Vitamin A and Osteoporosis

Experimental and Clinical Studies

BY

SARA JOHANSSON
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Abstract

Vitamin A in high doses is severely toxic to the rat skeleton, and the active metabolite retinoic acid (RA) can induce bone resorption in vitro. An excessive dietary intake of vitamin A has been associated with reduced bone mineral density and an increased risk of hip fracture. In this thesis, mechanisms of vitamin A toxicity have been investigated.

In the human osteosarcoma cell line MG-63 and in human primary osteoblast-like cultures, stimulation with RA decreased expression of osteoprotegerin (OPG), a potent inhibitor of osteoclast formation and activity. Expression of receptor activator of NF-κB ligand (RANKL), which stimulates osteoclastogenesis, was induced. This increase of the RANKL/OPG ratio is a likely mechanism of RA-induced bone resorption.

An interaction between vitamin A and D was demonstrated in humans for the first time. Fifteen mg retinyl palmitate antagonized the serum calcium-increasing effect of 2 μg 1,25-(OH)2-D3. This antagonism did not appear to be mediated via PTH.

Rats with subclinical hypervitaminosis A after 3 months’ exposure to approximately 9,000 IU retinyl palmitate per day had decreased bone strength, as measured by three-point-bending analysis of femur. Bone diameter and volume, but not bone mineral density, were reduced, suggesting the use of measurements other than BMD for evaluation of early hypervitaminosis A. Indirect mechanisms of toxicity may develop over time, since serum levels of other fat-soluble vitamins were decreased.

In summary, vitamin A can increase bone fragility in the rat at doses considerably lower than previously shown. The regulation of RANKL/OPG is a likely pathway for direct effects of vitamin A in bone. An antagonistic effect between vitamin A and vitamin D has been demonstrated in humans, suggesting indirect mechanisms for vitamin A toxicity.

Keywords: vitamin A, bone, osteoporosis

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To Gullan
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# ABBREVIATIONS

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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>α-MEM</td>
<td>alpha modification of minimum essential medium Eagle</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>BMC</td>
<td>bone mineral content</td>
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<tr>
<td>BMD</td>
<td>bone mineral density</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>CRABP</td>
<td>cellular retinoic acid-binding protein</td>
</tr>
<tr>
<td>CRBP</td>
<td>cellular retinol-binding protein</td>
</tr>
<tr>
<td>CrossLaps</td>
<td>C-telopeptide of type I collagen</td>
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<tr>
<td>CYP</td>
<td>cytochrome P450 enzyme</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbant assay</td>
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<tr>
<td>FAO</td>
<td>Food and Agriculture Organization of the United Nations</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
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<td>FGF</td>
<td>fibroblast growth factor</td>
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<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
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<td>IGF</td>
<td>insulin-like growth factor</td>
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<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>LRAT</td>
<td>lecithin-retinol acyltransferase</td>
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<tr>
<td>M-CSF</td>
<td>macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor kappa-beta</td>
</tr>
<tr>
<td>NHANES</td>
<td>National Health and Nutrition Examination Survey</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegerin</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
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<tr>
<td>PTH</td>
<td>parathyroid hormone</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pQCT</td>
<td>peripheral quantitative computed tomography</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>RAE</td>
<td>retinol activity equivalents</td>
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<tr>
<td>RANK</td>
<td>receptor activator of NF-κB</td>
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<tr>
<td>RANKL</td>
<td>receptor activator of NF-κB ligand</td>
</tr>
<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
</tr>
<tr>
<td>RARE</td>
<td>retinoic acid responsive element</td>
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</tbody>
</table>
RBP  retinol-binding protein
RDA  recommended dietary allowance
RDI  recommended dietary intake
RE   retinol equivalent
REs  retinyl esters
RIA  radio-immuno assay
RNA  ribonucleic acid
RXR  retinoid X receptor
RXRE retinoid X responsive element
S    serum
TGF-β transforming growth factor-beta
TNF-α tumor necrosis factor-alpha
TRAP tartrate-resistant acid phosphatase
VDR  vitamin D receptor
WHO  World Health Organization
10 × C, 50 × C 10 times control, 50 times control
1,25-(OH)2- vitamin D3, 1,25-dihydroxy-vitamin D3
INTRODUCTION

BONE

The skeleton gives stability to the soft parts of the body. It protects the brain and the internal organs of the thorax, and it provides muscular attachments and levers, thus enabling movement. The cavities of the long bones and vertebrae contain the bone marrow, where blood cells are formed. Bone tissue is also an important reservoir of calcium and other minerals and is therefore central in mineral homeostasis.

There are two types of mature bone, cortical and cancellous bone, which differ morphologically and functionally. Cortical bone is dominating in the diaphyses of the long bones. It is compact, and its most important task is to give mechanical strength to the skeleton. Cancellous bone is found in the trabecular bone network in the vertebrae, pelvis and in the epiphyses of the long bones. Despite its smaller volume, the surface of the cancellous bone is larger than the cortical surface. It is in close contact with the bone marrow, and is considered to be metabolically more active than cortical bone (1). Osteoblasts, osteoclasts and osteocytes are the bone cells of major importance for production and maintenance of bone tissue, and for regulation of its metabolic function. The precursors of the bone cells originate from the bone marrow cavity and, when needed, move to skeletal sites for differentiation and proliferation.

Osteoblasts and bone formation

Osteoblasts, the cells responsible for bone formation, are of mesenchymal origin. Bone formation starts with the secretion of an extracellular matrix, consisting mainly of type I collagen (90-95%), but also of several proteoglycans and non-collagenous proteins such as osteocalcin and bone sialoprotein (2). The deposition of hydroxyapatite crystals of calcium and phosphate salts converts the matrix into hard bone tissue. This combination of collagen and mineral salts makes bone tissue both elastic and strong.

The differentiated, mature, active osteoblasts lie together at the bone surface, and are characterized by a cuboidal shape, high alkaline phosphatase activity and expression of bone matrix proteins (2).

When the osteoblast has enclosed itself in mineralized matrix, it flattens and remains in its lacunae as an osteocyte, connected to other osteocytes and to blood vessels via cytoplasmic processes through bone canaliculi (1).
osteocyte is active in the maintenance of the bone tissue, and is proposed to be a sensor and mediator of the beneficial effect of mechanical stress on bone mass (3). The lining cells are inactive, flat cells resting on the bone surface. An ability of lining cells to differentiate into active osteoblastic cells upon mechanical stimulation has been reported (1,4).

**Osteoclasts and bone resorption**

Bone resorption is initiated by the activation of osteoclasts. They are derived from progenitor cells from the monocyte/macrophage lineage, which fuse and differentiate into multinucleated giant cells. Upon activation, the osteoclast cytoskeleton is rearranged and forms a tightly sealed compartment between the ruffled border, which is a highly folded part of the plasma membrane, and the bone surface. By the transport of H+ ions and the secretion of proteolytic enzymes, e.g. tartrate-resistant acid phosphatase (TRAP) and cathepsin K, into this resorption pit, the underlying bone is eroded (2). Degradation products are endocytosed, and transported via the osteoclast cytoplasm to the circulation (2).

The differentiated osteoclast is large and extensively branched. It lies on the bone surface and is characterized by its multiple nuclei and by the ruffled border. Measurement of TRAP activity is used as an osteoclast marker (5).

**Bone growth and remodeling**

In the beginning of life, bone formation occurs via two different mechanisms. Intramembranous ossification is a direct transformation of mesenchymal cells into osteoblasts, which synthesize bone tissue. The flat bones of the skull and a few other bones are formed this way. In endochondral ossification, the mesenchymal cells first develop into chondrocytes. They produce a cartilage matrix which is mineralized and used as a template for the new bone. After apoptosis of the chondrocytes, the cartilage is invaded by osteoblasts and transformed into bone tissue by the deposition of bone matrix. The long bones, spine and ribs are formed this way. Cartilage remains in two places: the articular cartilage and the epiphyseal plate, which connects the bony epiphyses with the diaphysis. By growth of the epiphyseal plate, the long bones grow longitudinally. The width of the long bones increases when formation of new bone on the periosteal, external surface of the diaphysis exceeds resorption on the endosteal side, a process called apposition. After closure of the epiphyseal plates at the end of puberty, longitudinal growth is not possible but apposition can still occur.

In the growing skeleton of children and adolescents, bone formation dominates over resorption, the metabolic activity is high and bone mass is increased. Peak bone mass is acquired around the age of 25-30 years. Later in life, bone mass is continuously removed and rebuilt in a process called bone remodeling (see Figure 1). Resorption always precedes formation, but the phases are tightly linked. Remodeling, which is going on simultaneously
at numerous sites throughout the skeleton, permits maintenance of bone shape during growth and repair of injuries, and is the prerequisite of the tight regulation of serum (S) calcium levels. The net effect of bone remodeling is determined by the balance between the activity of osteoclasts and osteoblasts, which is physiologically controlled by a complex network of

Figure 1. The remodeling sequence. Resting bone with the bone surface covered with lining cells (A). When bone resorption is initiated, the lining cells retract. Osteoclast precursor cells fuse and become activated osteoclasts, which move in and start to resorb bone (B). Meanwhile, osteoblastic precursors differentiate into osteoblasts. The osteoclasts are removed from the resorption pit, and are replaced by mature osteoblasts (C). The osteoblasts synthesize new bone matrix, which is subsequently mineralized (D). Some osteoblasts are captured in the bone tissue (osteocytes) and some remain at the bone surface (lining cells) (E).
endocrine hormones as well as paracrine and autocrine signaling (6,7). Bone mass is preserved in healthy young adults, but in the middle-aged and elderly, resorption dominates over formation, leading to a loss of bone mass.

**Regulation of bone remodeling – the RANK/RANKL/OPG system**

Bone remodeling begins with the activation of osteoclast progenitor cells to form osteoclasts. Fusion of the precursor cells and subsequent differentiation requires stimulation by M-CSF (8), but it has long been known that cell-cell interaction with cells of the osteoblastic lineage is also needed. In 1997-98 the molecular constituents of this interaction were identified. Receptor activator of NF-κB ligand (RANKL) is expressed by osteoblastic cells, and binds to its receptor RANK present on the surface of the osteoclastic progenitors (9-12), thereby stimulating the proliferation and differentiation of the progenitors and the activation of the mature osteoclasts. Osteoprotegerin (OPG), belonging to the same TNF receptor superfamily as RANK, is a soluble protein also produced by osteoblastic cells. It is secreted, and acts via binding to RANKL, thereby blocking its interaction with RANK and neutralizing its osteoclast activating effects (figure 2) (13-15). The importance of these factors as regulators of osteoclastogenesis is apparent. Over-expression of OPG in the liver of transgenic mice, causing high circulating levels of OPG generates an osteopetrotic phenotype (13) while the OPG knock-out mice have severe, early onset osteoporosis (16,17). Administration of RANKL increases bone resorption in mice, and is associated with systemic hypercalcemia (10), and both the RANKL and RANK knock-out mice have profound osteopetrosis and lack osteoclasts (18,19). After RANKL activation, RANK interferes with TNF receptor-associated family members (TRAFs), thereby stimulating intracellular signaling pathways leading to activation of transcription factors and transcription of osteoclast specific genes (11,12). The ability of pre-osteoblastic/osteoblastic cells to produce RANKL and activate osteoclastogenesis is greatest in the less differentiated cells. The more differentiated osteoblasts instead increase the production of OPG, thereby avoiding the stimulation of resorption at the moment when formation is about to begin (20).

Apart from the RANK/RANKL/OPG system, there are other molecules that act locally in bone. For example, the inflammatory cytokines IL-1 and TNF-α are potent inducers of bone resorption and they can also inhibit bone formation (21). On the endocrine level, several factors such as PTH, vitamin D, sex steroids, cortisol and thyroid hormone can affect the remodeling balance, with apparent effects on bone mass (22-28). However, the RANK/RANKL/OPG system constitutes the essential regulatory components in the paracrine signaling between bone cells. According to current knowledge, these molecules are the final regulators of bone remodeling and most of the other cytokines and hormones are considered to be modulators of bone remodeling rather than necessary factors.
Most of the modulators, though signaling via osteoblasts, primarily affect osteoclast differentiation and resorptive activity. Less is known about the regulation of bone formation. Direct stimulation of the proliferation and differentiation of osteoblastic progenitors is exerted by growth factors such as FGFs, IGF-1 and TGF-β released from the eroded bone or produced by the bone cells (29,30), and a regulatory effect of bone matrix proteins is also likely (31). Leptin was first described as neuro-endocrine inhibitor of bone formation, but may also act as an autocrine factor in bone tissue in a way that promotes bone formation (32). Mechanical stress has a positive effect on bone mass via mechanisms only partly known (33,34).

Mineralization, which completes the remodeling cycle, is not well characterized with regard to its regulation. A calcium-binding role for the bone matrix proteins, e.g. osteocalcin has been established (35).

Figure 2. Cells of the osteoblastic lineage stimulate osteoclast precursor cells to fuse and differentiate via production and cell surface exposure of receptor activator for NF-κB ligand (RANKL), which binds to its receptor RANK on the osteoclast precursor cells. Osteoprotegerin (OPG) is a secreted protein produced by osteoblastic cells. OPG acts via binding to RANKL, thereby blocking its interaction with RANK and neutralizing its osteoclast activating effects.
OSTEOPOROSIS

The bone remodeling process is essential for maintenance of the skeletal functions, but also makes the bone sensitive to disturbances leading to disease. Osteoporosis reflects an imbalance in the remodeling process in favor of bone resorption, leading to “low bone mass, microarchitectural deterioration of bone tissue and a consequent increase in fracture risk” (36). The increased risk of fracture after low-energy trauma is the major complication of the disease.

The hip, spine and distal radius are typical sites for osteoporotic fractures with hip fractures being particularly associated with functional impairment and increased mortality for the individual, and high costs for society (37,38). The absolute number of hip fractures in Sweden is around 18,000 per year, and the calculated cost is around 1.1% of the total direct medical care costs or 2-3 billion kronor per year (39). The incidence of hip fracture is remarkably high for both men and women in the Scandinavian countries (40-42). In Sweden, the life-time risk for a 50 year old person of suffering an osteoporotic fracture is approximately 50% for women, and 25% for men (39).

In 1994, WHO defined female osteoporosis as a bone mineral density (BMD) of 2.5 standard deviations or more below the mean value for healthy, young adults (43). However, low BMD alone can not explain all osteoporotic fractures (44). According to the Study of Osteoporotic Fractures, total hip BMD can predict only 28% of hip fractures (45), and elderly individuals have a several-fold increased hip fracture risk compared to young individuals with the same BMD (46). Thus, there are other factors of interest in the search for variables that explain and predict osteoporotic fractures. Among these are the intrinsic mechanical quality (material properties) of the bone tissue and its spatial distribution (geometry) (44). An increased rate of bone remodeling, apart from bringing about a loss of bone mass, is itself a risk factor for fracture, and has been proposed as a major cause of bone fragility (47). Also the quality of bone matrix and its degree of mineralization can be of importance (48,49).

Postmenopausal osteoporosis is caused by multiple factors, both genetic and environmental. It is a multigenetic disease, i.e. several genes with modest effects on bone mass are involved. Around 50–90% of the interindividual variance in BMD is considered to be genetically determined, but the genetic influence is less pronounced with increasing age (50-52). Advanced age and female sex are risk factors for osteoporosis which can, at least in part, be attributed to postmenopausal estrogen deficiency. However, men are also at risk for osteoporosis (53), and both men and women are dependent on estrogen for maintenance of bone mass (54). Other risk factors are height (39), low body weight (55), smoking (56), low physical activity or immobilization (34,57), low exposure to sunlight (58) and dietary factors such as calcium and vitamin intake (see below). Secondary osteoporosis can
be induced by corticosteroid treatment, hyperthyroidism, alcohol abuse and skeletal malignancies, among other factors (53). The fracture risk is also dependent on the tendency to fall, which is influenced by loss of balance, muscle weakness, visual impairment, and the environment (59,60).

CALCIUM, VITAMIN D AND BONE

Calcium
Calcium is important for cells throughout the body, as a co-factor for proteins, for control of neuronal excitability and as an intracellular 2nd messenger. Therefore the calcium level of the extracellular compartment is kept constant through delicate homeostasis. In the skeleton, deposition of calcium salts simultaneously increases bone strength and provides a store of calcium, and about 99% of the body’s calcium content is found in the tissues of bone and teeth. Calcium absorption and preservation in the body is not very efficient. Intestinal absorption can be as low as 15% (61), and the ability to adapt to a low calcium intake is reduced after menopause and in elderly people (62). Renal conservation of calcium also deteriorates after menopause (63).

The skeleton, intestine and kidney are central in the regulation of calcium homeostasis. Calcium absorption in the intestine is facilitated by vitamin D, especially under conditions of low calcium intake (64). Via a brush border calcium channel and the intracellular calcium-binding protein calbindinD9k, both largely vitamin D-dependent, calcium is actively absorbed and transported to the circulation (65). If abundant, calcium is deposited in bone tissue via mineralization. On the contrary, a low calcium level leads to an instantaneous release of PTH from the parathyroid glands. PTH increases activation of vitamin D in the kidney, with secondary positive effects on intestinal calcium absorption. PTH also increases reabsorption of calcium from the urine and mobilizes calcium via direct effects on bone (66). Via these mechanisms, the overall goal to increase serum calcium to normal is achieved. Since the need for calcium mobilization overrides other functions of the skeleton, calcium homeostasis is of major importance for the balance of bone remodeling (30).

Vitamin D
Vitamin D is produced endogenously in the skin where 7-dehydrocholesterol is converted to cholecalciferol (vitamin D3) in response to exposure to ultraviolet light (67). Vitamin D3 and the less common ergocalciferol (vitamin D2) can also be obtained from the diet, via absorption in the small intestine and transport in chylomicrons to the circulation. During the darker period of the year, people in Sweden and other countries at high latitude have a low
endogenous production of vitamin D, and hence become more dependent on dietary intake. Young people can use stores of vitamin D produced during the summer, but elderly people have a reduced capacity for endogenous production and therefore reduced storage capacity, and are at risk of vitamin D deficiency (64).

The active compound 1,25-(OH)₂-vitamin D₃ (calcitriol) is produced via successive 25- and 1α-hydroxylation in the liver and kidney, respectively (67). Since 25-hydroxylation in the liver is poorly regulated, 25-OH-vitamin D is commonly used as an indicator of vitamin D status. A 24-hydroxylase, present in the kidney, liver and target tissues, can also convert 1,25-(OH)₂-vitamin D₃ into an inactive metabolite, thus initiating the major catabolic pathway (68). 1,25-(OH)₂-vitamin D₃ exerts vitamin D activity via binding to the genome in complex with the vitamin D receptor (VDR), thereby regulating the expression of certain genes. For some very rapid responses to vitamin D treatment, among them the rapid stimulation of intestinal calcium absorption, an alternative signaling pathway including cell surface receptors has been proposed (69). The classic vitamin D target tissues include the intestine, the kidney, the parathyroids and bone. Vitamin D also has nonclassic actions in many other organs where it regulates cell differentiation and proliferation (64).

Despite the usage of vitamin D for the treatment of osteoporosis and other metabolic bone diseases, the mechanism by which it is beneficial for bone is not exactly known. 1,25-(OH)₂-vitamin D₃ can induce differentiation of osteoclastic cells, but indirectly via osteoblastic cells (64). Interestingly, a physiological vitamin D dose inhibits bone resorption while a pharmacological dose stimulates resorption (70-72). The characteristic disease of vitamin D deficiency is rickets or, in the adult, osteomalacia, i.e. incomplete mineralization. However, the skeletal symptoms of the VDR knock-out mice – hypocalcemia and rickets – are rescued by a high calcium diet (73,74). Therefore it has been assumed that the function of vitamin D is to provide calcium levels sufficient for mineralization rather than acting directly in the mineralization process.

**Intake levels in relation to bone health**

Several randomized trials have shown a positive effect of calcium supplementation on BMD ((75,76) and references therein) and animals fed a calcium-deprived diet suffer from a PTH-dependent loss of bone mass (61). It is suggested that an insufficient calcium intake leads to decreased bone gain during growth and bone loss in the adult (75). Despite this, a large number of observational studies have come to different conclusions about the effect of dietary calcium intake on bone health (77), and there is no consensus about the optimal level of calcium intake. Though an increased dietary intake of vitamin D has been associated with decreased risk of hip fracture, the few clinical trials about vitamin D supplementation and fracture risk are conflict-
Since no study has proven an effect of calcium supplementation independent of vitamin D on osteoporotic fractures, a combination of calcium and vitamin D supplement is recommended as osteoporosis treatment in many countries, including Sweden (39).

**VITAMIN A – A GENERAL BACKGROUND**

Vitamin A is a group of fat-soluble nutrients which are essential for cell proliferation and differentiation in many different tissues. They are involved in important physiological processes during embryogenesis, in the immune system, in reproduction, and for maintenance of the skin and mucous membranes. In the eye, the retinol metabolites have a unique role in the visual cycle (79).

In the diet, two main groups of vitamin A can be distinguished: retinoids or preformed vitamin A, including retinol and its metabolites, and carotenoids or provitamin A (see figure 3). Retinoids are found in animal products such as liver and dairy products, and vitamin supplements are also an important source (80-82). Carotenoids, for example \( \beta \)-carotene, come from vegetable sources (83).

**Nomenclature**

Biochemically, the term retinoid is defined as a substance that can exert biologic vitamin A activity, via binding to and activation of a specific receptor, “with the program for the biologic response of the target cell residing in the retinoid receptor rather than in the ligand itself” (84). This definition includes the dietary retinoids but also synthetic retinoids, substances that are often more active than the naturally occurring compounds. Concerning the carotenoids, only about 10% of them can exert vitamin A activity (85), and only after conversion in the body to retinol. They are therefore not included in the term retinoids.

In this thesis, both “vitamin A” and “retinoids” will be used as alternate, general terms for the group of naturally occurring substances that can readily exert retinoid activity, i.e. not including the synthetic retinoids or the carotenoids. Specific metabolites will be denoted by their chemical names.

**Definition of vitamin A activity**

FAO/WHO defined the golden standard for calculation of vitamin A activity in the diet. The unit 1 µg retinol equivalent (RE) was defined as equal to the activity of 1 µg retinol or 3.33 international units (IU) of vitamin A activity from retinol (86). Moreover 1 µg RE was defined as equal to 6 µg of beta-carotene or 12 µg of other carotenoids. Because of increasing knowledge about the poor bioconversion of provitamin A compounds, a new definition termed retinol activity equivalents (RAEs) has been proposed. One micro-
The recommended dietary intake (RDI) of vitamin A stated by FAO/WHO is 600 µg RE/d for men and 500 µg RE/d for women (87). However, there is an uncertainty about the adequate intake, and therefore quite large differences between countries. The Nordic recommendations are 900 µg RE/d for men and 800 µg RE/d for women (88). The actual intake in the population also varies considerably. In the Nurses’ Health Study, 14% of the women had an intake below the recommended dietary allowance (RDA, used in the United States) of 700 µg RAE/d, while 21% had an intake exceeding the tolerable
upper limit which is set at 3,000 µg RAE/d (82,89). The average vitamin A intake in Sweden is 1,300 µg RE/d for men and 1,100 µg RE/d for women (90), which is high compared to countries in southern Europe (91). In Norway, the intake is even higher (1,900 µg/d for men and 1,600 µg/d for women (92)).

**Vitamin A metabolism**

The uptake, transport and storage of vitamin A are well-regulated, with the goal of providing the body with correct amounts of retinoids even when dietary intake fluctuates. Retinoids occur in the diet mainly as retinyl esters (REs). They are hydrolyzed to retinol and free fatty acids in the intestine and absorbed from the lumen. Carotenoids, mainly β-carotene, diffuse into the epithelial cells where they are cleaved by a dioxygenase (93). The resulting two retinal molecules are reduced to retinol by a retinal reductase (94). In the luminal epithelial cell, retinol is bound to the cellular retinol-binding protein-II (CRBP-II) (95). Retinol is re-esterified, mainly with palmitate, by lecithin-retinol acyltransferase (LRAT) (96), incorporated into chylomicrons and transported via the lymph to the blood circulation (97). The chylomicron remnants are cleared by the liver where the REs are hydrolyzed and re-esterified by hepatic hydrolases and LRAT, respectively. During these processes, the free retinol is bound to CRBP-I, and transported to stellate cells where re-esterification and storage of the resulting REs occur. Some other organs, such as the kidneys, also have storage capacity (98). CRBP-I shares structural, genetic and biochemical properties with CRBP-II but is expressed in various tissues (99). CRBP-II is almost exclusively expressed in the cells of the small intestine where it is one of the most abundant proteins. Both proteins have a function in the enzymatic conversions of vitamin A metabolism, and CRBP-II possibly also in the absorption of retinol from the intestinal lumen (100). Apart from this generally accepted description of retinoid uptake and storage, there are also reports that small amounts of retinoic acid can be transported from the intestine via the portal vein (98,100).

When there is a need for retinoids in the body, the stored REs are hydrolyzed and released to the circulation as retinol bound to retinol-binding protein (RBP) (101). RBP has been considered to protect the body from toxic effects of free retinol. However, RBP-knockout mice were essentially unaffected (102). In the target tissues, retinol is transported across the plasma membrane through a mechanism that is still unclear. In the eye, retinol is transformed stepwise to 11-cis-retinal, which together with opsin forms rhodopsin, the visual cycle chromophore in photoreceptor cells (103). In most other tissues, including bone, the active metabolite is retinoic acid (RA).
Figure 4. Vitamin A metabolism. Retinoids (retinyl esters) from the diet are hydrolyzed to retinol and free fatty acids in the intestinal lumen before absorption. Carotenoids (beta-carotene) are cleaved to retinal after absorption, and further reduced to retinol. Retinol from both sources is esterified and incorporated into chylomicrons, which are transported via the lymph to the circulation. In the liver, retinyl esters are hydrolyzed to retinol, which is either released to the circulation bound to retinol-binding protein (RBP) or re-esterified and stored in the liver as retinyl esters. In the target tissues, retinol is converted to the active metabolite retinoic acid, which binds the retinoic acid receptor-retinoid X receptor (RAR-RXR) dimer. The receptor-ligand complex acts as a transcription factor in the nucleus.

In RA synthesis, retinoids bound to CRBP-I are believed to be the dominating substrates. A retinol dehydrogenase oxidizes retinol to retinal, which is further oxidized by retinal dehydrogenase to RA (104). The last step is irreversible, and ends with the transferal of RA from CRBP-I to cellular retinoic acid-binding protein (CRABP) (105). Tissue RA is inactivated by hydroxylation and oxidation to more polar and less active substances. The
conversion is catalyzed by cytochrome P450 enzymes (98), of which the most important is CYP26 whose expression is also induced by RA (106,107). RA and retinol can also be converted to retinoyl β-glucoronide and retinyl β-glucoronide, respectively, mainly in the liver and intestine (105). These compounds are less toxic and are secreted in the bile, but the physiological importance of this route of inactivation is uncertain.

**Serum levels of vitamin A metabolites**

Retinol, bound to RBP, is the most abundant of the retinoid metabolites in serum. The normal value is poorly defined, but is usually considered to be approximately 1-2 µM (108). S-retinol (< 0.7 µM) is used as a marker of retinoid deficiency, though it is not very sensitive. Not until liver levels of vitamin A are very low does S-retinol decrease (109). S-RA levels are low, around 4-14 nM (98), but may contribute to the delivery of retinoids to target tissues (110). S-REs normally increase postprandially, and there is a five-fold interindividual difference in clearance from serum (half-life 1.54-9.90 h after a single intake of 50,000 IU) (111). In cases of vitamin A intoxication, S-REs are elevated for extended periods. It is generally accepted that the normal S-RE level is <5-10% of circulating vitamin A and that S-RE >10% is an indication of chronic vitamin A toxicity (83). In absolute numbers, this corresponds to a normal value <244 nM (112).

**Molecular action of vitamin A – regulation of gene transcription**

The identification of the first retinoid receptor in 1987 was a breakthrough in the vitamin A field (113,114). Since then, five more receptors have been cloned and the retinoid receptors known today include retinoic acid receptor (RAR) α, β and γ, and the retinoid X receptor (RXR) α, β and γ (115). However, many more isoforms exist, due to different splicing and promoter usage. Both the all-trans-RA and 9-cis-RA metabolites can bind to the RARs while the RXRs bind only 9-cis-RA (116).

RARs and RXRs are members of the steroid-thyroid hormone receptor family, which also includes the vitamin D receptor (VDR) and the thyroid hormone receptor. These receptors act as heterodimers, and RXR is the common dimerization partner, acting together with RAR, VDR or any other receptor in the family (117). Binding of ligand, for example RA binding to RAR, activates the dimer complex. The RXR ligand 9-cis-RA has been shown to be a modulator of heterodimer action in vitro, and has been proposed as a possible modulator of vitamin D, thyroid and steroid hormone signaling (118). However, the physiological relevance of these findings is controversial. A recent in vivo study on mouse embryos indicates that transcriptional regulation by 9-cis-RA is a pharmacological phenomenon, but that 9-cis-RA is not necessary under physiological conditions (119). Even the existence of 9-cis-RA in vivo is questioned, since it has been undetect-
able after its initial discovery. RA will hereon be used to refer to all-trans-RA, unless otherwise is stated.

In the classical signaling pathway, ligand-binding activates the RAR-RXR complex, leading to conformational changes of the heterodimer (115). The activated complex binds to specific DNA sequences called retinoid response elements (RAREs or RXREs), which are transcription enhancers upstream or downstream the transcription start site. Ligand-activated RAR-RXR functions as a transcription factor with transactivating or, less commonly, transrepressive effects (115). In the absence of ligand, the heterodimer can repress transcription of target genes (120). Transcriptional control involves interaction with co-activator and co-repressor proteins (121).

The genes of many proteins of importance for retinoid metabolism, such as RARs, CRBPs and CRABPs, contain RAREs (115,122). However, some genes also lack RAREs, even though their expression is affected by retinoids. This regulation is called indirect, as opposed to the direct regulation via the classical RA pathway. Indirect regulation can entail activation of receptors other than RAR-RXR, influence on mRNA stability, interference with other transcription factors or regulation of an intermediary factor (122). Of course this distinction is not easily made for many of the known RA-regulated genes.

It is largely unknown how retinoid signaling is regulated. The uptake of retinol in the target cell, the rate of retinol esterification to REs or oxidation to RA, the expression of receptors and retinoid binding proteins are all possible regulative stages. In this perspective, Raldh2 and other retinal dehydrogenases have been pointed out as key factors during development (123). Regulation of LRAT and CYP26 expression can serve to maintain retinoid homeostasis (124). Cell-specific action can be achieved by variation of receptor expression, isoforms and co-factors.

The physiological role of vitamin A in bone
In general, a major role of vitamin A is to regulate proliferation and differentiation of cells, and this is a likely role of vitamin A in bone tissue as well. Both osteoblastic and osteoclastic cells express retinoid receptors. RARα, RARβ, RARγ and RXRα have been found in osteoblasts (125), which also express CRBP-I, CRABP-I and low levels of CRABP-II. In osteoclasts, the expression of RARα and RXRβ has been demonstrated (126). A direct, up-regulatory effect of RA is reported for at least the RARα, β and γ, CRABP-II and CRBP-I genes (122,127,128). The generation of mice with combined knock-outs of the retinoid receptors has emphasized the importance of vitamin A during development, but since the mutants died in utero or shortly after birth, our knowledge about how they function in the adult skeleton was hardly increased (reviewed in (129)).
In bone cells, known targets for RA gene activation in vitro are the genes for alkaline phosphatase (130,131), osteocalcin (132), osteopontin (133) and osteonectin (134) which are induced in osteoblasts, and the cathepsin K/OC-2 (126) and osteopontin (135) genes, which are induced in osteoclasts. RA treatment also influences the expression of collagen and collagenase (136,137), and vitamin A stimulates bone resorption and osteoclast number and activity both in vitro and in vivo.

The expression and regulation of genes involved in retinoid signaling and metabolism, and the induction of bone-specific genes by RA indicate a physiological role for vitamin A in bone tissue.

VITAMIN A TOXICITY AND BONE

Both deficiency and overdose of vitamin A are hazardous for the body. Vitamin A deficiency usually does not occur in well-nourished populations, but in the rest of the world it is one of the major nutritional problems. The opposite situation, vitamin A intoxication or hypervitaminosis A, with special regard to skeletal toxicity, is the subject of the rest of this thesis. Since carotenoids do not cause hypervitaminosis A in animal experiments, and there are no reports about vitamin A toxicity even after massive overdose of carotenoids (138,139), the following work will focus on the toxicity of retinoids.

General vitamin A toxicity

The first description of vitamin A intoxication, written in 1596, tells about a group of Dutch polar explorers complaining of headache, dizziness, and vomiting after eating polar bear liver (81). There are several more reports about acute toxic reactions, and even one death, after ingestion of bear and seal liver. Today the common cause is overdose of vitamin A supplements. However, acute toxicity is relatively rare. It occurs within a short time period (hours up to a few days) after ingestion of one single high dose, in adults usually of 500,000 IU or more (over 150 times the RDI) (140). Children are more sensitive. The symptoms include headache, nausea, mental confusion, fatigue, dry skin, bulging of the fontanel, hydrocephalus and toxic hepatitis (140). Chronic toxicity presents more insidiously, but with similar symptoms: dryness and desquamation of the skin and mucous membranes, alopecia, nausea and vomiting, loss of appetite and weight, fatigue, headache and bone and joint pain (81).

Because of the accumulation of retinoids in the liver, chronic toxicity can arise at intake levels much lower than those causing acute toxicity. The duration of intake is crucial, and chronic toxicity occurs after months or years of increased consumption (139). The lowest dose causing chronic hypervitaminosis A in humans is not known. It usually appears at doses of 100,000 IU (>30 times RDI) per day and above (81), but has been reported at intake
levels as low as 25,000-50,000 IU daily (141-143). The suggested threshold
dose for teratogenicity is 3 mg or 10,000 IU per day (144). Vitamin A in
water-miscible, emulsified and solid forms are more toxic than oil-based
preparations or retinol in liver, and result in higher peak plasma values,
higher liver concentrations and lower fecal losses (145).

The evaluation of reported human data is complicated. Intake of other nu-
trients or toxins (e.g. high alcohol intake), and the storage capacity of the
liver, which is affected by liver disease and prior vitamin A intake, influence
the dose and time needed for development of toxicity symptoms (139,145).
Moreover, it is difficult to estimate the dietary intake of vitamin A, and in
many studies the results have been blurred because the researchers have not
differentiated between retinoid and carotenoid intake. Subclinical adverse
effects are probably evident at intake levels considerably lower than those
reported for general, clinical toxicity.

**Bone toxicity of vitamin A in rats**

In 1925, the adverse effects of hypervitaminosis A in rat were described for
the first time by Takahashi et al who treated rats with fish-oil-concentrates in
daily doses around 10,000 times the levels required (146). Takahashi et al
described the general vitamin A toxicity syndrome, including paralysis of the
legs, hemorrhages, weight loss and alopecia of the head. The true skeletal
harmfulness of vitamin A was evident in 1933 when spontaneous fractures
were confirmed by X-ray examination in young rats receiving very high
doses of fish-oil concentrates (147,148). Moore and Wang carried out the
first study on intoxication with pure vitamin A (purified retinyl acetate) in
1945, thereby confirming that the fish-oil toxicity was caused by retinoids
(149). They also reported spontaneous fractures as the most prominent
symptom. Skeletal lesions have also been reported in dogs, pigs, rabbits and
chickens (150).

Fractures, which are found mainly at the diaphysis of the long bones
(147), seem to develop earlier in young rats than in adults, and the lowest
daily dose known to lead to fractures is around 3 mg (10,000 IU) in young
rats and 7.5 mg (25,000 IU) in adult rats (150). Other bone lesions include
thinning of the long bones (151), thinning of the cortex, premature epiphy-
seal closure and accelerated bone remodeling (reviewed in (150) and (143)).

More recent studies, using histomorphometry, have shown that the num-
ber of osteoclasts is increased and osteoid surface decreased in rats treated
with 50,000 IU per day for 6 weeks (152) and in rats treated with 10,000-
25,000 IU per day for 3 weeks (153). Increased S-alkaline phosphatase and
urinary hydroxyproline excretion, indicating increased bone turnover, were
also seen (153).

The effect of moderately elevated doses of vitamin A has rarely been
evaluated. Li et al saw no significant changes in bone ash content or bone
areas in adult rats fed 300 IU retinyl acetate per day for 14 months, although
histomorphometry showed changes in both fractional resorption and formation surfaces (154). This dose is around 5 times the calculated daily amount needed for normal growth of the rat (155). Decreased whole body BMD, as measured by DPX-α bone scanning and ash weight of femur, has been reported after administration of RA at doses ranging from 10 to 100 µg/day for 4 months in young rats (156). Since it is not known how IUs correspond to a certain amount of RA, it is difficult to compare the doses in this study with previous experiments.

**Bone toxicity of vitamin A in vitro**

A bone resorptive effect of vitamin A has been demonstrated both as shrinking of long bones of mouse and chick embryos cultivated in vitamin A-rich medium (157) and as increased ⁴⁵Ca release from cultured fetal rat and mouse calvaria (153,158). Both stimulation of osteoclast numbers and activity (159,160) and decreased growth of osteoblasts (161) have been reported. RA can regulate the expression of the genes for alkaline phosphatase, osteocalcin, and osteonectin in osteoblastic cells (130-132,134), and cathepsin K and osteopontin in osteoclasts (126,135). In embryonic chick calvaria, collagen expression is decreased (136) and collagenase synthesis and collagen degradation increased (137) by retinol.

It is difficult to compare the *in vitro* effects of vitamin A because of the variety of experimental systems (e.g. fetal calvarial cultures, resorption pit assays), cell types (e.g. fetal or adult, different species) and methods for isolation of osteoclasts (pieces of whole bone, bone cell suspensions including both osteoblastic and osteoclastic progenitors, isolated osteoclasts). One study even shows that RA inhibits bone resorption (162). However, altogether the *in vitro* data strongly indicate that vitamin A possesses bone tissue activity, and RA is generally considered to be a bone resorbing agent. The expression of RARs, RXRs, and retinoid binding proteins in bone cells, and their regulation by RA, support this statement (127,128).

**Bone toxicity of vitamin A in humans – case reports**

Several reports have described skeletal pain and abnormalities seen in accidental cases of chronic vitamin A intoxication (163-166). Severe bone pain, hypercalcemia, periosteal calcifications, exostoses and increased bone resorption are among the reported symptoms, and radiographic osteopenia has been seen. Patients with hypercalcemia have normal or decreased PTH levels, indicating that the high calcium level has arisen through a direct effect of vitamin A on bone rather than via PTH (166,167). Hypercalcemia does not always develop, but the reason for this is unclear.

The amount of ingested vitamin A differs between the cases, but is usually about 85,000-125,000 IU per day or above. For water-miscible, emulsified and solid forms of vitamin A, the lowest dose known to induce chronic toxicity is as low as around 43,000 IU per day (0.2 mg/kg per day) (145).
The duration of intake has usually been one or more years, but much shorter periods are reported for water-miscible, emulsified and solid preparations (143,145). One case of fracture in a 1 year old girl has been reported after intake of 50,000 IU daily for 7 months (168).

**Bone toxicity of vitamin A in humans – epidemiological studies**

A number of epidemiological studies have addressed the question of vitamin A and bone health. The information from some of these studies is limited, since they are too small or have not distinguished between retinoid and carotenoid intake.

The first study in which a separate analysis of retinoid and carotenoid intake was performed, came from Melhus et al in 1998 (169). They reported an association between a high intake of dietary vitamin A and reduced BMD and increased risk for hip fracture. BMD was reduced by 10% at the femoral neck and the risk for hip fracture was doubled when the group with an intake exceeding 1.5 mg retinol per day was compared with the group with an intake of 0.5 mg per day or less. This finding has been confirmed by others, using either dietary retinoid intake or S-retinol as a measure of vitamin A exposure (77,82,170,171). In one study, it was shown that men with a S-retinol level exceeding 3.6 µM had a relative risk of 7.14 for any fracture (77).

There are also studies which do not find an association (112,172,173). One explanation to this failure may be the difficulty to correctly estimate vitamin A intake, leading to miscalculations (174). The serum metabolites are not very sensitive markers of high vitamin A intake either. Another explanation for the inconsistency is the fact that vitamin A intake actually varies between populations. Accordingly, mean intake in the different studies varies considerably, and this is likely to affect the outcome. In two studies the association is U-shaped (170,171), and this dual relationship may also explain the failure to demonstrate a linear association in some studies.

**Synthetic retinoids in clinical use**

Pharmacological use of synthetic retinoids has been of great benefit for the treatment of dermatological and malignant diseases. However, this use also has an established skeletal toxicity, as evident from several reports of bone pain and artralgia, calcifications of ligaments and tendons, osteophytes, premature epiphyseal closure and radiographic changes in patients on etretinate or isotretinoin treatment (175-180). Hypercalcemia is reported after long-term treatment (181), but Kindmark et al found a transient decrease of S-calcium as well as of several markers of bone turnover during the first 14 days of isotretinoin treatment (182). Regarding effects on bone mass, this and some other studies found no harmful effects of isotretinoin (182-184), etretinate (185) or acitretin (186) while some studies show decreased BMD after treatment with etretinate (184,187) or isotretinoin (188). Large, pro-
spective studies, which are needed to thoroughly investigate the effects of synthetic retinoids on bone mass, have not been published yet.

In the rat, administration of 5 mg/kg (about 0.8 mg) etretinate daily for 15 days led to decreased tibial breaking strain (-11%) and spontaneous fractures in 1 of 5 rats, without affecting body weight gain (189,190). After administration of 50 mg/kg (about 8 mg) isotretinoin for 15 days, tibial breaking strain was reduced (-15%) but no spontaneous fractures were seen.

**Interaction between vitamin A and vitamin D**

An interaction between vitamin A and D has long been suggested, in part based on clinical observations and in part on the basis of the VDR-RXR heterodimer mediating the molecular action of vitamin D. Accordingly, allosteric receptor ligand interactions have been demonstrated *in vitro* (118), and *in vitro* studies have variously indicated antagonistic, additive or synergistic interaction between the vitamins (191). The recent finding that 9-cis-RA exhibits receptor-binding activity *in vivo* only at pharmacological doses, and the failure to isolate 9-cis-RA *in vivo* (119) makes a direct ligand-mediated interaction with the VDR-RXR complex less likely, but an antagonism can arise via competition between RAR and VDR for formation of RXR heterodimers. Many other mechanisms for interaction, during intestinal absorption, transport, metabolism or storage of the vitamins, are of course possible. For example, RA can induce expression of a 24-OH-hydroxylase which is a key catabolic enzyme for 1,25-(OH)₂-vitamin D₃ (192).

*In vivo*, a high level of vitamin A intake has been shown to reduce the toxicity associated with hypervitaminosis D in the rat (193) and the turkey poult (194) and to increase the need for dietary vitamin D in the chicken (195) and the poult (194). Hypervitaminosis D similarly diminishes vitamin A toxicity symptoms, such as reduced bone ash, in the chicken (196). In rats, an antagonistic relationship has been demonstrated in bone and intestine by Rohde et al 1999 (197). The possible interaction between vitamin A and D in man had not been investigated when this project was initiated. In 2003, a review of all hitherto published case reports of vitamin A intoxication found that the mean dose causing chronic toxicity was lower for patients taking vitamin A only, compared to patients taking supplements containing both vitamin A and vitamin D (145). This finding is in agreement with competitive antagonism between the vitamins.
CONCLUDING REMARKS

In summary, the skeletal toxicity of vitamin A is well documented in animal studies. The potential of vitamin A to affect the human skeleton is evident from case reports of human vitamin A intoxications, and several epidemiological studies have shown an association between an excessive dietary intake of vitamin A and increased fracture risk. However, our knowledge about how vitamin A functions in bone tissue is still limited. Also, the lowest level for vitamin A toxicity is not known.

The possible mechanisms for vitamin A action on bone tissue can be roughly categorized as direct or indirect. Direct effects on the skeleton are likely, since both osteoblastic and osteoclastic cells express retinoid receptors and other proteins involved in vitamin A metabolism. RA can regulate several bone specific proteins and stimulate bone resorption in vitro, but the molecular mechanisms of RA action in bone tissue are still largely unknown.

Among indirect mechanisms, the interaction between vitamin A and D has been in focus. In animals, an antagonistic relationship has been established. The corresponding relationship in man has not been investigated previously.
AIMS OF THE PROJECT

The starting point for this thesis was the publication in 1998 by my tutor Håkan Melhus, who had found that an excessive intake of dietary vitamin A was associated with increased risk of hip fracture. This finding naturally raised the question of a causal relationship, i.e. can vitamin A be involved in the pathogenesis of osteoporosis?

In this project, the effect of vitamin A on bone metabolism and bone quality in vitro and in vivo has been studied. The general aim was to identify possible mechanisms of vitamin A action and to investigate the lowest level for vitamin A toxicity. In specific, the aims were:

- to examine whether vitamin A can antagonize the effect of vitamin D in humans by studying the acute effects of vitamin A and D on calcium homeostasis.

- to study if a long-term excessive vitamin A intake at a level that does not cause general toxicity can adversely affect the rat skeleton and, if so, begin to investigate at what level of intake skeletal lesions manifest.

- to examine how BMD and bone geometry were affected in rats with subclinical hypervitaminosis A.

- to study in vitro the effect of RA on the expression of OPG and RANKL - central molecules in the regulation of osteoclastogenesis.
MATERIALS AND METHODS

The following is a brief summary of materials and methods used in this thesis. A detailed description can be found in the individual papers.

**Subjects (Paper I)**
Four men and five women (mean age 28 years) were recruited. Their healthy status was assessed by an interview and the presence of any undiagnosed parathyroid disease was ruled out by the biochemical analyses of serum performed on the first reference blood sample (see Design). Informed consent was obtained from all subjects volunteering for the study.

**Animals (Paper II and III)**
Forty-five female Sprague-Dawley rats, 3 months of age, were obtained from Möllegaards breeding centre Ltd. (Skensved, Denmark). They were kept in polycarbonate cages with hardwood chips as bedding, using a 12 hour light:dark cycle, and they had free access to water and commercial pellet diet (Lactamin R36, Stockholm, Sweden). Prior to use they were allowed to acclimatize for a minimum of 23 days.

**Cell Cultures (Paper IV)**
Cells from the human osteosarcoma cell line MG-63 and primary human osteoblast-like cultures were used. Primary cultures were isolated from bone fragments taken from patients undergoing surgery. Trabecular bone was cut into pieces, thoroughly rinsed and vortexed in PBS five times. Both cell types were cultured in α-MEM supplemented with 10% FBS, 2 mM L-glutamine and antibiotics. The osteoblastic phenotype of cells in the primary culture was verified by use of biochemical markers as previously described (198). RA was dissolved in 95% ethanol in a dark room under flow of nitrogen. The stock solution was stored at −70 °C and shielded from light until use. The high affinity pan-RAR antagonist (AGN 194310) and the RAR-β,γ-agonist (AGN 190299) were dissolved in dimethyl sulfoxide (DMSO).

**Study Design (Paper I-IV)**
All studies were approved by the Uppsala University ethics committee.
**Paper I**

Nine healthy subjects were included in a double-blind, randomized, cross-over study. On four different study occasions, every subject took one of four different oral vitamin preparations (Table 1).

<table>
<thead>
<tr>
<th>Occasion</th>
<th>Vitamin intake</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>15 mg retinyl palmitate</td>
</tr>
<tr>
<td>2.</td>
<td>2 µg 1,25-(OH)2-vitamin D3</td>
</tr>
<tr>
<td>3.</td>
<td>15 mg retinyl palmitate plus 2 µg 1,25-(OH)2-vitamin D3 (combined intake)</td>
</tr>
<tr>
<td>4.</td>
<td>Placebo</td>
</tr>
</tbody>
</table>

The vitamin intake was always at 22.00, and the effect was monitored by urine and blood samples the following day (Table 2). All samples were analyzed for S-calcium, S-albumin, S-parathyroid hormone and the degradation product of C-telopeptide of type I collagen (S-CrossLaps), and urine calcium and creatinine. Analyses of S-1,25-(OH)2-vitamin D3 and S-REs were performed for the eight o’clock fasting samples only.

<table>
<thead>
<tr>
<th>Time</th>
<th>Day 1</th>
<th>Day 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>08.00</td>
<td>Reference blood sample (fasting)</td>
<td>Blood and urine samples (fasting)</td>
</tr>
<tr>
<td>10.00</td>
<td>Blood sample</td>
<td></td>
</tr>
<tr>
<td>12.00</td>
<td>Blood sample</td>
<td></td>
</tr>
<tr>
<td>14.00</td>
<td>Blood sample</td>
<td></td>
</tr>
<tr>
<td>16.00</td>
<td>Blood sample</td>
<td></td>
</tr>
<tr>
<td>22.00</td>
<td>Vitamin intake</td>
<td></td>
</tr>
</tbody>
</table>

**Paper II and III**

Forty-five mature, female Sprague-Dawley rats were divided into three groups and fed diets with increasing amounts of vitamin A, in the form of retinyl palmitate and retinyl acetate. The diet was either a standard diet containing 12 IU vitamin A/g pellet (control, C) or standard diet supplemented with 120 IU (“10 × C”) or 600 IU (“50 × C”) vitamin A/g pellet. Clinical examination was performed weekly. Body weight was measured at the beginning and at the end of the study. After three months the rats were killed. Internal organs and serum samples were analyzed for retinoid content. The bone quality and strength was tested with three-point bending analysis of the femur and with peripheral quantitative computed tomography (pQCT) of the humerus, and humeral dimensions and composition were determined.
The human osteosarcoma cell line MG-63 and primary cultures were cultured in cell medium which was changed two times a week until subconfluence was achieved. Prior to stimulation with RA, cells were incubated in serum-free medium for 24 hours. RA, at final concentrations ranging from $10^{-6}$ to $10^{-10}$ M, or vehicle (ethanol, not exceeding 0.1%), RAR antagonist or RAR-β,γ-agonist was added in fresh serum-free medium. For measurement of OPG secretion, medium aliquots were collected after 24 hours and the number of cells in each well was counted manually. For RNA isolation, cultures were harvested 2, 4, 8 and 24 hours after stimulation. All sets of experiments were repeated twice.

**Biochemical analysis (Paper I-III)**

The biochemical analyses listed in table 3 are described in paper I, II and III, respectively.

Table 3. *Biochemical analyses*

<table>
<thead>
<tr>
<th>Analysis</th>
<th>Method</th>
<th>Paper</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-1,25-(OH)$_2$-vitamin D$_3$</td>
<td>immunoextraction and RIA</td>
<td>I</td>
</tr>
<tr>
<td>S-calcium, albumin</td>
<td>colorimetric spectrophotometry</td>
<td>I</td>
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<tr>
<td>S-parathyroid hormone</td>
<td>RIA</td>
<td>I</td>
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<tr>
<td>S-CrossLaps</td>
<td>ELISA</td>
<td>I</td>
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<tr>
<td>U-calcium</td>
<td>atomic absorption spectrophotometry</td>
<td>I</td>
</tr>
<tr>
<td>U-creatine</td>
<td>colorimetric spectrophotometry</td>
<td>I</td>
</tr>
<tr>
<td>S- RE</td>
<td>HPLC</td>
<td>I and II</td>
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<tr>
<td>Retinoids in liver and kidney</td>
<td>disisopropyl ether extraction, HPLC</td>
<td>II</td>
</tr>
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<td>25-OH-vit D$_3$, α-tocopherol,</td>
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<td></td>
</tr>
<tr>
<td>phylloquinone</td>
<td>HPLC</td>
<td>III</td>
</tr>
</tbody>
</table>

**Peripheral quantitative computed tomography (Paper II and III)**

The peripheral quantitative computed tomography (pQCT) system Stratec XCT 960A with version 5.20 software (Norland Stratec Medizintechnik, Pforzheim, Germany) was used. For cortical measurement, the humeral diaphysis was scanned at midshaft just distal to the deltoid tuberosity. For trabecular measurement, the humeral epiphysis was scanned 5 mm from the proximal end of the bone, an area rich in trabecular bone (figure 4). The left humerus was placed horizontally inside a glass tube and scanned using a voxel size of $0.148 \times 0.148 \times 1.25$ mm and an attenuation threshold coefficient of $0.930 \text{ cm}^{-1}$ for definition of cortical bone.
Dimensions and composition of humerus (Paper III)
Total length (defined as the distance from the upper edge of the humeral head to the medial epicondyle) and waist (defined as the diameter of the narrowest part of the humerus) were measured using an electronic sliding caliper. Bone volume of the right humerus was measured using Archimedes principles of displacement. Determination of bone composition was made with the bone ash method. The bones were dried at 100°C for 24 hours and finally burned to ashes at 800°C for 48 hours. The weight was determined at each step.

Measurement of OPG secretion (Paper IV)
The levels of OPG were analyzed using ELISA. A MaxiSorb micro titer plate was coated with anti-human OPG mouse capture antibody. After incubation for 2 hours with sample or standard recombinant human OPG protein, detection was performed with biotinylated anti-human OPG goat detecting antibody. After developing, the plate was read at 450 nm in a microplate reader. Protein concentrations were normalized to the number of cells, and expressed as pg/ml per 10⁶ cells.

RNA isolation and quantitative real time PCR analysis (Paper IV)
Total ribonucleic acid was isolated using RNeasy Midi kit. RNA (1 µg/sample) was reverse transcribed to cDNA with Superscript II. All RNA samples were transcribed at the same time and each cDNA was analyzed in duplicate. Real-time PCR analysis was performed using TaqMan 7700 with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as endogenous control. Gene-specific primers for RANKL and OPG were purchased as Assay-on-Demand from Applied Biosystems.

Statistical analysis (Paper I-III)
The StatView 4.5 or 5.0 software (Abacus Concepts, Berkeley, USA) was used for all statistical analyses. In every case, a P value less than 0.05 was considered significant.

Paper I
To analyze the effect of vitamin intake on S-calcium, the five serum samples from day two (8.00 to 16.00) were used. As a substitute for measurement of the area under curve, the mean level of S-calcium was calculated. This was done separately for each vitamin intake and for placebo. The effect of vitamin intake was defined as the mean S-calcium level after vitamin intake minus the mean level after placebo. Thus, for every individual three values, corresponding to the effect of retinyl palmitate intake, 1,25-(OH)₂-vitamin D₃ intake and combined intake, respectively, were calculated. These effect values were statistically analyzed using the Wilcoxon signed rank test. The
effect of vitamin intake on S-PTH and S-CrossLaps was analyzed in exactly the same way. The effect of vitamin intake on S-1,25-(OH)₂-vitamin D₃ and S-RE, and on urine calcium excretion was evaluated by the Wilcoxon signed rank test.

**Paper II and III**

The data were evaluated by one-way ANOVA followed by post-hoc Fisher’s PLSD.
RESULTS AND DISCUSSION

PAPER I

_Vitamin A antagonizes calcium response to vitamin D in man._

The effects of single doses of 1,25-(OH)₂-vitamin D₃ and retinyl palmitate on the S-calcium level were investigated. As expected, intake of 2 µg 1,25-(OH)₂-vitamin D₃ resulted in increased S-calcium and concomitantly decreased S-PTH. After the combined intake of 1,25-(OH)₂-vitamin D₃ and retinyl palmitate, the rise in S-calcium was significantly lower compared to the rise after intake of 1,25-(OH)₂-vitamin D₃ alone. Thus, the intestinal calcium response to vitamin D was antagonized by retinyl palmitate.

After intake of retinyl palmitate only, S-calcium but not S-PTH, was significantly decreased compared to placebo. Since the study subjects were not vitamin D depleted before the experiment, the decrease in S-calcium after intake of retinyl palmitate only may be due to an antagonistic effect on physiological vitamin D levels, and does not necessarily prove a direct effect of vitamin A on calcium homeostasis.

Possible mechanisms for the interaction are at the level of intestinal calcium absorption, vitamin absorption, degradation or transport, bone resorption or renal calcium reabsorption. In this study, the interaction is not obviously exerted in bone or kidney, or at the level of vitamin absorption since neither the bone resorption marker Cross-Laps nor urine calcium was significantly affected, and serum levels of retinyl palmitate and 1,25-(OH)₂-vitamin D₃ were not significantly affected after combined intake. In a study on rats, a similar antagonistic effect of retinyl acetate on the S-calcium response to a low dose of vitamin D₂ was eliminated in rats fed a rachitogenic diet (197), suggesting that the antagonism was exerted at the level of intestinal calcium absorption. Our data, although not conclusive, are in agreement with this study.
PAPER II and III

Subclinical hypervitaminosis A causes fragile bones in rats
Subclinical hypervitaminosis A: effects on fat-soluble vitamins and BMD

In this study on rats, the excessive vitamin A intake in the 10 × C group (diet supplemented with 120 IU retinyl palmitate/g pellet) and the 50 × C group (diet supplemented with 600 IU/g pellet) led to dose-dependently increased levels of serum and liver retinoids, indicating that intestinal uptake was adequate. In the 50 × C group kidney retinoids were also increased. No typical signs of general toxicity were evident, i.e. the intoxication was subclinical. No effect on the bone was seen in the 10 × C group but in the 50 × C group, bone strength was reduced as measured by three-point bending breaking force (-10.3%, p < 0.01). In this group, pQCT showed decreased cortical area and reduced diameter of the diaphysis of the long bones, i.e. bone changes typical for hypervitaminosis A (199). Thus, bone toxicity is evident at this level of subclinical hypervitaminosis A.

This is the first demonstration of increased bone fragility in rats with subclinical hypervitaminosis A. According to the literature, the lowest dose known to induce chronic toxicity and fractures in adult rats is around 25,000 IU/day, but the doses used are often even higher (150). Our data indicate that the negative effect on bone strength appears at a vitamin A intake of somewhere between 1,800 IU/day and 9,000 IU/day, a level considerably lower than previously reported. Similarly, a decreased serum vitamin D at this level of vitamin A intake has not been reported before. Previously published data show that administration of 50,000 IU vitamin A three times per week to rats decreased the level of 25-OH-vitamin D$_3$ (152) while no effect on S-1,25-(OH)$_2$-vitamin D$_3$ was seen in rats fed 10,000 IU vitamin A per day for 3 weeks (153) or 300 IU per day for 14 months (154). In our study administration of approximately 9,000 IU per day (50 × C group) for 12 weeks resulted in a reduction of both S-25-OH-vitamin D$_2$ and S-25-OH-vitamin D$_3$.

It is important to note that vitamin A is rapidly absorbed but slowly cleared from the circulation. It is accumulated in the liver until the storage capacity of the liver is exceeded. Therefore, toxicity is dependent on both dose and duration of vitamin A administration. Interestingly, in the 10 × C group, S-25-OH-vitamin D$_2$ and also S-α-tocopherol (vitamin E) were decreased, suggesting that subclinical toxicity is developing already at this level of vitamin A intake. Prolonged exposure would most likely result in adverse skeletal effects in this group as well, although this was not found in our study.

Like vitamin D$_2$ and vitamin D$_3$, S-vitamin E was decreased in the 50 × C group. A general antagonistic interaction among the fat-soluble vitamins has

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previously been demonstrated in the chicken (200,201). Our data suggest that a similar general interaction among fat-soluble vitamins is also present in the rat. Although the decrease in S-phyloquinone (vitamin K) only reached marginal statistical significance, an interaction between vitamin K and vitamin A seems likely. Clinical reports of bleedings and hypothrombinemia after intoxication with vitamin A and vitamin E, respectively, support this hypothesis (202,203). Vitamin D has well-known beneficial effects on the skeleton, and similar effects are proposed for vitamins K and E (204,205). Therefore, our findings suggest possible indirect mechanisms of vitamin A toxicity.

The antagonism causing reduced serum levels of other vitamins can occur during vitamin absorption in the intestine, or during transport, storage or degradation of the vitamins. Previous studies have suggested a competitive interaction at the level of intestinal absorption (195,206), and our data are in agreement with this hypothesis (see also discussion in paper III). However, other levels of interaction must also be considered. For example, the 24-OH-hydroxylase, a key catabolic enzyme for 1,25-(OH)2-vitamin D3, is up-regulated by RA (192).

The effect of vitamin A on bone mass was evaluated by the bone ash method and pQCT. No detectable changes in BMD could explain the increased bone fragility. However, the external diameter and cortical thickness were reduced. These variables are of major importance for determination of bone strength, since the bending strength is proportional to the fourth power of the radius (207).

In the 50 × C group, bone ash measurement even showed a slightly increased BMD (ash weight divided by bone volume). This increase is due to the fact that the volume decrease is larger than the ash weight decrease and does not reflect a true increase in bone mass. PQCT also showed decreased total bone mineral content at the thinned diaphysis but the corresponding BMD was not decreased. Thus, early vitamin A toxicity in the rat seems to affect bone geometry and bone strength before any negative effect is detectable by density measurements. For evaluation of early hypervitaminosis A in the rat, measurements other than BMD must be recommended. Since the diagnosis osteoporosis is based on BMD, further studies are warranted to examine whether this is also true in humans.
PAPER IV

Vitamin A Differentially Regulates RANKL and OPG Expression in Osteoblasts

In the human osteosarcoma cell line MG-63, OPG protein expression, measured by ELISA, was down-regulated by RA in a dose-dependent way. With quantitative real-time PCR, a similar down-regulation of OPG mRNA levels was detected. In both cases, the maximum effect was seen at a dose of $10^{-6}$ M. The down-regulation was evident in primary cultures of human osteoblast-like cells as well. Time-course experiments with $10^{-6}$ M RA showed a maximum effect at 4 hours. RANKL mRNA levels were up-regulated by RA stimulation in primary cultures. The maximum effect was at $10^{-6}$ M RA, after 8 hours. In MG-63 no RANKL expression was detected. This is consistent with previous studies (208). In summary, it was demonstrated that OPG is down-regulated and RANKL is up-regulated in osteoblastic cells by RA.

OPG and RANKL are essential regulators of osteoclastogenesis. Their effect on osteoclast formation and activation is determined principally by the relative ratio of RANKL/OPG in the bone marrow microenvironment (9,209). In our \textit{in vitro} system, the RANKL/OPG ratio was increased by RA stimulation. This differential regulation may be an important mechanism by which vitamin A induces bone resorption \textit{in vivo}.

RAR\(\alpha\), RAR\(\beta\) and RAR\(\gamma\) are all expressed in osteoblasts (125,210,211) and signaling via these receptors is the most likely signaling pathway for vitamin A activity in bone. In this study, the addition of a RAR antagonist, which inhibits the action of RAR\(\alpha\),\(\beta\) and \(\gamma\), could inhibit the effect of $10^{-6}$ M RA, while an agonist for RAR\(\beta\) and \(\gamma\) could mimic the RA effect. This was evident on both OPG and RANKL expression in both cell types, indicating that the RA effect is mediated by these receptors. To confirm that these are indeed transcriptional effects and do not represent a change in message stability, further experiments such as a reporter assay using RANKL and OPG upstream regulatory sequences will be needed.
GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Since the identification of vitamin A in 1913, its structure and function have been thoroughly investigated. In some fields, such as the phototransduction in the eye, this has led to detailed knowledge. In contrast, our knowledge about how vitamin A functions in bone tissue is still limited, even though it has been known for almost a century that high doses of vitamin A are severely toxic to the rat skeleton. The potential of vitamin A to affect the human skeleton is evident from case reports of human vitamin A intoxications. Since the publication by Melhus et al in 1998 (169), the possible relationship between high vitamin A intake and osteoporosis has gained increasing interest.

One question, maybe the most important in view of the pathogenesis of osteoporosis, is whether toxic effects of hypervitaminosis A occur at levels close enough to physiologically high intakes, i.e. of relevance for human nutrition. This is suggested by the epidemiological studies, showing that the level of vitamin A intake associated to increased fracture risk in humans is below the tolerable upper limit of 3,000 µg/d or only about two times the Swedish RDI. The average vitamin A intake in Sweden is between one and two times the RDI for both women and men (88,90). I have shown that adverse skeletal effects of vitamin A are demonstrable in rats where no clinical signs of toxicity are evident. Although the doses are many times the “RDI” for rats, it is likely that subclinical skeletal effects may arise at much lower doses. This field needs to be further investigated. As discussed previously, the duration of intake is crucial for vitamin A toxicity. In the pathogenesis of human osteoporosis, both peak bone mass and later bone losses are of importance, and exposure time can potentially be life-long. Therefore, when studying the lowest safe level of vitamin A intake, longer exposure times are recommended.

The relevance of animal models for human disease pathogenesis can naturally be questioned. In general, the rat is considered to be an appropriate model for human postmenopausal osteoporosis (212). In the experiments demonstrating spontaneous fractures in rats, the doses were extremely high. In the severe cases of human vitamin A intoxication, the dose per kg body-weight was lower, but instead continued for a longer time. In one patient, who ingested 500,000 IU per day for several years, x-rays showed os-
teopenia (163). In another patient, with lower doses and shorter exposure time, x-rays were normal but bone biopsy showed increased resorptive surfaces (164). When the lower dose is taken into account, these symptoms are consistent with the fractures seen in rats.

I report that RA can increase the RANKL/OPG ratio in osteoblastic cells in vitro. Considering the importance of the RANK/RANKL/OPG system for osteoclastogenesis and bone resorption, the regulation of these molecules is a likely signaling pathway for the direct effects of vitamin A in bone. It would be of interest to elucidate the effects on osteoclasts in a co-culture in vitro system, but in vivo studies are needed for certain confirmation.

An interaction between vitamin A and D in humans was also demonstrated, where the calcium response elicited by vitamin D was antagonized by simultaneous intake of vitamin A. In the rats with subclinical hypervitaminosis A, the serum levels of other fat-soluble vitamins were reduced, suggesting a general antagonistic effect on fat-soluble vitamins. These findings are consistent with previous literature and argue in favor of indirect effects in development of vitamin A toxicity.

It has previously been assumed from animal studies that the antagonistic action between vitamin A and D in bone is weak, since it is evident at high levels of vitamin A intake (193-195). However, at marginal dietary intake of vitamin D, the antagonistic effects occur at lower vitamin A doses (197). In vitamin D-depleted rats with a marginal dietary intake of phosphorous or calcium, the negative effect of vitamin A on bone ash was aggravated when vitamin D was added to the diet, compared to controls with a vitamin D-deficient diet (156). These data suggest that at least in certain conditions of limited intake of vitamin D and calcium and/or phosphorous, the antagonistic effect on vitamin D and other fat-soluble vitamins may also be of importance.

In humans, these indirect effects can possibly be more pronounced in the elderly, and in other individuals with poor intake of calcium and/or vitamin D. In Scandinavia, the average daily intake of vitamin D is below recommended levels in many groups (213-216) and the limited exposure to sunlight further increases the risk for hypovitaminosis D. I hypothesize that the high intake of vitamin A in Scandinavia may further aggravate the effect of hypovitaminosis D on calcium absorption and, possibly, contribute to the high incidence of osteoporosis.

Several new questions are raised: For which doses of vitamin A and D is the antagonistic effect evident in humans? How long after intake does vitamin A exert an antagonistic effect? What are the effects of long term high vitamin A intake on vitamin K and vitamin E in humans? It would also be interesting to see if the outcome of epidemiological studies would be different if, in addition to the level of vitamin A intake, consideration was taken to the vitamin D status of the individuals. Answers to these questions would
help in the effort to more precisely pinpoint the physiological relevance of the antagonistic effects in humans.

Although further studies are needed to clarify whether the past and current daily intake of vitamin A in Sweden and other countries has contributed to the high incidence of osteoporosis, the question is not if vitamin A is toxic for the skeleton, but rather at what level of intake this toxicity begins (143). It has become clear that vitamins cannot be considered magic pills that bring only benefit to health, and that the widespread misconception – “the more vitamins, the better” – is a fallacy.
CONCLUSIONS

In this project, it was found that:

- the serum calcium-increasing effect of vitamin D is antagonized by simultaneous intake of vitamin A in humans.

- in rats with increased intake levels of vitamin A but no clinical signs of vitamin A toxicity, bone fragility is increased. This bone toxicity appears at somewhere between 10 and 50 times the intake of vitamin A in the control rats.

- in rats with increased bone fragility, the bones have reduced diameter and cortical area, but BMD is not decreased. Moreover, levels of other fat-soluble vitamins are reduced.

- in osteoblastic primary cultures and in the osteosarcoma cell line MG-63, stimulation with RA decreases OPG levels while RANKL is increased.

In summary, these findings indicate that vitamin A can increase bone fragility in the rat at doses considerably lower than previously shown. Since geometrical variables, but not BMD, were negatively affected, measurements other than BMD are suggested for evaluation of early hypervitaminosis A. The regulation of RANKL/OPG in vitro provides a mechanistic explanation for the bone resorbing activity of vitamin A, and is a likely pathway for direct effects of vitamin A in bone in vivo. An antagonistic effect of vitamin A on vitamin D action has for the first time been demonstrated in humans, suggesting indirect mechanisms of vitamin A toxicity.
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SUMMARY IN SWEDISH

Vitamin A och osteoporos
Kliniska och experimentella studier

Utgångspunkten för den här avhandlingen var en artikel som min handledare Håkan Melhus publicerade 1998. Det var en epidemiologisk studie som visade på att ett högt intag av vitamin A i kosten var kopplat till en minskad bentäthet och en ökad risk för höftfraktur. Dessa resultat kan inte säkert kan bevisa ett orsakssamband, men har ändå väckt frågan: kan utvecklingen av sjukdomen osteoporos, som kännetecknas av minskad bentäthet och stor frakturrisk, påverkas av vitamin A?

Kort om ben och osteoporos


Lite förenklat kan man säga att ju äldre man blir, desto mer dominerande blir benedbrytningen, och alla människor tenderar att förlova benmassa när de blir äldre. Detta blir särskilt tydligt hos kvinnor efter klimakteriet, eftersom östrogen är en viktig faktor för att hålla remodelleringen i god balans. Sjukdomen osteoporos innebär en minskad benmassa, men också förändrad struktur i benet, så att hållfastheten minskar och risken för fraktur ökar kraftigt. De typiska osteoporos-frakturerna är fraktur i kotkropparna, handleden och höften. Särskilt höftfrakturen innebär mycket lidande och besvär för patienten, en minskad överlevnad och stora kostnader för samhället.
**Vitamin A**

Vitamin A är en grupp fettlösliga ämnen som är nödvändiga för många fysiologiska processer i kroppen, bland annat under fosterutvecklingen, i hunden och för immuntörsvar. De verkar genom att binda till speciella receptorer, som i sin tur påverkar vilka gener som uttrycks (används) i en cell. På så sätt är vitamin A ett av många ämnen som reglerar hur celler delar sig och mognar.


**Vitamin A och ben**

Vad finns då känt om hur vitamin A påverkar skelettet? Redan i början av 1900-talet, när vitamin A nyligen hade identifierats och man hade börjat undersöka effekten av över- och underdosering av olika vitaminer, visade det sig att råttor som fick mycket höga doser av vitamin A fick spontana frakturer. Hittills är vitamin A det enda ämne som har visat sig ge spontana frakturer hos råtta, och tillsammans med blödningar var detta det mest framträdande symtomet. Senare studier har visat att benen hos vitamin A-intoxikerade råttor är förtunnade, och har ett ökat antal osteoklaster. Man har också visat att vitamin A kan påverka olika benceller, och styr regleringen av flera ben- och specifika proteiner, och att benneddnyttningen ökar i benbitar som odlats i en vitamin A-rik miljö.

Hos människor som av misstag åtit för mycket av vitamin A (detta gäller dock inte överintag av karotenoider) under en lång tid, oftast i form av


Mål
Målet med avhandlingsarbetet har varit dels att studera olika mekanismer för hur vitamin A kan påverka benet, dels att undersöka gränsen för hur låga doser som är skadliga för skelettet.

Projekt I
En mekanism för vitamin A toxicitet skulle kunna vara att motverka effekten av vitamin D, som är av känd positiv betydelse för skelettet. Detta har påvisats hos djur, t ex rätta och kyckling, men har aldrig tidigare undersöks hos människa. I det här projektet har jag undersökt hur vitamin A och vitamin D påverkade nivån av kalcium i blodserum (S-kalcium).

Nio friska försökspersoner fick äta olika kombinationer av engångsdoser av vitaminerna vid fyra olika tillfällen. Följande dag fick försökspersonerna lämna blodprov varannan timme, samt ett urinprov.

Efter intag av endast vitamin D steg nivån av S-kalcium under dagen, en väntad effekt eftersom den viktigaste funktionen för vitamin D är att öka upptaget av kalcium i tarmen. Kombinationen av vitamin A och D gjorde att ökningen av S-kalcium blev signifikant lägre än efter enbart vitamin D-intag, och efter intag av enbart vitamin A sjönk kalciumnivån jämfört med placebo. Slutsatsen blir att vitamin A kan motverka den kalciumhöjande effekten av vitamin D, och att en tänkbar indirekt mekanism för hur vitamin A-effekter kan medieras har påvisats hos människa.
Projekt II och III

I de här projektten studerade jag råttor som under 3 månader fått extra tillskott av vitamin A i kosten. Tre grupper med 15 råttor/grupp fick antingen standardfoder (kontroll, C), standardfoder med ett ca 10 gånger ökat vitamin A-innehåll (10 × C) eller standardfoder med ett ca 50 gånger ökat vitamin A-innehåll (50 × C).

I tidigare studier där man påvisat spontana frakturer hos råttor har man använt mycket höga doser, som även medfört allmän avtackling och död. I projekt II visade jag att råttornas skelett kunde påverkas negativt av vitamin A, trots att råttorna inte uppsoades några allmänna tecken på vitamin A-toxikation (subklinisk toxicitet). Nedsatt hållfasthet i skeletten (-10,3%) påvisades med 3-punkts-böjnings-test.

I vårt försök verkade den lägsta skadliga nivån ligga någonstans mellan intaget i 10 × C- och 50 × C-gruppen. I det här sammanhanget är det av betydelse att levern har en stor förmåga att ta upp och lagra vitamin A från blodet. Detta innebär ett skydd både mot enstaka för höga intag av vitamin A och mot vitamin A-brist i tider av ett ojämnt intag. Det gör dock också att det kan ta lång tid innan toxiska symtom utvecklas vid medelhöga doser, eftersom det sker först när leverns förmåga till lagring har överskridits. Man kan anta att även råttorna i 10 × C-gruppen skulle få toxiska symtom om exponeringen fortsatte tillräckligt länge. Flera sommer studier krävs därför för att kunna utvärdera den nedre toxiska gränsen bättre.

I projekt III undersöktis råttornas ben vad gäller form och bentäthet. Det visade sig att benen i 50 × C-gruppen var förstennade, vilket är typiskt för vitamin A-intoxikation. Bentätheten däremot var inte minskad, trots att benets hållfasthet var nedsatt. Detta resultat antyder att andra faktorer än bentäthet, som t ex benets geometri, kan vara av betydelse för hållfastheten. Detta är i samstämmighet med tidigare studier om osteoporos, som visat att endast en del av alla osteoporosfrakturer kan förklaras av låg bentäthet.

I projekt III fann jag också att nivåerna av andra fettlösliga vitaminer i blodet, var nedsatta hos råttor med förhöjt vitamin A-intag. Detta skulle kunna bero på att höga doser vitamin A motverkar upptaget i tarmen av övriga vitaminer, eller t ex på att deras nedbrytning ökats. Liksom i projekt I antyder de här resultaten indirekta mekanismer för vitamin A-toxicitet.
**Projekt IV**

I djurförsök och i experiment med benbitar har man sett att vitamin A kan öka bennedbrytningen, men den exakta mekanismen för hur detta går till är inte känt. Eftersom osteoklastens aktivitet är avgörande för graden av bennedbrytning undersökte jag i det här projektet hur vitamin A påverkar två signalsubstanter, RANKL och OPG, av stor betydelse för regleringen av osteoklasterna. Både RANKL och OPG produceras av osteoblaster. RANKL stimulerar osteoklasternas tillväxt och aktivitet medan OPG motverkar denna stimulering, och därför hämmer osteoklastaktivitet och bennedbrytning (se figur 2, sid 13).

I försöket odlade jag osteoblaster, som stimulerades med vitamin A. Stimulering med vitamin A medförde att osteoblasternas produktion av RANKL ökade medan OPG-produktionen minskade. Detta är en förändring som skulle innebära en ökad stimulering av osteoklasterna i benvävnaden. Slutsatsen blir att reglering av nivåerna av RANKL och OPG är en trolig mekanism för hur vitamin A kan inducera benresorption.

**Sammanfattning**

Jag har visat att vitamin A-intoxikation ger minskad hållfasthet i skelettet på råttor vid doser betydligt lägre än vad som tidigare varit känt. Jag har också undersökt olika mekanismer för hur vitamin A kan påverka skelettet: dels genom att påverka hur de bennedbrytande osteoklasterna fungerar, dels genom att motverka effekten av vitamin D. Mina resultat tyder på att vitamin A är skadligt för skelettet, men för att säkert kunna besvara frågan om vitamin A har bidragit till den höga förekomsten av osteoporos i Sverige krävs fler studier.
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A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to October, 1985, the series was published under the title “Abstracts of Uppsala Dissertations from the Faculty of Medicine”.)