vitamin D Quality Assessment Scheme (DEQAS) monitors constantly the performance of different 25OHD assays. In addition, the Vitamin D Standardization Program (VDSP), organized by the National Institute of Health, strives to standardize internationally the measurement of 25OHD (Binkley et al. 2014). VDSP recognizes DEQAS as one of the acknowledged external quality assessment schemes. The laboratories that participated in DEQAS between 2000 and 2004 (N=88) showed relatively concordant 25OHD results, as the mean bias of the most commonly used individual methods differed less than 7% from All-Laboratory Trimmed Mean (ALTM) (Carter et al. 2004). Along with an increasing number of participants in DEQAS the inter-laboratory precision has increased (Carter et al. 2010).

Vitamin D-binding protein

Both monoclonal and polyclonal antibody-based immunoassays are available for assessing DBP concentrations. Although the use of monoclonal antibodies usually results in high specificity, in DBP assessment, due to abundant genetic variation in DBP (Malik et al. 2013), polyclonal antibody-based assay may be more accurate (Bouillon et al. 2014). Unlike polyclonal antibodies, monoclonal antibodies may have variable affinity for different haplotypes of DBP (Hollis and Bikle 2014, Hoofnagle et al. 2015). In order to minimize the effect of different DBP isoforms in assessing DBP concentrations, a liquid chromatography-tandem mass spectrometric assay was recently introduced (Henderson et al. 2015).

Free 25-hydroxyvitamin D

Since the mid-1980s the assessment of bioavailable and free 25OHD concentrations has been based on mathematical formulas which take into account vitamin D-binding protein and albumin concentrations (Bikle et al. 1986). A direct two-step immunoassay for quantifying free 25OHD concentration has been available for a short time, and based on the first findings with this assay, mathematical formulas may overestimate the true concentration of free 25OHD (Schwartz et al. 2014). However, this is a novel assay with scarce reports so far, and the results need to be interpreted with caution.

2.3.2 Vitamin D intake and 25OHD levels in Finnish children and adolescents

The National FINDIET survey by the National Institute of Health and Welfare reports adults' dietary habits and nutrition intake at five-year intervals. The latest report is from 2012 (Helldan et al. 2012). Unfortunately, it does not provide data on children and adults younger than 25 years. In Finland, fortification of dairy products with vitamin D started in 2003 due to low vitamin D intake in all age groups in the National FINDIET 2002 study (Männistö et al. 2003). The initiation of food fortification, however, did not improve the situation sufficiently (Lehtonen-Veromaa et al. 2008). Thereby, a major change in the fortification was implemented in 2010 when the recommended fortification doubled. Dietary fats presently contain 20 µg vitamin D/100 g and liquid dairy products 1 µg vitamin D/100 ml. After this increment, no systematic survey has evaluated the intake of vitamin D in children or young adults. FINDIET 2012, however, showed that adults reached the vitamin D intake recommendations better than before. Of men, 33% used vitamin D supplements regularly, while the corresponding number for women was 55%. Moreover, the daily vitamin D intake exceeded the current recommendation (7.5 µg), as mean daily vitamin D intake was 11 µg in men and 9 µg in women (Helldan et al. 2012).

Two studies among Finnish adolescent girls reported alarming data on the prevalence of vitamin D deficiency in the late 1990s (Lehtonen-Veromaa et al. 1999, Cheng et al. 2003). At that time, the cut-off value for vitamin D deficiency was 37.5 nmol/l (Lehtonen-Veromaa et al. 1999) and 40 nmol/l (Cheng et al. 2003). In these two studies, the prevalence of vitamin D deficiency was 68 and 78%, respectively. Table 3 summarizes Finnish pediatric studies in recent decades reporting either the use of vitamin D supplements or 25OHD concentrations, or both.

After launching of the fortification of some food items with vitamin D in 2003, vitamin D status improved. Higher serum 25OHD concentration was evident in children with genetic susceptibility for type 1 diabetes participating in the Type 1 Diabetes Prediction and Prevention (DIPP) study: between 1998 and 2002, serum 25OHD concentration in children 2-12.2 years was on average 62 nmol/l, and between 2003 and 2006, 82 nmol/l (Mäkinen et al. 2014). During the DIPP study the increased use of supplements was also evident (Räsänen et al. 2006, Kyttälä et al. 2010). Moreover, data on vitamin D intake in the DIPP study from food and supplements in children 1–6 years were collected between 2003 and

2005. The mean (± SD) daily intake of vitamin D from food and supplements was 12.2 (± 4.6) µg in 1-year-olds. After that, the intake decreased considerably, the mean daily intake being 7.0 (± 4.4) µg in 3-year-olds and 5.9 (± 3.4) µg in 6-year-olds (Kyttälä et al. 2010). The decrease is probably a result of a decreased use of supplements, as the proportion of supplement users declined from 86 to 21%.

From 2006 to 2008, altogether 195 Finnish children aged 7 to 19 years participated in a cross-sectional study: 34% received less than the recommended daily intake of vitamin D (7.5 µg/day) and the prevalence of vitamin D deficiency (25OHD <50 nmol/l) was as high as 71% (Pekkinen et al. 2012). In a cohort of 124 newborns (2007) mean (± SD) 25OHD concentration in cord blood was 51 (± 15) nmol/l (Viljakainen et al. 2010b). Of these, 86 participated in a follow-up visit at 14 months. Then, the daily vitamin D intake of the children was on average 12.3 (± 3) µg and mean serum 25OHD concentration 64 (± 21) nmol/l (Viljakainen et al. 2010a). There are no data on vitamin D status in children and adolescents in Finland after doubling the vitamin D fortification of food and changing the recommendations for supplement use. It is worth noticing that dietary assessment methods may vary between studies and this may complicate comparison of dietary intakes.

Table 3. *Pediatric studies on vitamin D status and use of supplements in Finland.*

First author	Year	Age	N	Supplement users (%)	Mean S-25OHD (nmol/l)	S-25OHD <50 nmol/l (%)	
Lagström	1992–1994	8 mo	1,062	96	-	-	STRIP study
		13 mo		92	-	-	
		24 mo		75	-	-	
Lehtonen-Veromaa	1996–1997	9-15 y	191[†]	22	37	68[a]	
Cheng	1999–2000	10-12 y	193[†]	-	32	78[b]	
Viljakainen	2001–2002	11 y	196[†]	18	46	9[c]	
Viljakainen	2008–2009	14 mo	87	100	64	21	
Räsänen	1998–2001	3 mo	69	91	-	-	DIPP study
		6 mo	118	91	-	-	
		12 mo	267	81	-	-	
		24 mo	233	42	-	-	
		36 mo	209	26	-	-	
Kyttälä	2003–2005	12 mo	455	86	-	-	DIPP study
		24 mo	230	70	-	-	
		36 mo	471	47	-	-	
		4 y	554	31	-	-	
		6 y	713	21	-	-	
Mäkinen	1998–2002	0-1 y	314	-	76	70	DIPP study
		2-12 y	356	-	62		
	2003–2006	0-1 y	314	-	90	37	DIPP study
		2-12 y	356	-	82		
Pekkinen	2006–2008	7-19 y	195	-	43	71	

[†]Girls; 25OHD, 25-hydroxyvitamin D; S-25OHD cut-offs [a]37.5 nmol/l, [b]40 nmol/l, [c]25 nmol/l

STRIP, Special Turku Coronary Risk Factor Intervention Project; DIPP, Type 1 Diabetes Prediction and Prevention Study

2.3.3 Seasonal and age-related variation in 25OHD

Cutaneous synthesis of vitamin D has a considerable effect on serum 25OHD concentration. Factors influencing this include skin pigmentation, the use of sunscreen, age-related decrease in epidermal 7-dehydrocholesterol (a precursor for vitamin D), genetics, and the penetrance of ultraviolet B radiation to the earth surface (Holick 1995). Finland is located in the north (latitude >60°N). For comparison, in Edmonton, Canada (52°N), between October and April no cutaneous vitamin D synthesis was observable (Webb et al. 1988). Hence, in Finland cutaneous synthesis of vitamin D is relevant for vitamin D status only during the summer. Seasonal variation in vitamin D status is well known (Holick et al. 2007, Prentice 2008, Kumar et al. 2009, Michel et al. 2015), and age also has an effect on vitamin D status. In Boston, USA, the prevalence of vitamin D deficiency (25OHD <50 nmol/l) was evaluated among healthy children: vitamin D deficiency was more common in adolescents (42%) (Gordon et al. 2004) compared with toddlers and infants (12%) (Gordon et al. 2008a). During childhood a downward trend in 25OHD concentration with age is apparent in several Western countries (Lapatsanis et al. 2005, Cashman 2007, Vidailhet et al. 2012)

2.4 Vitamin D and chronic illness in childhood

Due to the profound skeletal and several non-skeletal effects of vitamin D, its deficiency associates with several diseases, and the risk of osteoporosis is true already in pediatric patients (Rosen et al. 2012, Palermo and Holick 2014, Högler and Ward 2015). The most common conditions that associate with vitamin D deficiency in childhood are (in alphabetical order): chronic renal failure, immobility, inflammatory and infectious diseases, malabsorption, and obesity (Chapter 5) (Palermo and Holick 2014). In addition to the disease itself, the required medication and other treatments may also increase risk for vitamin D deficiency (Zhou et al. 2006).

2.4.1 Chronic diseases influencing vitamin D status

As diet (food and supplements) is the main source of vitamin D in the northern latitudes, any condition affecting intake or absorption of vitamin D from the intestine may cause vitamin D deficiency. In Finland the main source of dietary vitamin D is fortified dairy products (Helldan et al. 2012). Hence, milk allergy and reduced consumption of dairy products may cause vitamin D deficiency (Fox et al. 2004, Yu et al. 2006, Barreto-Chang et al. 2010). Moreover, cystic fibrosis and intestinal failure due to short bowel syndrome impair vitamin D absorption (Tangpricha et al. 2012, Wozniak et al. 2015).

Other intestinal morbidities, associated with vitamin D deficiency, include celiac disease (Mager et al. 2012) and inflammatory bowel diseases (IBD), including Crohn's disease and colitis ulcerosa. IBD patients are at risk for osteoporosis partly due to inflammation, and

partly due to vitamin D deficiency (Mouli and Ananthakrishnan 2014). Celiac disease, on the other hand, is an autoimmune disease like diabetes mellitus type 1, both being relatively common diseases of childhood, especially in Finland. In such conditions, vitamin D may play a role as either a causal factor (Hyppönen et al. 2001) or a consequence of the disease, or both (Bellastella et al. 2015).

Chronic kidney disease (CKD) may lead to vitamin D deficiency (Seeherunvong et al. 2009). In kidney failure, hyperphosphatemia due to reduced glomerular filtration rate leads to increased iFGF23, and further increased inactivation of 25OHD and $1,25(OH)_2D$, in addition to reduced renal activation of $1,25(OH)_2D$ (Quarles 2012). Such alterations lead to a disorder known as "chronic kidney disease-mineral bone disorder" (Khouzam et al. 2014, Kazama et al. 2015).

Several diseases may affect vitamin D status directly (see 4.1), but often the medication given also increases the catabolism of vitamin D. Such medications include for example antiepileptic drugs and glucocorticoids, which both stimulate the degradation of 25OHD and $1,25(OH)_2D$ (Zhou et al. 2006). Impaired growth and bone mineralization are common in pediatric patients after transplantation (Taskinen et al. 2007, Valta et al. 2008), with immobility (Kilpinen-Loisa et al. 2010), and in conditions characterized by chronic inflammation such as arthritis (Markula-Patjas et al. 2012).

2.4.2 Vitamin D deficiency as a risk factor for chronic disease

Vitamin D exerts regulatory effects on both innate and adaptive immune responses (Rosen et al. 2012). As already mentioned, vitamin D deficiency may increase the risk of autoimmune diseases, such as celiac disease, although the causal relationship is still unconfirmed (Dong et al. 2013, Vondra et al. 2015). Poor vitamin D status relates to recurrent wheeze in small children (Devereux et al. 2007, Camargo et al. 2011), and to later risk of asthma (Erkkola et al. 2009) and asthma exacerbations (Brehm et al. 2010). Vitamin D supplementation may reduce such exacerbations (Pojsupap et al. 2015, Xiao et al. 2015). In addition, maternal vitamin D supplementation may reduce wheezing in small children (Chawes et al. 2016, Litonjua et al. 2016). Furthermore, low 25OHD concentration associates with childhood allergies and atopic eczema (Sharief et al. 2011, Jones et al. 2012), and vitamin D supplementation may improve these conditions (Camargo et al. 2014). Further studies are necessary to verify the causality.

2.5 Vitamin D and obesity

2.5.1 Vitamin D status and response to supplementation

The association between obesity and impaired vitamin D status is well established (Jorde et al. 2010, Brock et al. 2010, Saneei et al. 2013). A recent meta-analysis supports the association between vitamin D deficiency and obesity in all age groups (Pereira-Santos et al. 2015). The prevalence of obesity in childhood is increasing (Gordon-Larsen et al. 2004, Cunningham et al. 2014, Lobstein et al. 2015), and already at that time an inverse correlation exists between adiposity-related parameters and 25OHD concentration (Rajakumar et al. 2011). Despite lower vitamin D status, the fracture risk in obese individuals is not linearly increased (Premaor et al. 2013, Johansson et al. 2014), although interaction between adipose tissue and the skeleton is evident (Viljakainen et al. 2011, Pollock 2015). Such a finding has raised a question of whether obesity affects bioavailable or free 25OHD concentration.

As obesity increases the risk for cardiovascular complications (Ayer et al. 2015), and low vitamin D status associates with increased risk for cardiovascular outcomes (Reddy Vanga et al. 2010, Rosen et al. 2012), the combination of vitamin D deficiency and obesity may significantly increase the risk for obesity-related morbidity. Low vitamin D status may also associate with an increased risk for metabolic syndrome, a well-known risk factor for cardiovascular morbidities (Joergensen et al. 2010, Mitri et al. 2014). Although a previous systematic review could not confirm the correlation between vitamin D supplementation and cardiovascular outcomes (Pittas et al. 2010), in a more recent cohort with almost 11,000 patients, treatment of vitamin D deficiency resulted in improved survival (Vacek et al. 2012).

Several studies in different populations have documented lower response to vitamin D supplementation in obese individuals than in normal-weight subjects (Gallagher et al. 2013, Didriksen et al. 2013). In obese adolescents, previous studies have confirmed this finding with both daily and weekly dosing of vitamin D (Harel et al. 2011, Aguirre Castaneda et al. 2012, Rajakumar et al. 2015). Obese individuals are at risk for vitamin D deficiency, and its treatment may require larger doses of vitamin D than in normal-weight individuals.

2.5.2 Bioavailable (free) 25-hydroxyvitamin D

Bioavailable 25OHD means the part of circulating 25OHD that is not bound to DBP (Chun et al. 2014). As DBP binds most of the circulating 25OHD, and albumin approximately 10%, free 25OHD makes up less than 1% (Bikle et al. 1985). The impact of obesity on the concentration of vitamin D-binding protein, and the bioavailable 25OHD, is poorly characterized and the available data conflicting. Some studies have shown a positive correlation between obesity and DBP, while several others have failed to demonstrate any correlation between DBP and BMI or body fat content (Powe et al. 2011, Ashraf et al. 2014, Karlsson et al. 2014). Both adipose tissue and muscle are vitamin D storage sites in the body

(Vieth 2007). In obese individuals fat-soluble vitamin D is diluted in a larger fat mass (Drincic et al. 2012), and the bioavailability of vitamin D may be affected (Wortsman et al. 2000).

The free hormone hypothesis

The free hormone hypothesis, by Mendel 1989, states that the unbound form of a hormone is responsible for its biological actions (Mendel 1989). In clinical use, this approach has proved useful in measuring free thyroid and free testosterone concentrations, but concerning vitamin D scientific evidence is still inadequate (Faix 2013). However, bioavailable 25OHD may influence mineral metabolism (Bhan et al. 2012), and BMD has associated with calculated bioavailable 25OHD (Powe et al. 2011, Powe et al. 2013a).

3 Aims of the study

Normal growth requires adequate intake of vitamin D. As diet and cutaneous vitamin D synthesis are insufficient to secure the need for vitamin D in infants, continuous supplementation is essential. In addition to infants, chronically ill children are also prone to vitamin D deficiency. Therefore, the specific risk factors predisposing to vitamin D deficiency and the appropriate dose for vitamin D supplementation need to be determined. This doctoral thesis aimed to:

I Examine the prevalence of vitamin D deficiency in pediatric risk groups.

II Evaluate specific risk factors for vitamin D deficiency in children with a chronic illness.

III Study the efficacy and safety of vitamin D supplementation in newborns and individuals with childhood-onset obesity.

IV Explore the impact of vitamin D supplementation on mineral metabolism and bone growth in healthy newborns.

4 Subjects and methods

4.1 Subjects and study design

This thesis work is based on four studies involving three study populations (Table 4). In a cross-sectional study in chronically ill children (I) we examined the prevalence of and risk factors for vitamin D deficiency. We conducted two randomized vitamin D interventions: one in infants (II, III) and one in young adults (IV).

Table 4. *Study populations and design.*

Study	N	Time	Baseline age (years)	Sex (F/M)	Study Design
I	1,351	1/2007–12/2010	10.6 (5.2)	49.5%/ 50.5%	Register-based cross-sectional
II, III	113	9/2010–5/2011	0 (0)*	49.6%/ 50.4%	Randomized double-blinded intervention
IV	42	11/2012–5/2013	20.5 (2.7)	45.2%/ 54.8%	Randomized double-blinded intervention

age: mean (SD), *0-3 months; F/M: female/male

4.1.1 Vitamin D deficiency in chronically ill children (I)

To evaluate vitamin D status in children with a chronic illness, data on serum 25OHD measurements performed at Children's Hospital Helsinki between 2007 and 2010 for patients who had visited the outpatient clinic were collected from the hospital's laboratory database. These children required follow-up at a tertiary hospital and suffered from one or several diseases, including asthma, allergies, gastrointestinal diseases, cancer, renal diseases, diabetes and other endocrine diseases, chronic inflammatory or infectious diseases, eating disorders or metabolic bone diseases. We included children aged 0 to 18.0 years, and selected the first measurement of 25OHD during the given time period. Serum 25OHD measurements were made as part of patients' normal follow-up, based on the decision of an individual clinician, specialized in pediatrics. Altogether 1,351 children were included in this register-based cross-sectional observational study (Figure 9). In order to avoid misleading values caused by renal insufficiency and impaired 1-α hydroxylation, we

excluded 25OHD concentrations from children with serum creatinine exceeding the upper normal limit by 20%. Since baseline characteristics did not include ethnic background or data on diseases or medications, we conducted a post-hoc analysis searching for factors predisposing for and predicting vitamin D deficiency; we included children with the 50 highest and 50 lowest 25OHD concentrations. Clinical characteristics, collected from hospital records for each subject, included ethnicity, anthropometric measurements, diagnosis and features of the underlying illness, and the use of vitamin D supplements.

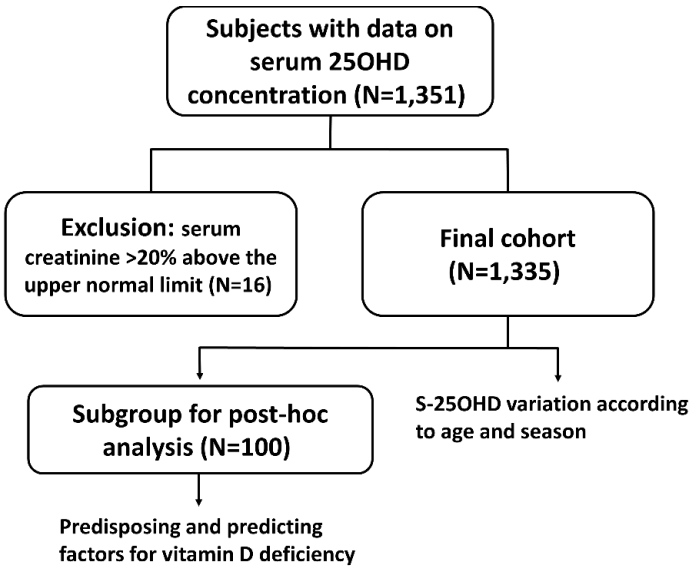

Figure 9 *Flow chart of study I. 25OHD, 25-hydroxyvitamin D.*

4.1.2 Vitamin D intervention in infants – pilot study (VIDI-P) (II, III)

VIDI-P was a randomized, controlled and double-blinded interventional study. Children were randomized into three groups, stratified by gender (Figure 10). The vitamin D doses were 10 µg, 30 µg or 40 µg, given daily from two weeks to three months. Randomization and blinding was carried out by Helsinki University Hospital Pharmacy. The study preparation replaced the otherwise recommended vitamin D supplementation for infants, and the use of other vitamin D products was not allowed.

Recruitment of healthy term infants started in September 2010 at Helsinki Maternity Hospital, and continued until February 2011. Families participating in routine pre-labor hospital visits during the third trimester (H33-36) received information about the study. Written informed consent from parents was collected by midwifes who met the families before the child was born. Inclusion criteria were verified before entering the study; these included healthy Caucasian mother with uneventful pregnancy, labor at gestational weeks

37+0 to 42+0, and children's birth weight appropriate for gestational age (-2.0 to +2.0 SD) without signs of postnatal illness or major malformations. The final study cohort comprised 113 healthy term infants.

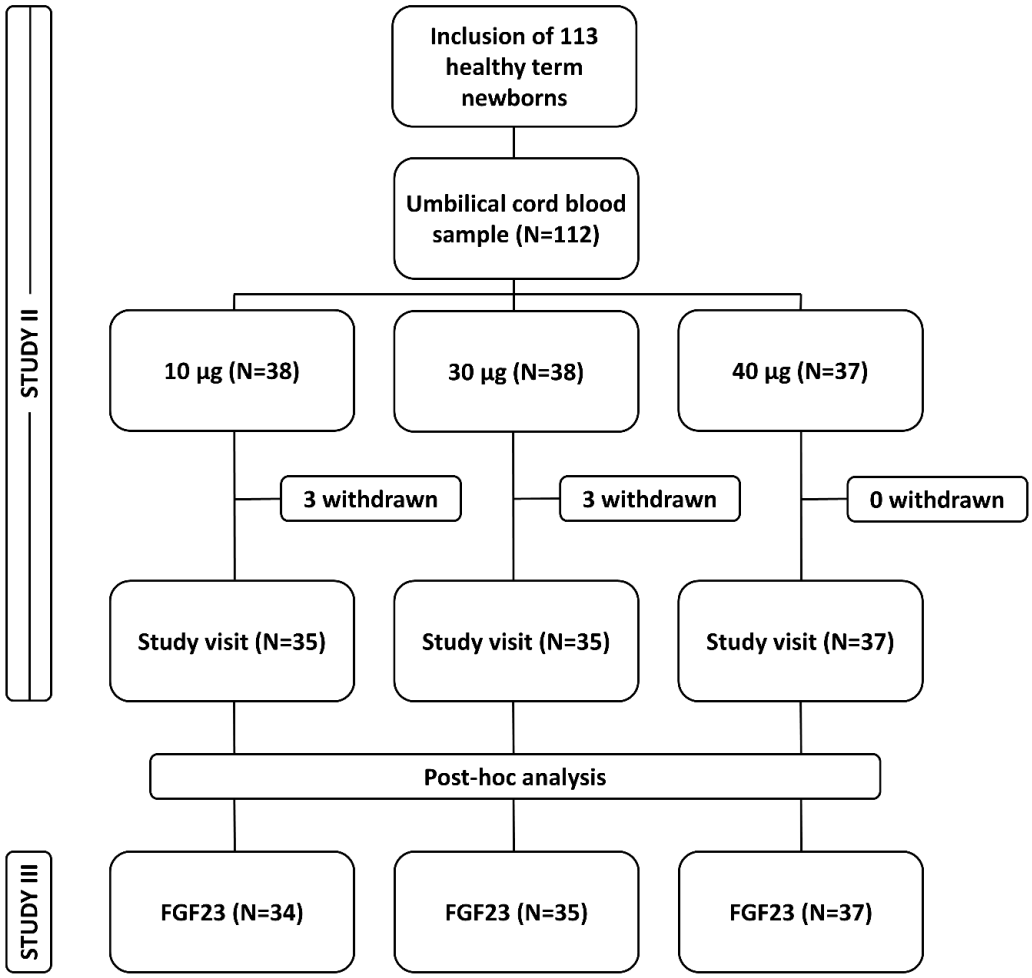

Figure 10 *Flow chart for studies II and III. FGF23, fibroblast growth factor 23.*

4.1.3 Vitamin D intervention in obese young adults (IV)

To evaluate vitamin D status and response to supplementation in obese young adults we recruited altogether 42 subjects to a 12-week vitamin D intervention (Figure 11). The study was a part of a larger follow-up study (ELLU) assessing the effects of lifestyle factors and obesity among adolescents and young adults. The study was conducted between November

2012 and May 2013. Controls with a similar age range, without history of obesity, were collected from the national population register (N=24). Obese subjects (N=18) had a medical history of long-term severe obesity, defined as weight-for-height ratio exceeding 60% for at least 3 years, from early childhood (diagnosis before age 7 years). Patients with endocrine or genetic disorders underlying obesity were excluded. Hospital records at Children's Hospital provided detailed information of diagnosis and follow-up. Participants, or guardians of those <18 years, gave their written informed consent before entering the study.

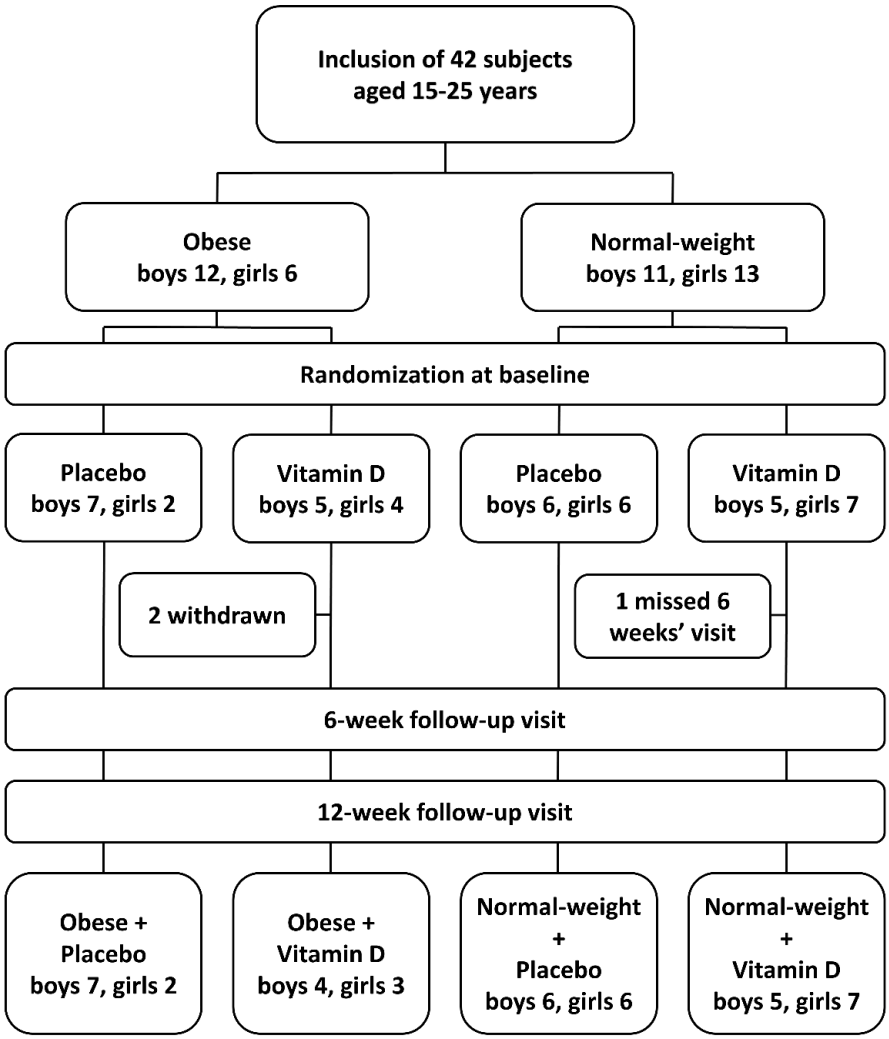

Figure 11 *Flow chart of study IV.*

Randomization, without stratification by gender, was performed prior to study initiation. Subjects received in a double-blinded fashion placebo or vitamin D_3 50 µg daily for 12 weeks. Habitual use of vitamin D containing supplements was allowed. A Finnish pharmaceutical company, Verman (Kerava, Finland), donated the preparation used and ensured blinding, but had no role in study conduction or data analysis.

4.2 Ethical considerations

Study design, conduction and recruitment practices, including information about the study protocol, potential discomfort and written informed consents, were performed in accordance with the World Medical Associations' Declaration of Helsinki, a statement of ethical principles for clinical research. The Research Ethics Committee of the Hospital District of Helsinki and Uusimaa, and Children's Hospital Helsinki approved each study protocol before inception of any given study. The Finnish Medicines Agency (Fimea) gave approval for study II (EudraCT 2009-015940-40), which was also registered in ClinicalTrials.gov (NCT01275885). Study IV was registered in ClinicalTrails.gov after the completion of the intervention (NCT02549326).

4.3 Methods

4.3.1 Vitamin D preparation (II, III, IV)

In VIDI-P, the vitamin D preparation was vitamin D_3 dissolved in medium-chain triglyceride (MCT) oil (Vitamin D_3 Forte®; Renapharma, Uppsala, Sweden). Helsinki University Hospital Pharmacy prepared the three different concentrations (10 µg/ml, 30 µg/ml and 40 µg/ml), and the Finnish Food Safety Authority Evira confirmed appropriate stability of the preparation. Parents gave the daily dose with a 1 ml syringe. The intervention began when the children were 2 weeks and lasted until they were 3 months. In study IV, 50 µg vitamin D_3 tablets (Minisun®; Verman, Kerava, Finland) were used. Placebo was otherwise equal in composition, but without cholecalciferol. The intervention began at the first follow-up visit and lasted 12 weeks.

4.3.2 Questionnaires (II, III)

Data on family background, infant feeding, allergies, infectious or other diseases, medications, and abdominal symptoms were collected with a questionnaire. The questionnaires were reviewed in detail during the follow-up visit. The vitamin D follow-up form served as a prospective way of recording adherence; guardians documented every

vitamin D dose given to the infant during the study. Overall compliance was calculated based on reported use of vitamin D supplements and returned vitamin D preparations.

4.3.3 Anthropometric data

Anthropometric data were collected at every follow-up visit. We measured height as centimeters (cm), and the result was recoded at millimeter (mm) accuracy. In studies II and III, the infant was lying down during height measurement. Weight was recorded in grams (g) in infants, otherwise as kilograms (kg). In studies II&III, head circumference (cm), and in study IV, waist and hip circumference (cm) were measured. In study I, anthropometric data were obtained from patient records for the patients with the 50 lowest and 50 highest 25OHD concentrations.

4.3.4 Laboratory measurements

Table 5 summarizes the biomarkers analyzed in studies I-IV and the methodological aspects. In order to increase the comparability and accuracy of the results, 25OHD concentrations in the intervention studies were analyzed with IDS-iSYS chemiluminescent-based automated analyzer. The manufacturer of the analyzer, Immunodiagnostic Systems Ltd., has harmonized the method along VDSP recommendations (Simpson CA 2015). Moreover, the research laboratory of Children's Hospital has participated in the DEQAS. During 2013 the policy changed, and NIST is currently the reference. Our results were on average 11% higher than NIST. The assay for iFGF23, provided by Kainos Laboratories (Tokyo, Japan), detects only the full-length, i.e., active FGF23. C-terminal assay (Immunotopics International, San Clemente, CA, USA), however, measures both full-length and C-terminal fragments. The sensitivity of the used FGF23 assays has been good in previous studies (Imel et al. 2006). Both FGF23 analyses were performed in duplicate. Values below the detection level for iFGF23 (3 pg/ml) were recoded as 3 pg/ml, and values exceeding the upper detection level for cFGF23 (1400 RU/ml) were recoded as 1400 RU/ml (n=15).

4.3.5 Bone mineral density and body composition

In study II, bone mass and other skeletal markers were measured with pQCT from distal tibia. Although complete DXA analyses were available for young adults in study IV, only parameters of body composition were utilized.

pQCT (II)

Peripheral quantitative computed tomography (pQCT) measurements were performed during the follow-up visit in study II. The length of the left tibia was measured and pQCT-

scan was performed at sites of 65% and 10% (measured from the distal end of the tibia). The majority of the measurements at the 10% site, located near the region of growth plate, failed due to lack of solid bone tissue. Measurements at the 65% site were included in the final analysis. Movement artifacts resulted in disqualification of 25% of the measurements.

We used voxel size of 0.2 mm^2 and CT scan speed of 20 mm/s. The threshold used for cortical bone was 480 mg/cm^3, for total bone 180 mg/cm^3, and for muscle 40 mg/cm^3. CALCBD refers to the analysis of total and trabecular bone; contour mode 1 was used for detection of outer bone edge, and peel mode 1 for distinguishing subcortical bone from trabecular bone. CORTBD refers to analysis of cortical bone, and separation mode 1 was used to distinguish cortical bone from trabecular bone. In polar stress and strain index (SSI) threshold of 480 mg/cm^3 was used. No reference values exist for children under 5 years.

DXA (IV)

Dual-energy X-ray absorptiometry measurement was performed with Lunar Prodigy Advance on each subject participating in the larger follow-up study ELLU. In the intervention study (IV) (N=42) data on total body fat (%) and fat-mass index [fat mass (kg)/ cm^2] were used in order to evaluate the effect of body composition on vitamin D status.

Table 5. *Laboratory analyses used in original studies I-IV*

Biomarker	Test	Study	Intra-assay CV	Site of analysis
S-25OHD	HPLC	I		HUS
	CLIA	II, III	<8%	PS
	CLIA	IV	<5%	CH
S-iPTH	CLIA	I		HUS
	CLIA	II, III		PS
	CLIA	IV	<7%	CH
P-Ca	Spectrophotometric	I-IV		HUS
P-Pi	Spectrophotometric	I-IV		HUS
S-iFGF23	ELISA	III	<5%*	UU
S-cFGF23	ELISA	III		UU
S-25OHD free	ELISA	IV	<8.5%	CH
S-DBP	ELISA	IV	8% (mean)	CH
S-PINP	CLIA	II, III		PS
S-CTX	CLIA	II, III		PS
S-OC	IFMA	IV	4.4%	UT

25OHD, 25-hydroxyvitamin D; iPTH, intact parathyroid hormone; Ca, calcium; Pi, phosphate; i/cFGF23, intact/C-terminal fibroblast growth factor 23; DBP, vitamin D-binding protein; PINP, type 1 collagen N-terminal propeptide; CTX, type 1 collagen C-terminal telopeptide; OC, osteocalcin

HPLC, high-performance liquid chromatography; CLIA, chemiluminescence immunoassay; ELISA, enzyme-linked immunosorbent assay, IFMA, immunofluorescence assay; CV, coefficient of variation

*assessed in the performing laboratory in previous studies

HUS, University Hospital Laboratory; PS, Pharmatest Services Ltd.; CH, Research Laboratory of Children's Hospital; UU, Uppsala University, Sweden; UT, University of Turku

4.3.6 Statistical analysis

Statistical analyses were performed with IBM SPSS Statistics 19 (II), 22 (III, IV) and PASW 18 (I) (IBM, Armonk, USA). P-values <0.05 indicated statistical significance. Distribution of the variables was tested with Kolmogorov-Smirnov test. If a variable was non-normally distributed, a logarithmic transformation was performed or a non-parametric test was used.

For continuous variables, baseline differences between means of two groups were analyzed with Independent samples T-test or Mann-Whitney U test, appropriate for the distribution. Respectively, analysis of variance (ANOVA) or Kurskal-Wallis test was used in the case of three or more comparable groups. For categorical variables, Pearson's chi-squared test (χ^2) was used. In sub-study I, seasonal or age-related differences in S-25OHD were analyzed with analysis of covariance (ANCOVA), as covariates were included in the analysis. In the interventional studies (II, III, IV) repeated-measures ANCOVA was used in analyzing the impact of the intervention, allowing to take into consideration the effect of covariates. Covariates were associated with the dependent variable in simple linear regression models.

In order to test the strength of the association between two variables, either Pearson's or Spearman's (both variables non-normally distributed) correlation coefficient was used. In sub-study III, association between FGF23 and phosphate required non-parametric partial correlation due to covariates (25OHD, PTH).

In studies II, III and IV, intention-to-treat (ITT) analysis was used. In order to illustrate how adherence affects dose-response in study II, results in subjects receiving >80% of the study preparation were also reported. Compliance-based dose-response was calculated in the following way: change in S-25OHD (nmol/l)/by dose (µg) *compliance (%).

5 Results and Discussion

This doctoral thesis comprises four studies involving healthy term infants (N=113), children and adolescents aged ≤18 years with a chronic illness (N=1,335), and both normal-weight (N=24) and obese (N=18) adolescents and young adults. Hence, it was possible to extensively evaluate vitamin D status in young population at risk for low 25OHD concentration and the prevalence of vitamin D deficiency (25OHD <50 nmol/l) and to compare the impact of vitamin D supplementation in different populations. On the other hand, due to heterogeneous study populations with respect to age, health, and study year (Table 4), comparison of absolute serum 25OHD concentrations between different study populations was inappropriate. Age groups narrowly overlapped between the studies: in studies II and III infants were less 4 months, in study I only 8 children were ≤4 months and 60% were between 10.1 and 18.0 years, and in study IV the proportion of subjects >18.0 years was 74%. Study I involved chronically ill children, whereas the infants in studies II and III were healthy. The timing of the studies may also affect 25OHD concentrations, as vitamin D fortification of foods increased in 2010, together with revised recommendations for supplemental vitamin D intake in children, pregnant and breastfeeding mothers in 2011 (www.ravitsemusneuvottelukunta.fi).

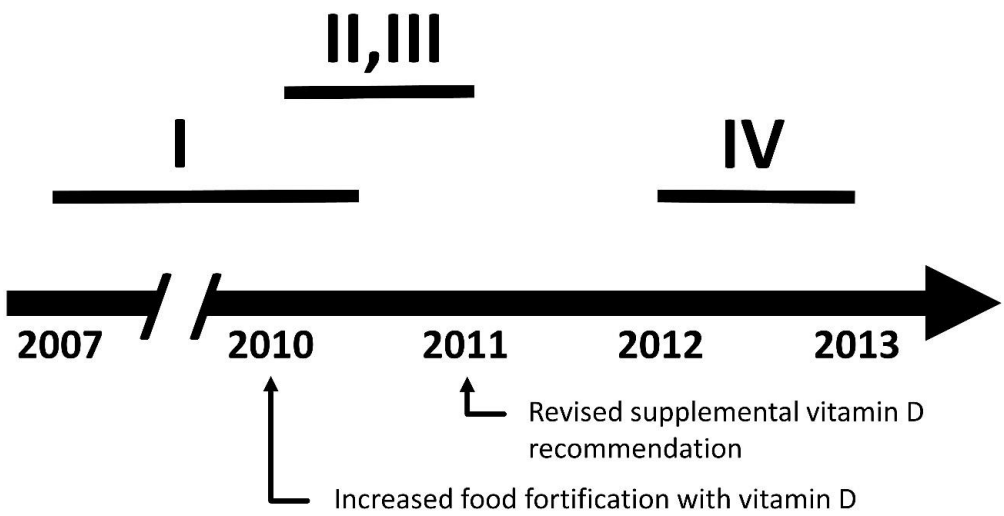

Figure 12 *Timeline illustrating the timing of data collection in the studies.*

5.1 Vitamin D status and the prevalence of vitamin D deficiency

More than 40% of all subjects suffered from vitamin D deficiency, and the prevalence increased with age: more than half of school-aged chronically ill children were vitamin D deficient. Table 6 summarizes mean 25OHD concentrations (±SD) and the prevalence of vitamin D deficiency, defined as serum 25OHD <50 nmol/l, in studies I-IV. Even 44% of cord blood 25OHD concentrations were below 50 nmol/l, reflecting the proportion of vitamin D deficient newborns before the initiation of vitamin D supplementation. In young adults, obesity associated with lower 25OHD concentration than in normal-weight subjects, the prevalence of vitamin D deficiency being in study IV 56% and 29% in the obese and normal-weight groups, respectively. Seasonal variation of 25OHD in chronically ill children was evident in school-aged subjects. Serum 25OHD concentrations were lowest and the prevalence of vitamin D deficiency highest during winter and spring.

Table 6. *Baseline concentrations of 25OHD and the prevalence of vitamin D deficiency (25OHD <50 nmol/l) in studies I-IV.*

Study	N	year	mean (SD) 25OHD (nmol/l)	25OHD <50 nmol/l (%)	Season
I					
0–2.0 year	129	2007–2010	74 (27)	19	all seasons
2.1–6.0 year	185	2007–2010	64 (23)	26	all seasons
6.1–10.0 year	229	2007–2010	56 (20)	40	all seasons
10.1–15.0 year	473	2007–2010	49 (20)	54	all seasons
15.1–18.0 year	319	2007–2010	47 (18)	56	all seasons
II, III					
cord blood	112	2010–2011	53 (14)	44	September-February
IV					
obese	18	2012–2013	49 (15)	56	November-March
normal weight	24	2012–2013	62 (24)	29	November-March

5.1.1 Cord blood (II, III)

In studies II and III, mean 25OHD concentration was on average 53 nmol/l (±14) and the prevalence of vitamin D deficiency 44%. Altogether, 88% of mothers in studies II and III reported regular use of supplemental vitamin D, the daily intake of supplemental vitamin D being on average 11 µg. Despite the well-adopted maternal recommendation of vitamin D

supplementation during pregnancy, almost half of the newborns were vitamin D deficient. In a previous Finnish study (Viljakainen et al. 2010a), mean cord blood 25OHD concentration was 51 nmol/l, which is almost identical with studies II and III. This is surprising, because in the study by Viljakainen, 80% of the mothers used daily vitamin D supplementation, and the mothers' mean daily intake of supplemental vitamin D was 4.8 µg. Thus, the cord blood 25OHD concentrations were similar even though in studies II and III maternal vitamin D intake from supplements was more than double that in the study by Viljakainen. Both studies were conducted in the same maternal hospital in Helsinki during winter, and 25OHD assay was similar (IDS-iSYS). Hence, despite higher fortification of foods and greater self-reported intake of supplemental vitamin D, the cord blood 25OHD concentration did not improve. Different study populations undoubtedly complicate direct comparison between the studies, and vitamin D intake from foods would be of interest: after the increment of vitamin D fortification of foods in 2010 in Finland, the mean daily intake of vitamin D from food has increased from 6 µg in pregnant women to 9 µg in women of reproductive age (Marjamäki et al. 2010, Helldan et al. 2012).

The aforementioned studies included mothers who had likely adopted vitamin D recommendations better than the average population. Thus, vitamin D deficiency may be even more prevalent in the general population. In the late 1990s, pregnant women in Finland were recommended to use supplemental vitamin D during wintertime. At that time, only 40% of pregnant women reported taking vitamin D supplements, and only 15% reached the recommended total daily vitamin D intake (Arkkola et al. 2006). However, self-reported vitamin D supplementation among pregnant women whose children were at risk for type 1 diabetes increased from 63% in 2004 to 71% in 2010 (Aronsson et al. 2013). In the same population, the use of vitamin D supplements was more common if the mother was pregnant with the first child, and less common if the mother was ≥35.0 years. In our cohort, parity did affect the adherence, since the average compliance was 89% in families with the first child and 80% in others (p=0.002). On the other hand, maternal age did not affect the use of supplements. The use of supplemental vitamin D has remained relatively stable, as in a recent report among mothers at high risk for gestational diabetes the proportion of pregnant women using regular vitamin D supplement was 72% (Meinilä et al. 2015).

Because 25OHD passes the placenta readily, low maternal 25OHD concentration before the delivery inevitably leads to low 25OHD in the newborn: cord blood 25OHD concentration is on average 75% of maternal concentration (Kovacs 2012). Genetic factors play a limited role in regulating neonatal 25OHD concentration (Novakovic et al. 2012). Data on how maternal 25OHD concentration changes during pregnancy are conflicting: some report a decline towards the end of the pregnancy, others an increase, or no change at all (Novakovic et al. 2012, Grant et al. 2014). The bioavailable 25OHD, i.e., not bound to DBP, may decrease towards to end of the pregnancy, probably due to increased DBP concentration (Zhang et al. 2014). With an extensive maternal supplementation with vitamin D, however, cord blood 25OHD increases (Kovacs 2008). In fact, in studies II and III the cord blood 25OHD concentration correlated with maternal supplemental vitamin D intake (r=0.215, p=0.024). In conclusion, sufficient maternal vitamin D supplementation is an effective means to prevent vitamin D deficiency in the newborn.

5.1.2 Chronically ill children (I)

Serum 25OHD concentrations in different age groups

The mean serum 25OHD concentration in children with a chronic disease decreased with age, and the prevalence of vitamin D deficiency increased (Table 6). The prevalence of vitamin D deficiency, defined as serum 25OHD <50 nmol/l, was in total 45%: of children less than 2 years, <20% had vitamin D deficiency, compared with 56% of adolescents; concurrently, the mean 25OHD concentration decreased from 74 nmol/l to 47 nmol/l, respectively. In 2010 mean 25OHD concentrations were higher than between 2007 and 2009 (Table 7): the increase in mean 25OHD concentrations was highly significant in children <2 years and adolescents >15 years (both p=0.001).

Previous studies among healthy children have reported a decrease in 25OHD concentration with age (Prentice et al. 2008, Kumar et al. 2009). However, reports in pediatric patients are still relatively scarce (Robien et al. 2011, Lerner et al. 2012). The age-dependent variation in 25OHD concentrations in study I was striking: the prevalence of vitamin D deficiency in children <2 years was only one third of the prevalence in adolescents >15 years. It is likely that the well-established recommendation of vitamin D supplementation to all infants and small children resulted in a relatively good vitamin D status in the age-group of 0 to 2 years (mean 25OHD 74 nmol/l). As seen in a previous Finnish study, the proportion of supplement users decline with age: the child's 1st year 86%, 2nd year 70%, 3rd year 47%, 4th year 31%, while only 21% of 6-year-old children received vitamin D supplements (Kyttälä et al. 2010). Such a decrease could explain, at least partly, the dramatic age-related change in the mean 25OHD concentrations seen in study I. Along with the decline in the use of supplements, the increased intake of vitamin D from food in older children was not sufficient to cover the nutritional need for vitamin D. The unselected study population in study I complicates the interpretation of the results. Baseline characteristics did not include data on underlying clinical conditions, duration of the illness or the medication given, which likely differ between age groups, and may also directly affect 25OHD concentrations. Moreover, since approximately 25,000 children have follow-up at Children's Hospital in Helsinki, the total number of 25OHD measurements was relatively low.

The overall prevalence of vitamin D deficiency (45%) was a notable finding, as vitamin D is essential for normal growth and development, and chronically ill children may be even more vulnerable to the adverse effects of vitamin D deficiency due to their chronic disease or the medication they receive. Fortunately, higher 25OHD concentrations were evident in measurements made in 2010 (Table 7). In fact, the overall prevalence of vitamin D deficiency in 2010 was 36%. Especially in the youngest and oldest age groups, the increase in mean 25OHD concentration over time was significant. However, almost half of the adolescents were still vitamin D deficient. Improved 25OHD concentrations in 2010 could at least partly be a result of increased intake of vitamin D due to the recommended increase in vitamin D fortification of foods in 2010. In 2011, revised recommendations stated that

all children <18 years should receive regular vitamin D supplementation. The sufficiency of these recommendations remains to be elucidated in future studies.

Table 7. *Mean 25OHD concentrations, adjusted for the summer season, in study I according to age groups and sampling year.*

		Mean 25OHD (nmol/l)				
Age group (y)	**N**	**2007**	**2008**	**2009**	**2010**	**p†**
0–2.0	129	70	68	60	85	0.001
2.1–6.0	185	57	65	66	71	0.084
6.1–10.0	229	53	54	59	61	0.026
10.1–15.0	473	47	51	46	53	0.028
15.1–18.0	319	45	44	47	55	0.009

25OHD, 25-hydroxyvitamin D; y, year
†ANCOVA, using summer season as a covariate

Infants are prone to vitamin D deficiency. In study I, almost every fifth child aged 0 to 2 years presented vitamin D deficiency. Breastfeeding of the newborn is common in Finland: in the DIPP study, 92% of the mothers reported that they breastfed their child at 1 month, and 58% at 6 months (Erkkola et al. 2009). The WHO recommendation for the duration of exclusive breast-feeding is 6 months (http://who.int/topics/breastfeeding). Breast milk is scarce in vitamin D (Vieth Streym et al. 2015). Mothers would need high doses (50 to 100 µg) of daily supplemental vitamin D in order to achieve increased vitamin D content in their breast milk (Hollis and Wagner 2004, Wall et al. 2015). In fact, in a Finnish study, non-breast-fed children had higher mean intake of vitamin D compared with breast-fed children (17 vs. 10 µg at 3 months, and 16 vs. 10 µg at 6 months) (Räsänen et al. 2006). Infants are dependent on regular vitamin D supplementation. Considering the frequency of vitamin D deficiency in this population, in spite of the supplementation that most of the children probably received, the question of the optimal dose of supplemental vitamin D is debatable.

The prevalence of vitamin D deficiency exceeded 50% in school-aged children, which is lower compared with the prevalence of 71% among healthy Finnish school-aged children in 2006 to 2008 (Pekkinen et al. 2012). This could be a result of individual counseling that patients have received during follow-up visits at the outpatient clinic, in addition to increased awareness of the potential health benefits of vitamin D. It is worth noting that both in the study by Pekkinen and study I, the 25OHD measurements were performed in the

same laboratory and with the same assay. Between 2007 and 2009, the results from HPCL assays resulted in ca. 5% higher concentrations than All-Laboratory Trimmed Mean (ALTM). For comparison: In 2013 the IDS-iSYS method resulted on average in 15% higher concentrations compared with ALTM. It is therefore likely that the aforementioned studies give a reliable estimation of the true prevalence of vitamin D deficiency in this population. Despite the limitations of study I, it demonstrated that in this unselected population of children with an illness requiring follow-up at a tertiary center the prevalence of vitamin D deficiency was considerably high.

Parallel to the reduced use of vitamin D supplements, bone mineral accrual and rapid bone growth occur during adolescence, leading to increased need for vitamin D (Bonjour et al. 1991, Lehtonen-Veromaa et al. 2002, Pekkinen et al. 2012). Hence, vitamin D deficiency in this age group may have long-term adverse effects on bone growth and bone quality. Moreover, during puberty, as a result of hormonal changes, the body composition changes, and fat mass increases in both sexes, though it may be more prominent in girls than in boys (Kang et al. 2015, Medina-Gomez et al. 2015). Such an increase in fat mass could also lead to volumetric dilution of 25OHD concentration (Drincic et al. 2012).

Predisposing and preventing factors of vitamin D deficiency

In order to further examine the predisposing and preventing factors of vitamin D deficiency, we included children with the 50 highest and 50 lowest 25OHD concentrations in a post-hoc analysis. Compared with children in the group of 50 highest 25OHD concentrations, children in the group of 50 lowest concentrations were older (6 vs. 14 years, $p<0.001$), more commonly of non-Finnish ethnicity (4 vs. 36%, $p<0.001$), used less vitamin D supplements (88 vs. 23%, $p<0.001$), and tended to have higher height-adjusted weight (-4 vs. +5%, $p=0.054$).

Identified risk factors of low 25OHD concentration were consistent with the established risk factors for vitamin D deficiency in other populations. As discussed above, the prevalence of vitamin D deficiency increased with age. Gender did not affect the risk for vitamin D deficiency. Instead, ethnicity was an important factor: as much as one third of the 50 patients with the lowest 25OHD were of non-Finnish ethnicity, compared with 4% in the group of high 25OHD. In several studies conducted in the USA, blacks have presented lower 25OHD concentrations than whites (Ginde et al. 2009, Powe et al. 2013b). Pigmentation of the skin reduces cutaneous synthesis of vitamin D (Holick et al. 2007). Two surveys by the Institute of Health and Welfare showed that in addition to the reduced cutaneous vitamin D synthesis of dark-skinned patients, eating patterns (including the use of dairy products) and the adoption of Finnish nutritional recommendations may also differ from those of Finnish ethnicity (Castaneda et al. 2012, Alitolppa-Niitamo et al. 2014). In fact, the use of supplements was less frequent in those with non-Finnish ethnicity and with low 25OHD.

Unfortunately, due to the relatively small sample size and wide variety of diseases, it was not possible to evaluate how single diseases affect the risk: only eating disorders were

exclusively prevalent among those with low 25OHD. In fact, one of the most interesting findings was that despite eating disorders, prevalent in the low 25OHD group, height-adjusted weight tended to be higher in the low 25OHD groups than in the high 25OHD group. This indicates that, also in this population, obesity is a risk factor for vitamin D deficiency.

Seasonal variation in 25OHD

We did not observe seasonal variation in 25OHD concentration in children under 6 years in study I. On the other hand, it was notable in older age groups. The prevalence of vitamin D deficiency in chronically ill children according to season is illustrated in Table 8. The overall prevalence was highest in the winter and spring, as more than 50% of all subjects presented serum 25OHD <50 nmol/l, and among adolescents aged 15 to 18 years the prevalence was as high as 65%. In the summer, the prevalence of vitamin D deficiency was on average 27%, and of adolescents approximately one third still suffered from vitamin D deficiency.

Table 8. *Prevalence of vitamin D deficiency (25-hydroxyvitamin D <50 nmol/l) in study I according to season.*

		Season					
Age group (y)	**N**	**Winter**	**Spring**	**Summer**	**Autumn**	**Total**	**p***
0–2.0	129	19%	23%	14%	21%	19%	0.830
2.1–6.0	185	37%	27%	23%	19%	26%	0.210
6.1–10.0	229	44%	55%	23%	34%	40%	0.006
10.1–15.0	473	62%	63%	36%	49%	54%	<0.001
15.1–18.0	319	65%	65%	28%	57%	56%	<0.001
Total	1,335	52%	52%	27%	42%	45%	<0.001

y, year; *Pearson Chi-Square

Although it was not possible to examine seasonal variation in cord blood 25OHD in studies II and III, such variation has been evident in mothers and neonates in different populations (Bowyer et al. 2009, Godang et al. 2014), as well as in infants and toddlers (Michel et al. 2015). Small children are usually protected against direct sunlight exposure with clothing and sunscreen, both of which inhibit cutaneous vitamin D synthesis (Holick et al. 2007). On the other hand, chronic disease or medication may require protection against sunlight

regardless of the age group, and this should be taken into consideration when treating chronically ill children. Notably, in this study population, sunlight exposure was insufficient to prevent vitamin D deficiency. Thus, dietary intake of vitamin D is essential, and a survey is needed on current vitamin D status after the increment of vitamin D fortification and the revision of guidelines for supplementation.

5.1.3 Obese young adults (IV)

Study IV involved young adults aged 15 to 21 years with severe childhood-onset obesity and normal-weight subjects with a similar age range. The mean 25OHD concentration was lower in obese than in normal-weight subjects (49 vs. 62 nmol/l, p=0.041), and the prevalence of vitamin D deficiency was 56% and 29%, respectively (Table 6). Compared with the difference in total 25OHD concentration between obese and normal-weight subjects, the difference in free 25OHD was even greater (2.8 vs. 4.7 pg/ml, p=0.001). Furthermore, obesity-related parameters (waist circumference, BMI, fat%, and fat-mass index) correlated inversely only with free 25OHD, not with total 25OHD or DBP.

Several studies have indicated that obesity and low 25OHD concentration associate with each other (Saneei et al. 2013). In study IV, more than half of the obese and almost one third of the normal-weight individuals suffered from vitamin D deficiency. This study was conducted during winter and early spring, which probably increased the prevalence of vitamin D deficiency. On the other hand, compared with the extremely high prevalence of vitamin D deficiency in school-aged children in 2006 to 2008 (71%) (Pekkinen et al. 2012), the prevalence in this population was lower.

Is the low 25OHD concentration (total or free) a reason for obesity or a consequence of it? Dilution of 25OHD due to increased fat mass explains, at least partly, the low 25OHD concentrations in obese subjects (Drincic et al. 2012). Instead, the interaction between obesity and DBP is still unconfirmed. Although hyperinsulinemia and insulin resistance showed an inverse correlation with DBP concentrations (Ashraf et al. 2014), body fat mass or obesity has not been consistently associated with DBP: some studies have failed to observe any correlation while others have reported an inverse or positive correlation (Taes et al. 2006, Bolland et al. 2007, Winters et al. 2009, Powe et al. 2011, Karlsson et al. 2014). In study IV, DBP did not associate with obesity-related parameters.

The fact that only free 25OHD concentration, not total 25OHD, correlated with obesity-related parameters at baseline could be due to the relatively small sample size. Anyhow, obesity seems to affect both total and free 25OHD concentrations. Reduced free 25OHD (or bioavailable 25OHD) has been apparent in previous studies among obese individuals (Wortsman et al. 2000, Karlsson et al. 2014). However, in those studies a mathematical formula was used when calculating free 25OHD. The direct method for assessing free 25OHD has been available for a couple of years. It is likely that the calculated free 25OHD overestimates the true free 25OHD concentration (Schwartz et al. 2014, Aloia et al. 2015b).

Study IV was the first one to report differences in directly measured free 25OHD in obese compared with normal-weight individuals.

Obese subjects in study IV had all suffered from severe childhood-onset obesity, and do not represent all overweight or obese individuals. In addition, a specialized pediatric endocrinologist had organized their follow-up and individual counseling during childhood. This unique population is prone to various long-term health problems; impaired bone development being just one of them. Obesity in childhood may impair bone mineral accrual (Mughal and Khadilkar 2011, Mosca et al. 2014), and obesity-related metabolic syndrome may also reduce BMD (Nobrega da Silva et al. 2014). In a similar population as in study IV, obesity affected metabolic activity and the quality of the bone (Viljakainen et al. 2014, Viljakainen et al. 2015).

The free hormone hypothesis

Free 25OHD correlated inversely with PTH ($r=-0.345$, $p=0.025$) but lacked correlation with calcium, phosphate, and osteocalcin. Total 25OHD concentration did not correlate with any of the markers of bone metabolism.

In order to study the free hormone hypothesis, which states that the unbound form of a hormone is responsible for its biological actions, many groups have examined correlations between calculated or directly measured free 25OHD concentration, or bioavailable 25OHD, and parameters of calcium and bone metabolism. The results have not been consistent and the hypothesis is still questionable. Previously, correlations between PTH and free 25OHD have occurred either with or without concomitant correlation with total 25OHD, but the study populations have been heterogeneous in terms of age, ethnicity, way to assess free 25OHD, and state of health (Bhan et al. 2012, Ponda et al. 2014, Lai et al. 2015, Aloia et al. 2015a, Schwartz et al. 2016). Considering the small sample size in study IV, the correlation between free 25OHD and PTH that is only moderate provides weak support for the free hormone hypothesis.

5.2 Response of 25OHD concentration to vitamin D supplementation

In study II, a randomized vitamin D_3 intervention in infants, due to the critical role of vitamin D in the overall postnatal development and growth it would have been unethical to perform a placebo-controlled study. We examined the effect and safety of a daily dose of vitamin D_3 of 30 and 40 µg compared with the currently recommended daily dose of 10 µg. Instead, study IV was a placebo-controlled intervention of 50 µg vitamin D_3, and participants were allowed to continue the habitual use of vitamin D containing supplements during the follow-up.

The average duration of interventions in studies II and IV was 11 and 12 weeks, respectively. The overall adherence was good in both studies, as 82% of infants (II) and 81% of young adults (IV) received more than 80% of the originally planned doses. In study II, 14% of the parents were at most high-school graduates, 26% had bachelor's degree, and 60% had a higher university degree. Unlike in study II, the level of education in the families of young adults was notably different between the groups: parental education was higher in the families with normal-weight subjects than in those with obese subjects (Viljakainen et al. 2015). The overall adherence to the intervention in study IV, however, was equal in obese and normal-weight subjects (95% vs. 99%, p=0.495).

In the general population the adherence to vitamin D supplementation, and hence the dose-response, would likely be lower (Kyttälä et al. 2010). In study II, parents' high level of education may also cause bias. Interestingly, however, pregnant women in Finland used equally dietary supplements regardless of their level of education, while in the USA and in Sweden the use of supplements associated with higher education (Aronsson et al. 2013). Overall awareness of health issues and motivation to participate in the study are likely to be better than on average, and such a selected study population may result in abnormally favorable results.

5.2.1 Infants (II)

Mean cord blood 25OHD concentration was similar in the three intervention groups (Table 9). After the intervention, however, mean 25OHD concentration differed significantly between the groups: the higher the daily vitamin D_3 dose, the higher the mean 25OHD concentration. The overall dose-response was highest with the lowest daily dose: the increase in 25OHD for each 1 µg of vitamin D was 3.6 nmol/l with 10µg daily dose, 2.3 nmol/l with 30 µg, and 2.5 nmol/l with 40 µg (Table 9).

Table 9. *Serum 25-hydroxyvitamin D (25OHD) concentrations in study II at baseline (BL) and at three months (3 mo), including minimum, maximum, and dose-responses at three months, according to intervention groups.*

	Intervention group			
	10 µg	**30 µg**	**40 µg**	**p**[†]
S-25OHD (nmol/l)				
BL mean (SD)	52 (14)	54 (15)	54 (13)	0.772
3 mo mean (SD)	88 (18)	124 (30)	153 (40)	<0.001
minimum	49	57	86	-
maximum	125	198	230	-
mean dose-response (nmol/l*μg^{-1})	3.6	2.3	2.5	<0.001

[†]ANOVA

As baseline 25OHD concentrations were similar in the groups, gender alone served as a covariate in repeated measures ANCOVA. Serum 25OHD increased from baseline to three months in each group ($p<0.001$), and the increase in 25OHD was more pronounced the higher the dose was ($p<0.001$) (Figure 13).

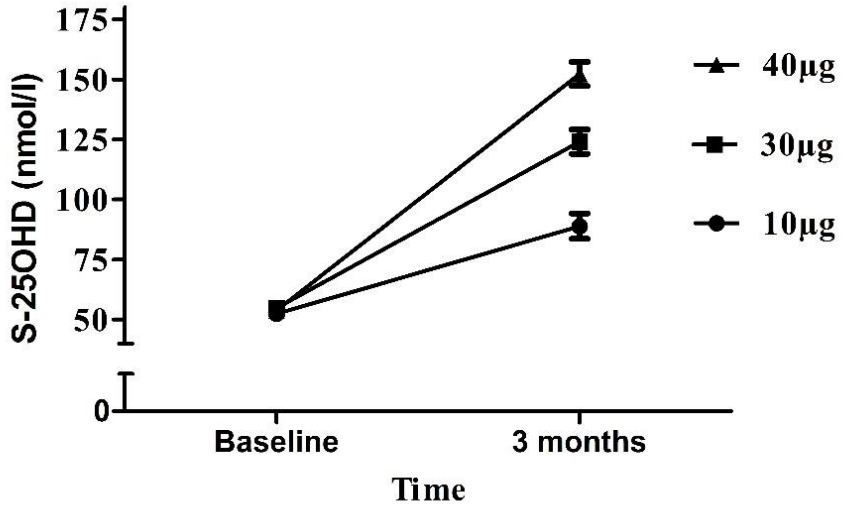

Figure 13 *Serum 25-hydroxyvitamin D (25OHD) in the three intervention groups in study II from baseline to 3 months. Repeated measures ANCOVA using gender as a covariate, p<0.001. Values are estimated marginal means.*

Vitamin D intervention studies in healthy infants are scarce. After study II, only one large intervention has been published (Gallo et al. 2013). This study included 132 healthy infants who received either 10, 20, 30 or 40 µg of vitamin D_3 daily from 1 month to 11 months. The group of 40 µg daily dose was discontinued prematurely because of high 25OHD concentrations (>250 nmol/l) observed at 3 months without hypercalcemia. Notably, the decision to discontinue the 40 µg arm was based on an enzyme immunoassay, and the final results were assessed with liquid chromatography tandem mass spectrometry (LC-MS/MS). At three months with LC-MS/MS mean 25OHD concentration with 40-µg dosing was 180 nmol/l (95% CI 154-207 nmol/l).

In countries with recommendations for vitamin D supplementation, placebo-controlled studies are unethical. In Australia, however, a large placebo-controlled vitamin D intervention is now recruiting (Allen et al. 2015). Their primary outcome includes allergic sensitization and prevalence of infections at 12 months, in addition to prevalence of vitamin D deficiency.

Several factors affect dose-response: baseline 25OHD concentration, dose of vitamin D, the preparation in use (vitamin D_2 vs. vitamin D_3), and the method of administration (Viljakainen et al. 2010a, Tripkovic et al. 2012, Gallo et al. 2013, Tan et al. 2015). In the study by Viljakainen et al. (2010), the highest increment in 25OHD concentration was evident in those with the lowest baseline 25OHD concentration. In a recent meta-analysis, vitamin D_3 proved to be better than vitamin D_2 in increasing 25OHD concentration

(Tripkovic et al. 2012). Vitamin D_3 is most commonly available in Finland, and due to the national recommendations, daily supplementation is widely used compared with more infrequent dosing.

The pre-defined target 25OHD concentration in study II was 80 nmol/l. All of the infants who received 40-µg dosing achieved this target concentration. On the other hand, 10% of those infants showed 25OHD concentration >200 nmol/l which was considered to be unnecessarily high. If the compliance was good, over >80%, 30-µg dosing was also sufficient to achieve 25OHD concentration ≥80 nmol/l. To conclude, in order to achieve 25OHD concentration at or above 80 nmol/l with regular use of supplemental vitamin D_3, a daily dose of 30 µg is sufficient.

Safety issues

Plasma calcium concentration and urine calcium/creatinine (U-Ca/Cr) ratio at three months served as safety measurements. Plasma calcium concentration remained within the reference range 2.22-2.82 mmol/l, and it did not correlate with 25OHD concentration. In study II the cut-off for abnormally high U-Ca/Cr was 2.2 mmol/l, and 39% of all infants exceeded this. However, hypercalciuria occurred in all intervention groups, it did not correlate with 25OHD concentration, and the mean U-Ca/Cr was similar in all groups. The reference range for U-Ca/Cr in this age group is poorly defined, and infants have much higher values than older children or adults (Sargent et al. 1993, Matos et al. 1997). A 24-h urine collection is preferable to a spot urine sample but impossible to perform in a study like this. In the Canadian study in infants with a high dose of vitamin D (up to 40 µg /day) no hypercalcemia was observed (Gallo et al. 2013). Moreover, in older children daily doses of vitamin D_3 up to 100 µg did not increase calcium absorption or cause hypercalcemia (Lewis et al. 2013). In conclusion, vitamin D_3 supplementation in infants with up to 40 µg daily is safe in the short term but 25OHD concentrations may rise to an unnecessarily high level.

5.2.2 Young adults (IV)

In study IV with young adults, mean baseline total and free 25OHD concentrations were lower in the obese than in the normal-weight subjects, without any differences in DBP concentrations (Table 10). The dose-response to 50 µg of vitamin D_3 supplementation also differed between obese and normal-weight subjects, resulting in lower total and free 25OHD concentrations in the obese subjects at the end of the intervention. In repeated measures ANCOVA (both total and free) baseline 25OHD concentration served as a covariate. Figure 14 illustrates the change of total and free 25OHD concentration over time. Total 25OHD concentration increased during the follow-up period ($p<0.001$) and differed between the four intervention groups ($p<0.001$). The increase in the placebo groups was not significant ($p=0.058$). Free 25OHD increased in both obese and normal-weight subjects receiving

vitamin D supplementation (p=0.006). Unlike total 25OHD, the increase in free 25OHD did not differ between obese and normal-weight subjects (p=0.487).

Table 10. *Total and free 25-hydroxyvitamin D (25OHD) concentrations [mean (SD)] at baseline and after 12 weeks' intervention in study IV.*

	Group				
Baseline 25OHD	**Normal weight**		**Obese**		**p**[†]
t25OHD (nmol/l)	62 (24)		49 (15)		0.041
f25OHD (pg/ml)	4.7 (2.0)		2.8 (1.4)		0.001
DBP (µg/ml)	346 (98)		309 (87)		0.212
Final 25OHD	**Placebo**	**50 µg D_3**	**Placebo**	**50 µg D_3**	**p**[†]
t25OHD (nmol/l)	85 (28)	112 (22)	63 (19)	89 (24)	<0.001
f25OHD (pg/ml)	4.5 (1.9)	6.5 (2.0)	2.7 (1.5)	4.9 (2.0)	0.001
DBP (µg/ml)	333 (101)	362 (86)	372 (48)	375 (110)	0.708
Minimum t25OHD (nmol/l)	46	67	38	54	-
Maximum t25OHD (nmol/l)	129	147	97	118	-
t25OHD dose-response (nmol/l*μg^{-1})	0.2	1.2	0.1	0.9	<0.001[‡]

[†]ANOVA, [‡]Kruskal-Wallis test

t, total; f, free; DBP, vitamin D-binding protein

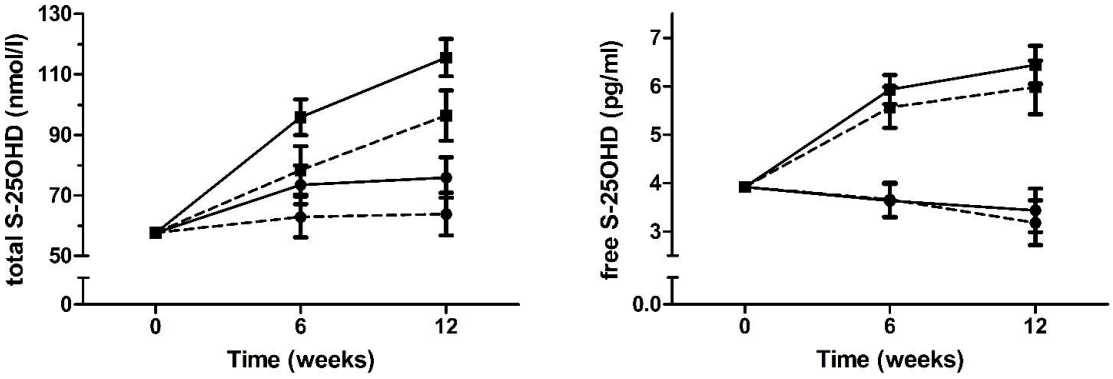

Figure 14 *Study IV. Changes in total (left) and free (right) 25-hydroxyvitamin D (25OHD) concentrations with time and according to intervention group. In repeated measures ANCOVA using baseline 25OHD concentration as a covariate: temporal change in total 25OHD ($p<0.001$) and difference between intervention groups ($p<0.001$), and temporal change in free 25OHD ($p=0.021$) and difference between intervention groups ($p<0.001$). Solid lines represent normal-weight subjects and dashed lines obese subjects, squares represent vitamin D_3 groups and circles placebo groups. Values are estimated marginal means.*

DBP concentrations were similar between obese and normal-weight subjects at baseline and did not differ between intervention groups at the end of the follow-up (Table 10). The change in DBP concentration with time ($p<0.001$) is illustrated in Figure 15.

In study IV the response of both total and free 25OHD concentrations was of interest, as there are no previous studies examining changes in directly measured free 25OHD in obese subjects. When comparing the response of total 25OHD concentration between those obese and normal-weight who received vitamin D_3 supplementation, a notable difference was evident: normal-weight subjects responded better than obese ones ($p=0.027$). In normal-weight individuals the mean increment of 25OHD concentration/1 µg vitamin D_3 was 1.2 nmol/l, and in obese individuals 0.9 nmol/l. Thereby, supplementation in obese subjects resulted on average in 13.8 nmol/l (95% CI: 1.8, 25.8) lower 25OHD concentration than in normal-weight subjects. A trend towards an increase in total 25OHD concentration in placebo groups was evident ($p=0.058$), without a difference between obese and normal-weight subjects ($p=0.074$). Moreover, at the end of the intervention obese subjects merely achieved equal total 25OHD concentrations to normal-weight subjects in the placebo group; 89 vs. 85 nmol/l, respectively. The finding of a lower response to vitamin D_3 supplementation in obese individuals is similar as seen previously in other populations (Dhaliwal et al. 2014).

Correspondingly, at the end of the follow-up free 25OHD concentrations were similar in obese subjects who received vitamin D_3 supplementation and in normal-weight subjects in the placebo group. Unlike total 25OHD concentration that showed a trend towards increase

in placebo groups, free 25OHD concentration remained stable during the follow-up (p=0.924), and no difference was observed between obese and normal-weight subjects in placebo groups (p=0.758). Hence, free 25OHD concentration may be more tightly regulated.

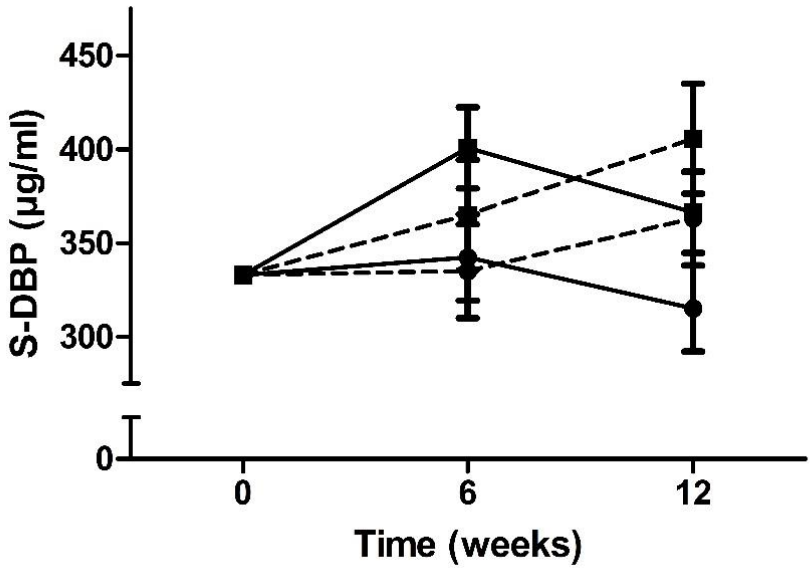

Figure 15 *Study IV. Changes in vitamin D-binding protein (DBP) over time and according to intervention groups. In repeated measures ANCOVA using baseline DBP concentration as a covariate: temporal change p<0.001 without differences between intervention groups (p=0.193). Solid lines represent normal-weight subjects and dashed lines obese subjects, squares represent vitamin D_3 groups and circles placebo groups. Values are estimated marginal means.*

Vitamin D_3 intervention had an impact on DBP concentrations: DBP tended to be at its highest at 6 weeks in normal-weight subjects, and at 12 weeks in the obese (Figure 15). At the end of the intervention, among those who received vitamin D_3, obese subjects had higher DBP concentration than normal-weight ones (p=0.025). Due to the large variation in DBP concentrations the importance of this finding needs to be verified in other studies. Moreover, the differences between monoclonal and polyclonal antibodies in the detection of DBP require further studies (Powe et al. 2014). Only few studies have examined the response of DBP concentration to vitamin D supplementation, and the results are conflicting. Among elderly vitamin D deficient patients (25OHD <50 nmol/l) with hip fracture, vitamin D supplementation with both vitamin D_2 and D_3 resulted in increase in DBP concentration (Glendenning et al. 2013). This patient group is, however, very different from that in study

IV. On the other hand, vitamin D_3 supplementation did not affect DBP concentrations in adults with vitamin D deficiency or in adults with prediabetes (Ponda et al. 2014, Sollid et al. 2016). In addition, DBP phenotype affects 25OHD concentrations (Lauridsen et al. 2005, Pekkinen et al. 2014, Ashraf et al. 2014, Braithwaite et al. 2015) and may also affect the response to vitamin D supplementation (Fu et al. 2009, Didriksen et al. 2013). And as earlier, obesity and DBP concentrations may be associated (Powe et al. 2011, Ashraf et al. 2014, Karlsson et al. 2014).

In conclusion, we observed an increase in both total and free 25OHD concentrations in those who received vitamin D_3. The response varied between total and free 25OHD concentrations: the response of free 25OHD was similar in normal-weight and obese subjects, but the response of total 25OHD was greater in normal-weight than in obese subjects. As this was the first study to examine the response of directly measured free 25OHD to vitamin D_3 supplementation in obese subjects, further studies are needed to verify the finding and to examine the clinical significance. The impact of DBP remains uncertain.

Safety issues

The highest total 25OHD concentration in study IV was 147 nmol/l, i.e., well below the concentrations seen in vitamin D intoxication. A reference range for directly measured free 25OHD concentration does not exist yet. Plasma calcium (P-Ca) and U-Ca/Cr served as safety measurements. No hypercalcemia existed, and neither P-Ca nor U-Ca/Cr correlated with total 25OHD concentrations. P-Ca and U-Ca/Cr did not differ between placebo and vitamin D_3 groups. Thus, daily supplementation with 50 µg vitamin D_3 was safe.

5.3 Mineral metabolism and bone growth

Studies I-IV included data on serum 25OHD concentration and markers of mineral metabolism, namely PTH, calcium and phosphate, enabling the examination of vitamin D impact on mineral metabolism. Table 11 summarizes the results of correlation analyses between 25OHD and PTH, calcium and phosphate. In infants and small children, in addition to young adults, 25OHD and PTH concentrations lacked mutual correlation. In school-aged children a weak inverse correlation between 25OHD and PTH concentrations was evident. Serum 25OHD concentration lacked consistent correlation with either calcium or phosphate.

Although in studies I-IV it was possible to examine correlations between 25OHD concentration and markers of mineral metabolism – phosphate, calcium, and PTH – it should be noted that these studies differed from each other, which must be taken into consideration when comparing the results. In studies II and III correlations are analyzed at three months, at the end of the vitamin D_3 intervention. In study IV, on the other hand, correlations are examined before the intervention, and in study I without any intervention, in a retrospective

fashion. In studies III and IV also other biomarkers were included: FGF23 (III) and OC (IV).

Table 11. *Pearson correlations between 25-hydroxyvitamin D (25OHD) and markers of mineral metabolism in studies I to IV. Significant correlations are in bold.*

		PTH	P-Ca	P-Pi
Study I (total 25OHD)				
0–2 years	r	-0.128†	-0.104†	0.116†
	p	0.279	0.447	0.270
2–6 years	r	-0.118†	0.188	0.002†
	p	0.218	0.097	0.979
6–10 years	r	**-0.347**†	0.099†	**-0.212**
	p	**0.000**	0.312	**0.009**
10–15 years	r	**-0.174**†	0.067	-0.047
	p	**0.003**	0.299	0.396
15–18 years	r	**-0.189**†	-0.066	**-0.176**
	p	**0.015**	0.490	**0.017**
Study II&III (total 25OHD)				
3 months	r	-0.175†	0.160	0.054
	p	0.107	0.102	0.585
Study IV (age 15 to 25 years)				
total 25OHD	r	-0.086	-0.220	0.295
	p	0.590	0.162	0.058
free 25OHD	r	**-0.345**	-0.123	0.129
	p	**0.025**	0.437	0.414

PTH, parathyroid hormone; Ca, calcium; Pi, phosphate
†Spearman's correlation coefficient

5.3.1 FGF23 and bone turnover markers (II and III)

In cord blood, iFGF23 was mainly below the detection level, and cFGF23 considerably high (Table 12). At three months a rise in iFGF23 and a concurrent decrease in cFGF23 was evident. The increase in iFGF23 differed between sexes: in girls the iFGF23 concentration was almost double than in boys (51 vs. 26 pg/ml, p<0.001). Vitamin D_3 intervention did not affect FGF23 concentrations. Correlation between iFGF23 and phosphate was observed only in girls, not in boys (r=0.433, p=0.004 and r=0.069, p=0.327). Instead, boys presented an inverse correlation between cFGF23 and phosphate (r=-0.373, p=0.006).

Bone formation marker procollagen type 1 N-terminal propeptide (P1NP) concentration and bone resorption marker collagen type 1 cross-linked C-telopeptide (CTX) concentration was similar in both sexes, and vitamin D_3 intervention did not affect their concentrations. CTX and iFGF23 correlated inversely, though the correlation was weak (Spearman's r=-0.262, p=0.007). P1NP and iFGF23 did not correlate (r=-0.057, p=0.559), and neither did cFGF23 and CTX or P1NP.

Table 12. *Sex-related differences in mean (SD) concentrations of intact and C-terminal fibroblast growth factor 23 (i/cFGF23) at baseline, at 3 months, and the change over time in study III.*

	Girls	**Boys**	**p†**
Cord blood			
iFGF23 (pg/ml)	3.0 [10.7][a]	3.0 [2.3][a]	0.314
cFGF23 (RU/ml)	536.2 [731.7][a]	605.9 [842.9][a]	0.970
3 months			
iFGF23 (pg/ml)	**51.4 [30.0][a]**	**25.9 [48][a]**	**<0.001**
cFGF23 (RU/ml)	106.9 (64.0)	105.4 (52.1)	0.573
Change from baseline to 3 months			
iFGF23 (pg/ml)	**44.9 [35.3][a]**	**15.7 [46.2][a]**	**0.001**
cFGF23 (RU/ml)	-574.7 (442.4)	-581.2 (430.1)	0.970

†Mann-Whitney U-test; [a]Median [Interquartile Range]

An increase in iFGF23 and concomitant decrease in cFGF23 in previous studies support the hypothesis that regulation of mineral metabolism starts after birth, as a result of

physiological hypocalcemia which leads to secretion of PTH, and further to $1,25(OH)_2D$ activation in the kidneys (Takaiwa et al. 2010, Ohata et al. 2011). Because FGF23 does not cross the placenta (Ma et al. 2014), the high concentration of cFGF23 in cord blood reflects the capability of the fetus to secrete iFGF23, which is mainly cleaved to C- and N-terminal fragments (Kovacs 2014). The physiological role of FGF23 during fetal development is unknown. The regulation of FGF23 metabolism after birth is also inadequately understood.

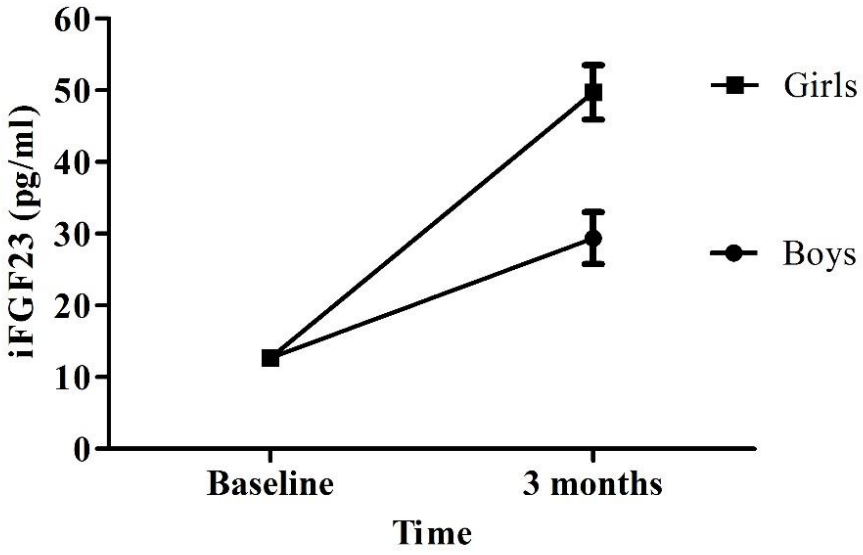

Figure 16 *Increase in intact fibroblast growth factor 23 (iFGF23) in boys and girls separately. In repeated measures ANCOVA using baseline iFGF23 as a covariate, iFGF23 increased with time p<0.001, and differed between boys and girls p<0.001.*

A striking finding was the considerable sex difference in iFGF23 at three months (Figure 16). This was a novel finding, and no other similar findings have been published. On the other hand, studies on sex-related differences in FGF23 are infrequent (Gkentzi et al. 2014). What could explain the sex difference? Minipuberty and high concentration of sex steroids could be one reason. During neonatal minipuberty, at 1 to 3 months after birth, testosterone concentrations may rise up to pubertal levels (Kuiri-Hänninen et al. 2014), and testosterone plays a significant role in bone development (Vanderschueren et al. 2014, Carson and Manolagas 2015). Unlike at birth, bone parameters differ between sexes at 14 months (Viljakainen et al. 2010a). We hypothesized that male bones grow faster and require more phosphate than in girls. Both sexes receive abundant phosphate from milk. As iFGF23 is a phosphaturic hormone, the increased need for phosphate could explain the low concentration of iFGF23 seen in boys (Figure 17).

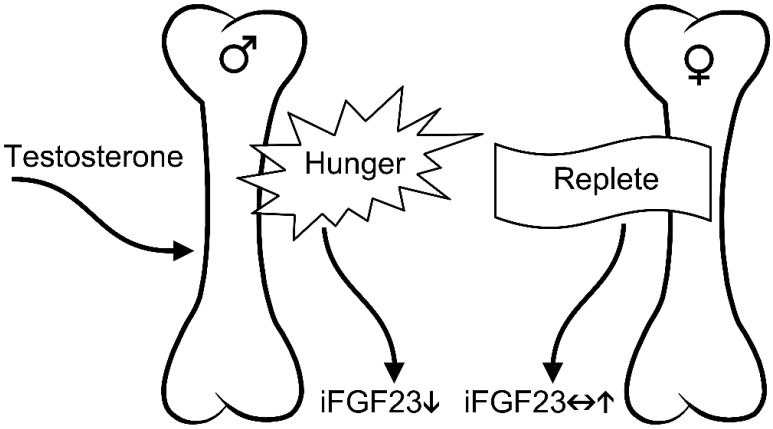

Figure 17 *Hypothesis of sex difference in intact fibroblast growth factor 23 (iFGF23) metabolism.*

One of the limitations in study III was that we used serum samples. iFGF23 is more stable in plasma than in serum, and the use of serum for measurements may result in decreased concentrations of iFGF23 (Smith et al. 2014). In fact, altogether 22 measurements of iFGF23 were below the detection level at baseline and at three months. Due to several low iFGF23 concentrations, we performed sensitivity tests: after omitting iFGF23 measurements that were below the detection level at baseline and at three months, we were able to verify the significant sex difference and change over time in iFGF23. Moreover, the blood samples in both sexes were equally processed.

5.3.2 Osteocalcin (IV)

In young adults OC was slightly lower in obese than in normal-weight subjects (14 vs. 17 ng/ml, p=0.027) at baseline, and differed between intervention groups at the end of the follow-up. The focus was on the correlation between OC and 25OHD concentrations. Correlation between OC and free 25OHD concentration would support the free hormone hypothesis. However, we failed to observe any correlation between OC and either free or total 25OHD. In this population, OC and its connection to energy metabolism has been published previously (Viljakainen et al. 2014).

5.3.3 Peripheral quantitative computed tomography (pQCT) (II)

In order to examine BMD and other bone parameters in infants in study II, we included 75% of pQCT measurements and omitted 25% due to poor quality. BMD was similar in the three intervention groups (vitamin D_3 10 µg, 30 µg or 40 µg/day), but a trend towards greater total and trabecular bone area (p=0.069), cortical area (p=0.053), and higher stress and strain index (p=0.070) was evident with the 40 µg daily dose.

pQCT measurements at three months were challenging due to lack of the infants' co-operation as motion affects the quality of the pQCT image. We evaluated the quality of the measurement visually. Recently a quantitative motion assessment methodology was introduced in order to manage the motion artifacts (Blew et al. 2014). Moreover, this is a small study with limited sample size and short follow-up period. Changes in bone microarchitecture, gross structure, and growth require time, and individual variation may be great. Maternal 25OHD concentration may affect newborn bone parameters, and supplementation with vitamin D has an impact on bone health already in infancy (Viljakainen et al. 2010a, Viljakainen et al. 2010c). The dose-dependent effect of vitamin D supplementation on bone parameters needs to be confirmed in a longer intervention with a larger cohort.

5.4 Future considerations

Along with the increasing data on the multiple physiological roles of vitamin D, awareness of its health benefits has increased, and the use of vitamin D supplements has become popular in Finland. In addition, detailed age-specific national recommendations for nutrient intakes and the increased vitamin D fortification of foods introduced in 2010 have likely increased the overall intake of vitamin D in all age groups. In fact, the FINDIET survey in 2012 reported increased vitamin D intake and increased prevalence of supplement use among adults >25 years (Helldan et al. 2012). However, data on vitamin D intake, the prevalence of supplement use, and most importantly, serum 25OHD concentrations in adults less than 25 years of age, adolescents and younger children are lacking. It would be important to study the impact of current recommendations in all these populations. Moreover, the prevalence of vitamin D deficiency was considerably high in children with a chronic disease. It would be of interest to examine if the increase in mean 25OHD concentrations seen in 2010 has continued and resulted in improved 25OHD concentrations in pediatric patients. Serum 25OHD measurements are commonly based on decisions by individual clinicians, not performed as part of treatment or follow-up protocol. For example, Current Practice Guidelines (Käypä hoito, Duodecim) commonly include vitamin D recommendations of the National Nutrition Council, but detailed recommendations for vitamin D supplementation or the follow-up scheme of serum 25OHD concentration are often lacking.

The currently recommended vitamin D supplementation is sufficient to prevent rickets and osteoporosis in otherwise healthy Finnish children. It has previously been discussed that in order to achieve e.g. immunological benefits of vitamin D supplementation, the given dose and the target concentration of 25OHD may need to be higher than what is needed for optimal bone health. In order to study the long-term effects and safety of higher than currently recommended vitamin D_3 supplementation, a prospective vitamin D intervention in healthy infants (VIDI) was started in 2013. The comparison dose (30 µg/day) was defined based on the findings of study II. Primary outcomes of the VIDI study include vitamin D_3 effects on bone and infections. Altogether nearly 1,000 healthy infants are included in the

study, and the 2-year follow-up ended in May 2016. In this cohort it is possible to examine the dose-response of vitamin D_3 supplementation on pQCT parameters in order to verify the findings of study II. Moreover, sex differences in iFGF23 concentrations needs to be confirmed in a larger cohort, although the age group will differ from that in study III.

In rodent models $1,25(OH)_2D$ does not cross the placenta, whereas 25OHD crosses it readily (Kovacs 2014). The transport mechanism is unknown: it is not known whether it is DBP-bound 25OHD or free 25OHD that crosses the placenta. Thus, it would be interesting to examine the impact of maternal free 25OHD concentration on cord blood 25OHD concentration. Although the role of vitamin D during fetal bone development may be minor, other functions of fetal vitamin D are still poorly studied. Additionally, the attempt to examine the free hormone hypothesis in study IV with a relatively small sample size requires further confirmation in a larger and more homogenous population. Considering free 25OHD concentration, the understanding of DBP polymorphism is essential and needs to be taken into consideration.

6 Summary and conclusions

This doctoral thesis provides data on the prevalence of vitamin D deficiency in specific risk groups: infants, children with a chronic disease, and adolescents and young adults with obesity. Exploring specific risk factors for vitamin D deficiency in chronically ill children revealed seasonal- and age-related variation in 25OHD. Further, vitamin D interventions examined the effect and safety of higher than currently recommended vitamin D_3 doses in both infants and obese individuals, and allowed us to deepen our understanding of the factors influencing mineral metabolism.

The main results and conclusions can be summarized as follows:

I The prevalence of vitamin D deficiency, defined as serum 25OHD <50 nmol/l, in all cohorts exceeded 40%, the prevalence being highest among adolescents (10 to 18 years) and obese individuals.

II In chronically ill children higher age, winter and spring seasons, non-Finnish ethnicity, and earlier sampling (between 2007 and 2009) were associated with inferior vitamin D status, defined by serum 25OHD concentration.

III Vitamin D supplementation in infants from 2 weeks to 3 months with 30 or 40 µg vitamin D_3 daily was effective in raising serum 25OHD >80 nmol/l, without hypercalcemia. Long-term safety and benefits warrant further studies.

IV In young adults with severe childhood-onset obesity, daily supplementation with 50 µg vitamin D_3 resulted in similar serum 25OHD concentration as in normal-weight individuals receiving placebo. Obesity associates with lower total and free 25OHD concentrations and increases the requirements for supplemental vitamin D.

V Vitamin D intervention did not affect serum FGF23 concentrations. Sex difference in serum iFGF23 concentration may reflect differences in skeletal growth and mineral metabolism in boys and girls during infancy.

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