Boning up on Vitamin D

Observational Studies on Bone and Health

GRETA SNELLMAN
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Abstract

The primary function of vitamin D in humans is to maintain sufficient circulating calcium concentrations. Low vitamin D levels could result in excessive calcium resorption from bone. Vitamin deficiency may therefore decrease bone mineral density (BMD), resulting in an increased risk of fracture. This thesis sought to determine the association between vitamin D intake and bone health and to estimate circulating levels of vitamin D optimal for bone health without increasing the risk for non-bone disease. Furthermore, the thesis assessed the difference in performance between common serum vitamin D assays and the genetic influence of vitamin D status.

In prospective population-based cohorts, blood concentrations <40 nmol/L (lowest 5%) increased the risk of fracture in elderly men. Low levels were further associated with a slight decrease in lumbar spine BMD. Both high (≥98 nmol/L) and low (<46 nmol/L) vitamin D levels were associated with higher cancer and overall mortality. In another cohort, also of older men and women, no association was found between vitamin D levels and fracture. Low vitamin D levels were weakly associated with decreased total body BMD in men but not in women.

Dietary intake of vitamin D over a 20-year period in more than 60,000 Swedish women was not associated with osteoporosis or fracture, regardless of calcium intake. During summer, dietary vitamin D intake and other life style habits are of minor importance for the variation in vitamin D levels relative to sun exposure and genes. In summer time, genes explain about half of the variation in vitamin D levels, but none of the variance in winter time. The variability between vitamin D assays was substantial. Three assays classified 8, 22 and 43% of the same study population as vitamin D insufficient if <50 nmol/L was set as the insufficiency level.

Based on the results in this thesis, low 25(OH)D levels and low dietary vitamin D intake are not a major cause of fractures in community-dwelling elderly Swedish women and men. Differences in assay performance and potential negative health outcomes of high 25(OH)D levels need to be considered.

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To my family
This thesis is based on the following studies, which are referred to in the text by their Roman numerals.


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<th>Description</th>
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<tr>
<td>ALTM</td>
<td>All-laboratory trimmed Mean</td>
</tr>
<tr>
<td>APCI</td>
<td>Atmospheric pressure chemical ionization</td>
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<tr>
<td>BMD</td>
<td>Bone mineral density</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
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<tr>
<td>CLIA</td>
<td>Chemiluminescent immunoassays</td>
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<tr>
<td>CV</td>
<td>Coefficient of variation</td>
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<tr>
<td>DBP</td>
<td>Vitamin D binding protein</td>
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<tr>
<td>DEQAS</td>
<td>Vitamin D external quality assessment scheme</td>
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<tr>
<td>DXA</td>
<td>Dual-energy X-ray absorptiometry</td>
</tr>
<tr>
<td>DZ</td>
<td>Dizygotic</td>
</tr>
<tr>
<td>FFQ</td>
<td>Food Frequency Questionnaire</td>
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<tr>
<td>HPLC</td>
<td>High-pressure liquid chromatography</td>
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<tr>
<td>HR</td>
<td>Hazard ratio</td>
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<tr>
<td>ICC</td>
<td>Intraclass correlation coefficient</td>
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<tr>
<td>IOM</td>
<td>Institute of medicine</td>
</tr>
<tr>
<td>IU</td>
<td>International units</td>
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<tr>
<td>LC</td>
<td>Liquid chromatography</td>
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<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<tr>
<td>MZ</td>
<td>Monozygotic</td>
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<tr>
<td>OR</td>
<td>Odds ratio</td>
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<tr>
<td>PIN</td>
<td>Personal identification number</td>
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<tr>
<td>PIVUS</td>
<td>Prospective Investigation of the Vasculature in Uppsala Seniors</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized controlled trial</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
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<tr>
<td>SMC</td>
<td>Swedish Mammography Cohort</td>
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<tr>
<td>SMCC</td>
<td>Swedish Mammography Cohort Clinical</td>
</tr>
<tr>
<td>ULSAM</td>
<td>Uppsala Longitudinal Study of Adult Men</td>
</tr>
<tr>
<td>UVB</td>
<td>Ultra violet B</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
<tr>
<td>25(OH)D</td>
<td>25-hydroxyvitamin D</td>
</tr>
<tr>
<td>1,25(OH)₂D</td>
<td>1,25-dihydrovitamin D</td>
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In the 17th century, the first clinical description of a vitamin D-related disease was presented. Curved legs had become increasingly common in England, where as much as 90% of children working in mines were affected. Because of the relatively high prevalence in England, this illness was called Morbus Anglorum. Today, the disease is known as rickets.  

Vitamin D is actually a steroid hormone but through a historical accident it became classified as a vitamin. In the 1920s, it was discovered that dogs raised exclusively indoors developed a bone disease similar to rickets. It was thought that the disease was caused by a deficiency of a trace component present in the diet, probably a fat-soluble vitamin. Because it was the forth vitamin to be discovered, it was called vitamin D. The chemical structure was determined in the 1930s, and in the early 20th century the therapeutic use of cod liver oil, containing vitamin D, led to a dramatic decrease in the incidence of rickets.  

Vitamin D is needed for optimal calcium and phosphorus absorption. Because calcium ions and phosphorus ions combine to form hydroxyapatite crystals that mineralizes bone matrix, they are primarily associated with bone tissue: severe deficiency can cause rickets in children and osteomalacia in adults. In recent years, vitamin D has also been suggested to play a role in a variety of other medical conditions, such as cancers, cardiovascular, diabetes, neurological and psychiatric disorders and autoimmune diseases.  

Osteoporosis and subsequent osteoporotic fractures are common. Every second middle-aged Swedish woman will eventually suffer from a fracture. These fractures contribute to substantial suffering for the patient, as well as a severe economic burden for society. The cause of osteoporosis is multifaceted but vitamin D deficiency is considered one possible risk factor, although the importance of vitamin D for bone health, especially in a general population, is not well understood. Even so, supplementation with vitamin D and calcium is a well established treatment for osteoporosis and prevention of osteoporotic fractures. The main aim of this thesis is therefore to clarify the role and importance of vitamin D for health, and for bone health in particular, in the general adult population.
Vitamin D metabolism

Vitamin D can be obtained from diet, supplements or from endogenous production in the skin. The skin consists of two primary layers: the inner layer (called the dermis), composed largely of connective tissue, and the outer, thinner epidermis. The epidermis is composed of five strata. Vitamin D is produced in the two innermost strata, the stratum basale and stratum spinosum. In these strata, a derivative of cholesterol, 7-dehydrocholesterol, is photolyzed by ultra violet B (UVB) light to previtamin D at wavelengths between 290 and 320 nm. The solar elevation needed to produce UVB at these wavelengths rarely occurs at the artic poles, only during the warm season in temperate regions but all year round at the equator. The dermal production is very effective and even a few minutes in the sun will increase circulating vitamin D concentrations.\(^8,9\) Tanning beds mainly produce UVA, but typically 4-10% of the UV light is UVB. 7-dehydrocholesterol is produced in adequate amounts in humans and is not a reason for vitamin D deficiency. Vitamin D intoxication does not occur from intensive sun exposure because excessive pre-vitamin D is instantly degraded.\(^2\)

Pre-vitamin D is thermodynamically unstable, and because of the heat produced by the radiation, immediately transformed into vitamin D. Vitamin D is translocated from the skin to the blood where it binds to a vitamin D-binding protein (DBP). Besides the dermal production, vitamin D can be
obtained from natural food sources and supplements that are absorbed from the intestine into the blood stream where it binds to DBP. Vitamin D exists in two forms, namely ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃). Ergocalciferol is obtained from plants, in particular mushrooms, whereas all other dietary sources as well as the cutaneous production of vitamin D are cholecalciferol.

Figure 2. Schematic diagram of the structure of 7-dehydrocholesterol (left), and previtamin D (right)

Vitamin D is hydroxylated to 25-hydroxyvitamin D (25(OH)D) in the liver. 25(OH)D has a direct negative feedback on the hydroxylation, but this feedback is not tightly regulated and therefore an increased intake or extensive cutaneous production will increase the level of 25(OH)D in the blood. Excess production can be stored in body fat and mobilized when needed. Because 25(OH)D represents the amount of vitamin D available for our body to use, it is the analyzed indicator of vitamin D status. 25(OH)D is regarded as mainly biological inert and is further hydroxylated to 1,25-dihydroxyvitamin D (1,25(OH)₂D) by 1α–hydroxylase. This process mainly takes place in the kidney but many other tissues (such as the colon, breast, prostate, macrophages, osteoblasts and keratinocytes) express 1α–hydroxylase. Vitamin D has a half-life of approximately 2 months, 25(OH)D a half-life of 15 days and 1,25(OH)₂D a half-life of only a couple of hours.

1,25(OH)₂D is the active metabolite. It enters the cells and acts as a ligand for the vitamin D receptor (VDR). The VDR is a classic transcription factor that exerts its effects via the formation of a complex with the retinoid X receptor (RXR). The VDR-RXR heterodimer interacts with DNA response elements on target genes. The VDRs are found in many different tissues in our body but the classic target organs are bone, intestine and kidney, stimulating calcium transport from these organs to the blood. 1,25(OH)₂D also down-regulates inflammatory markers and has an antiproliferative effect. Extrarenal synthesis of 1,25(OH)₂D, important for the paracrine regulation of cell differentiation and function, occurs under the influence of cytokines. This may explain why vitamin D deficiency can play a role in the patho-
genesis of cancers and autoimmune diseases such as multiple sclerosis and diabetes type 1. 10

1,25(OH)2D is essential for optimal calcium absorption in the gut. When 1,25(OH)2D binds to the VDR in the intestinal cells, calcium channels are stimulated to absorption calcium. A high oral intake or high-dose supplementation with calcium can keep serum calcium at normal levels, even in a vitamin D deficient individual because there is a passive diffusion of calcium from the gut to the capillaries. This diffusion does not have a maximum but depends on the calcium intake. 10 VDR knockout mice are normal at birth but develop abnormalities of mineral ion homeostasis because of impaired intestinal calcium absorption. 14 As the mice grow, the skeletal manifestations are the same in VDR knockout mice as in humans with vitamin D deficiency, i.e. expanded and hypomineralized growth plates. If mineral homeostasis, however, is kept normal by parenteral calcium supplementation, the mice will develop histologically and biomechanically normal bone. The same effect is seen in children with rickets caused by VDR mutation. 15

Figure 3. Schematic diagram of vitamin D metabolism (Holick 2004 2)

Low blood calcium levels stimulates the secretion of parathyroid hormone (PTH). PTH stimulates the hydroxylation of 25(OH)D to 1,25(OH)2D, the tubular resorption of calcium in the kidney and activates osteoclasts to resorb calcium from calcified bone tissue. Both calcium and 1,25-dihydroxyvitamin D have a negative feedback on PTH excretion. In bone, severe long-term
vitamin D deficiency can cause osteomalacia in adults and rickets in children when the osteoid (new bone) is not mineralized. At this stage, the bone becomes soft and painful.

Besides the role of calcium as building material for bone mineralization, normal calcium levels are required for the functioning of the neuromuscular junction, nerve transmission, vasodilatation and hormonal secretion.\textsuperscript{16}

In addition to insufficient dietary intake and impaired cutaneous production of vitamin D, malabsorption and many other diseases can alter the process of mineralization of cartilage and bone. Several of these result from a relative or absolute insufficient supply of vitamin D or its metabolites. Impaired hydroxylation can be caused by renal failure, liver diseases and hypoparathyroidism. Phenytoin, barbiturates, rifampin, isoniazid and ketoconazole are medications that may effect vitamin D metabolism.\textsuperscript{17}

Besides vitamin D deficiency, rickets can be caused by resistance to vitamin D and insufficient intake or increased renal wasting of mineral ions. Pseudovitamin D-deficiency rickets is an autosomal recessive disorder leading to impaired or absent activity of 1α–hydroxylase and thereby impaired hydroxylation of 25(OH)D to its active metabolite.\textsuperscript{18} This condition is treated with supplementation of 1α–hydroxylase, although bone formation in these patients is often affected even with treatment. Hereditary vitamin D resistant rickets is caused by a mutation in the vitamin D receptor. 1,25(OH)\textsubscript{2}D levels are high but have no effect.\textsuperscript{19} X-linked hypophosphatemia is an X-bound genetic disorder, resulting in an increase in fibroblast growth factor (FGF)-23 levels, a phosphaturic hormone, causing decreased phosphate levels owing to decreased reabsorption in the kidneys.\textsuperscript{20}

Sources of Vitamin D

UVB radiation

UVB is the primary source of vitamin D in humans.\textsuperscript{21-23} The absolute percentage of circulating 25(OH)D that arises from oral intake versus cutaneous synthesis is hard to specify because it is not possible to separate 25(OH)D derived from food sources, supplements and skin production. The amount of UVB radiation differs widely between different latitudes and seasons. In Sweden and other northern located countries, UVB radiation is low during winter and the production of vitamin D is non-existent.\textsuperscript{24,25} Therefore, 25(OH)D levels are generally lower in winter.\textsuperscript{9} In southern countries, there is enough UVB radiation for satisfactory dermal production all year round.
Among women in the Swedish Mammography Cohort (SMC), sun-seeking behavior in summer increased 25(OH)D levels by 28% and in winter time a 1-week sun vacation contributed to 16% increase. 27,28 The skin capacity to produce vitamin D decreases with age. 29 When exposed to the same amount of UVB radiation, persons over 70 years of age will produce less than 30% of the amount of vitamin D compared with a young adult. 2 Heavily pigmented skin also decreases vitamin D synthesis because melanin competes with 7-dehydrocholesterol for UVB photons. 30 Sunscreen efficiently absorbs UVB radiation and thereby decreases the dermal production of vitamin D. A sun protection factor of 8 might reduce the production by 95% when applied in sufficient amounts. 31 Because sunscreen is seldom applied at the recommended concentration (i.e. 2 mg/cm²) but more likely about 0.5 mg/cm², even frequent usage will probably not result in deficiency. 32

Supplements

Supplemental vitamin D is usually measured in international units (IU). Dietary intake and food content is normally measured in µg. 1 µg corresponds to 40 IU. In Sweden, supplements are most often subscribed as a combination of vitamin D and calcium if the indication is osteoporosis. The dose is usually 800 IU of vitamin D and 1 g of calcium daily. Vitamin D is also included in most over-the-counter multivitamins. Children in Sweden are given vitamin D substitution, 400 IU, from birth until the age of 2 years. Supplements are widely used in Sweden and vitamin D supplement use seemed to contribute to a 17% increase in 25(OH)D during winter. 28 In many other western countries, supplements are even more common. 33
Serum levels of 25(OH)D increase with approximately 1-2 nmol/L for every 100 IU of vitamin D given. The dose-response relationship differ depending upon the persons baseline serum 25(OH)D level, and the duration of the supplementation. A baseline level <40 nmol/L will result in a steeper increase than if baseline levels are >40 nmol/L. Further, the increase is steeper the first 3 month of supplementation.  

Both vitamin D2 and D3 are used for supplementation. In Europe, D3 is exclusively used while D2 is the most common available vitamin D supplement in the USA. There are studies suggesting that vitamin D3 is more effective than D2 in maintaining vitamin D status and should be used as supplementation, although these results have been questioned.

Diet

Vitamin D is rare in foods. Natural sources of vitamin D3 are fatty fish, such as salmon, herring (~ 400 IU/100 g) and fish liver oils. Small amount is also found in eggs and certain meats. In Sweden, low fat dairy products and margarine are the main sources of fortified foods. One liter of low-fat milk is supplemented with 150 IU and 100 g of margarine contains 400 IU. Dietary intake is important for those who seldom spend time outdoors but is of less importance for others. Two to three servings of fatty fish per week increased serum vitamin D concentrations by 26 nmol/L among women who did not use supplement and did not go on sun vacations but only 10 nmol/L if attending a sun holiday during winter. In summer time, diet was not correlated with 25(OH)D concentrations.

Genetic influence

Only a modest proportion of the variance in 25(OH)D levels has been explained by dietary intake and sun exposure. Despite high latitude resulting in insufficient UVB radiation during winter time, Scandinavian inhabitants have higher 25(OH)D levels than inhabitants in Southern Europe. Differences in sun-seeking habits, clothing and fortified dairy products have been suggested as explanations for this difference.
Whether a genetic influence exists that could explain the variance in 25(OH)D status that cannot be explained by diet, supplements and sun exposure is less well studied. Few studies on genetic influence on 25(OH)D status are available. 41-44 Hunter et al. found a 43% (95% confidence interval (CI) 28-57%) unique genetic variance on 25(OH)D levels in a twin study of 1068 adult twin pairs, independent of season. In a family study, Shea et al. reported a heritability estimate of 29%, although a family study cannot separate familial influences in common from individual genetic influences.

Recommended intake of Vitamin D

The current recommended adequate intake for adults in Sweden is 7.5 µg (300 IU) per day and 10 µg (400 IU) for the elderly. 45 In 2011, the Institute of Medicine (IOM) in the USA published the report Dietary Reference Intakes for Calcium and Vitamin D. 46 The purpose of this report was to assess current data and to update the daily reference intakes for vitamin D and calcium. All health aspects of vitamin D in all life stages were taken into consideration. In this report, the estimated average requirements (EAR) for adults and elderly was 10 µg (400 IU) per day. The recommended dietary allowance (RDA) was 15 µg (600 IU) per day for adults and 20 µg (800 IU) per day for elderly. The EAR is defined as the daily nutrient intake level that is estimated to meet the requirement of 50% of the healthy individuals in a defined group. The RDA is defined as the average daily dietary intake level
that is sufficient to meet the nutrient requirements of 97.5% of the healthy individuals in a defined group. The report emphasized that these recommendations are based on the assumption that sun exposure is very low.

**Measurement and reference levels of vitamin D**

25(OH)D is the analyzed indicator of vitamin D status because it reflects the amount of vitamin D in our body that is available to be converted to the active metabolite 1,25(OH)2D. 25(OH)D can be measured in nanomol per liter (nmol/L) or nanogram per milliliter (ng/ml). To convert nmol/l to ng/, nmol/L should be divided by 2.495.

Several assays for 25(OH)D exist, including high-pressure liquid chromatography (HPLC) and mass spectrometry (MS), 47 radioimmunoassay (RIA), 48 enzyme immunoassays (EIA), 49 competitive protein binding assays (CPBA), 50 automated chemiluminescence protein-binding assays (CLPBA) 51 and chemiluminescent immunoassays (CLIA). 52 There is evidence that diversity between assays exists, 53-58 but these studies have been limited by few participants, a non-population-based setting or with selective and only partially overlapping analyses of the samples included. This diversity is caused by both inter-assay and laboratory disagreement and can contribute to uncertainty when comparing results from studies investigating the prevalence or clinical consequence of vitamin D insufficiency. Therefore, the need for assay-specific reference levels or the use of a gold standard method have been suggested. 46 Currently, HPLC is considered the gold standard. 47,59

True vitamin D deficiency is generally considered to exist at a 25(OH)D level below 25 nmol/L as it is associated with rickets and osteomalacia. 60 The limit for the more common vitamin D “insufficiency” is under intense debate, with recommendations ranging from 40 nmol/L to 100 nmol/L. 61-65 It has also been suggested that the level for deficiency should be at the point where PTH starts to rise, 66 but such a threshold has not been defined because studies are inconsistent. 67-71

Several studies have investigated the prevalence of insufficiency and deficiency in diverse populations. 25,40,72,73 These studies have shown huge variations between regions and populations, although the results are hampered by the previously discussed problem with heterogeneity between assays. Defined risk groups, independent of nationality, are people with highly pigmented skin, 30,74 obese, 75 veiled and elderly. 29

The IOM report concluded that, regarding bone health, there is no scientific evidence for aiming at higher levels than 50 nmol/L. This recommendation is with reference to the general population. 76
Vitamin D and bone

Vitamin D intake and BMD

Observational studies on large adult populations regarding dietary vitamin D intake and the association with bone mineral density (BMD) are rare. The only large-scale prospective study, by Nieves et al, estimated in 76,507 white postmenopausal women the relation between vitamin D intake and BMD. The incidence of osteoporosis was significantly lower in women with a daily intake of >15 µg (600 IU) compared with women with an intake of <5 µg (200 IU). Potential problems with this study are that different methods were used for the measurement of BMD and dietary intake was partly based on questions regarding intake during childhood and adolescence. Prospective cohort studies have assessed the association between intake of dairy products and BMD without specifically analyzing the intake of vitamin D. The majority of these studies failed to indicate a protective effect of dairy products on bone mass.

Most randomized controlled trials (RCTs) using vitamin D supplements include calcium, and therefore the independent effect of vitamin D is hard to determine. Results from RCTs using vitamin D alone are inconsistent. In 247 postmenopausal American women, supplementation with 700 IU vitamin D alone increased 25(OH)D levels and decreased the rate of bone loss but 25(OH)D levels per se did not correlate to BMD at any site. In Pakistani immigrants from Denmark, vitamin D supplement without calcium did not increase BMD although almost all participants had 25(OH)D levels <25nmol/L at baseline. Some RCTs report a preventive effect on bone loss by dietary calcium intake and in the observational study SMC a calcium intake of <750 mg increased the risk for osteoporosis.

The IOM report concluded that there is good evidence that vitamin D plus calcium supplementation results in small increases in BMD of the spine, total body, femoral neck and total hip. They further concluded that it is unlikely that vitamin D alone has a significant effect on BMD.

Vitamin D intake and fractures

Whether dietary vitamin D intake is associated with risk for fracture is not clear. Only three large-scale prospective observational studies have assessed the association between estimated dietary intake of vitamin D and fracture. All studies included women only and reported the risk for hip fractures. In The Nurses Health Study, the authors included over 70,000 postmenopausal women in a non-fixed cohort. The women were followed for 18 years, resulting in a study of 860,000 person years. Using repeated food frequency questionnaires (FFQ), vitamin D and calcium intake from natural food sources and supplements were estimated. The risk for hip fracture was
significantly lower in women with a high vitamin D intake. Interestingly, neither milk nor fatty fish alone, the main sources of dietary vitamin D, was associated with reduced fracture risk. The other two studies did not report any association between vitamin D and risk for fracture. These two studies depended on a single FFQ. The study by Michaëlsson et al. is based on the first FFQ in the SMC, the cohort studied in paper V. The study by Nieves et al. had a shorter follow-up time than the Nurses’ Health Study (thereby also relatively few fractures).

Table 1. Prospective cohort studies with vitamin D intake as exposure and fracture as outcome

<table>
<thead>
<tr>
<th>First author</th>
<th>Publication year</th>
<th>Number of participants (hip fractures)</th>
<th>Positive association (yes/no)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feskanich 86</td>
<td>2003</td>
<td>72 000 (603)</td>
<td>Yes</td>
</tr>
<tr>
<td>Nieves 77</td>
<td>2008</td>
<td>76 000 (337)</td>
<td>No</td>
</tr>
<tr>
<td>Michaëlsson 87</td>
<td>2003</td>
<td>61 000 (1535)</td>
<td>No</td>
</tr>
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</table>

Vitamin D in combination with calcium is a well-established first treatment of osteoporosis. Most RCTs assessing fracture prevention with vitamin D have used vitamin D in combination with calcium, but results from these studies have been inconsistent. The seminal Decalyos I study by Chapuy on institutionalized elderly women demonstrated a significantly decreased risk of both hip and non-vertebral fractures in the women randomized to 1,200 mg calcium and 800 IU vitamin D daily for 1 year. Ten years later, the Decalyos II study confirmed the results of Decalyos I. Two RCTs reported a fracture preventive effect using vitamin D only and three others did not. In a study of more than 2,000 community-dwelling women aged 70 years, an annual oral dose of 500,000 IU vitamin D increased the risk of falls by 15% and the risk of fracture by 26%. The finding that vitamin D supplements could increase the risk of fracture is reported in another study using 300,000 IU as an annual injection. Most studies used a vitamin D supplement dose within the range 400-800 IU per day, although some studies have given high doses less often. The DIPART group analysed the effect from seven major randomized trails, and found that calcium supplements reduced the risk of fracture but vitamin D per se did not.

The overall conclusion drawn in meta-analyses, Cochrane reviews and the IOM report with regards to supplementation with vitamin D and calcium is that a combination of vitamin D and calcium reduces the risk for non-vertebral fractures in old frail women living in nursing homes and may prevent fractures in community-dwelling elderly as well, but vitamin D alone is unlikely to have a fracture preventive effect.
Figure 6. Cumulative incidence for any fracture (left) and hip fracture (right) for vitamin D treated (darker lines) and controls (lighter lines). Vitamin D and vitamin D in combination with calcium are presented separately. Insert shows treatment effect and 95% CI (The DIPART Group 2010\textsuperscript{101}).

Blood 25(OH)D levels and BMD

Many observational studies have reported a positive association between 25(OH)D levels and BMD, especially in hip BMD.\textsuperscript{67,106-118} The positive association in observational studies is most commonly seen when comparing the participants with the very highest levels to the participants with the very lowest 25(OH)D levels. Concentrations below 30 to 80 nmol/L are associated with decreased BMD in these studies. One study that did not find a positive association (the OFELY study) looked at BMD only at the distal radius, and not at the clinically more important hip region.\textsuperscript{117} Ensrud et al found that a 25(OH)D level <50 nmol/L was associated with greater rate of hip bone loss, but there was no additional benefit from levels above 50 nmol/L. In a Swedish study of 1,044 independently living elderly women, no association was detected between 25(OH)D levels and BMD measured with dual-energy x-ray absorptiometry (DXA). Only 4% of these women had levels below 50 nmol/L, which could explain the negative result. Despite this finding, the women with low 25(OH)D levels had an increased risk of fracture. The authors suggested that inferior physical activity and postural stability, rather than a decreased BMD, could explain the increased risk of fracture.\textsuperscript{118}

Six RCTs using vitamin D supplements also analyzed the association between 25(OH)D levels and BMD.\textsuperscript{81,82,84,119-121} All RCTs included postmenopausal women only. The only study that found an association between 25(OH)D levels used vitamin D without calcium supplementation. In this study, the positive association between circulating 25(OH)D levels after vitamin D supplementation on hip BMD was independent of baseline 25(OH)D, which could be explained by the fact that a majority (70%) had levels below 30 nmol/L.
The IOM report concluded that, based on the results from observational studies, there is fair evidence for an association between 25(OH)D and BMD at the femoral neck and a possible weak association with BMD at the lumbar spine but not for other sites. 

Table 2. Prospective cohort studies with 25(OH)D levels as exposure and BMD as outcome

<table>
<thead>
<tr>
<th>First author</th>
<th>25(OH)D Assay</th>
<th>Publication year</th>
<th>Number of participants</th>
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<td>Rosen 113</td>
<td>-</td>
<td>1994</td>
<td>18</td>
<td>Yes</td>
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<tr>
<td>Ooms 67</td>
<td>-</td>
<td>1995</td>
<td>330</td>
<td>Yes</td>
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<tr>
<td>Melin 116</td>
<td>RIA</td>
<td>2001</td>
<td>64</td>
<td>Yes</td>
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<td>del Puente 112</td>
<td>-</td>
<td>2002</td>
<td>139/ F</td>
<td>Yes</td>
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<tr>
<td>Bishoff-Ferrari 106</td>
<td>RIA</td>
<td>2004</td>
<td>13,432</td>
<td>Yes</td>
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<td>Gerdhem 118</td>
<td>CLIA</td>
<td>2005</td>
<td>1,044</td>
<td>No</td>
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<tr>
<td>Hagström 110</td>
<td>HPLC</td>
<td>2006</td>
<td>78</td>
<td>Yes</td>
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<td>Saquib 111</td>
<td>CBP</td>
<td>2006</td>
<td>414</td>
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<td>2007</td>
<td>669</td>
<td>No</td>
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<td>Macdonald 109</td>
<td>HPLC</td>
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<td>Bishoff-ferrari 107</td>
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<td>9,961</td>
<td>Yes</td>
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<tr>
<td>Ensrud 108</td>
<td>LC-MS</td>
<td>2009</td>
<td>1,279</td>
<td>Yes</td>
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</table>

Blood 25(OH)D levels and fractures

Because fracture is the clinically important consequence of osteoporosis, many observational studies have focused on the association between 25(OH)D levels and the risk of fracture. Although a majority of these studies reported a positive association between high 25(OH)D levels and decreased risk of fracture, the results are inconsistent. Differences in study design, study populations and 25(OH)D assays complicate comparison of the studies. The studies that have identified a threshold above which the risk of fracture starts to rise have suggested levels starting at 30 nmol/L and upwards. The lower limit, 30 nmol/L, is reported from a study in Dutch community-dwelling men and women, whereas higher limits (>60 nmol/L) are suggested in studies from NHANES III and WHI-OS, cohorts including community-dwelling elderly Americans. Gerdhem et al. found, as described earlier, an increased risk for fracture in the 4% of women with 25(OH)D levels below 50 nmol/L. This study was conducted in elderly Swedish women. Only one study have used the gold standard assay HPLC-MS or LC-MS for determining 25(OH)D levels. In this nested case-
control study in elderly men (MrOs), one standard deviation (SD) decrease in 25(OH)D levels increased the risk of hip fracture by 60%, in men with 25(OH)D levels <50nmol/L. 128

The conclusion drawn, based on the results from observational studies and RCTs, in the IOM report is that there is inconsistent evidence to support an association between 25(OH)D levels and an increased risk of fracture. Considering the available data from observational studies, a 25(OH)D level >50 nmol/L is likely to cover the need of most people regarding bone health. 76

Table 3. Prospective cohort studies with 25(OH)D levels as exposure and fractures as outcome

<table>
<thead>
<tr>
<th>First Author</th>
<th>25(OH)D Assay</th>
<th>Publication year</th>
<th>Number of participants/fractures</th>
<th>Positive association (yes/no)</th>
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<tr>
<td>Woo 122</td>
<td>-</td>
<td>1990</td>
<td>283/9</td>
<td>No</td>
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<tr>
<td>Cummings 123</td>
<td>RIA</td>
<td>1998</td>
<td>9704/271</td>
<td>No</td>
</tr>
<tr>
<td>Gerdhem 118</td>
<td>CLIA</td>
<td>2005</td>
<td>986/109</td>
<td>Yes</td>
</tr>
<tr>
<td>Roddam 124</td>
<td>EIA</td>
<td>2007</td>
<td>1445/730</td>
<td>No</td>
</tr>
<tr>
<td>Garnero 117</td>
<td>CBP</td>
<td>2007</td>
<td>669/134</td>
<td>No</td>
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<tr>
<td>Looker 125</td>
<td>RIA</td>
<td>2008</td>
<td>1917/156</td>
<td>Yes</td>
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<tr>
<td>Van Schoor 126</td>
<td>CBP</td>
<td>2008</td>
<td>1311/115</td>
<td>Yes</td>
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<tr>
<td>Cauley 127</td>
<td>RIA</td>
<td>2008</td>
<td>800/400</td>
<td>Yes</td>
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<tr>
<td>Cauley 128</td>
<td>MS</td>
<td>2010</td>
<td>1608/436</td>
<td>Yes</td>
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</table>

Because falls is a risk factor for fracture, the association between 25(OH)D and risk for falls has also been addressed. Deficiency has also been associated with muscular weakness and impaired balance, which, in turn, increases the risk of fall and fracture. 129,130 A meta-analysis presented in the IOM report could not detect a risk reduction for falls per 10 nmol/L increase in 25(OH)D concentration (relative risk 0.92, 95% CI 0.80-1.05). Further, vitamin D supplements did not yield a preventive effect on falls. 76

Vitamin D and mortality

Vitamin D has been suggested to play a role in a variety of other medical conditions, including cancers and cardiovascular and autoimmune diseases. 2,64,131-137

Many tissues (e.g., colon, breast, prostate, macrophages, osteoblasts and keratinocytes) express 1 α–hydroxylase and have a local production of 25(OH)D. This production is not, with the exception of macrophages, involved in calcium homeostasis but may play a part in regulation of a wide variety of biological functions, such as cell growth, apoptosis, angiogenesis,
differentiation and regulation of the immune system. Therefore, in recent years, attention has been paid to the relation between vitamin D status and mortality, a particularly important measure of health. Vitamin D supplementation reduced total mortality by 7% in a meta-analysis of randomized trials in which mortality were not the main outcome. In a community-based cohort NHANES III, 25(OH)D levels < 45 nmol/L were associated with a 26% increased rate of all-cause mortality. In women, an increased total mortality was seen not only at low levels of vitamin D but also at high levels (> 125 nmol/L). When the same study population was followed for another 6 years and only cancer deaths were considered, low vitamin D levels were no longer associated with increased mortality, and men with levels > 100nmol/L had a 85% higher risk of dying. In another population-based cohort study of 1,260 community-dwelling elderly Dutch men and women, 25(OH)D below 25 nmol/L had an approximately 50% higher multivariable-adjusted mortality rate. However, after adjustment for a large number of health indicators, the association was no longer significant.
Aims of the studies

General aim
To investigate the importance of vitamin D for health, bone health in particular, and to examine to what extent this is influenced by dietary habits and genes

Specific aims
To assess the seasonal impact of genetic influence on 25(OH)D concentrations (Study I)

To consider the performance of three common commercially available 25(OH)D assays and the clinical consequences of differences in assay results (Study II)

To determine a possible threshold at which low 25(OH)D concentrations are associated with increased risk of fractures in elderly men and to clarify the contribution of low 25(OH)D levels on total fracture burden (Study III)

To assess whether 25(OH)D concentrations are associated with BMD and incident fractures in elderly Swedish men and women (Study IV)

To clarify the importance of long-term dietary and total vitamin D intake on the risk of osteoporosis and fractures in Swedish women (Study V)

To examine if 25(OH)D status relates to overall morality and cause-specific mortality in elderly Swedish men (Study VI)
Materials and Methods

Study population

The Swedish twin registry (Study I, II)
The Swedish twin registry, established in the 1960s, includes all twins born and living in Sweden from 1886 and onwards. The Swedish twin registry is described in detail in a review by Lichtenstein. 143

For study I and II, all like-sexed intact twin pairs born in 1965 or earlier, living in Uppsala County in central Sweden at northern latitude 60º, were invited to participate. In total, 172 twin pairs were found eligible and asked to participate. Of these, 102 twin pairs (59 female and 43 male), i.e. 204 participants, accepted to participate in the studies. 144

Uppsala Longitudinal Study of Adult Men-ULSAM (Study III and VI)
Between 1970 and 1973, all 50-year-old men (i.e. born 1920 to 1924) and living in Uppsala County in Sweden were invited to participate in the cohort. Of 2,841 men invited, 82% (n=2,322) accepted to participate in the study.

The screening examination program at baseline included a medical questionnaire and interview, blood and urine sampling, blood pressure and anthropometric measurements, an intravenous glucose tolerance test, an ECG recording, a chest X-ray and pure tone audiometry. The participants were reinvestigated at age 60, 70, 77, 82 and 88 years.

The studies included in this thesis are based on the investigation at 70 and 82 years of age. 145
Figure 7. Flow chart of the ULSAM cohort. All 50-year-old men living in Uppsala in 1970 were invited to the baseline examination. All men were re-invited to another investigation at age 60, 70, 77 and 88 years. For the 82-years investigation, those who participated in the 70 and/or the 77 years investigation were invited.

The Swedish Mammography Cohort-SMC (study V)

Women born between 1914 and 1948 and living in Uppsala and Västmanland County in Sweden were asked to respond to a comprehensive six-page FFQ between March 1987 and December 1990 when invited to a mammography screening. Completed questionnaires were obtained from 66,651 (74%) women in the source population. The questionnaire also included questions about age, self-reported current weight and height, marital status and educational level. After exclusions, 61,433 women remained in the cohort.

In 1997, a second FFQ and extended questionnaire, including information on physical activity, medical history, use of dietary supplements, age at menarche, history of oral contraceptive use, age at menopause, postmenopausal hormone use and lifestyle factors was sent to all participants who still lived in the study area. A subcohort of 5,022 randomly selected women who answered the second questionnaire formed the Swedish mammography cohort clinical (SMCC). The participants in this subcohort underwent measurement of BMD by DXA. They also completed a third questionnaire.

In study V, we included all women in both the SMC and SMCC. All questionnaires were included.
Prospective Investigation of the Vasculature in Uppsala Seniors-PIVUS (Study IV)

Between 2001 and 2004, 2,025 70-year-old men (49%) and women (51%) living in Uppsala County in Sweden were randomly recruited from the general population. Of these 2,025 men and women, 1,016 (50%) accepted to participate. The primary aim was to investigate the predictive power of different measurements of endothelial function and arterial compliance. In addition to numerous cardiac measurements, the variables included 7-day food intake recordings, DXA measurement and frozen serum, plasma and blood samples for analysis of serum 25(OH)D, calcium, PTH and other bone metabolism biomarkers. Medical/drug history and information about lifestyle factors (e.g., smoking habits and physical activity) were also collected. 1,002 had measurable S-25(OH)D levels.

Between 2006 and 2009, a reinvestigation of the cohort was performed. The major measurements performed at age 70 years were repeated at age 75 years. Of the initial 1,016 subjects, 52 had died during the 5-year interval and 827 attended the re-examination at age 75 years. The information from the first survey was used in study V. \(^{147}\)
25-hydroxyvitamin D assays

High performance liquid chromatography and mass spectrometry atmospheric pressure chemical ionization (HPLC-APCI-MS) (Study I-III, VI)

Plasma contains a collection of various bio-molecules, including proteins, lipids, salts, nutrients and waste products. These molecules differ vastly in terms of both size (molecular weight) and abundance (e.g., between albumin at ~50 mg/mL in plasma down to the extremely low levels of hormones and vitamins). Thus, bio-analysis often requires some means of separating this type of very complex mixture in order to simplify or even enable the analysis. HPLC is a widely used separation method for such samples. It is based on the fact that different molecules (analytes) have different properties based on their molecular composition. In HPLC analysis, this is exploited because the molecular properties of an analyte will determine its partitioning between two “compartments” of the system (i.e. what is called the stationary phase and the mobile phase). The stationary phase consists of very small particles (coated to possess different absorption properties) that are packed into tubes called “columns”. The mobile phase (e.g., water, organic solvent or solvent-water mixtures) is pumped through the column. If a sample is injected into the mobile phase entering the column, different bio-molecules will bind with different affinity to the column. Thus, some will be retained longer on the column than others, and accordingly, will be detected at longer “retention times”.

APCI allows the high flow rates in the HPLC method to be used directly and therefore a smaller amount of plasma is needed. For detection and quantification of the separated components, MS was used. 47

Determination of 25(OH)D$_2$ and 25(OH)D$_3$ in plasma with HPLC-APCI-MS was done at Vitas, Oslo, Norway. The Coefficient of Variation (CV) for inter-assay analyses is 7.6% at a plasma 25(OH)D concentration of 47.8 nmol/L and 6.9% at a plasma 25(OH)D concentration of 83.0 nM.

Radioimmunoassay (RIA) (Study II)

A known quantity of antigen is labeled with a radioactive isotope and mixed with a known amount of antibody for the antigen. A sample of serum containing an unknown quantity of that same antigen is added, and the unlabeled antigens from the serum thereby compete with the radio-labeled antigen for antibody binding sites. As the concentration of unlabeled antigen increases, more of it binds to the antibody, displacing the radio-labeled antigen. The bound antigens are then separated from the unbound, and the radioactivity of the unbound antigen is measured using a gamma counter.
Using known standards, a binding curve can then be generated that allows the amount of antigen in the patient's serum to be derived.\textsuperscript{48}

In study II, 25(OH)D\textsubscript{2+3} in serum was measured at a research laboratory in Uppsala using Gamma-B 25(OH)D RIA (IDS, Boldon, England). The CV for inter-assay analyses was 7.9\% for the assay.

Chemiluminescent immunoassay (CLIA) (study II and IV)
Specific antibody to vitamin D is used for coating magnetic particles (solid phase) and vitamin D is linked to an isoluminol derivative. During incubation, 25(OH)D is dissociated from its binding protein and compete with labeled vitamin D for binding sites on the antibody. After incubation, the unbound material is removed with a wash cycle. The starter reagent is then added and a flash chemiluminescent reaction initiated. The light signal, which is inversely proportional to the concentration of 25(OH)D present in samples, is measured by a photomultiplier as relative light units.\textsuperscript{52}

In study II and IV, 25(OH)D\textsubscript{3} in serum was measured at Uppsala University Hospital laboratory. The LIAISON® 25(OH)D Assay (DiaSorin) which uses chemiluminescent immunoassay technology. CVs for inter-assay analyses were 18.4\% at a 25(OH)D level of 39.5 nmol/L and 11.7\% at 121.25 nmol/L.

Dietary vitamin D intake (study V)
Vitamin D intake from diet and supplements and intake of other nutrients were calculated from a self-administered FFQ that includes 67 food items commonly eaten in Sweden. The women were asked how often, on average, per day during the past 6 months they had consumed these foods. Eight pre-defined frequency categories that ranged from “never/seldom” to “four times a day” were used. Additional open-ended questions about consumption of dairy products were included (for example type of milk and number of glasses of milk per day, number of slices of cheese per day). The frequency categories of the daily food items were converted to frequencies per month. For nutrient calculation, we used age-specific portion sizes (40-52, 53-65 and 66-74 years) based on scale-weighed portions that were recorded for a total of 5,922 days by 213 women randomly selected from the study base. Nutrient data were obtained from the Swedish National Food Administration database.\textsuperscript{148} Nutrient intakes were adjusted for total energy intake (mean 1700 kilocalories in the study population) using the residual method.\textsuperscript{149}

Information on vitamin D supplements was not obtained in the baseline questionnaire, but reported frequency of supplement use within the cohort during the first years of follow-up was less than 5\%.\textsuperscript{150}
A validation of vitamin D intake from the FFQ was carried out with four 7-day food records every third month in 104 of the women. There were only small systematic errors related to intake level between the methods with an average difference of 0.11 µg vitamin D per day (95% CI -0.15-0.38) \((r=0.72)\), an indication of good accuracy.

**Identification of outcomes**

**Bone mineral density and osteoporosis (study III, IV and V)**

BMD \((g/cm^2)\) was measured with DXA (Lunar Prodigy, Lunar corp., Madison, WI, USA). The body is exposed to x-ray beams of two different energies and the attenuation caused by the tissues is registered by a detector. The two energies are absorbed differently by soft tissue and bone, allowing bone mass parameters to be calculated. Because this is a 2D technique, there may be misinterpretation of the results in that no compensation is made for the volume of the bone and the amount of subcutaneous fat.\(^{151}\)

In the ULSAM, PIVUS and SMCC cohorts, total body, total hip, femoral neck and the lumbar spine (vertebrae L1-L4) BMDs were measured. When applicable, both extremities were used in the calculation. By triple measurements in 15 participants, the precision error of the DXA measurements in our laboratory has been calculated to be between 0.8 and 1.5% for BMD, depending on site. Daily scans of a lumbar spine phantom have been performed. The long-term precision error \((CV\%)\) was less than 1% during all study periods.

We defined osteoporosis as a DXA score at either the total hip, the femoral neck or the lumbar spine of less than -2.5 SD below the reference standard provided by the manufacturer.\(^{152}\)

**Fractures (Study III, IV and V)**

PIVUS: First time fractures that occurred within the cohort after enrolment were registered. Using the unique personal identification number (PIN) provided to all Swedish residents, the study cohort was matched to the patient registers within the county council of Uppsala to identify all fracture cases that were treated as in- or outpatients.

ULSAM: Using the PIN of every participant, we linked the study cohort to the National Patient Registry (NPR) to identify all cases of fractures admitted to hospitals. With the use of the PIN, fractures were also confirmed by linkage to radiographic records and county outpatient registries. All orthopedic records at the local hospitals in areas where the participants in the initial investigation resided were reviewed to identify fractures.
SMC: Fracture events were collated through linkage to the NPR and outpatient treated fractures were collected from the local outpatient registers by use of the PIN.

Fractures included ICD 10 codes S12, S22, S32, S42, S52, S62, S72, S82 and S92.

Cancers and death (Study VI)
Every participant’s PIN was linked to the Swedish National Cancer Registry and the Cause of Death Registry (CDR). The Cancer Registry contains all diagnosed cancer cases in Swedish residents since 1958. Since 1952, the National Board of Health and Welfare has collected information on the causes of death for all Swedish residents in the CDR. The underlying cause of death within the CDR was used to define all-cause, cardiovascular (ICD-9 codes 390-459, or ICD-10 codes I00-I99) and cancer mortality (ICD-9 codes 140-208, or ICD-10 C-codes), as well as mortality from other causes. The completeness of ascertainment and accuracy of classification of causes of death in the Swedish CDR are high. 153

Statistics
Statistical analyses were performed by using Mx (script rawVC1a.mx) in study I, STATA in study II, IV, V and VI and SAS (SAS 9.1, SAS Institute, Cary, NC, USA) in study III and VI.

Genetic influence (study I)
The classical twin method is based on the knowledge that monozygotic (MZ) twins share 100% of their genome, whereas dizygotic (DZ) twins share, on average, 50% of their segregating genes. A higher correlation, expressed as the intraclass correlation coefficient (ICC) within MZ twin pairs than within DZ twin pairs, provides a first indication of genetic influences on a trait. Information concerning shared genetic and environmental influences is best estimated by structural equation modelling techniques that fit models over all types of twins to best describe the causes of variation in a phenotype. The total variance in the trait can be partitioned into genetic variance (A), shared environmental variance that includes shared (familial) environmental variance (C) and individual-specific variance (E), which also includes measurement error. To estimate the parameters of interest, the equation for one of the twins can be written as:

\[ V_{p1} = a \times A_1 + c \times C_1 + e \times E_1 \]  

(1)

\( V_{p1}, A_1, C_1 \) and \( E_1 \) are the total phenotypic variance, additive genes, shared environments and unique environments, respectively, for the first twin in the
pair. A similar equation can be written for the second twin. The theoretical expectations for variance and covariance within twin pairs can be described with the following equations:

\[ V_p = a^2 + c^2 + e^2 \]  \hspace{1cm} (2)  
\[ \text{Cov (MZ)} = a^2 + c^2 \]  \hspace{1cm} (3)  
\[ \text{Cov (DZ)} = 0.5 \times a^2 + c^2 \]  \hspace{1cm} (4)

The parameters \( a, c \) and \( e \) can then be estimated with maximum likelihood methods and the relative importance of genes and environments can be evaluated. Heritability (\( A \)), the relative importance of genetic influences for variation in a trait, is defined as genetic variance \( (a^2) \) divided by the total phenotypic variance.

**Measures of association**

Linear regression analysis was used to determine the association between the dependent variable and the explanatory variables when the dependent variable was continuous.

Logistic regression analyses were used to determine the association between the dependent variable and the explanatory variables when the dependent variable was dichotomized.

Cox regression analysis was used if the explanatory variable is time dependent as it relates the time that passes before the dependent event occurs.

To gain additional insights into potential nonlinear associations, the nonlinear trend in the risk of the outcome was assessed by a restricted cubic-spline Cox regression analysis in study III, IV, V and IV. We used five "knots" at 25(OH)D percentiles 5, 27.5, 50, 72.5 and 95. The result can be illustrated as a smoothed plot with 95% CI for the overall risk of fracture.

In all analyses, we first analyzed the crude and age-adjusted estimates. Thereafter, we adjusted for potential confounders by adding them to the crude model. A confounder is defined as an extraneous variable that correlates positively or negatively with both the dependent and independent variable. Variables were therefore thought of as potential confounders when they were considered pathophysiologicaly associated with both the exposure and outcome, not only if they changed the estimate when included in the model.
Results

Study I. Seasonal genetic influence on serum 25-hydroxyvitamin D levels: a twin study

Relatively little is known about genetic influences on vitamin D levels and virtually nothing about seasonal differences in these effects. 42,43 We therefore conducted a classical twin study to estimate the importance of genetic factors on 25(OH)D levels by season.

Our results show a tendency of a stronger correlation for S-25(OH)D within MZ twin pairs (ICC 0.73; 95% CI, 0.57-0.84) than within DZ twin pairs (ICC 0.61; 95% CI 0.41-0.75), indicating a genetic trait. During the summer season, the correlation within MZ twin pairs was 0.64 (95% CI, 0.39-0.81) and within DZ twin pairs 0.51 (95% CI, 0.27-0.70). There was no difference in the correlation within MZ and DZ twin pairs during winter time. By structural equation modelling techniques, we could further estimate the proportion of the variance that is derived from genetic variance, shared environmental variance and individual-specific environmental influences in summer and winter, as well as independent of season. Non-shared environmental influence includes diet, supplements and sun-seeking behavior, but also measurement error.

Independent of season, there was a modest heritability of S-25(OH)D and an equally strong shared environmental influence. The individual-specific environmental influence on variation in serum 25(OH)D contributed to approximately one fourth of the variation independent of season. A considerable seasonal difference in 25(OH)D heritability was seen. Half of the total variance during the summer season was explained by the genetic variance compared with non-existing heritability estimates during the winter season.

In summary, genes explain about half the variation in vitamin D levels in summer time, but none of the variance in winter time.
Table 4. The age-adjusted genetic, shared and individual-specific environmental influences all year and by season on variance of S-25(OH)D levels (95% CI)

<table>
<thead>
<tr>
<th></th>
<th>Genetic influence</th>
<th>Shared environmental influence</th>
<th>Individual-specific influence</th>
</tr>
</thead>
<tbody>
<tr>
<td>All year</td>
<td>0.39 (0.32-0.75)</td>
<td>0.38 (0.04-0.42)</td>
<td>0.23 (0.15-0.36)</td>
</tr>
<tr>
<td>Summer</td>
<td>0.48 (0.00-0.85)</td>
<td>0.25 (0.00-0.64)</td>
<td>0.26 (0.14-0.51)</td>
</tr>
<tr>
<td>Winter</td>
<td>0.00 (0.00-0.77)</td>
<td>0.72 (0.00-0.82)</td>
<td>0.28 (0.14-0.52)</td>
</tr>
</tbody>
</table>

Study II. Determining vitamin D status: a comparison between commercially available assays

Although numerous 25(OH)D assays are available, their comparability is uncertain. We therefore aimed to investigate the differences in performance between three common commercially available assays. We used the same study population as in Study 1 and could therefore take advantage of the twin study design. The assays included a HPLC-APCI-MS, a RIA and a CLIA assay.

Our results revealed low inter-assay agreement. HPLC-APCI-MS measured a mean 25(OH)D level of 85 nmol/L (95% CI, 81-89), RIA 70 nmol/L (95% CI, 66-74) and CLIA 60 nmol/L (95% CI, 56-64). Furthermore, HPLC-APCI-MS was better at detecting differences between summer and winter samples than RIA or CLIA.

There were considerable differences between the methods in the proportion of participants classified as vitamin D insufficient. Using a 50-nmol/L cut-off, only 8% of our participants were classified as vitamin D insufficient with the HPLC-APCI-MS method, 22% with RIA and 43% with CLIA.

Bland-Altman plots demonstrated that RIA and CLIA had a non-proportional bias relative to HPLC-APCI-MS. Both positive and negative bias became more accentuated with increasing 25(OH) values, i.e. the inter-assay disagreement increases with an increasing serum level of 25(OH)D. This non-uniform variability at different serum levels of 25(OH)D between the methods was confirmed by linear regression analyses, where the relation between absolute differences in serum values between the methods against the mean of the two values was tested. Both parameter estimates were positive and highly statistically significantly different from zero, and accordingly, the differences in variance between the methods were higher at increasing levels of serum 25(OH)D.
Figure 9. Mean serum 25(OH)D levels by assay independent of season (left) and by season (right). The error bars indicate 95% CI.

As measures of assay accuracy, the ICC for within twin pair similarity in 25(OH)D levels were calculated. Because we know that there is a genetic trait in 25(OH)D levels, we hypothesized that a high ICC is an indirect measure of accuracy. HPLC-APCI-MS had a significantly higher ICC (r=0.7) relative to both RIA (r=0.5) and CLIA (r=0.4). The sample precision error of the assays was determined by SCV. HPLC-APCI-MS had the lowest SCV, 32.3 (95% CI, 28.5-36.0), RIA the next lowest, 34.2 (95% CI, 29.8-38.3) and CLIA the highest, 43.5 (95% CI, 37.7-48.9).

In summary, substantial inter-assay disagreement exists, which needs to be considered when comparing 25(OH)D studies or when determining thresholds for vitamin D deficiency and insufficiency.

Study III. Plasma 25-hydroxyvitamin D levels and fracture risk in a community-based cohort of elderly men in Sweden

Although blood levels of 25(OH)D are the generally accepted indicator of vitamin D status, no universal reference level regarding bone health has been reached. Therefore, in a cohort of 1,194 Swedish elderly men, the aim of this study was to determine the threshold at which low plasma 25(OH)D levels
are associated with fractures. Further, we aimed to clarify the importance of low levels on total fracture burden.

25(OH)D in plasma was determined with a HPLC-APCI-MS assay. During the follow-up period (median 11 years), 309 of the 1,194 participants suffered from at least one fracture. Mean 25(OH)D levels did not differ between men with and without fracture. We did not observe any association between 25(OH)D in continuous form and fracture risk: hazard ratio (HR) 0.98 (95% CI, 0.87-1.09) per SD increase in plasma 25(OH)D. The multivariable-adjusted HR for fracture was higher than the reference only for 25(OH)D values below approximately 40 nmol/L, which corresponded to the 5th percentile of 25(OH)D.

![Figure 10](image)

**Figure 10.** Smoothed plot of HR for fracture by level of plasma 25(OH)D. The HR (solid line) and 95% CI (dotted lines) were estimated by restricted cubic-spline Cox regression analysis. The 25(OH)D percentile 80 was used as the reference value.

We therefore compared the risk of fracture for individuals with 25(OH)D <40 nmol/L with those having levels >40 nmol/L. The comparison revealed a divergence of the Kaplan-Meier curves throughout the follow-up period (Figure 11). The multivariable-adjusted HR for fracture was 1.65 (95% CI 1.09-2.49) for individuals with 25(OH)D levels <40 nmol/L when compared with individuals with >40 nmol/L. The HR decreased slightly after additional adjustment for co-morbid conditions to 1.58 (95% CI, 1.04-2.41). If we only analyzed first fractures (n=207), crude HR was 1.84 (95% CI, 1.12-3.02) and adjusted HR 1.69 (95% CI, 1.03-2.80). The adjusted population attributable risk of fracture was 0.03 (95% CI, 0.01-0.08).
BMD with DXA was measured in 507 of the men at 82 years. In comparison with individuals with 25(OH)D levels >40 nmol/L, those with levels <40 nmol/L had a lower adjusted BMD at the lumbar spine (adjusted BMD 1.20 (95% CI, 1.09-1.32) vs. 1.32 (95% CI, 1.30-1.35) g/cm², p=0.04), but no significant difference in BMD emerged at the proximal femur.

In summary, P-25(OH)D levels <40 nmol/L were associated with increased risk of fracture. Because only 1 in 20 men had these low levels, we conclude that low vitamin D levels are not a major cause of fracture in community-dwelling Swedish men.

Study IV. Serum 25-hydroxyvitamin D in relation to BMD and fractures in a Swedish cohort of women and men

To further consider the relationship between 25(OH)D and bone health, the aim of this study was to assess whether circulating vitamin D is associated with BMD and incident fractures in a cohort of elderly Swedish men and women.

25(OH)D in serum was measured with a CLIA assay. The mean S-25(OH)D level was 58 (SD 20) nmol/L, with 38% of the participants having levels <50 nmol/L. During follow-up (median 7 years), 155 (15%) of the participants sustained a first fracture. Twice as many women as men had a
fracture. For each SD increase in S-25(OH)D, the rate of fracture did not change. No differences in fracture rates between quintiles of S-25(OH)D were detectable, with a multivariable-adjusted HR of 1.13 (95% CI, 0.65-1.94) for quintile 1 compared with the highest quintile. Moreover, no effect modification by sex was discovered. Nonlinear trends in the rate of fracture using restricted cubic-spline Cox regression revealed no further association. After multivariable adjustments, 25(OH)D was only weakly associated with total body BMD in men (P=0.03). Each SD increase in S-25(OH)D (approximately 20 nmol/L) conferred a 1% increase in total body BMD. Low 25(OH)D levels were not associated with lower BMD at the total hip or the lumbar spine in men or women.

![Multivariable-adjusted spline curve showing the relation between serum 25(OH)D and a first fracture. The HR is indicated by the solid line and the 95% CI by dashed lines.](image)

In summary, in a general population of elderly Swedish men and women, 25(OH)D was not associated with the risk of fracture. A weak association with low total body BMD in men was detected. This weak association, however, probably lacks clinical relevance.

**Study V. Dietary intake of vitamin D in relation to BMD and fracture among Swedish women**

The importance of dietary vitamin D intake for bone health in adults is uncertain, and only a few studies have addressed this issue. This uncertainty is reflected by the wide range of intake recommendations for middle-
aged and older individuals in different countries. The aim of this study was therefore to clarify the associations between long-term dietary intake of vitamin D with risk of fractures and osteoporosis in Swedish women.

In the 66,651 women in the SMC, 14,738 experienced any type of first fracture during follow-up, with 3,871 of these being hip fractures. Further, 5,043 women had at least a second fracture. Mean dietary vitamin D intake was 4.3 µg. By quintiles of vitamin D intake, the multivariable-adjusted HR and 95% CI for any first fracture was 0.96 (95% CI, 0.92-1.01) for the lowest and 1.02 (95% CI, 0.96-1.07) for the highest quintile when compared with the third quintile that was set as a reference. The corresponding HR for a first hip fracture was 1.02 (95% CI, 0.96-1.08) for the lowest and 1.14 (95% CI, 1.03-1.26) for the highest quintile. Multiple fracture events did not alter our estimates, nor did stratification by calcium intake. A vitamin D intake below 5 µg per day compared with an intake higher than 10 µg per day did not affect the rate of fracture of any type (HR 1.02; 95% CI, 0.92-1.13). In fact, individuals with high intake even had a slightly higher rate of hip fracture (HR 1.27; 95% CI, 1.03-1.57).

Twenty percent of the women in the subcohort were classified as osteoporotic. The odds ratio (OR) of osteoporosis by quintiles of vitamin D intake was 1.20 (95% CI, 0.85-1.71) for the lowest and 0.99 (95% CI, 0.78-1.25) for the highest quintile. BMD was 2% higher at the lumbar spine and 0.3% higher at the total hip in women with highest vs. women with lowest intake of vitamin D (p<0.0001).

In summary, dietary vitamin D intake in middle-aged and elderly Swedish women was not associated with fracture risk or with osteoporosis.
Table 5. HR with 95% CI for a first any fracture and hip fracture and OR with 95% CI for osteoporosis by quintiles of vitamin D intake. Adjusted for age, BMI, height, total energy intake, retinol, potassium, protein, alcohol and calcium intake, calcium and vitamin D supplementation, nulliparity, educational and physical activity level, smoking status, previous fracture, and Charlson’s comorbidity index

<table>
<thead>
<tr>
<th>Dietary vitamin D intake (µg/day)</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
<th>Q5</th>
</tr>
</thead>
<tbody>
<tr>
<td>First event any fracture</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of fractures</td>
<td>2884</td>
<td>2813</td>
<td>2952</td>
<td>2855</td>
<td>3234</td>
</tr>
<tr>
<td>Person-years at risk</td>
<td>212 880</td>
<td>212 240</td>
<td>213 360</td>
<td>202 290</td>
<td>210 510</td>
</tr>
<tr>
<td>Rate per 1000 person-years</td>
<td>13.5 (13.1-14.1)</td>
<td>13.3 (12.8-13.8)</td>
<td>13.8 (13.3-14.3)</td>
<td>14.1 (13.6-14.6)</td>
<td>15.4 (14.8-15.9)</td>
</tr>
<tr>
<td>Age-adjusted HR (95% CI)</td>
<td>1.01 (0.96-1.07)</td>
<td>0.99 (0.94-1.05)</td>
<td>1 (reference)</td>
<td>1.01 (0.96-1.06)</td>
<td>1.00 (0.95-1.05)</td>
</tr>
<tr>
<td>Multivariable-adjusted HR (95% CI)</td>
<td>0.96 (0.92-1.01)</td>
<td>0.98 (0.93-1.03)</td>
<td>1 (reference)</td>
<td>1.01 (0.96-1.07)</td>
<td>1.02 (0.96-1.07)</td>
</tr>
<tr>
<td>First event hip fracture</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of fractures</td>
<td>734</td>
<td>686</td>
<td>723</td>
<td>774</td>
<td>954</td>
</tr>
<tr>
<td>Person-years at risk</td>
<td>228 580</td>
<td>227 490</td>
<td>230 000</td>
<td>217 700</td>
<td>227 830</td>
</tr>
<tr>
<td>Rate per 1000 person-years</td>
<td>3.2 (3.0-3.5)</td>
<td>3.0 (2.8-3.2)</td>
<td>3.1 (2.9-3.4)</td>
<td>3.6 (3.3-3.8)</td>
<td>4.2 (3.9-4.5)</td>
</tr>
<tr>
<td>Age-adjusted HR (95% CI)</td>
<td>1.10 (0.99-1.21)</td>
<td>1.03 (0.93-1.14)</td>
<td>1 (reference)</td>
<td>1.09 (0.98-1.20)</td>
<td>1.07 (0.97-1.18)</td>
</tr>
<tr>
<td>Multivariable-adjusted HR (95% CI)</td>
<td>1.02 (0.96-1.08)</td>
<td>0.94 (0.93-0.96)</td>
<td>1 (reference)</td>
<td>1.10 (0.99-1.21)</td>
<td>1.14 (1.03-1.26)</td>
</tr>
<tr>
<td>Osteoporosis (SMCC)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Women without osteoporosis. n (%)</td>
<td>213 (76.1)</td>
<td>437 (76.9)</td>
<td>754 (81.2)</td>
<td>902 (81.6)</td>
<td>1704 (79.6)</td>
</tr>
<tr>
<td>Women with osteoporosis. n (%)</td>
<td>67 (23.9)</td>
<td>131 (23.1)</td>
<td>175 (18.8)</td>
<td>203 (18.4)</td>
<td>436 (20.4)</td>
</tr>
<tr>
<td>Age-adjusted OR (95% CI)</td>
<td>1.37 (0.98-1.90)</td>
<td>1.36 (1.04-1.76)</td>
<td>1 (reference)</td>
<td>0.94 (0.75-1.19)</td>
<td>0.95 (0.78-1.16)</td>
</tr>
<tr>
<td>Multivariable-adjusted OR (95% CI)</td>
<td>1.20 (0.85-1.71)</td>
<td>1.21 (0.92-1.60)</td>
<td>1 (reference)</td>
<td>1.03 (0.81-1.32)</td>
<td>0.99 (0.78-1.25)</td>
</tr>
</tbody>
</table>
Study VI. Plasma vitamin D and mortality in older men: a community-based prospective cohort study

Vitamin D is important for bone health but may also affect the development of several chronic diseases, including cancer and cardiovascular diseases, two major causes of death.\textsuperscript{157,158} We therefore aimed to examine how vitamin D status relates to overall and cause-specific mortality among 1194 elderly men in the ULSAM cohort.

25(OH)D in plasma was determined with a HPLC-APCI-MS assay. During follow-up (median 12.7 years), 49% of the participants died. By restricted cubic-spline Cox regression analyses (Figure 14) we observed a U-shaped association between 25(OH)D concentrations and total mortality (p=0.001 for 25(OH)D as a quadratic term) as well as cancer mortality (p=0.001 for vitamin D as a quadratic term). That is, men with both low and high plasma vitamin D levels had higher total and cancer mortality rates than those with intermediate values. For cardiovascular mortality, the mortality curve showed a tendency of elevations only at low 25(OH)D levels (p=0.52 for vitamin D as a quadratic term).

Analysis of risks in the low and high ends of the range of vitamin D levels confirmed these graphical findings. Overall mortality was increased among subjects with the lowest 10% and highest 5% of the serum vitamin D distribution (multivariable adjusted HR 1.43 (95% CI 1.11-1.84) and 1.67 (95% CI 1.19-2.35) respectively. Cancer mortality, after excluding prevalent cancers and cancer deaths within the first 5 years after baseline, was more than two-fold higher at both low and high extremes (multivariable adjusted HR 2.24 (95% CI 1.39-3.58) and 2.52 (95% CI 1.28-4.96) respectively). Cardiovascular mortality tended to be increased only in the bottom 10% (multivariable adjusted HR 1.53 (95% CI 0.97-2.41).

In summary, both high and low vitamin D levels were associated with increased risk of cancer mortality and overall mortality.
**Figure 13.** Smoothed plot of HR for all-cause mortality (A), cancer mortality (B) and cardiovascular mortality (C) by 25(OH)D levels. The dotted lines represent the 95% CI.
Conclusions

The following conclusions can be drawn from the studies presented in this thesis and in previously published research:

Circulating vitamin D levels are partly determined by our genes. In the summer, among Caucasians living at northern latitudes, genes are more important for vitamin D status than the diet, probably due to genetic adaptation of dermal vitamin D synthesis.

Differences in performance and disparate results between assays for 25(OH)D determination require assay-specific reference levels. Better, a gold standard should be used in both scientific and clinical settings. HPLC/MS should be regarded as the gold standard method.

Low 25(OH)D levels are not a major cause of osteoporosis or fracture in Swedish community-dwelling elderly men and women. Only levels <40 nmol/L is associated with increased risk of fracture. Therefore, regarding bone health, aiming at a higher level of 25(OH)D than 50 nmol/L does not seem beneficial in this population.

Dietary intake of vitamin D in Swedish middle-aged and elderly women is low. The average intake is less than half of the current recommendations. Despite this, a higher dietary intake than recommended is not associated with decreased risk for osteoporosis or of fracture in community-dwelling middle-aged and elderly women.

Low levels of plasma 25(OH)D, but also high levels, are associated with elevated risks of overall mortality and cancer mortality. The potential harm of high vitamin D levels should be considered before recommending high-dose supplements.
Discussion

The overall aim of the studies in this thesis is to provide information to the public and health care providers on what constitutes an optimal vitamin D status. Recommendations on dietary vitamin D intake, vitamin D supplementation and safe sun exposure, as well as the range of circulating levels of vitamin D optimal for bone health that does not infer an increased risk for disease needs to be established. These recommendations must be based on scientific evidence derived from high quality studies on cohorts that can be generalized to entire populations or, in the case of vitamin D supplementation, on RCTs. Furthermore, because no study will ever be flawless, a sound analysis of strengths and limitations of the available evidence is necessary. This thesis sought to determine the association between variations in vitamin D intake and risk for fracture and the association between the levels of circulating 25(OH)D and risk for fracture. Further, the thesis identified clear variability between assays commonly used both in clinical settings and in research. This thesis should therefore contribute to the overall goal of establishing sound recommendations on vitamin D status and bone health.

25(OH)D assays

Before discussing the role of vitamin D in health, the problem of inconsistencies between assays commonly used for determination of 25(OH)D levels should be addressed. HPLC/MS or LC-MS/MS is currently considered as the gold standard, but the technique is complex and available in only a few laboratories. Older and many recent studies have used other assays. Disagreement between these assays may partly explain some of the discrepancies in study results. Calibration between assays would facilitate a reliable comparison of study results, but in study II we demonstrated that this can be complicated and, in many cases, not a realistic alternative. The quality of future studies and the interpretation of their results would improve if a gold standard assay was generally applied. Accordingly, using a HPLC or LC-MS/MS assay is generally considered as strength of a study. Both the National Diet and Nutrition Survey in Great Britain and the NHANES cohort in the USA are now using LC-MS/MS as their preferred method. In clinical settings, it is not probable that HPLC or LC-MS/MS will be the dominating assay in the near future. The demand for analyzing 25(OH)D is rising dra-
matically because of the general awareness of the proposed “epidemic vitamin D deficiency”\textsuperscript{158}. In a still unpublished study from Denmark presented at the ASBMR 2011 annual meeting, it was reported that the number of analyzed 25(OH)D samples in Copenhagen has increased manifold over the past years\textsuperscript{159}. A similar pattern is seen at Uppsala university hospital laboratory. Therefore, assay-specific reference levels are desirable.

Another potential problem regarding vitamin D assays is the way they are validated. Most laboratories send samples to a Vitamin D External Quality Assessment Scheme (DEQAS)\textsuperscript{160} and many studies on assay performance are reports from DEQAS.\textsuperscript{55,59,161,162} The performance target was set by an advisory panel in 1997 and requires participants to get 80\% or more of their results within +/-30\% of the All-Laboratory Trimmed Mean (ALTM). If achieved, the method is considered to have good accuracy and thereby certified as having met the standards set forth by the DEQAS. In 2003, only 59\% of participants met the criteria,\textsuperscript{163} even though the performance target is relatively wide. The ALTM is the mean 25(OH)D concentration from all the samples analyzed. Therefore, if one assay is more common than others, the result of this assay will push the ALTM toward its own mean. The DiaSorin CLIA assay, which performed worst in our study (study II), contributed to 38\% of the samples sent to DEQAS recently, whereas HPLC only contributed to 3\% (personal communication with DiaSorin, Sweden). Therefore, the CLIA assay will bias the ALTM and results from DEQAS should be interpreted with this information in mind.

Vitamin D and bone health

There has been, and still is, an intense debate regarding as to what circulating 25(OH)D level should be considered sufficient. Concerning bone health, a range between 40 to 75 nmol/l has been suggested as a lower limit,\textsuperscript{61-63,65,108,164} although 50 nmol/L is suggested in the IOM report.\textsuperscript{46} Considering that the clinically important endpoint of low BMD and osteoporosis is fracture, vitamin D trials designed to study fracture as outcome are of more interest than BMD as outcome. A decrease in BMD may not be clinically relevant because a small decrease did not increase the risk of fracture in study III and IV. Looking at the results in study III in conjunction with previous studies,\textsuperscript{27,118} the general Swedish adult population has a low prevalence of vitamin D insufficiency if a 50 nmol/L reference is used. 25(OH)D levels in study IV were relatively low, but we concluded in study II that the assay used in study IV underestimates 25(OH)D levels. More importantly, only 3\% of the participants in study III had a fracture that could be explained by low vitamin D concentrations (<40 nmol/L), and no association was found in study IV. Accordingly, our results support the conclusion in the IOM report, that levels >50 nmol/L are sufficient for maintaining bone health. Monitor-
ing vitamin D blood concentrations are probably unnecessary for the general Swedish population.

In study V, which includes over 60,000 women, no association between vitamin D intake and fracture or osteoporosis was detected. This was not surprising in view of the weak effects that have been found in RCTs. Thus, dietary intake does not seem to be an important determinant for vitamin D status, but the intakes in our study might be regarded as too low to detect an association. Mean intake in our cohort was only 4.3 µg (172 IU) and the inter-quintile range was 2.1 µg (82 IU). The vitamin D doses used in most clinical trials ranges from 400-800 IU. However, we did consider intakes as high as 12.5 µg without detecting an association with fracture risk. Because the results from observational studies and the RCTs conducted up to now are mainly non-beneficial regarding the effect of vitamin D intake and supplementation, a large RCT with higher doses of vitamin D alone is warranted in order to find causality. In vitamin D and calcium combination trials, the interpretation is confounded by the effect of calcium. This is because calcium alone seems to have a modest effect on both BMD and fracture. The ongoing Vitamin D and Omega-3 Trial (VITAL) study aims to include 20,000 persons in the USA, randomized to either placebo or 50 µg (2,000 IU) vitamin D once daily as an oral supplement. The outcome of this study will hopefully show whether or not high daily oral doses of vitamin D prevent fractures. Recently, a single annual dose of 300,000 IU (averaging 34 µg daily) in older community-dwelling women was surprisingly shown to increase the number of falls and increase fracture risk. Further, large scale studies on certain populations (such as dark-skinned, obese, males and children) are needed as they are strongly underrepresented.

In study II, we concluded that solar altitude and genetic influences are probably the most influential factors on 25(OH)D concentrations. A genetic influence could theoretically be important on several levels, including the capacity to synthesize vitamin D in the skin, to assimilate vitamin D from the diet and to store it in body fat. Because our results reveal a genetic influence in summer but not in winter, it is reasonable to conclude that it is the skin’s capacity to produce vitamin D that is mostly affected by our genes. This result could explain why inhabitants in Scandinavia have higher 25(OH)D levels than inhabitants in countries with more constant UVB radiation exposure. Previous studies have concluded that diet is only a minor determinant of vitamin D status, especially during summer time. One recently published study found, in contrast to our study results, that genes are important in winter but not in summer. This study was conducted in California with sufficient UVB radiation all year round, which could explain the discrepancy with our results.

UVB radiation can cause skin cancer and therefore sun exposure cannot be recommended as a vitamin D source without caution. Studies on the amount of UVB radiation that increases S-25(OH)D levels as much as possi-
ble without increasing the risk of skin cancer would contribute to safe recommendations for healthy sun exposure. It would also be of interest to determine how 25(OH)D levels in old, fragile men and women, who would possibly benefit from vitamin D supplementation, are affected by diet in relation to sun exposure. These people may have limited exposure to sunlight and therefore dietary intake could be of more importance in this elderly group. Even more likely, they would benefit more from regular sun exposure than from diet and supplements. Because these persons are old, the risk of developing skin cancer is probably not an overriding concern.

The cause of decreased BMD, osteoporosis and low energy fracture is multifactorial. Decreased BMD and osteoporosis are, per se, not a burden for a person but are a defined risk factor for fracture. In younger persons, osteoporosis is a more defined risk factor of fracture than for elderly, where such risk factors as falls and frailty are more important. Frailty is not readily defined but could indirectly be measured as gait speed. The association between vitamin D, falls and muscle strength has gained increasing interest but, as for other outcomes, results are inconsistent. A still unpublished study by Glendenning, Prince and colleagues presented at the ASBMR annual meeting in 2011 has used a high oral dose of vitamin D (150,000 IU per 3 months). Despite the high dose, the authors could not find increased muscle strength or a decreased fall rate after 1 year. Future studies designed to assess risk factors for fracture should include risk factors (e.g., balance and frailty) other than vitamin D.

Potential harm of Vitamin D

Few studies have been designed to specifically evaluate the safety of vitamin D intake and 25(OH)D concentrations. Nonetheless, there is accumulating evidence that not only low, but also high levels of vitamin D may have negative health outcomes. Additionally, in the unpublished study from Denmark (presented at the ASBMR annual meeting in 2011), mentioned earlier in this thesis, a U-shaped association was reported between 25(OH)D concentrations and mortality. Because most RCTs on vitamin D supplement have not revealed a fracture preventive effect with moderate doses, attempts are now being made to use high-dose supplementation. Because there are ethical issues associated with conducting clinical trials designed to study the adverse effect of vitamin D, the ongoing trials should also address potential negative health outcomes. The VITAL trial described above addresses, in addition to bone health, the association between vitamin D and many medical conditions (e.g., cardiovascular diseases, cancers and fractures).

Concerning the absolute upper limit of vitamin D intake that can be tolerated without acute toxic effect, the best option is to define the intake where
hypercalcemia occurs. Further, there is a need for subgroup-specific recommendations for the upper limit of vitamin D intake and 25(OH)D levels. For example, renal insufficiency, common in the elderly, can make a person more sensitive and susceptible to excess vitamin D.

Methodological considerations

All our cohorts are population-based. In other words, they are a designated group of people that should represent the general population in age, sex, lifestyle, socioeconomic status and health. Results from the ULSAM cohort in study III and VI can therefore be generalized to elderly community dwelling men, but may not be generalized to women or to men with another ethnicity. The larger the cohort, the more improved are the precision and internal validity, as well as the external validity of the results, provided there is no selection bias in the sample. Selection bias is introduced when there are systematic errors in the procedures used to select a study person from the study population or if there are factors influencing the participation. A low participation rate is a potential selection bias. In the PIVUS study, the participation rate was relatively low, i.e. 50%. The participation rate was high in the SMC study (74%), but the age span of the invited women was wide (39-76 years), and it is possible that the oldest and sickest women were less willing to participate. Contrariwise, it could be that younger women do not find the time to answer the questionnaire. ULSAM had a high participation rate (82%) and included only men of the same age and could therefore be thought of as having high external validity at start. However, in both these cohorts, the participation rate declined in subsequent investigations and it is likely that a selection toward healthy participants occurred.

Observational studies cannot prove causality because the participants are not randomized to exposure. The advantage of prospective cohort studies is that they enable us to study large populations at a relatively low cost. These studies are valuable in that they make it possible to detect associations that can be further analyzed in a RCT. Critique that is often pointed out is that it is more likely that old and frail persons seldom go outside and therefore have lower vitamin D levels when they sustain a fracture or get a cancer diagnosis, rather than the opposite, i.e. a reverse causation of the phenomenon. However, in comparison with the cross-sectional design, the prospective cohort design can distinguish between exposure and outcome in that the participants are exposed at baseline, before the outcome has occurred. They are then followed over time until they eventually have the outcome or become censored. To avoid confounding by current undiagnosed cancers in study VI, we excluded patients who had a cancer diagnosed within the first 5 years after baseline. In study III, IV and V, we adjusted for previous fracture because one fracture is a strong risk factor for a subsequent fracture and a
fracture event could affect the decision to change lifestyle. Although our statistical models were adjusted for potential confounders, residual confounding remains a possible limitation in our studies. In study V, information about serum vitamin D, sun exposure and sun-seeking behavior would have been preferable so that they could be construed as effect modifiers in the analysis.

FFQs are sometimes considered a method with high information bias because intake of nutrients is based on self-reported intake of certain foods. The information bias, i.e. that some women overestimate while others underestimate the amount of foods they have consumed, is unavoidable. Even if that is the case, the size of the cohort (over 60,000 women) provides sufficient power to detect even small effects. The questionnaire has also been validated with four 7-day food records every third month in 104 of the women, with high validity. Finally, the women answered repeated questionnaires, where the data indicated that vitamin D intake was relatively stable over time.

**Future research**

Considering the above discussion, future research should aim at:

Improving the standardization of vitamin D assays and set assay-specific reference levels to be used until a gold standard assay is more common in clinical settings.

Identifying the genetic differences that contribute to inter-individual differences in circulating 25(OH)D levels.

Defining insufficiency level for 25(OH)D for skeletal health outcomes at different life stages and for different racial/ethnic groups.

Defining the role for vitamin D in non-skeletal health outcomes.

Clarifying the potential negative effects of high-dose vitamin D supplementation on bone and general health.
Sammanfattning


Jag har i min avhandling studerat om vitamin D nivåer i blodet eller vitamin D intag från kosten har betydelse för benhälsa men även för generell hälsa. Studierna är utförda på stora kohorter av medelålders och äldre svenska män och kvinnor. En kohort är en grupp av människor som representerar den befolkningsgrupp man vill studera och som följs över en bestämd tidsperiod. Jag har därför i en kohort kunnat studera om en misstänkt riskfaktor, i mitt fall exempelvis bristfälligt kostintag av vitamin D, på sikt leder till benbrott. Jag har även studerat om våra gener påverkar nivån av vitamin D i blodet. Eftersom det finns många olika metoder för att mäta vitamin D i blodet har jag också undersökt om dessa är jämförbara eller om det finns skillnader mellan metoderna som vi måste ta hänsyn till när vi ska bestämma om en person har D vitamin brist eller som måste beaktas när vi jämför studier om D vitamin.

Resultaten från mina studier visar att kostintaget av vitamin D inte verkar spela någon roll för risken att drabbas av ett benbrott och inte heller för benskörhet. Kostintaget är hos de flesta lågt och har troligtvis en underordnad betydelse för vitamin D nivån jämfört med hudens produktion. När jag undersökte sambandet mellan vitamin D nivån i blodet och risken att drabbas av benskörhet och benbrott kunde jag i en studie inte se något samband, men
i en annan studie fann jag att låga vitamin D nivåer ökade risken för att få ett benbrott. Det var dock endast 5% av deltagarna i den undersökta kohorten som hade så låga nivåer. D vitaminbrist av den grad att det leder till benbrott verkar alltså inte vara vanligt i Sverige. Vidare fann jag att både låga, men även höga nivåer av vitamin D i blodet ökade risken för att dö i för tid, ett tecken på en generellt ökad sjuklighet.

De tre olika metoder för att analysera vitamin D i blodet som jag jämförde skiljde sig åt väsentligt i dels de vården de uppmätte, och dels i tillförlitlig-
het. Skillnaderna var så stora att en person kunde bedömas lida av vitamin D brist om den ena metoden användes, men inte med de andra metoderna. Sto-
ra skillnader mellan analysmetoder kan också innebära problem att jämföra resultat från olika studier om olika metoder används.

Då solljuset är den viktigaste vitamin D källan borde innevånare i Norden ha lägre vitamin D nivåer i blodet än innevånare i t ex Sydeuropa, eftersom solen står så lågt vintertid i Sverige att vi inte kan bilda något D vitamin. Flera studier har dock visat att så inte är fallet. Jag fann att våra gener spelar stor roll för vitamin D nivåer i blodet sommartid men inte vintertid. Detta skulle kunna vara en anpassning av vår hud till ett nordligt klimat för att kunna producera så mycket D vitamin som möjligt på sommaren och därmed skapa en reserv inför vintern. Att våra gener påverkar vitamin D nivån skulle också kunna förklara varför vi har högre nivåer i Norden än i Sydeuropa.

Sammanfattningsvis visar min avhandling att, förutom solexponering och kostintag, har våra gener betydelse för vitamin D nivåer i blodet. Vid analys av vitamin D i blodet måste den metod som använts beaktas. Låga, men även höga, nivåer av vitamin D kan vara skadliga. Vitamin D brist är inte en stor orsak till benskörhet och benbrott bland svenska män och kvinnor.
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