Calcium homeostasis and Vitamin D in Obesity and Preeclampsia

HELLA HULTIN
Dissertation presented at Uppsala University to be publicly examined in Grönwallssalen, ing 70, Akademiska sjukhuset, Uppsala, Friday, April 8, 2011 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract

Normal physiological functioning is highly dependent of calcium and the concentration range is very narrow. Normal calcium levels are so crucial to survival that the body will de-mineralize bone if the levels are insufficient. A prerequisite for normal calcium uptake is a normal Vitamin D level. Insufficient levels of Vitamin D are associated to several diseases.

The aims of this thesis were to study the relationship between pregnancies and hyperparathyroidism (pHPT) (I), between pHPT and pregnancy with preeclampsia (II) and also to determine if disturbances in calcium homeostasis with vitamin D deficiency are apparent in preeclamptic women (III). The aim was also to study calcium homeostasis in obese patients before and after bariatric surgery (IV and V) with emphasis on vitamin D status, parathyroid secretion and bone mineral density (BMD).

A correlation was found between a history of pHPT and pregnancy with preeclampsia, with an odds ratio of 6.89 ( 95% CI 2.30, 20.58). Parathyroid hormone was significantly raised in preeclamptic pregnancies but vitamin D deficiency was present both in preeclamptic and healthy pregnancies. A certain polymorphism of the Vitamin D receptor (baT haplotype), overrepresented in pHPT, was not over expressed in preeclampsia. Hypovitaminosis D was present in more than 70% of bariatric patients preoperatively, which did not change after surgery, despite great weight loss and start of Vitamin D supplementation. BMD was significantly lower in bariatric patients with a negative correlation to the time elapsed since surgery. A small increase in BMD could be noted 10-13 years after bariatric surgery, possibly due to gradual weight gain. CiCa-clamping in obese patients demonstrated a disturbed calcium homeostasis with a left-shifted calcium-PTH relationship and a lower set-point of calcium. This disturbance persisted one year postoperatively.

In conclusion, derangements in calcium homeostasis with decreased levels of Vitamin D are present in preeclampsia and obesity. A history of pHPT should be viewed as a risk factor for preeclampsia. Life long follow-up is necessary after bariatric surgery, and an individually adjusted high dose Vitamin D substitute is probably needed to avoid a development of osteoporosis.

Keywords: Calcium homeostasis, vitamin D, preeclampsia, obesity, parathyroid hormone

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A Sunshine Story
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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### Abbreviations

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<tr>
<td>1.25-OH-D</td>
<td>1.25-dihydroxyvitamin D$_3$</td>
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<td>7-DHC</td>
<td>7 dehydro cholesterol</td>
</tr>
<tr>
<td>25-OH-D</td>
<td>25 hydroxy vitamin D</td>
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<td>CaR</td>
<td>Calcium sensing receptor</td>
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<td>CiCa-clamp</td>
<td>Citrate-calcium clamping</td>
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<tr>
<td>cPLA$_2$</td>
<td>Cyclic phospholipase A$_2$</td>
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<tr>
<td>EPE</td>
<td>Early onset preeclampsia</td>
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<tr>
<td>HPT</td>
<td>Hyperparathyroidism</td>
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<td>IBD</td>
<td>Inflammatory bowel disease</td>
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<tr>
<td>LPE</td>
<td>Late onset preeclampsia</td>
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<tr>
<td>P</td>
<td>Phosphate</td>
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<tr>
<td>PA</td>
<td>Parathyroid adenoma</td>
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<tr>
<td>PE</td>
<td>Preeclampsia</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
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<tr>
<td>RDI</td>
<td>Recommended daily intake</td>
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<tr>
<td>sHPT</td>
<td>Secondary hyperparathyroidism</td>
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<td>VDR</td>
<td>Vitamin D receptor</td>
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Introduction

Vitamin D

Vitamin D and its metabolites are all cholesterol derivates. Ergosterol, which is converted to vitamin D$_2$ when exposed to sunlight (1) was produced in phytoplankton living in the sea more than 750,000,000 years ago. As vertebrates evolved and left the ocean they retained the photosynthetic capacity in their skin. The ability to facilitate absorption of dietary calcium became more important as dinosaurs and animals with larger skeletons developed, and vitamin D has therefore played a crucial role through the eons of evolution.

Although Glisson first described osteomalacia in 1651, the importance of vitamin D for humans was probably unknown until the industrialization of northern Europe. At that time pollution from coal burning caused less sun exposure in children, which created severe vitamin D deficiencies and rickets. Adolf Windaus from Germany was awarded the Nobel Prize in chemistry 1928 for the discovery of vitamin D as an effective treatment for this disease. In the beginning of the 20$^{th}$ century it was estimated that more than 80% of children in Leiden, Netherlands and in Boston, USA, suffered from rickets. Rickets was later eradicated in USA and Europe, by educating parents in sensible sun exposure for their children, and also by irradiation and fortification of milk. Currently northern countries with limited sunlight fortify selected dairy products, and in several countries all infants are given vitamin D.

Today, vitamin D has become a most popular subject. Approximately two thirds of the global population is suspected to be vitamin D deficient, and there is a constant flow of reports on associations between vitamin D deficiency and disease in the most diverse of fields. However, surprisingly few interventional studies are performed, maybe due to the fact that vitamin D is not protected by a patent.
Calcium homeostasis

Calcium

Calcium is the most common mineral in the human body. The greater part (99%) is found in its solid form, within the mineral phase of bone and teeth. The remaining soluble 1% is found in blood and soft tissues, thereby playing an essential role in cell signalling, and as a cofactor for enzymes and proteins optimizing their activities. Normal physiological functioning is highly dependent of calcium and the concentration range is very narrow. Normal calcium levels are so crucial to survival that the body will demineralize bone if the levels are insufficient (2).

Regulation of calcium concentrations in blood and extracellular fluids is controlled by a complex feedback system. A decrease in blood calcium concentration will be detected by calcium sensing receptors (CaR) on the surface of the parathyroid glands. The following immediate secretion of parathyroid hormone (PTH) stimulates the conversion of vitamin D to its active form in the kidneys. The active vitamin D, calcitriol, increases the calcium uptake from the small intestine and the reabsorption in the kidneys. PTH and calcitriol also affect bone by activating osteoclasts, thereby releasing calcium. An increase in blood calcium will trigger off the opposite reaction, by terminating the secretion of PTH and instead excreting excess calcium via the kidneys. A tight and rapid control of calcium levels is maintained through this system (3).
Figure 1. Vitamin D synthesis, stepwise hydroxylation and PTH actions.

Serum calcium
Almost 50% of the calcium in blood is ionized (free calcium), fully 40% is protein bound and the rest is bound to other ions; magnesium, phosphate, and citrate (4). Although albumin is the predominant protein, levels of albumin may vary in different circumstances and the binding of calcium to albumin changes dependent on the pH-value. It has been shown that serum total calcium concentration increases with age while the ionized fraction remains stable throughout life. For these reasons it is considered more reliable to measure the ionized form of calcium with adjustment to pH 7.4, and not the total calcium. Normal levels of calcium are considered to be between 2.20–2.50 mmol/L with minor intra-individual variations. However, seasonal variations are seen and depend on variations in dietary intake and sun exposure.

Hypercalcemia
The most common reasons for hypercalcemia are malignancy with skeletal metastases, primary hyperparathyroidism (pHPT), immobilization, sarcoidosis, thyreotoxicosis and calcium or vitamin D intoxication. A mild chronic hypercalcemia (2.50–2.80 mmol/L) gives few if any symptoms, but in the long term, as in mild pHPT, fatigue, depression and cardiovascular
Symptoms may be seen. Further pronounced hypercalcemia often presents with thirst, fatigue and gastrointestinal symptoms. The susceptibility to hypercalcemia varies but levels above 3.0 mmol/L may give nausea, renal failure and confusion, whereas higher levels may lead to hypercalcemic crisis with coma and CNS symptoms of various degrees. The initial treatment for hypercalcemia is rehydration and diuretics, followed by close examination in order to determine causality.

Hypocalcemia

The most common reason for hypocalcemia is hypoparathyroidism, which may be transient or permanent, after surgery of thyroid or parathyroids. Hypocalcemia may also develop in vitamin D deficiency and can be intermittent due to seasonal variation. Severe hypermagnesemia can cause hypocalcemia by inhibiting PTH secretion (5).

Chronic mild hypocalcemia is asymptomatic, while acute decreases in calcium levels involve increased neuromuscular irritability which reveals itself through paresthesias and numbness of fingertips and/or perioral area. Severe hypocalemia causing prolonged QT interval, tetany, hypotension and bronchospasm can be fatal.

Parathyroid hormone (PTH)

PTH is secreted by the four parathyroid glands, normally symmetrically situated in two pairs, the superior and the inferior parathyroids, on the dorsal surface of the thyroid gland. The normal weight is approximately between 30-60 mg and the size and shape of a normal parathyroid gland is comparable to a grain of rice. The glands consist mainly of chief cells with the addition of oxyphil and fat cells. PTH is synthesized by the chief cells and then stored in secretory granules (3).

The synthesis of PTH occurs initially as a 115-amino acid chain polypeptide, pre-pro PTH. Pro-PTH forms rapidly (within a few minutes) by cleavage of a 25 amino-acid sequence from the amino terminal. After approximately 15-20 minutes 1-84 PTH is formed by a new cleavage of a 6 amino acid sequence from the amino-terminal of the pro-PTH. 1-84 PTH is then stored in secretory granules, until delivered by exocytosis. The 34 amino acids in the N-terminal part compose the biologically active domain by their affinity to the PTH-receptor. A mixture of C-terminal, N-terminal and mid-molecule fragments are present after degradation in the liver. The bioactive N-terminal fragments then degrade rapidly and do not accumulate to any high degree. The main bioactive ability in PTH relates to 1-84 intact PTH (6).

Basal secretion of PTH is low but continuous (7). The physiological level of serum calcium partially activates the CaR, a seven transmembrane
receptor which thereby decreases the PTH secretion. When the CaR recognizes hypocalcemia, cellular signal transduction occurs and PTH is secreted. More than 90% of the produced PTH is never secreted but degrades in the cell.

Measurement of PTH
PTH was initially measured using immunoassays detecting C-terminal fragments, both active and inactive. Mid-molecule assays, and then NH2-terminal assays upgraded accuracy. Currently two-site assays are used to target the NH2-fragment, and so-called intact PTH (iPTH) is analyzed. As it became clear that fragments of PTH containing amino acids 7-84 also are immunoreactive assays targeting the whole 1-84 peptide, bio-active PTH, have developed. The ratio between 1-84 and 7-84 PTH is normally more or less constant, but bio-intact PTH is said to be more sensitive to detecting primary HPT.

A normal PTH concentration is 1.1-6.9 pmol/L. An age-related rise in PTH levels is connected to decreasing renal function. Colored populations have higher PTH concentrations, which is compensatory due to lower levels vitamin D synthesis from their pigmented skin. First trimester pregnancies show lower levels of maternal PTH, while later on the levels gradually normalize. In the fetus the parathyroid function is suppressed and this may produce hypocalcemia in a premature baby.

Synthesis of vitamin D
The main forms of the hormone are vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol). These are obtained by irradiation of plants and/or foodstuffs, and synthesized in skin after sun exposure, respectively (8). Although nutritional sources such as fatty fish contribute to vitamin D uptake, most cholecalciferol synthesis is obtained from the skin during summer. Cholecalciferol is hydroxylated in the liver into 25-hydroxyvitamin D3 (25-OH-D3) which is also an inactive form. Subsequent hydroxylation, through 1-α-hydroxylase activity, into 1,25-dihydroxyvitamin D3 (1,25-OH-D) occurs in the kidney. PTH enhances the production of 1,25-OH-D, and there is a negative feedback system in the opposite direction through calcium, which decreases PTH. The active metabolite 1,25-OH-D stimulates calcium absorption from the small intestine, kidneys and bone. This is a result of binding to the vitamin D receptor (VDR) which is expressed on essentially every cell in the body (9, 10). Activated VDR functions as a transcription factor. By heterodimer formation with retinoic acid receptors (RAR) VDR has capability to affect more than 200 genes by its influence on promoter regions (11). The active form of 1,25-OH-D also has a direct
effect on parathyroid cells, inhibiting PTH transcription, secretion and cell proliferation (8).

Measurement of vitamin D

The most reliable method of diagnosing vitamin D status is measuring of plasma 25-hydroxy vitamin D concentration (25-OH-D). The concentration of 1.25-OH-D is often normal or even increased in hypovitaminosis D, due to enhanced 1-α-hydroxylase activity in vitamin D-deficiency or in secondary hyperparathyroidism.

Measuring is difficult because of the presence of two forms of the vitamin in plasma [ergocalciferol (vitamin D$_2$) and cholecalciferol (vitamin D$_3$)], and also because of the hydrophobic nature of vitamin D. There are also a variety of methods used for the analysis and an inter-laboratory variation of results. The methods used are HPLC (high performance liquid chromatography), liquid chromatography tandem mass spectroscopy (LCMSMS) and RIA (radio immuno assay) methodologies (12). The Vitamin D External Quality Assessment Scheme (DEQAS) is an organized control function in which more than 200 laboratories world wide participate to ensure reliability of vitamin D assays. Recently Snellman et al published a report in which blood samples from twins were analyzed by three different methods (HPLC; RIA and CLIA). A high degree of inter-assay disagreement was found (13).

Debate continues about optimal levels of plasma 25-OH-D in humans (14, 15). Based on the level of 25-OH-D that keeps PTH at a minimum steady state, optimum serum vitamin D concentration is 75-250 nmol/L. A level between 25 and 75 nmol/l is considered insufficiency and levels <25nmol/L define vitamin D-deficiency.
Hypovitaminosis D

It is estimated that one billion people worldwide are vitamin D deficient or insufficient. Elderly, postmenopausal women, people with osteoporosis and non-white groups are over-represented, as are children and devoted sunscreen consumers.

There is a clear seasonal variation in vitamin D levels, particularly in the northern countries where the UV radiation is inadequate during winter (October-March). The lowest concentrations in normal population in Sweden are found in April; concentrations peak is in September. Skin pigmentation offers an efficient sun screen and colored people require 5-10 times longer sun exposure than Caucasians to produce the same amount of vitamin D (10, 16). Non-white immigrants in high latitude countries are at high risk for vitamin D depletion, particularly veiled women.

Vitamin D deficiency/insufficiency obstructs intestinal calcium uptake. As normal levels of ionized calcium are a top priority for the body, this will trigger a safety system to provide for enough calcium. The CaR on the parathyroids will immediately register a low level of ionized calcium and stimulate the parathyroids to express, produce and secrete PTH. This will stimulate the kidneys to produce more 1.25-OH-D. Moreover, PTH will increase the calcium re-absorption in the kidney and also mobilize calcium from bone. A prolonged shortage of vitamin D will lead to development of osteopenia, osteoporosis with risk for fractures and destruction of the skeleton (10, 17). Phosphate excretion is augmented with an increased PTH level, which will cause mineralization defects of the skeleton. Osteomalacia may develop in adults and rickets in children. Osteomalacia is often associated with bone pain and muscle weakness, and many such patients are misdiagnosed as suffering from fibromyalgia, chronic fatigue syndrome or dysthymia (18).

Cancer

Low vitamin D status is associated to fatal cancer in general (19), and there are strong associations with breast, prostate, colon, rectal and ovarian cancer (20). Signaling from 1.25-OH-D and CaR/Ca$^{2+}$ suppresses growth and promotes differentiation in human colon cancer cells, and in breast cancer 1.25-OH-D has anti-proliferative effects by changing expression in
oncogenes and tumor suppressor genes (21). The 1.25-OH-D also induces apoptosis in breast cancer cells (22).

Infectious and inflammatory disease
Macrophages, monocytes and dendritic cells all express VDR and have 1α-hydroxylase activity; a circumstance rendering vitamin D a potent role in innate immunity. In skin wound infections or in chronic infections (e.g. periodontitis, gingivitis), adequate vitamin D levels are required to achieve a cure especially in infections caused by Mycobacterium tuberculosis (23, 24).

In one study the risk for women of developing autoimmune diseases as multiple sclerosis (MS) or rheumatoid arthritis was reduced by more than 40% if they ingested more than 400IU/day of vitamin D (25, 26). Moreover, vitamin D deficiency seems to increase the risk for pre-eclampsia in pregnancy, a 50 nmol/l decline in 25-OH-D doubled the risk for pre-eclampsia when 270 pregnancies were followed (27).

Diabetes
In a group of more than 10000 Finnish children, who all received 2000 IU of vitamin D /day, the risk of type I diabetes was reduced by 78%, compared to the normal population (28). Metabolites of vitamin D have down-regulating effects on dendritic cells and Th1 cells, and suppress the antigen presenting capacity of macrophages and dendritic cells. Moreover, these metabolites promote Th2 lymphocytes and influences β-cell function and may by that reason protect against Type 1 diabetes. There is also a positive relationship between 25-OH-D, calcium and insulin sensitivity (29, 30).

Hypertension
Many studies have been conducted in order to establish a relationship between hypertension and vitamin D levels. A recent review article demonstrates that eight out of ten observational studies and three randomized controlled trials strongly support an inverse relation between vitamin D level and blood pressure (31). 1.25-OH-D decreases the production of renin in the kidneys, as well as decreasing the inflammatory factors involved in chronic heart disease, including C-reactive protein and IL-10 (32).

Inflammatory bowel disease
A local vitamin D endocrine system, consisting of immune cells and mucosal epithelial cells, works in the gastrointestinal tract. Both cell types are capable to convert 25-OH-D into 1.25-OH-D by 1α-hydroxylase (33), a
circumstance that is important when considering the protective role of vitamin D against IBD. In addition, in studies on mice, vitamin D has demonstrated protective effects on damage from inflammatory reactions resembling those in ulcerative colitis (34).

Neuromuscular disease

It is well known that both vitamin D and calcium have important effects on neuromuscular function, but the underlying mechanisms remain unclear. Muscular cells express VDR, but not CaR or 1α-hydroxylase. Neurons and glial cells that express 1α-hydroxylase and CaR are widely distributed in the central nervous system (35). Vitamin D or calcium supplementation produces beneficial effects on balance, reaction time and postural swaying in elderly and also reduces the number of falls (36).

Hypervitaminosis D

Since the main portion (90-100%) of the vitamin D supply in humans result from sun exposure, one could suspect that excessive sun bathing may cause vitamin D intoxication. However, no reports indicate this, either from the sun or from artificial tanning beds. Skin pigmentation provides an effective protection in gradually decreasing the vitamin D synthesis. The formation of pre vitamin D₃ and vitamin D₃ in the skin allows the UVB and UVA radiation to be absorbed and degraded into different photoproducts that do not affect calcium metabolism (37).

Vitamin D intoxication was described in England in the 1950s and was thought to result from high amounts of vitamin D in milk. This led to a prohibition against the fortification of milk. Serum levels of >250 nmol/L are potentially toxic, since hypercalcemia occurs.

Phosphate

Phosphate is the most common form of phosphorus, and in the human body it is the most abundant intracellular anion. Approximately 1% of the total body weight of an adult consists of phosphate, and more than 85% of this is found in the bones and teeth. The remaining 15% is found as phospholipids, nucleic acids, enzyme co-factors and glycolytic intermediates within muscle and soft tissues. Phosphate is crucial in several areas, for example in signal transduction, energy metabolism, muscle contraction and cellular structure. Phosphate is found in nutritional products with a high degree of protein, such as meat, fish, dairy products and eggs. A diversified nutritional intake provides normal phosphate levels.

RDI of phosphate is 600-800 mg which is absorbed in the small intestine, a process that is increased by 1.25-OH-D. Phosphate is excreted by
glomerular filtration, but is reabsorbed to a high degree (80%) in the proximal tubule in the kidney. Re-absorption is dependent on the serum concentrations of phosphate and PTH. PTH is highly phosphaturic, an effect also seen in other hormones such as vasopressin, norepinephrine and dopamine.

Magnesium
Magnesium is another important factor in regulation of PTH secretion. Mg$^{2+}$ acts on the CaR with a lower affinity than does Ca$^{2+}$ and in hypomagnesemia PTH secretion is restrained until serum Mg$^{2+}$ is corrected (5).
Primary hyperparathyroidism (pHPT) is caused by tumor development in one or more of the parathyroid glands. Benign single adenomas (PA) are most common (80%), but hyperplasia is also seen (20%). Parathyroid cancer is very rarely diagnosed (< 1%).

PHPT is a common disease, found in all ages and both sexes, with an overall prevalence of about 1%. It increases with age and most commonly strikes postmenopausal women, where the incidence is higher (38).

Historically, pHPT presented with renal stones, osteoporosis and constipation; “stones, bones, moans and groans”. In the early 1970s automated serum calcium analyses became cheap and available at all care institutions, and currently most patients are diagnosed with milder disease (3). The picture has changed from an illness with severe physical symptoms to a presentation of mainly neuropsychiatric and cognitive problems. Complaints about depression, fatigue and muscular weakness are common (38, 39). The etiology of pHPT is not fully known.

Although often a mild disease long-term studies of pHPT show correlations with osteoporosis, cardiovascular disease, diabetes and cancer (40, 41). A diagnosis of pHPT requires repeated measurements of elevated serum calcium and parathyroid hormone levels. Many patients experience a long delay until the diagnosis is established, since the history often is vague and the physical examination mostly normal. Treatment for pHPT is limited to surgery for the removal of the adenoma. This is a safe operation with a high success-rate of 90-99%, low morbidity and almost no mortality. Studies have shown reversible effects on dyslipidemia, bone mineral density and vasodilatory dysfunction after parathyroidectomy (41-43). A pharmacological treatment has recently been developed, cinacalcet, which is a calcimimetic that can be used in selected cases where surgical approach is not possible.
Secondary hyperparathyroidism

If the calcium homeostasis system fails in maintaining normocalcemia, a state of secondary hyperparathyroidism (sHPT) develops (44). The decrease in plasma ionized calcium is restored by a response through CaR expressed on parathyroid cell surface, and secretion of PTH is increased. This can be seen in chronic renal failure, liver failure, vitamin D deficiency, malabsorption and pseudo hyperparathyroidism. Some, very rare patients may have acute symptoms from hypocalcemia, while the majority has a slowly developing disorder with lingering hyperparathyroidism.

In renal failure, decreased calcitriol production, hypocalcemia and phosphate retention are the main factors in the development of sHPT (45). Hypocalcemia is probably the dominating factor for sHPT development in malabsorption and vitamin D deficiency. The vitamin D deficiency obstructs intestinal calcium uptake and thus parathyroid activation occurs. Lower levels of calcitriol lead to decreased calcium absorption and higher PTH, with initially decreased renal phosphate reabsorption. But as renal failure progresses phosphorus is retained, leading to enhanced activation of parathyroid cells. Monoclonal cell proliferation of parathyroid tissue occurs as a consequence of the permanent stimulation and the initial formation of small nodules later develops into nodular hyperplasia. A reduction of CaR on cells can be seen in nodular hyperplasia, which explains the higher calcium concentration that is needed to suppress PTH secretion in sHPT.
The PTH-Ca relationship

The inverse sigmoidal relationship between serum calcium and PTH was first described by Mayer and Hurst (46). Before their work the relationship was thought to be inversely linear. Their findings confirmed the sensitivity of the parathyroids to very small changes in serum calcium concentration. Minimal decreases in calcium trigger PTH secretion, while a slight increase will reduce parathyroid activity, within minutes. This relationship is bifunctional; PTH also regulates the serum calcium concentration, through its calcemic actions. The PTH regulates the serum calcium level while serum calcium controls the PTH secretion, simultaneously (47, 48).

CiCa-clamp

Citrate-calcium clamp is a standardized method for quantifying of intact parathyroid hormone secretion during sequential induction of hypo- and hypercalcemia. It was first established and described by Schwarz et al 1993 (49). Brown introduced the mathematical four-parameter model to describe the inverse sigmoidal relation between PTH release and extracellular Ca\(^{2+}\) concentrations (50). This model was used to calculate the so called set-point, which implies the Ca\(^{2+}\) concentration corresponding to 50% inhibition of maximal PTH secretion.

The CiCa-clamping is without risk for the patient but takes 2-3 hours to complete. Through intravenous access in one arm a citrate-solution with gradually lower concentration is infused, the total amount calculated according to body weight. Citrate decreases ionized calcium levels, and blood samples measuring calcium and intact PTH are taken intermittently during the complete process, via intravenous access in the other arm. Some patients experience symptoms related to hypocalcemia, such as pricking sensations in hands and face. After 50 minutes (calculated time to establish steady state between calcium and PTH-secretion) the citrate infusion is stopped, and after five minutes an infusion of calcium is started. In this way calculations of PTH secretion capacity as well as inhibition of PTH secretion can be made.
PTH-Ca set-point

The PTH-Ca set-point is the ionized serum calcium level at which the maximal PTH secretion is inhibited to 50%. The set-point is used to indicate the sensitivity of the parathyroid glands to serum calcium concentration. Certain circumstances have been associated with changes in the PTH-calcium curve and set-point. A higher set-point, as seen in pHPT, indicates a lower sensitivity to calcium and a right-shifted PTH-Ca curve. A study in healthy postmenopausal women who were given estrogen treatment for 23 weeks demonstrated a reduced ionized calcium concentration. As a result the set-point was decreased and the curve shifted to the left (51). Several studies have demonstrated that treatment changing the existing calcium concentration entail changes in the set-point of calcium combined with a shift of the PTH-calcium relationship to the left or to the right. The direction of this change depends on the corresponding serum calcium concentration change (47).
Normal pregnancy is a complicated process, placing a great deal of strain on the female body. Numerous complications and adverse outcomes are possible, although the vast majority of women complete their pregnancies without problems. A combination of adaptive metabolic responses protects the maternal skeleton, while simultaneously providing the growing fetus with an adequate delivery of minerals. By end of the last trimester of pregnancy 25-30g of elemental calcium is obtained by the fetus, and through the lactating period 250 mg calcium is consumed daily for maternal milk production. Thus a considerable share of the maternal calcium store (approximately 1000g in the average woman) is drained in each pregnancy. The circulating levels of total calcium decrease with albumin in pregnancy, while ionized calcium levels rise. During pregnancy the maternal intestinal calcium uptake doubles and vitamin D activation increases (52) as a protecting mechanism towards calcium depletion. PTH is normally slightly decreased in the first trimester but normalizes later on. Bone mass is not usually lost but may decrease with sustained lactation. Complete restoration is seen if lactation ceases within 9 months.

Parathyroid hormone related peptide – PTHrp

The PTH related peptide was first discovered in the 1980s, in tumours causing humoral hypercalcemia of malignancy (53). In its first amino-terminal sequence it is very similar to PTH but the protein is not detectable by radioimmunoassays for PTH. Nevertheless it binds effectively to PTH receptors and its actions mimic the bone-resorbing, phosphaturic and hypocalciuric effects of PTH. PTHrp expression is found in a variety of tissues, yet its normal function remains unclear. In pregnancy PTHrp seems to have several roles and widespread expression is found during embryogenesis. It is involved in maternal-fetal calcium transport, cell differentiation, fetal skeletal development, onset of labor and milk production (52). As PTH normally decreases during early pregnancy, it seems as PTHrp contributes to a state of maternal functional hyperparathyroidism to ensure adequate fetal mineral supply.

Normal pregnancy is associated with an increased blood volume, a generalized vasodilatation and a drop in peripheral vascular resistance, all with the intention to keep blood pressure low and to enhance peripheral
blood flow. Blood pressure is kept low by PTHrp actions in several ways and the levels increase during normal pregnancy. Low levels of PTHrp have been found in pregnancies complicated by intrauterine growth retardation (54).

Pregnancy-induced hypertension

Pregnancy-induced hypertension (PIH) is defined as a significant rise of blood-pressure $>140/90$ after 20 weeks of gestation. Risk factors are pre-existing hypertension, family history of PIH, diabetes, obesity, teenage pregnancy, age $>35$ and multiple gestation. Patients who develop PIH need close monitoring to facilitate the early detection of preeclampsia.
Preeclampsia

Preeclampsia is a multisystem disorder of unknown etiology, unique to human pregnancy. It is a major cause of maternal and perinatal mortality and morbidity worldwide, especially in developing countries (55). The disorder is characterized by an abnormal vascular response to placentation. The clinical consequences may be maternal and/or fetal, with hypertension and proteinuria, with or without other systemic manifestations in the mother, or fetal growth restriction, reduced amniotic fluid and abnormal oxygenation in the fetus (56).

The disorder is heterogeneous and multi-factorial and probably consists of at least two different maternal diseases. The preeclampsia that appears in early pregnancy, before the 33rd week, is associated with increased maternal and perinatal morbidity and mortality, as is preeclampsia in women with pre-existing medical disorders or women from developing countries. Late-onset preeclampsia, developing later than 36 weeks, is usually mild, without severe perinatal consequences.

Epidemiology, risk factors and diagnosis

Preeclampsia affects between 2-7% of healthy nulliparous women, and in this group 75% have mild disease with late onset. Women with previous preeclampsia, chronic hypertension, diabetes mellitus, multifetal gestation or pre-existing thrombophilias are at much higher risk (57). Limited sperm exposure is a risk factor for preeclampsia, confirmed by the increased risk in first pregnancies and younger women, and by the increased risk that is associated with a change of partner (58).

Obesity is a definite risk factor for preeclampsia, and there is a positive correlation to BMI (59). Obesity is associated with insulin resistance, which is also associated to preeclampsia, but the exact mechanism is not known. Several studies have indicated that women with preeclamptic pregnancies are at higher risk for developing cardiovascular complications later in life. Indeed, many risk factors and pathophysiological features for preeclampsia are similar to those of cardio vascular disease (60).

Vitamin D deficiency during pregnancy, particularly in the early stages, was associated with a higher risk of preeclampsia (27) and the risk for preeclampsia was halved if the mother had been given vitamin supplementation during infancy (61).
Preeclampsia is diagnosed if new-onset of hypertension, at least 140/90 mm Hg, combined with proteinuria is present on at least two occasions, in women not previously diagnosed with hypertension. The proteinuria is defined as excretion of 300 mg or more protein per 24 hrs.

Prevention of preeclampsia

Several randomized trials have been conducted over the years studying various methods of preventing preeclampsia; salt restriction, zinc, calcium, magnesium, fish oil, aspirin, vitamin E supplementation, the use of diuretics or antihypertensive medication in at-risk women, but the results have been limited. However, calcium supplementation has been associated with reduced hypertension and preeclampsia, especially if base-line intake was low. No side-effects were recorded, but in a larger trial no convincing result from calcium supplementation was found, so the real benefit from this is still in question (62). Low-dose aspirin has been used in many trials for the prevention of preeclampsia, and small to moderate benefits have been found. The treatment is still limited to delivery, but identification of women at high risk and close monitoring of at-risk pregnancies decrease the fatal consequences of this treacherous disease.
Obesity

Obesity is a rapidly increasing global health problem associated with high morbidity and mortality. The most commonly used method to describe body composition is to calculate BMI (body mass index, \( \text{kg/m}^2 \)). Thus BMI gives a measure of body weight per \( \text{m}^2 \) body surface. This method does not differentiate between muscular or adipose tissue, which entails that a trimmed individual may have a high BMI without excess fat and therefore without increased health risks. The WHO definition of overweight is BMI >25, while obesity requires a BMI >30, and a BMI >40 is referred to as morbid obesity.

Globally, there are more than one billion overweight adults; 300 million of those are obese. More than 50% of the European population is overweight. The etiology is multifactorial, but consumption of energy-dense foods combined with reduced physical activity are key-causes. The disorder is widely spread in all social groups but highly overrepresented in lower socioeconomic groups (63, 64).

Obesity poses a major risk for a variety of chronic diseases, e.g. type 2 diabetes, hypertension, cardiovascular disease, stroke, various types of cancers, strain injuries, cholelithiasis, obstructive sleep apnoea and early death. Despite the excessive calorie intake that presupposes obesity, micronutrient deficiencies are common in the obese population (65, 66). Calcium, thiamine, vitamin B12, folate, vitamin A, E and C, magnesium, iron, zinc, selenium and chromium are all reported to be low in obese populations. Vitamin D deficiency in the obese population is well known and frequently reported, and it results in a kind of sHPT although not related to renal failure (67-70). The vitamin D deficiency in the obese population has been suggested to be the cause of obesity (71), but a more likely reason is that the fat-soluble and hydrophobic character of vitamin D causes sequestration of vitamin D in fatty tissue. The stores of vitamin D become inaccessible. Despite its several disadvantages, increased risk for a variety of diseases and association with early death, obesity has been considered protective towards osteoporosis (72, 73). Heavy loads stimulate bone formation and a lower incidence of osteoporotic fractures is seen in obese. However, conflicting results are reported and recently (74, 75) it has been suggested that obesity is deleterious to bone.

Long-term weight loss reduces the risks for disease, improves the cardiovascular profile substantially, and ultimately results in reduced overall
mortality (76). Conventionally, weight reduction is attained via lifestyle modification programs of various types. However; diet, physical exercise, intensive guidance in groups or individually have produced no convincing long-term effects. Even though a 15-25% weight reduction can be achieved after 3-6 months on a very low caloric diet, the long-term results are meagre: 9% weight reduction after one year and 5% after 4 years (77).

Currently accessible pharmacological treatments fall into two categories, appetite suppressants and nutrient uptake inhibitors. Although these are frequently prescribed no convincing long-term effects have yet been reported. Four years of orlistat combined with physical exercise produced a modest weight loss of 5-6 kg (78).

Bariatric surgery
The term bariatric originates from Greek, *baros* meaning fat. Surgery has proven to be the most effective method to counter severe obesity and the demand for these procedures is increasing all over the world. Although effective in terms of permanent weight loss, bariatric surgery has life-long consequences for the patients. It involves major rearrangements to the gastrointestinal tract in order to achieve the massive weight loss, and life-long follow up is needed in order to keep track of post-operative nutritional deficiencies.

Two types of surgical techniques are used, either malabsorptive or restrictive. Some of the earlier methods, as jejunoileal bypass, are no longer performed because of severe long term complications. Roux-en-y Gastric Bypass (GBP) and bileopancreatic diversion (BPD) are both surgical procedures that combine a partial gastrectomy with a gastro-jejunoostomy or a gastro-ileostomy, thus combining the restrictive and the malabsorptive techniques. The length of the common limb is the factor that determines the time when nutrient absorption and digestion can occur.
Side-effects and complications

The early mortality rate after bariatric surgery is between 0.1-2% depending on surgical method and patient characteristics (79, 80). Restrictive methods appear to have lower mortality than malabsorptive procedures. Pulmonary thromboembolism and anastomotic leak are the most feared post-operative complications. Close post-operative supervision of bariatric patients is mandatory.

When the first weeks after surgery have passed, other complications may occur. Vomiting (due to anastomotic stenosis), dumping, endogenous hyperinsulinemic hypoglycaemia with nesidioblastosis and internal hernias have been reported.

B12 and iron deficiencies are common after gastric bypass and oral supplementation is often needed. Bone resorption markers are increased after three months and there have been descriptions of loss of bone density occurring less than a year post-operatively. Vitamin D deficiency is often still present after bariatric surgery (81, 82).
Main hypothesis

There is an association between calcium demands of pregnancy and the development of parathyroid adenoma. This is related to vitamin D receptor polymorphism, baT haplotype in particular, which is associated with higher levels of serum calcium and PTH, and may lead to development of primary hyperparathyroidism.

Disturbances of calcium homeostasis in obese individuals may be related to hypovitaminosis D. A possible cause is sequestration of vitamin D in fatty tissue and thus an improvement would be expected after bariatric surgery with normalization of body weight. The degree of disturbance of calcium homeostasis is correlated to BMI.
Aims

• to investigate the relationship between pregnancy and primary hyperparathyroidism

• to investigate the relationship between primary hyperparathyroidism and pregnancies with pre-eclampsia

• to study the vitamin D receptor status in preeclamptic women compared to women with normal pregnancies

• to investigate the calcium homeostasis with emphasis on the parathyroid secretion, in obese patients, before and after bariatric surgery

• to study bone mineral density in obese patients before and after bariatric surgery
Patients and methods

This thesis is based on two retrospective register-based papers and three clinical papers.

Table 1. *Overview of the five studies*

<table>
<thead>
<tr>
<th>Patients and controls</th>
<th>Issue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study I</td>
<td>1822 patients with PA and noted in the Fertility Register, 9092 controls from the Fertility Register</td>
</tr>
<tr>
<td>Study II</td>
<td>52 patients with PA and noted in the Medical Birth Register, 519 controls from the Medical Birth Register</td>
</tr>
<tr>
<td>Study III</td>
<td>39 patients with preeclampsia, 18 EPE, 21 LPE, 38 healthy controls</td>
</tr>
<tr>
<td>Study IV</td>
<td>108 patients selected for bariatric surgery, a subgroup of 11 individuals underwent CiCa-clamp</td>
</tr>
<tr>
<td>Study V</td>
<td>143 women with a history of bariatric surgery, six underwent CiCa-clamping pre-and post-operatively, 335 healthy controls</td>
</tr>
</tbody>
</table>
The Swedish Cancer Register

The Cancer Register has recorded all malignant tumours in Swedish citizens from 1958 onward and is approximately 98% complete (83). The incidence of benign endocrine tumors such as parathyroid adenomas (PA, the code provided by the International Classification of Diseases, ICD-7 and 8, was 195.1 and later 252.0), defined as one single pathological gland, are also reported to the Register. Parathyroid hyperplasia, which accounts for approximately 15% of pHPT diagnoses, is not reported.

Notification is mandatory for both clinicians and pathologists, resulting in very complete records. The date recorded in the Cancer Register is the date of registration which may not precisely correspond to the date of diagnosis or of surgery. Registration is usually made within months of surgery.

The Fertility Register

The Fertility Register is based on women born between 1925-60 who were resident citizens in Sweden at the time of the 1960 census. Reproductive data in this register encompasses nulliparity as well as the number and dates of live births of these women, from 1943 onwards. Later birth cohorts of Swedish women have been added continuously, with their own births being recorded annually via vital statistics records. The quality of data concerning numbers and dates of birth is generally high.

The Medical Birth Register

The Medical Birth Register has recorded information on almost 100% of live births and stillbirths in Sweden since 1973 (84), including maternal characteristics and details of the pregnancy and the birth.

Biochemistry

Basal serum or plasma values for calcium, albumin, creatinine, intact PTH and 25-OH-Vitamin D were determined, with all testing performed by the clinical chemistry laboratory at the University Hospital in Uppsala.

Total serum calcium was measured spectrophotometrically with a compleximetric method using orthocresolphthalein (normal range 2.15-2.50 mmol/L), and the total serum calcium was corrected to serum albumin (normal range 37-48 g/L; correction calculation: serum calcium + 0.019*(46 -plasma albumin)). Ionized plasma calcium (normal range 1.10–1.30 mmol/l) was determined with an ion-sensitive electrode (Kone Instruments, Espoo, Finland).
Serum albumin was determined by spectrophotometry using Bromine Bresol Breen (normal range 37-48 g/L). Serum creatinine was measured by spectrophotometry using Jaffe’s reaction (normal range 60-106 μmol/L). Intact plasma PTH (normal range 1.1-6.9 pmol/L) was measured with a chemiluminescent solid-phase two-site immunoassay using an Immulite 2500 (Diagnostics Product Corporation, Los Angeles, USA).

25-OH-Vitamin D was determined immunometrically using a Diasorin, Liaison-equipment (Saluggia, Italy). Vitamin D2 and D3 were measured without differentiation between the two forms. What should be regarded as the normal range for 25-OH-vitamin D is under debate. In these studies, we have used the standard range of 75-250 nmol/L, where 25-75 nmol/l represents vitamin D insufficiency, and < 25 nmol/L vitamin D deficiency (85).

Insulin Growth Factor 1 (IGF-1) was measured on an Immulite 2500 (Siemens Healthcare Diagnostics, Deerfield, IL, USA). The CV for the IGF-1 method was 2,3% at 99 μg/L and 1,5% at 710 μg/L.

CiCa clamp

CiCa clamp is an accepted and validated method for quantifying the secretion of PTH in relation to a certain ionized plasma calcium level, which has been proven to be reliable and reproducible with small inter-individual variations (49). The method has been performed in Uppsala University Hospital over the last 16 years. Hypocalcemia is induced by intravenous infusion of a calculated (body weight related) amount of citrate during 50 minutes. After establishing a steady state of hypocalcemia, hypercalcemia is induced by calcium infusion during the following 60 minutes. Intact plasma PTH and ionized plasma calcium are measured throughout the investigation.

Values are described in real values as well as after normalization of the response to 100%, where 100% is set at the highest plasma PTH. The set-point (SP) of calcium is calculated, as described by Bas et al (86). The SP is equal to the plasma ionized calcium concentration at which 50% of the maximum secretion of PTH is inhibited, measured as the plasma ionized calcium concentration at the mid-range of the PTH maximum – PTH minimum curve. Basal PTH secretion (before manipulation) is determined as well as maximal PTH secretion (PTHmax), minimal PTH secretion (PTHmin) and the ratio of basal PTH / maximum PTH (PTH b/m). Ionized calcium concentration at PTHmax (Ca at PTHmax) is also measured. This is defined as the ionized calcium concentration when PTHmax is reached, and no further reduction in ionized calcium led to any increase in PTH. In addition, the ionized calcium concentration at PTHmin (Ca at PTHmin), defined as the ionized calcium concentration at which PTH reached PTHmin, and no further increase in ionized calcium led to any further reductions in PTH.
Dual energy X-ray Absorptiometry (DXA)

Bone mineral density (BMD) (g/cm2) of the total body, femoral neck region of the hip, total proximal femur, and the lumbar spine (vertebrae L1-L4) was measured by DXA (Lunar Prodigy for the bariatric cases and Lunar DPX-IQ for controls, Lunar corp., Madison, WI, USA). When applicable, both extremities were used in the calculation. By triple measurements in 15 subjects, 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 the site. Daily scans of a lumbar spine phantom were performed. The long-term precision error CV% was less than 1% during the study period.

Bone density results from DXA are reported in a variety of ways; as BMD (g/m²), bone mineral content (BMC g/cm), bone area (cm²), T-scores and Z-scores. Different manufacturers of DXA equipment use different reference populations (87) (Lunar – white American females 20-40 years, NHANESIII – 20-29 years Caucasians) and no global agreement has been obtained. T- and Z-scores are derived from statistical units of the standard deviation (SD). The T-score is the number of SDs below the average for a young adult of the same race at peak bone density. The Z-score is the number of standard deviations below an average person of the same age. An individual may have different T-scores at femoral neck, spine and at the total hip. The results in this study are reported in g/cm² only; neither T- nor Z-scores are shown. Since two DXA densitometers from the same manufacturer are used, one fan-beam scanner (Prodigy) and one pencil-beam scanner (DPX-IQ), a cross-calibration was made (only in BMDN) to make the results comparable (88), \[ DPX_{BMD} = 0.08 + 0.906 \times \text{Prodigy}_{BMD}. \]

DNA analysis

DNA was prepared from peripheral leukocytes. VDR genotypes denoted BB, Bb and bb were determined after BSMI restriction cleavage of genomic DNA amplified by the polymerase chain reaction (PCR) as previously reported (89).

A 740 bp fragment of the VDR genome, including the Apa I and Taq 1 restriction sites, was amplified by PCR using specific primers 5’-CAGAGCATGGACAGGGAGCAA-3’ and 5’-GCAACTCCTCATGGCTGAGGTCTC-3’. All PCR were run at 95° for 2 min followed by 40 cycles of 95° for 20 sec, 70° for 20 sec, 72° for 40 sec, and final extension at 72° for 7 min. The PCR products were digested with Apa I (5 U at 25°C ) or Taq 1 (3 U at 65°) for 4 hours. Apa I digestion reveals genotypes denoted AA (740 bp), Aa (740, 530, 210 bp) or aa (530, 210 bp), and Taq I genotypes TT (495, 245 bp), Tt (495, 290, 245, 205 bp).
or tt (290, 245, 205 bp). PCR products were separated on agarose gels and compared to a defined DNA ladder.

Studies I and II

The first two studies in this thesis were based on three nationwide registries, the Swedish Cancer Register, the Fertility Register and the Medical Birth Register. In Study I a total of 1822 women were identified with a diagnosis of PA, and a registration in the Fertility Register. In a nested case-control design all PA-cases were matched with five women randomly selected from the Fertility Register, the controls having the same year and month of birth as the case, and being free of a PA-diagnosis at that time.

In study II 573 women were identified with a diagnosis of PA and a registration of a singleton birth recorded in the Medical Birth Register between 1964 and 1997. Each of those cases was matched with up to ten randomly chosen women, giving birth at the same calendar year at the same delivery unit, altogether 5669 controls. One woman was excluded since she could not be matched with any reference woman, and 4 % were matched with fewer that 10 women, depending on the opening and closing of smaller delivery units during the study period. To ensure that treatment for parathyroid adenoma predated pregnancy the analysis was restricted to women who were treated more than 2 years before delivery.

1. The main analysis was based on cases (and the matched controls) who received a diagnosis of parathyroid adenoma before their first singleton birth during the study period. A total of 52 cases and 519 controls were used for the main analysis.
2. The second analysis included cases diagnosed at least 5 yr before delivery, limiting the number of cases and controls to 30 and 300, respectively.
3. The third analysis included cases diagnosed at least 2 yr after delivery, limiting the number of women to 454 (14 had preeclampsia) and 4488 controls (70 had preeclampsia).
4. The fourth analysis included cases diagnosed at least 5 yr after delivery, resulting in 410 cases (11 with preeclampsia) and 4052 controls (66 with preeclampsia).

Conditional logistic regression was used for matched analysis and odds ratio as estimates of the relative risk were obtained.

Study III

All patients with preeclampsia (PE) (defined as new onset of blood-pressure $\geq 140/90$ and proteinuria $\geq 0.3g/24$ hrs after 20 gestational weeks) admitted to
Uppsala University Hospital between 2001-2005 were asked to participate. Established hypertension, diabetes, renal failure and duplex pregnancy were exclusion criteria. In all, 39 patients accepted to participate in the study. They were divided into two groups according to gestational length at onset of disease (early preeclampsia, EPE, and late preeclampsia, LPE; diagnosis at week 24-32 and week 36-42, respectively). The control group consisted of 38 women with normal pregnancies who delivered at the Uppsala University Hospital during the same period.

Student’s t-test was used to determine differences between groups. Odds ratios (OR) with 95% confidence intervals (CI) as an estimate of relative risk were calculated with logistic regression for VDR polymorphism analyses.

Studies IV and V

Study IV included 108 consecutive patients (76 women) aged 39.1±10 years with a mean BMI of 46.6±6 selected for bariatric surgery during 2006. They all contributed blood samples pre-operatively. Eleven of those patients (8 women) with a mean age of 47±9 years and a BMI of 44.7±4 agreed to participate in further investigations, and all underwent CiCa-clamping prior to surgery. They were compared to 21 healthy volunteers of normal weight as well as to 15 patients diagnosed with primary hyperparathyroidism.

All pre-menopausal females (below 50 years of age) who had undergone Roux-en-Y Gastric Bypass surgery during 1996-2006 at Uppsala University Hospital were invited to participate in Study V. This included 58 women from study IV and a total of 143 (out of 208) agreed to participate. All women contributed blood samples and 89 underwent Dual energy X-ray Absorptiometry (DXA), 3-13 years postoperatively.

Statistical significance was set as p<0.05. Values were presented as mean±SD. In study IV PASW Statistics version 18.0 was used for calculations and DeltaGraph for visualization. Student’s unpaired and paired t-tests were used to determine changes, and Spearman rank correlation was used for calculation of dependence between two variables.

The statistical calculations in study V were performed using SAS (SAS 9.2; SAS Institute Inc., Cary, NC). For bone mineral density variables (femoral neck, total hip and lumbar spine), adjusted means with 95% confidence intervals among bariatric patients and controls were estimated using the GLM (general linear models) procedure in the SAS package.
Results

Study I

Overall, there was no difference between nulliparous and parous women in the risk of developing PA. Amongst premenopausal women (<50 years), the estimated risk of PA was lower amongst ever-parous than nulliparous subjects (OR 0.8395%; CI 0.68±1.02). In older women (>50 years), being ever-parous was associated with an elevated risk for PA (OR 1.1895%; CI 0.96±1.45).

Relative to uniparity, however, multiparity was associated with elevated risk estimates both amongst pre- and post-menopausal women. An inverse association between age at first birth and the risk of PA was only present in the pre-menopausal group.

Study II

This study showed that women with PA diagnosed and treated more than two years prior to delivery are at a statistically significant (P<0.001) higher risk of PE. The magnitude of the association was not diminished by adjustment for age at delivery, parity or for sex of the offspring. The association between PE and PA diagnosed and treated more than five years prior to delivery was also statistically significant.

The association of PE with a subsequent diagnosis of PA produced an unadjusted odds ratio of 2.11 (1.14, 3.88) and an odds ratio adjusted for potential confounding factors of 2.54 (1.35, 4.81). The analysis of women with a diagnosis of PA more than five years after delivery did not show a statistically significant association: 1.71 (0.87, 3.37).
<table>
<thead>
<tr>
<th></th>
<th>With PA</th>
<th>Without PA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
</tr>
<tr>
<td><strong>Mother’s age (years)</strong></td>
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<td></td>
</tr>
<tr>
<td>&lt; 21</td>
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<td>2</td>
</tr>
<tr>
<td>21-25</td>
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<td>21</td>
</tr>
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<td>26-30</td>
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</tr>
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</tr>
<tr>
<td>&gt; 40</td>
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</tr>
<tr>
<td>No</td>
<td>45</td>
<td>87</td>
</tr>
<tr>
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<td>7</td>
<td>13</td>
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<tr>
<td><strong>Median duration</strong></td>
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<td></td>
</tr>
<tr>
<td>from PA diagnosis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>to delivery (years)</td>
<td>6</td>
<td>(median)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>52</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Characteristics of women (and their pregnancies) with parathyroid adenoma (PA) and a matched comparison group. The diagnosis of PA occurred more than two years prior to delivery.

* Women without PA were included in the study in the same year as PA was registered in the women they were matched with. The length of time from study entry to pregnancy was thereby identical for those with and without a diagnosis of PA.
<table>
<thead>
<tr>
<th></th>
<th>Unadjusted OR (95% CI)</th>
<th>Adjusted OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA diagnosis more than 2 years prior to delivery</td>
<td>6.35 (2.44, 17.30)</td>
<td>6.89 (2.30, 20.58)</td>
</tr>
<tr>
<td>PA diagnosis more than 5 years prior to delivery</td>
<td>7.69 (2.43, 24.31)</td>
<td>10.76 (2.78, 41.61)</td>
</tr>
</tbody>
</table>

Table 3. The odds ratios for pre-eclampsia associated with a diagnosis of parathyroid adenoma (PA) more than two and five years prior to delivery. This analysis is matched by risk-set, such that women with PA are only compared with women without a diagnosis of PA who were matched for the date of PA diagnosis, the date of delivery and the delivery unit.

Study III

Statistically significant differences were found between PE versus healthy pregnancies in weight, BMI, albumin, total serum calcium, PTH and creatinine, but not in 25-OH-D (table 4). The increase in PTH remained robust also when adjusted to creatinine. There were no statistically significant differences between early and late preeclampsia in those parameters.

The DNA analysis of VDR could not highlight any differences in polymorphisms in the VDR gene between normal Swedish population and this selection of women with preeclampsia. Vitamin D levels were lower than normal in both pregnant groups.
## Table 4

Students t-test of PE-cases versus healthy controls. S-ca = total calcium, corr ca is total calcium corrected to serum albumin; \((46\text{-albumin})*0.02) + \text{total calcium. crea=serum creatinine, 25OHD=25-hydroxy vitamin D.}\n
### Study IV

All except four subjects had normal serum calcium and creatinine levels, and 14% showed increased levels of PTH. One patient was diagnosed with pHPT and underwent surgery for parathyroid adenoma before the bariatric procedure.

CiCa-clamping in the obese individuals demonstrated a left-shifted calcium-PTH relationship and a decreased set-point compared to healthy controls as well as to patients with pHPT. There was a statistically significant positive correlation between PTH and duration of obesity.
Study V
The postoperative CiCa-clamping showed a persistent left-shift of the calcium-PTH relationship and a decreased set-point compared to healthy controls, although a slight tendency towards normalization was detectable. The difference was not significant.

Post-operative levels of vitamin D were unchanged, despite the start of a daily vitamin D supplementation immediately after surgery. PTH showed a significant increase from 5.5 pre-operatively to 6.9 pmol/L post-operatively, with 38% displaying levels above the upper normal range. Serum calcium was decreased from 2.4 mmol/L preoperatively to 2.2 mmol/L after GBP.
Figure 4. CiCa-clamping in bariatric patients, pre- and postoperatively, in comparison with healthy controls and individuals with pHPT. Postoperatively the obese group is normalizing, the altitude of the curve is decreasing and shifting to the right.

Investigation by DXA of the women who underwent surgery demonstrated a pronounced decrease in bone mass in all sites, compared to healthy women. All measurements were adjusted to age, BMI and height. If the women were divided into groups according to time elapsed since surgery, 3-5 years, 6-9 years and 10-13 years, there was a significant negative correlation between BMD and time since surgery in the first to intervals. After that a slight increase in bone mass was seen, but BMD was still significantly decreased compared to non-operated women.
**Figure 5.** DXA investigation in cases (n=89) and controls (n=335), all differences statistically significant. Mean values are shown as filled circles, 95% confidence intervals are indicated as vertical bars.
Conclusions

- There is an association between childbearing and the risk of parathyroid adenoma. High parity (four or more live births) is associated with an increased risk.
- There is an association between history of parathyroid adenoma and preeclampsia, producing an adjusted odds ratio of 6.89 (95% confidence interval, 2.30-20.58).
- PTH is significantly increased in preeclampsia. No significant pattern of pHPT associated polymorphisms could be detected in VDR in preeclampsia, and hypovitaminosis D was present in both groups.
- There is a left-shifted relationship between calcium and PTH in obesity, and set-point for calcium is decreased. Hypovitaminosis D is present in obesity and there is a positive correlation between duration of obesity and PTH.
- The left-shifted relation between calcium and PTH persists post-operatively. Calcium is decreased and PTH is increased, and there is a pronounced decrease in bone mass that is correlated to time since surgery in long-term follow-up of bariatric patients.
Discussion

Maintaining a constant calcium level is crucial for a living being, and a variety of protective systems prevent alterations in either direction. Up to 99% of the calcium is found in its solid form in man (in bones and teeth) and only 1% is found in blood and soft tissues. It is playing an essential role in cell signaling, and as a cofactor for enzymes and proteins, optimizing their activities. The normal range of serum calcium is very narrow, 2.20-2.50 mmol/L.

A prerequisite for an adequate calcium uptake from food is a sufficient level of vitamin D. In this thesis we have studied calcium homeostasis and vitamin D in two populations; patients selected for bariatric surgery and pregnant women. Both groups are overweight, although for different reasons. Disturbances in calcium homeostasis with decreased levels of serum calcium and increased levels of PTH were detected in both groups. Vitamin D levels in a group of 148 healthy individuals with a mean age of 57 living in Uppsala was in mean 62.7 nmol/L (95% CI 58.0-76.3), and both of our study groups demonstrated lower levels than that.

Optimal range of vitamin D

The 14th Vitamin D Workshop (Bruges 2009) consensus could not agree on an optimal level of vitamin D (90), but declared that a minimum of 50 nmol/L is necessary in all individuals in order to support and maintain all the classic actions of vitamin D on bone and mineral health. Newer data which show associations between vitamin D status and several other diseases such as cardiovascular disease, hypertension, colon and breast cancer, multiple sclerosis, and also involvement of vitamin D in immunological functions and muscle strength, indicate that the optimal range is higher, 75-100nmol/L (19, 22, 26, 28, 91). Many of the assembly of invited scientists advocated this range.

Whichever range chosen at least half of elderly North Americans and Western Europeans, and probably also two thirds of the rest of the world are vitamin D deficient as judged by their inability to maintain a healthy bone density (15, 91). At Uppsala University Hospital normal range of 25-OH-D is considered 75-250 nmol/L.
Vitamin D in the obese

The reports of hypovitaminosis D in obese are several (67, 70, 92, 93), and our results (studies IV and V) confirm earlier findings. Since vitamin D is fat-soluble and has a hydrophobic nature, it sequestrates in fatty tissue, and is not easily accessed from there. Blum et al showed that the concentration of vitamin D in fat is approximately ten times higher than in the blood stream (92).

Secondary hyperparathyroidism is associated to vitamin D deficiency but in study IV we made clear that there were no obvious signs of secondary hyperparathyroidism in our obese group. The CiCa-clamping in the obese group, which to our knowledge had not been performed previously in such a group, demonstrated a very high sensitivity for calcium and increased ability of secreting parathyroid hormone at hypocalcemia. This was a surprising result although earlier animal trials have shown that such a left-shifted relation between calcium and PTH may appear in early development of sHPT (47, 86). Our interpretation is that the “locked in” vitamin D prevents a sufficient calcium uptake in the obese, and therefore creates this left-shifted relation and lower set-point as a sign of latent hypocalcemia.

We also speculate that a longer duration of obesity may bring about an overt hyperparathyroidism due to development of nodular hyperplasia of the parathyroids. The reason for persistent hypovitaminosis D postoperatively despite prescription of oral supplementation must most likely be assigned to anatomic alteration due to the surgical procedure. The shortened gastrointestinal canal with the excluded duodenum probably precludes vitamin D uptake from food. Furthermore, the ability to sequestrate vitamin D in fatty tissue is still there. Despite the often huge weight loss after bariatric surgery most of the patients are still overweight or even obese. Moreover, ten years after surgery the majority of the patients are gaining weight again. The mean BMI 3-13 years postoperatively was 31 in our material.

Calcium and BMD

In the 3-13 year follow-up after bariatric surgery (study V) DXA investigations were performed on 89 women. They were compared to a group of 335 healthy Swedish women 20-40 years of age, after adjustment for age, height and BMI. Calcium levels were lower, PTH levels were higher and BMD was significantly lower in all sites in the operated women.
Figure 6. Statistically significant decrease in serum calcium levels postoperatively in the bariatric group

Main calcium uptake is normally performed in the duodenum where the majority of vitamin D receptors are expressed and only limited amounts are absorbed distally. Moreover, calcium absorption is strictly dependent on sufficient levels of vitamin D. In the case of an excluded duodenum complicated by a decreased level of vitamin D, the calcium absorption is strictly limited. These conditions are evident in the long term follow-up of bariatric patients. A total of 38% had PTH levels above normal, as a sign of activation of bone resorption in order to maintain adequate calcium levels.

Our study group contained no menopausal women, and yet their BMD levels were more than 10 % lower than in non-operated women (in femoral neck). A further decrease may be expected in connection to menopause. Sedentary habits are also overrepresented in this group, a fact that exacerbates the risk for later osteoporosis development.

PTH and preeclampsia

Study I-II disclosed associations between pregnancy and pHPT that are not previously described. In study III we found elevated PTH levels in preeclampsia as compared to healthy pregnancies, a result which remained robust after adjusting for creatinine.

Until recently the role of PTH in fetal development has been uncertain. Fetal serum calcium level is well above the maternal level, both total calcium and the ionized fraction. This circumstance has led to the conclusion
that calcium influx to the fetus is primarily driven by active transport pathways. PTHrp is expressed both in placenta and in the growth plate, and is also present in high levels in fetal circulation. It has major importance in fetal bone formation (94). However, PTH is expressed in fetal parathyroids as well as in placenta, and unlike PTHrp, it increases with fetal hypocalcemia. PTH is important for maintaining fetal blood calcium levels and skeletal mineralization, and it seems to act in concert with PTHrp (95). It is not fully known how this collaboration is regulated but animal models have shown decreased mineralization of fetal skeleton with loss of either protein. In preeclampsia the placental circulation is impaired, which influences fetal calcium transport. The increase in maternal PTH in preeclampsia may thus be a sign of a compensatory mechanism due to fetal hypocalcemia as well as to maternal hypovitaminosis D.

The actions of PTHrp in preeclampsia is not fully known, but this protein has long been suspected to have a role in PE scenario and lower levels of PTHrp is described in pregnancies with adverse outcomes (54).

**Vitamin D and preeclampsia**

Several reports describe associations between hypovitaminosis D and/or maternal obesity and preeclampsia (27, 96-98). In Study III we could confirm decreased vitamin D levels in the PE group and also a higher BMI, but hypovitaminosis D was equally present in the healthy pregnancies. The elevated risk for preeclampsia in mothers with a history of pHPT (study II) is interesting from many aspects. Parathyroidectomy because of parathyroid adenoma is a treatment with a usually high success rate, and calcium levels are normalized within days. PTH levels on the other hand may be elevated for months post-operatively. For this reason we excluded mothers who had delivered less than two years after parathyroidectomy from the material, to avoid influence from persistent raised PTH levels postoperatively. Vitamin D deficiency can be involved in development of HPT and thus be present in the women even after surgery, but since there are no available data on biochemistry in these women (Study II), this cannot be evaluated.

Study I showed a slight overrepresentation of pHPT development in women with many children, but in study II the pHPT preceded pregnancy and here the association was very strong. In this population (Study II) we also investigated the association between pregnancy with preeclampsia and a subsequent development of pHPT, and found a weak association which disappeared with adjustment for confounding factors.

Because pHPT mainly is a disease of the postmenopausal woman, and pHPT in fertile women is rare, these results may indicate the presence of two different disturbances in the parathyroids. One appears in the older woman, and may be associated with her pregnancies/lactation periods (the disease being subsequent to the pregnancies). The other seems to affect the younger...
woman, and might thus be a sign of another type of disturbance, also involved in development of preeclampsia.

Clinical implications
The association found between a history of parathyroid adenoma and subsequent pre-eclampsia in Study II is of such strength, with an adjusted OR of 6.89 (95% CI 2.30, 20.58) that a history of PA should be viewed as a risk factor for preeclampsia.

The increased part of the bariatric group (38%) in study V showing raised levels of PTH is a distinct sign of the progressive disturbance in calcium homeostasis post-operatively. Clear evidence of bone resorption in this group, with lower levels of BMD in all sites demonstrates that bariatric surgery poses a risk for developing osteoporosis post-operatively. The supplementation with multi vitamin tablets prescribed to these patients is obviously not sufficient. Patients who have undergone bariatric surgery should be subjected to life-long follow-up, including DXA and calcium homeostasis mapping. Oral supplementation may not be sufficient in this category of patients.

Limitations
The first two papers are based on register-based retrospective studies. We could establish the association between childbearing and subsequent pHPT on one hand, and between a history of parathyroid adenoma and subsequent preeclampsia on the other hand. However, there was no data on maternal calcium and PTH levels and we had no information on maternal BMI, which could have further strengthened the results.

In study III-V, vitamin D was analyzed, as described in Patients and Methods. The method available (CLIA) at Uppsala University Hospital is indeed a validated one but recent studies have shown its shortcomings, including a tendency to underestimate vitamin D levels (13). This may of course have influenced the degree of vitamin D deficiency reported in our material.

Also, we did not take into account the seasonal variation in vitamin D status. The difference between summer and winter is probably as high as 20nmol/L, as no synthesis in the skin is possible between October and March due to inadequate UV radiation (13). In Study IV blood samples for vitamin D were taken all year around, but in Study IV all blood samples were drawn in the summer and early autumn when vitamin D levels peak in Sweden. Because of this we conclude that the vitamin D levels in the women in Study V may be even lower.

A limited number of patients participated in Study III, a drawback that may have influenced the results, especially in the DNA analysis. In Studies
IV and V the CiCa-clamping was performed in a limited number of patients, which may have influenced the results.

Future perspectives
Sufficient levels of vitamin D seem to be of importance in the diseases studied. Hitherto all efforts to offer a substitute form of vitamin D to the bariatric patients show meager results, and the upcoming effects of a progressive depletion are disquieting. To our knowledge providing oral substitution of vitamin D substitutes to pregnant women who are at risk for preeclampsia has not been studied, although several trials have been performed where calcium substitutes have been provided (and found protective in selected populations).

To study causality in the disturbed calcium homeostasis in bariatric patients supplementation of vitamin D to depleted individuals preceded and followed by CiCa-clamping must be performed. Since oral vitamin D substitutes seem ineffective, injections of vitamin D or UVB-treatment may have a greater impact on vitamin D levels.
Övervikt och fetma är tillstånd som ökar över hela världen; enligt WHO finns för närvarande omkring en miljard överviktiga människor, och mer än 2,6 miljoner dödsfall årligen orsakas av detta. Definitionen på övervikt är ett body mass index (BMI, kg/m²) >25, medan BMI >30 innebär fetma. Orsakerna till övervikt och fetma är flera, det finns i alla befolkningsgrupper,


I denna avhandling har vi studerat den förändrade kalkbalansen hos överviktiga och hos gravida med PE. Vi har använt oss dels av data från register och dels av undersökningar av patienter och frivilliga kontrollpersoner.

I delarbete I analyserades med hjälp av register sambandet mellan antalet genomgångna graviditeter och senare utveckling av överfunktion i en bisköldkörtel (parathyroideaadenom PA), ett tillstånd som leder till för höga kalknivåer i blodet. Kvinnor som opererats på grund av PA identifierades i Svenska Cancerregistret och jämfördes med data från Svenska
födelseregistret där alla förlossningar i Sverige registrerats sedan 1943. Det visade sig att risken för PA ökade ju fler barn man fött, och att vid fler än tre graviditeter fanns en tydligt ökad risk att senare i livet utveckla PA.

I delarbete II analyserades med hjälp av register sambandet mellan pre-eklampsii och pHPT. Kvinnor som opererats på grund av PA identifierades i Svenska Cancerregistret och fick ingå i studien om de också hade en förlossning mellan 1973 och 1997 registrerad i Svenska födelseregistret. På detta sätt hittades 573 kvinnor, som matchades med 10 friska kontroller vardera, all förlösta samma år vid samma sjukhus. I denna grupp fanns 52 kvinnor som opererats pga PA mer än 2 år före graviditeten. Sju (13%) av dessa utvecklade PE under sina graviditeter, medan bara 2% av kontrollerna drabbades. När man tog hänsyn till kvinnans ålder, antal tidigare födda barn samt barnets kön visade sig risken för PE vara mer än tiodubblad för kvinnor med tidigare PA. Detta gällde om PA var behandlat mer än fem år före förlossningen. Om kvinnan behandlats för PA 2-5 år före förlossningen var risken för PE nästan sjufaldig.


ett tydligt samband mellan bisköldkörtelhormonnivån och hur lång tid de varit överviktiga. CiCa-clamp visade att de överviktiga var mer känsliga för kalknivån i blodet än friska och patienter med överfunktion av bisköldkörtlarna, och även hade en hög sekretionsförmåga (av bisköldkörtelhormon). Set-point, den nivå av blodkalk där bisköldkörtlarna är ”avstängda” till hälften, var lägre hos de överviktiga jämfört med både de friska kontrollpersonerna och patienterna med överfunktion av sina bisköldkörtlar. Detta tolkades som ett led i en tidig utveckling av sekundär överfunktion av bisköldkörtlarna. En möjlig orsak till tillståndet är bristen på D-vitamin.

Slutsatser


II. Det finns ett samband mellan bisköldkörtelsjukdom och havandeskapsförgiftning, pre-eklampsi. Risken att drabbas av pre-eklampsi är nästan sju gånger högre för en kvinna som tidigare haft bisköldkörtelsjukdom.

III. Bisköldkörtelhormon finns i förhöjd halt i blodet hos kvinnor med pre-eklampsi. Låga D-vitaminnivåer finns hos både friska gravida samt hos kvinnor med pre-eklampsi, men ingen skillnad i utseendet på D-vitaminreceptorerna hos de sjuka kunde hittas.


V. D-vitaminbrist kvarstår efter överviktsoperation. Dessutom uppstår kalkbrist och därigenom ökad stimulans av bisköldkörtlarna. Detta leder till urkalkning av skelettet och beror sannolikt på den försämrade upptagsförmågan av näringsämnen i den urkopplade tarmen.
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Johan my love, for everything
The cover picture is a photograph of the Venus of Willendorf, a tiny statuette of a female, just 11 cm high. Discovered in 1908 in Lower Austria near the city of Krems, it is thought she was made between 22,000 and 21,000 A.D. She is carved from an oolitic limestone that is not local to the area, and tinted with red ochre. Her history is unknown; why her face is covered, why her arms are so thin, whether she is pregnant or just obese. The right hand version of her is a photomontage after an imaginary bariatric procedure, or maybe a delivery
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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 Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)