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# Calcium Homeostasis in Patients with Graves' Disease

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### **Abstract**

Annerbo, M. 2016. Calcium Homeostasis in Patients with Graves' Disease. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1212. 51 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-554-9552-7.

Patients with Graves' Disease (GD) have a higher risk of developing more severe and prolonged hypocalcaemia after total thyroidectomy (TT) than patients who undergo surgery for benign atoxic goitre. Since TT is the most effective treatment for GD, it is crucial to identify mechanisms for postoperative hypocalcaemia. The aim of this thesis was to study the mechanisms of calcium metabolism in patients with GD.

It is safe to operate on GD patients with TT. Results in Paper I showed fewer recurrences and equal complication rates compared to patients who underwent subtotal thyroidectomy (ST). The transient lowering of PTH seen in the hypocalcaemic patients was fully restored one month after surgery (Papers II and V).

The calcium-sensing receptor (CaSR) is crucial for maintaining plasma calcium, and single nucleotide polymorphisms (SNPs) in the gene may alter the sensing function. Thus, we analysed SNPs in CaSR in GD patients (Paper II) and showed that they had a more left-shifted calcium-PTH set-point compared to controls, implicating higher sensitivity. This is also supported by the results in the group of postoperatively hypocalcaemic patients. They already had lower plasma calcium preoperatively (Papers II, IV and V) and lacked the T/G G/A G/C, a haplotype shown in Paper III to have a close relationship to higher p-calcium levels. Moreover, a lack of the T allele in rs1801725 was seen in the group of patients needing permanent treatment with calcium and vitamin D, i.e. > 12 months, (paper V).

Patients who became hypocalcaemic (p-calcium < 2.00 mmol/L) on day one postoperatively, had lower preoperative levels of thyroid stimulating hormone (TSH) and higher levels of T3, this was also applied to the patient groups requiring temporary or permanent postoperative treatment (Papers II and V). In addition, hypocalcaemic patients treated for less than six months with anti-thyroid drugs had higher levels of bone metabolism markers CTX and PINP than normocalcaemic patients (Paper V).

In conclusion, the postoperative period of hypocalcaemia seen in patients with GD is a complex medical condition, caused by a combination of surgical trauma, different SNPs in CaSR, and high bone metabolism related to preoperative thyroid metabolism.

*Keywords:* Graves' Disease, Calcium homeostasis, Total thyroidectomy, Bone metabolism, Calcium sensing receptor

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*Till min kära familj*



# List of Papers

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

- I Annerbo M, Stålberg P, Hellman P (2012) Management of Grave's Disease Is Improved by Total Thyroidectomy. *World Journal of Surgery*, 2012. Doi10.1007/s00268-012-1617-x.
- II Annerbo M, Hultin H, Stålberg P, Hellman P (2013) Left-shifted relationship between calcium and parathyroid hormone in Graves' Disease. *The Journal of Clinical Endocrinology and Metabolism*, 2013. Doi10.1210/jc.2013-2500.
- III Annerbo M, Lind L, Björklund P, Hellman P. Association between Calcium Sensing Receptor Polymorphisms and Serum Calcium in a Swedish Well-Characterized Cohort. Submitted
- IV Annerbo M, Azadi A, Stålberg P, Björklund P, Hellman P. Calcium-Sensing Receptor Polymorphisms and their Relationship to Postoperative Hypocalcaemia in Graves' Disease. Manuscript.
- V Annerbo M, Carlsson E, Björklund P, Hellman P. Biochemical Markers in Bone Metabolism in Patients with Graves' Disease. Manuscript.

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# Abbreviations

GD	Graves' Disease
TSH	Thyroid Stimulating Hormone, Thyrotropin
TSHR	Thyroid Stimulating Hormone Receptor/ Thyrotropin Receptor
TRAb	Thyroid Receptor Antibody
T4	Thyroxine
T3	Triiodothyronine
MNG	Multinodular goitre
ST	Subtotal Thyroidectomy
TT	Total Thyroidectomy
DO	Dysthyroid Ophthalmopathy
HLA	Human leukocyte antigen
CD40	Cluster of differentiation 40
CTLA4	Cytotoxic T lymphocyte-associated protein 4
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
FCRL3	Fc receptor-like protein 3
ECF	Extra cellular fluid
PTH	Parathyroid hormone
RANKL	Receptor activator of nuclear kappa-B ligand
ECD	Extra cellular domain
VFTD	Venus fly trap domain
GPCR	G-protein coupled receptor
OMIM	Online Mendelian Inheritance in Man
CiCa	Citrate Calcium Clamping
CaSR	Calcium Sensing Receptor
SP	Set-point
DEXA	Dual Energy X-ray Absorptiometry / Bone Densitometry
PIVUS	Prospective Investigation of the Vasculature in Uppsala Seniors
ADH	Autosomal dominant hypocalcaemia
FHH	Familial Hypocalciuric Hypercalcaemia
NSHPT	Neonatal Severe Primary Hyperparathyroidism
PHPT	Primary Hyperparathyroidism
SNP	Single Nucleotide Polymorphism
p	Plasma

PCR Polymerase chain reaction  
MAF Minor Allele Frequency  
CTX Beta-Crosslaps  
P1NP Procollagen 1 N-terminal Propeptide  
GWAS Genome Wide Association Study  
DEQAS Vitamin D External Quality Assessment Scheme

# Preface

What are the mechanisms behind the hypocalcemia seen in some of the patients with Graves' Disease (GD)? Why do not all patients who undergo surgery with total thyroidectomy (TT) become hypocalcaemic? And why only the patients with GD? When operating a patient with GD all endocrine surgeons know that this will be a more difficult, and in some ways a more hazardous operation than if this patient had an ordinary multi nodular goiter (MNG) from Dalecarlia, a so called "Dalastruma".

We know that we are using the same surgery technique as always. Performing capsule close dissection and always proximal ligation of the vessels to the parathyroid glands, those tiny healthy-brownish organs. But still the parathyroid glands hosted in a patient with GD seems to dislike the operation trauma. Why is that? Reduced bloodflow, autoimmunity unknown plasma related factors, bone-hunger, surgical damage to the parathyroid glands or maybe genetical differences in the calcium sensing receptor (CaSR)?

In this thesis I have made a serious effort to clarify the mechanisms behind the hypocalcaemia.

As an endocrine surgeon in this huge field of endocrinology I have often become stressed and confused but also thrilled, by the complexity concerning this small molecule  $\text{Ca}^{2+}$  and its relations to bone, kidney skin and intestinal functions. It has been fascinating to work within the huge field of genomics and to get to know the significance of small SNP changes.

# Introduction

Thyrotoxicosis is defined as an excess of thyroid hormones with clinical manifestations. The two main hormones synthesised and released by the thyroid follicular cells are thyroxine (T4), which presents at a higher concentration and counts for as a storage and resource inside the thyroid follicular cells, and triiodothyronine (T3). T4 is a prohormone and is modulated in nearly all cells in the body by deiodinases to T3, the biologically most active form. The synthesis of thyroid hormones requires iodine and the hormone levels are regulated by a feed-back loop including thyroid-stimulating hormone (TSH) secreted from the anterior pituitary gland and thyrotropin releasing hormone (TRH) from the hypothalamus.

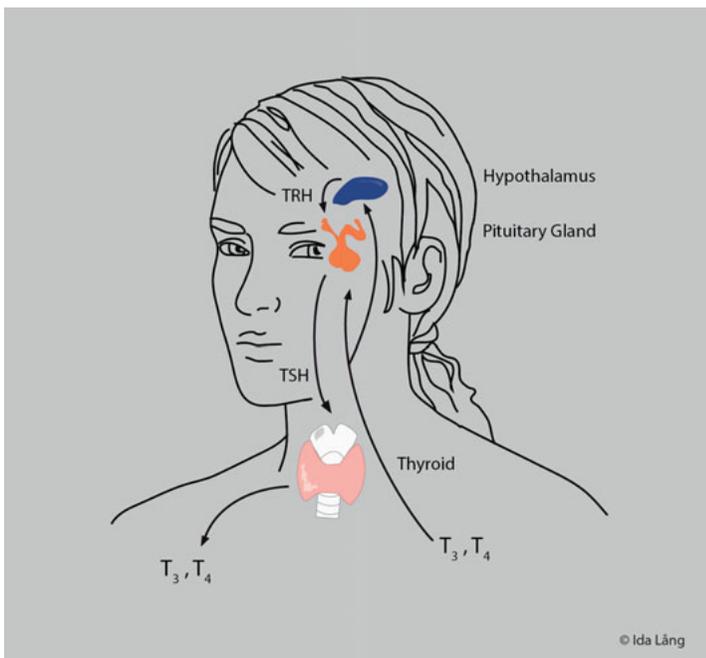


Figure 1. Regulation of thyroid hormones TSH feedback-loop.

Thyrotoxicosis can be caused by various diseases, such as toxic multinodular goitre (MNG), toxic adenoma and thyroiditis, but the most common one is Graves' Disease (GD) which counts for 75-90% of cases, depending on the level of iodine intake [1, 2].

## Graves' Disease

The excess of thyroid hormones will affect every physiological system and result in hyperactivity, increased cardiac frequency and risk of atrial fibrillation, hypertension, weight loss, raised body temperature with increased perspiration and, typically, humid shaking palms and fingers, decreased fertility and libido [1, 3], and osteoporosis [4-6]. In general, the symptoms are more aggravated in younger patients and related to the severity of the disease. In the elderly, few classical symptoms may be seen and GD is more often presented with cardiovascular complications such as atrial fibrillation or merely with fatigue and loss of appetite [7].

GD is an autoimmune disorder, characterized by stimulating autoantibodies directed against the thyrotrophic receptor (TSH-R), TRAb. The TSH-R is situated on the surface of the follicular cells of the thyroid, but it is also expressed in other organs including the heart, kidney, testes, pituitary, fibroblasts, adipocytes, lymphocytes and immune system cells [8-11]. In the skeleton the TSH-R is expressed in osteoblasts as well as in osteoclasts [12, 13], suggesting that TSH might have a direct action in the bone remodelling cycle.

TSH-R is also expressed by the preadipocyte subpopulation of orbital fibroblasts [14, 15] leading to Graves' Ophthalmopathy or Dysthyroid Ophthalmopathy (DO).

In GD, as many as 70% of patients complain of symptoms from their eyes, most commonly in a mild form with excessive tearing, or a dry and gritty ocular sensation, but also with double vision and photophobia. Approximately 3-5% of patients with GD will develop severe symptoms with intense pain, inflammation and sight-threatening corneal ulceration or compressive optic neuropathy, defined as severe DO [12, 16]. A rarer manifestation of GD are thyroid dermopathy (or pretibial myxedema) seen in 1.5% of patients with GD or 4-13% of those who are suffering from DO. Thyroid acropathy, which manifests as clubbing of the distal phalange of finger or toes, occurring even more rarely in just 20% of those with thyroid dermopathy or 0.3% of patients with GD [17].

## Incidence

As with other autoimmune diseases, more women develop GD than men. The incidence is 0.2-0.6 per 1000 women and 0.1 per 1000 men [14, 15, 18], which makes GD one of the most common autoimmune disorders. Peak incidence of GD is between the ages of 20 to 50 years old in women. Incidence has increased over the past 20 years, and contributing to this may be an awareness of the disease connected to more sensitive laboratory methods. In addition, there is an increased incidence of other autoimmune diseases,

such as Type 1-diabetes and coeliac disease seen in children, with possible unknown etiological mechanisms contributing to this trend. GD frequently co-occurs with other autoimmune disorders such as rheumatoid arthritis, suggesting a shared pathogenesis [19].

## Etiology

Studies so far indicate strong evidence that GD runs in families. Almost half of all patients have relatives with thyroid disorders [20]. Twin studies suggest that genes may be responsible for as much as 80% of the risk of developing GD [21]. Other studies support this genetic predisposition for GD but also claim that there are still unknown non-inherited etiological determinants [22]. *Yersinia enterocolica* infection might be involved in the pathogenesis of GD by molecular mimicry to the TSH-R [19, 23]. Smoking is a well-established risk factor for developing GD, especially contributing to DO [24, 25], and perhaps associated with a nonspecific suppression of T-cell activation, reduction of natural killer T-cells and impairment of humoral and cell-mediated immunity [26].

Since the early 1800s, it has been well-established that there is a relationship between stress and stressful life events and the onset of GD. In the literature, GD is mentioned as *Schreckbasedow* and *la glande d'emotion* when patients have developed exophthalmos and thyroid enlargements after a sudden fright. There has also been an increased incidence of GD after every major war, giving rise to another German term, *Kriegsbasedow*. There are several studies concluding that a close relationship exists between stress and GD [27-32]. These studies are all retrospective and observed more negative stressful life events, such as prevalence of divorce, severe illnesses of family members and unemployment in the patients with GD than in the controls. Due to the retrospective study design, they can all be criticized for lack of evidence linking the stressful events and onset of GD. However, there is one prospective study from Serbia that identifies stable GD incidence before the 1992-1995 war and remarkably higher incidence in GD during it [33]. One of the possible mechanisms discussed in that study is the role of stressors on the autoimmune system [34].

Several gene loci have been identified as consistently linked to GD: the human leucocyte antigen region-DR3 (HLA-DR3), CD40, cytotoxic T lymphocyte-associated antigen 4 (CTLA4), protein tyrosine phosphatase non-receptor type 22 (PTPN22) and Fc receptor-like protein 3 (FCRL3), all of which encode for proteins somehow involved in immune function [35-37]. Other strong associations to GD have been seen with the TSHR gene and thyroglobulin genes [11, 38].

GD is thus likely a multifactorial disease. In genetically susceptible individuals, environmental factors such as iodine, infections, gender, smoking,

stressors and periods of immune reconstitution are thought to be involved and to contribute to the loss of immune tolerance to thyroid antigens [39].

## Diagnosis

Measurement of serum TSH is the most appropriate screening test. Serum TSH is undetectable ( $< 0.01$  U/L) because of the negative feedback of the thyroid hormones on the anterior pituitary gland. Diagnostic accuracy is improved by analysing free T<sub>4</sub>, and often the active form T<sub>3</sub>, at the same time. Elevated TRAb levels support the diagnosis of GD. TRAb is present only in about 95-96% [40] of patients with GD, presumably due to the analytical methods used.

## Treatment

Besides reducing thyrotoxic symptoms with anti-thyroid drugs, there are two definitive treatments, namely radioiodine and surgery. Interestingly, there are overt geographical differences in the treatment of GD. In the USA, anti-thyroid drugs and radioiodine therapy is the preferred treatment, presumably due to anxiety about surgical complications [41]. On the contrary, the Japanese, due to their history of nuclear radiation, prefer surgery.

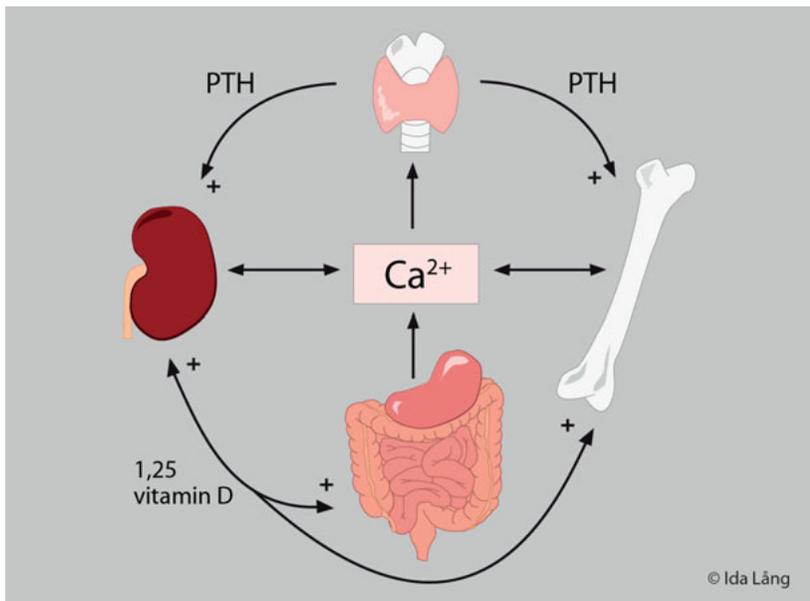
In Sweden, most centres usually offer treatment with anti-thyroid drugs for 12-18 months (carbimazole or propylthiouracil) and, when needed, beta-receptor blockers for symptom control. About 50% of GD patients treated will recur within a couple of years. In this situation, surgery may be chosen as a definitive treatment. Surgery may also be needed when there are allergic reactions to medical treatment or in the case of a huge goitre.

At the end of the past millennium, surgery was usually a subtotal thyroidectomy (ST), most often performed as a Dunhill operation, which leaves tissue at one of the upper poles. Among the benefits of ST is the possibility of avoiding the need for levothyroxin substitution and minimizing the risk of complications, such as damage to the recurrent laryngeal nerve or permanent hypocalcaemia. At the end of the 1990's, several studies indicated that total thyroidectomy (TT) was safe and presumably resulted in less recurrence than ST, and thus most endocrine surgeons in Sweden switched from ST to TT [42, 43].

# Calcium Homeostasis

## Calcium

Calcium ( $\text{Ca}^{2+}$ ) is crucial for normal function in all cell and organ systems in the body. All land vertebrates have developed a large skeleton as storage for calcium in the form of hydroxyapatite to be used in times of calcium deficiency. The level of calcium in the extracellular fluid (ECF) is thoroughly regulated by calcium itself through the calcium-sensing receptor (CaSR) and by integration with the functions of parathyroid hormone (PTH) and vitamin D. The main organs involved are the parathyroid glands, kidneys, gut, bone and skin.



*Figure 2.* Calcium homeostasis: Calcium is tightly regulated by close interaction by PTH and vitamin D including the parathyroid glands, kidneys, bone and intestines.

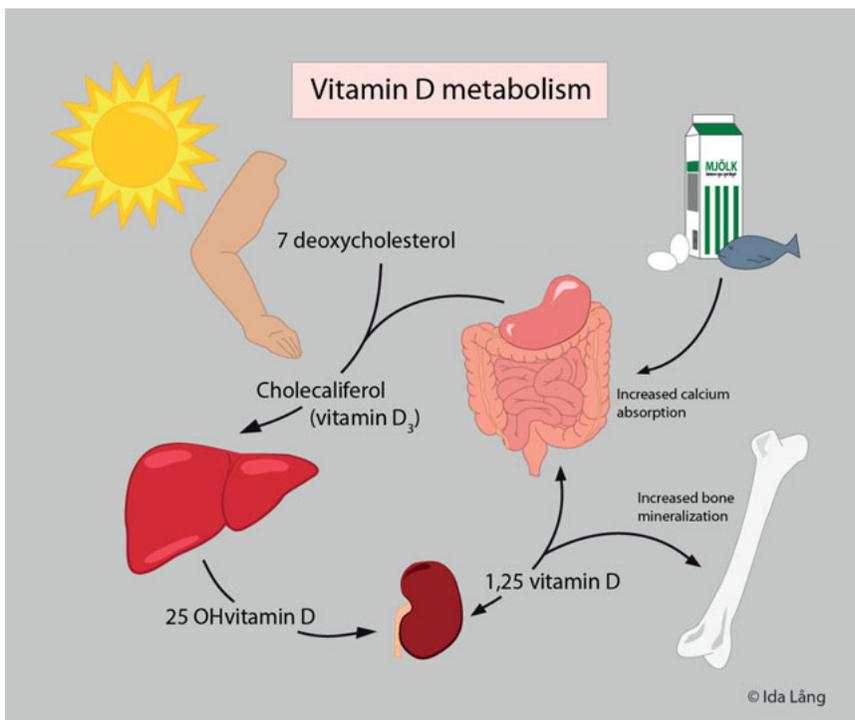
This close interaction maintains the total calcium range between 2.15 and 2.50 mmol/L. In the ECF, 50% of the calcium circulates as free ionized calcium, the biologically active form; 40% is protein-bound mainly to albumin and cannot be filtered by the kidney. The remaining 10% of the calcium is aggregated to anions as bicarbonate, lactate, phosphate, sulphate and citrate. The pH in ECF also affect the binding of calcium to albumin [44]. Measuring the ionized calcium level is probably the best way to guide therapy as it is the biologically important fraction, but the transition to measuring ionized calcium is slow because of past technical difficulties and the higher cost. Ionized calcium levels are tightly regulated between 1.15 and 1.32 mmol/L.

## Parathyroid Hormone

The current model of calcium homeostasis includes the ability of parathyroid chief cells to secrete PTH in response to subtle changes in calcium concentration, and the ability of PTH to activate its receptor PTH1R. Basal secretion of PTH is low but continuous [45]. PTH acts mainly in the kidney and bone. In the kidney, PTH promotes calcium reabsorption and acts phosphaturically by stimulating phosphate excretion in proximal tubules. PTH also stimulates conversion of 25-OH vitamin D into 1,25 dihydroxy vitamin D, which takes place in the kidney. In bone, calcium is recruited by PTH, catabolically, when PTH is at high stable levels as in primary hyperparathyroidism. If PTH is given as pulsatile treatment, osteoporotic treatment, the net effect on bone will be anabolic. PTH enhances osteoclastic activities by increasing osteoblastic expression of the RANK ligand (RANKL) (receptor activator of nuclear factor kappa-B ligand). These actions are well demonstrated by the therapies of bisphosphonates and monoclonal antibody denosumab (XGEVA<sup>®</sup>) which binds to and neutralizes the activity of the RANKL thereby suppressing osteoclastogenesis and bone resorption. The actions of PTH on osteoblasts are mediated by cell-autonomous responses as well as locally produced cytokines such as IGF-1 and fibroblast factor-2 [46].

## Vitamin D

Vitamin D is unique among human hormones because sunlight (UV light) can convert 7-dehydrocholesterol to vitamin D<sub>3</sub>. In the winter months, November to April, the amount of UV light is sparse in northern countries such as Sweden and the dietary source of the vitamin is more important. Vitamin D is most prominent in fatty fish such as salmon, mackerel and herring, as well as in dairy products such as cheese, eggs and fortified milk products. Vitamin D<sub>3</sub> whether from skin or diet is biologically inactive and needs a double hydroxylation, first in the liver modified by D-25-hydroxylase into 25-OH vitamin D, then in the kidney into the most active form, 1,25 dihydroxy vitamin D (also called calcitriol). 1,25 dihydroxy vitamin D activates calcium absorption from the gut: without 1,25 dihydroxy vitamin D, only 10-15% of dietary calcium is absorbed. In bone, 1,25 dihydroxy vitamin D interacts with the intracellular vitamin D-receptor (VDR) in the osteoblasts, stimulating RANKL and suppressing bone resorption. In the kidney, 1,25 dihydroxy vitamin D stimulates calcium reabsorption [46, 47].



*Figure 3.* Vitamin D metabolism, involving vitamin D from sunlight, skin and diet, intestines and stepwise hydroxylation in liver and kidney. 1,25 vitamin D increases  $\text{Ca}^{2+}$  absorption in the intestines and reabsorption in the kidneys. In bone 1,25 vitamin D has positive effect on mineralization.

## Bone

Collagen type 1 represents over 90% of the protein in bone. In the bone-formation-process, procollagen converts to collagen by removal of N- and C-terminals from the procollagen by proteases. There are extensions from C-terminal propeptides (PICP) and from the N-terminal propeptides (PINP). PINP appears to be the most sensitive for measuring the bone formation rate in osteoporosis. For measuring the effects of osteoporosis therapy, antibodies are developed against collagen containing cross-links, C-telopeptide of type 1 collagen (CTX, Crosslaps), and cross linked N-terminal telopeptide type 1 collagen (NTX, Osteomark). These antibodies are markers of the bone resorption process [48].

## Magnesium

Magnesium is believed to be another important factor in PTH regulation.  $Mg^{2+}$  deficiency leads to modulation of the PTH receptor's sensitivity and diminished PTH secretion which is suppressed until the  $Mg^{2+}$  levels are normal [49, 50].  $Mg^{2+}$  also binds to CaSR but with lower affinity than  $Ca^{2+}$  and competes with  $Ca^{2+}$  at the transporter level in the renal tubules. Clinically, it is obvious that hypomagnesaemia makes the correction of hypocalcaemia difficult or impossible until magnesium levels are corrected [51-53]. Hypomagnesemia is often associated with hypocalcaemia due to reduced intestinal absorption or poor dietary intake [44]. There is a high frequency of hypomagnesemia (70% after TT) and in combination with hypocalcaemia patients will have more symptoms [53-55]

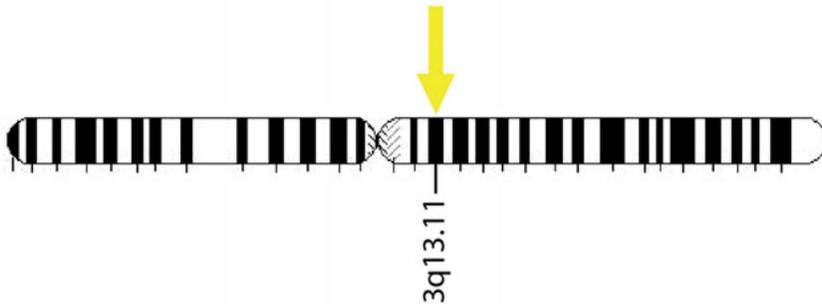
## Calcitonin

In humans calcitonin processed from precursor procalcitonin released from the thyroid C-cells plays a minor role if any, in normal calcium homeostasis[56].

## CaSR

Calcium sensing receptor (CaSR) is the molecular basis by which specialized cells (parathyroid glands, kidney, bone marrow, intestine, brain, lung, skin, and stomach) [57-60] detect and respond to changes in the extracellular (ECF) calcium concentrations. There is thus a strong minute-to-minute regulation of PTH secretion to ECF calcium concentration ruled by the set-point of the CaSR [61-63].

The discovery and cloning of the CaSR in 1993 by Brown [64] threw new light on our understanding of the regulation of calcium homeostasis, and opened up new possibilities, but much of the work was done years before [62, 65, 66]. CaSR is situated on chromosome 3q13 [67] and belongs to the family of G-protein coupled receptors (GPCR) (Fig 4).



*Figure 4.* The CASR gene is located on the long (q) arm of chromosome 3 at position 13. Image free from U.S National Library of Medicine.

The receptor is constructed with 3 different domains. The extracellular domain (ECD) consists of 612 amino acids constructed as a bilobed Venus fly trap domain (VFTD). In the ECD there are 5  $\text{Ca}^{2+}$  binding sites (CaBS1-5) all located in the cleft between the lobes in the VFTD [56]. The transmembrane part, amino acids 613-862, consists of seven domains that are characteristic of all GPCRs. The intracellular domain with 216 residues, and a hydrophilic carboxyl-terminal tail, are shown to have a role in cell surface expression of the CaSR [68].

Calcium binding to the VFTD of CaSR results in the activation of multiple signalling cascades for example including the  $\text{G}_q$  and  $\text{G}_{11}$ -proteins, dependent on stimulation of phospholipase C activity, causing an accumulation of inositol 1,4,5-triphosphate and a rapid increase in intracellular  $\text{Ca}^{2+}$  concentrations [69, 70]. These changes in turn lead to reduced PTH concentrations and increased urinary calcium excretion.

The way in which calcium acts on the CaSR varies depending on the cell type. In the parathyroid cell, activation of CaSR leads to a decrease in PTH secretion. In the kidney, CaSR activation is thought to have several different actions, leading to enhanced reabsorption of sodium chloride and increased calcium and magnesium excretion in the renal tubules [71, 72].

In bone, CaSR is expressed and involved in almost all cell types in bone metabolism, such as osteoblasts, osteoclasts as well as their precursors. CaSR and calcium levels are in close interaction with PTH and its receptor PTH1R, maintaining a steady state in the bone-forming balance [46].

CaSR is also found in other tissues in the body that are not involved in calcium homeostasis, suggesting that the CaSR has actions that are not associated with calcium homeostasis.

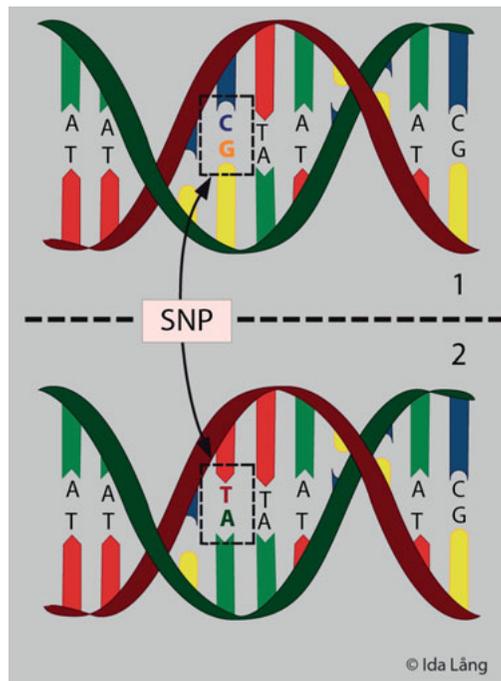
The importance of CaSR in calcium metabolism is also shown by findings in inherited diseases. There are activating and inactivating CaSR mutations. Activating or gain-of-function mutations are seen in autosomal dominant hypocalcaemia (ADH) and in a form of Bartter syndrome, type V. Inactivating or loss-of-function mutations are seen in familial hypocalciuric hypercalcaemia (FHH), neonatal severe hyperparathyroidism (NSHPT) [73-75] and primary hyperparathyroidism (PHPT) [76-78]. To date, several hundred naturally occurring mutations have been reported in the CaSR [79].

Different SNPs in CaSR and their relationship to calcium levels in healthy people have been discussed since 1993. The most frequently studied SNPs in CaSR are all situated at exon 7: rs1801725, rs1042636 and rs1801726 related to codons A986S, R990G and Q1011E respectively. These SNPs encode for non-conservative changes of single amino acids in the COOH-terminal tail of CaSR. They are linked to total calcium levels, ionized calcium levels [80] as well as kidney stone production [81, 82]. Among these polymorphisms, the A986S is the most commonly and widely studied variant in Caucasian subjects [82-86].

## Single Nucleotide Polymorphism (SNP)

All genetic information is kept in the chromosomes. Hard work by many scientists from Friedrich Miescher (1868), to Gregor Mendel 1870, finally revealed the DNA double helix as constructed by the four nucleotides A=Adenine, T=Thymine, C=Cytosine, G=Guanine. James Watson, Francis Crick, and Maurice Wilkins were honoured with the Nobel Prize in 1962. Rosalind Franklin was never nominated (Nobel Media 2015), but it was she and her studies with X-ray-diffraction and the “Photograph 51” (taken by Raymond Gosling in 1952) who finally revealed the helical structure of DNA presented by Watson and Crick in Nature 1953 [46].

A single nucleotide polymorphism, SNP is an exchange of a single nucleotide in a sequence, for example GAT to AAT (Fig. 5). The different nucleotide sequences constitute different alleles. Each verified SNP is given an r-number, and several openly accessible databases such as dbSNP ([www.ncbi.nlm.nih.gov/project/SNP](http://www.ncbi.nlm.nih.gov/project/SNP)) and Ensemble ([www.ensembl.org](http://www.ensembl.org)) handle information from researchers all over the world and make the data easy to reach and use.



*Figure 5.* DNA  $\alpha$ -helix with nucleotide exchange in one position, a so called single nucleotide polymorphism, SNP.

The SNPs will more often occur in noncoding areas of the genes, the introns, but they may still be responsible for a change in the resulting protein building. When a nucleotide change gives rise to a symptom, illness or syndrome it is called a mutation. Mutations have been catalogued since 1960 in the Mendelian Inheritance in Man, a register created by Dr. Victor A. McKusick. Each mutation has an MIM number. Since 1995, they can also be accessed on-line, and at that time the mutations were renamed OMIM (Online Mendelian Inheritance in Man) [87].

# Materials and Methods

## Laboratory Methods

Basal serum or plasma values for albumin, calcium, creatinine, intact PTH, phosphate, TSH, T4, T3 and 25-OH-vitamin D were measured at the clinical chemistry laboratories in Uppsala and Falun, while anti-TSH receptor antibodies (TRAb) were only determined at the clinical chemistry laboratory at the University Hospital in Uppsala. All spectrophotometrical measurements were performed on an Architect Ci8200 analyser (Abbot Laboratories, Abbott Park, IL, USA) except for TSH, T4, T3 in Uppsala, analysed on a Cobas Immuno602 or 8000 (Roche, Basel Switzerland).

Plasma albumin was determined by spectrophotometry using bromine cresol green (normal range = 36-45g/l). Total plasma calcium (p-calcium) was measured spectrophotometrically using a compleximetric method with orthocresolphthalein (normal range = 2.15-2.50 mmol/L) and was adjusted to the serum albumin level by using the formula: total p-calcium+[0.019 x (43-patient albumin)] in Papers I-III, and the formula: total p-calcium+[0.02 x (39-patient albumin)] in Papers IV and V. Hypocalcaemia was defined as an albumin-corrected plasma calcium level of <2.15 mmol/L, and severe hypocalcaemia as plasma levels below 2.00 mmol/L.

A chemiluminescent method (CLIA) was used to measure both intact plasma PTH (normal reference range 1.1-6.9 pmol/L; Liasion 1-84 PTH; DiaSorin, Saluggia, Italy), and 25-OH vitamin D (Liasion 25-hydroxyvitamin D assay; DiaSorin, Saluggia, Italy). The 25-OH vitamin D values were compared to a reference population analysed at the same laboratory and with the same method [88].

In Falun, a chemiluminescent assay was set up on Architect Ci8200 at 2011 for analyses of 25-OH vitamin D and samples from Falun dated after 2012 have been analysed by this method. There are known inter-assay disagreements and separate analyses were performed accordingly [88].

Thyroid receptor antibody (TRAb) were determined with immunoassays: before 2011 with an ELISA using human monoclonal antibody (HuMab-TRAb; reference range < 0.6 U/L; RSR Ltd, Cardiff, UK), and after 2011 using a Cobas8000 (reference range < 1.75 E/L Roche, Basel, Switzerland).

Analyses were performed for bone metabolism markers serum-beta crosslaps (CTX) and procollagen 1 N-terminal propeptide (P1NP). CTX was analysed by a chemiluminescent method at the clinical chemistry laboratory in Uppsala. Normal reference range for men <50 years and premenopausal women was <580 ng/L. P1NP was analyzed at our local research laboratory at Uppsala University Hospital, with a Sandwich - ELISA kit (range 15.6-1000 pg/ml), My BioSource, San Diego, CA, USA.

## CiCa Clamp

CiCa clamp is a method measuring parathyroid glands function in vivo focused on sensing of external calcium. The method was first established and described by Schwartz et al. in 1993 and has been proven possible to replicate and reliable with minimal inter-individual variations [89, 90]. In 1983, Brown et al. presented the four-parameter model describing the inverse sigmoidal relationship between PTH release and extracellular  $\text{Ca}^{2+}$  concentrations [65]. This model was then used to calculate the so-called set-point (SP) as described by Bas et al. [91] The SP is equal to the plasma ionized calcium concentration at which 50% of the maximal secretion of PTH is inhibited, measured as the plasma-ionized calcium concentration at the midrange of the PTH maximum - PTH minimum curve.

A body weight-related citrate infusion is given over the course of 50 minutes. When hypocalcaemia is established, calcium is infused over 60 minutes to create hypercalcaemia. Intact plasma PTH and ionized-plasma calcium are collected at predefined time intervals during the procedure.

Basal PTH secretion (before infusions started), maximal PTH secretion (PTH max), minimal PTH secretion (PTH min), and the ratio of basal PTH/maximum PTH (PTH b/m) are determined. Furthermore, ionized calcium concentration at maximum PTH (Ca at PTHmax), defined as the ionized calcium concentration when maximum PTH is reached and no further reduction in ionized calcium leads to any increase in PTH, is measured. In addition, the ionized calcium concentration at minimum PTH (Ca at PTHmin) defined as the ionized calcium concentration at which PTH reaches minimum PTH, and no further increase in ionized calcium leads to any further reductions in PTH, is measured.

We have been using this method at our institution for over 20 years.

## DNA Preparation

DNA was prepared from whole blood using DNAeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) and from FFPE sections by Allprep DNA/RNA FFPE kit (Qiagen, Hilden, Germany).

## PCR and Sanger Sequencing

In Paper III, we analysed the SNPs rs1801725, rs1042636 and rs1801726 corresponding to codons A986S, R990G and Q1011E respectively in CaSR. The samples were prepared and sequenced at the SNP&SEQ Technology Platform in Uppsala, Sweden.

(<http://molmed.medsci.uu.se/SNP+SEQ+Technology+Platform>).

In Papers IV and V, the same SNPs as in Paper III were analyzed. Polymerase chain reaction (PCR) and sequencing reactions were performed using designed primers for CaSR, resulting in a product including the codons for rs1801725, rs1042636 and rs1801726. Primers, FW 5'-CAC AGC AAC GAT CTC AG-3', and RW 5'-GAC GGT CAG ATC TAA GTC CG-3', were synthesized by TAGC AS (Copenhagen, Denmark). DNA fragments were directly sequenced using automated Sanger sequencing (Beckman Coulter Genomics, Takeley, UK). All PCR were run at 95°C for 2 min followed by 40 cycles of 95°C for 20 sec, 57°C for 20 sec, 72°C for 20 sec and final extension at 72°C for 7 min. PCR products were separated on an agaros gel and compared to a defined ladder. Obtained chromatograms were analysed in house using Codon Code Aligner software (CodonCode Corporation, Dedham, MA, USA).

Minor allele frequencies (MAF) were analysed in comparison to databases dbSNP and NHLBI Exome Sequencing Project (Ensemble).

## PIVUS cohort

Prospective Investigation of the Vasculature in Uppsala Seniors, PIVUS is a well-characterized cohort of 1061 healthy 70 year old seniors from Uppsala (49.3% women). This study was conducted with the primary aim to evaluate effects on the vascular endothelium with the power to predict future cardiovascular events. The subjects were invited in a randomized order, 2025 subjects were invited and 1016 participated giving a participation rate of 50.1%. All subjects answered questions about their medical history, smoking habits and regular medication. No one had previously been diagnosed with primary or secondary HPT, and none was taking calcium or vitamin D supplementation. A number of measurements related to calcium homeostasis were performed including: calcium, PTH, phosphate, magnesium, creatinine and vitamin D. All samples were drawn in the morning after an overnight fast [92, 93].

# Aims

General aim:

To discover the crucial mechanism for the postoperative period of hypocalcaemia in patients with GD and from this, develop suitable treatment and follow-up of these patients to avoid this clinical problem.

The aims of the studies in this thesis were:

- I To investigate whether patients undergoing total thyroidectomy (TT) for GD have lower plasma calcium in the postoperative period than patients with MNG undergoing the same operation, and to evaluate whether TT is safe and leads to reduced frequency of recurrence than subtotal thyroidectomy (ST).
- II To examine whether the parathyroid gland function is disturbed in GD.
- III To examine three well-defined SNPs, A986S, R990G, and Q1011E in CaSR in a normal cohort, PIVUS, and their relationship to plasma calcium and pHPT.
- IV To investigate whether patients with GD have any differences in three well-defined SNPs, A986S, R990G, and Q1011E in CaSR.
- V To evaluate clinical data concerning calcium homeostasis i.e. bone turn-over, in a prospective, observational study of patients with GD.

# Results

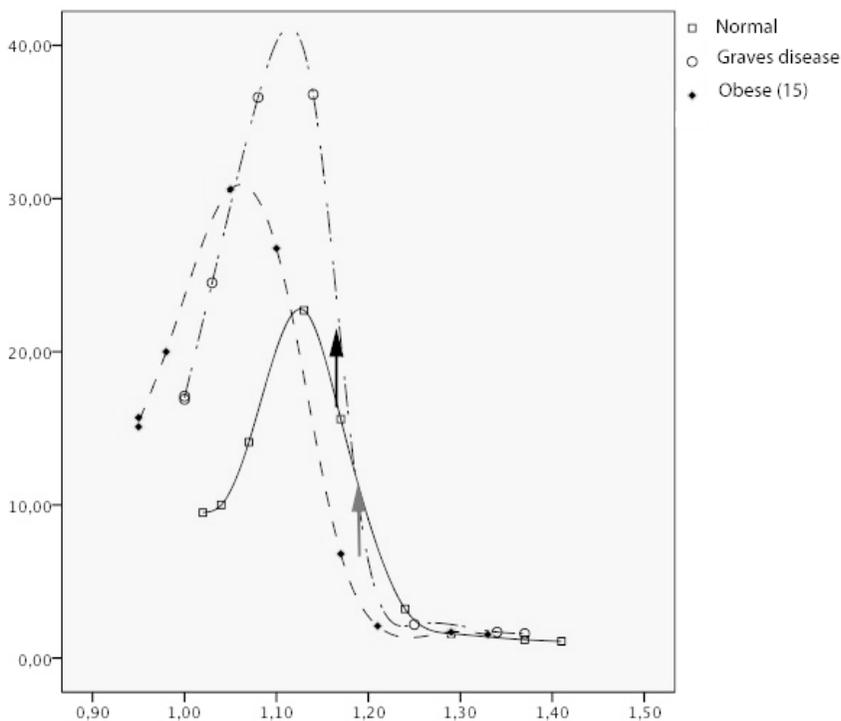
## Paper I

In this retrospective study we compared 267 patients with Graves' Disease who underwent surgery in Falun and Uppsala between 2000 and 2006 with a previously published study from the same region (Winsa et al. 1995) which included 176 patients operated on between 1980 and 1992. The change of surgical method from subtotal thyroidectomy (ST) to total thyroidectomy (TT) rendered significantly fewer recurrences, 1.7% vs 20.4%. Mean time to follow up was five years. There was no significant increase in complications rates. The change in surgical method did not seem to have any implication for the progression of dysthyroid ophthalmopathy. Notably four patients operated with TT had a recurrence in their disease, all high up at the hyoid bone.

## Paper II

In this prospective study 56 patients with Graves' Disease were enrolled to investigate the in vivo function of the parathyroid glands. Blood samples were drawn preoperatively; on days one to five postoperatively, and at follow up at one, six and 12 months postoperatively. Fourteen patients gave their permission to participate in a CiCa-clamp, in order for us to scrutinize their calcium metabolism. Their results were compared to 21 healthy controls.

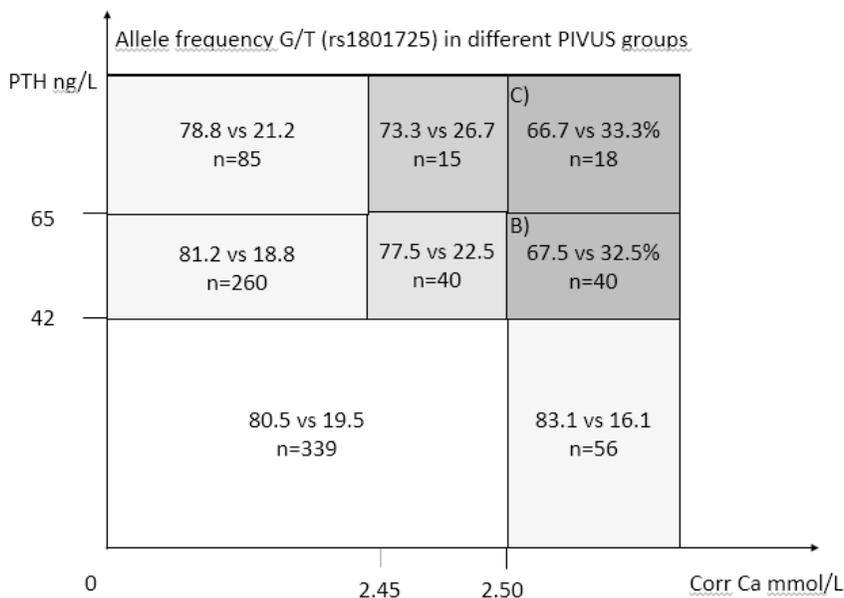
The results showed that patients with GD had a more left-shifted set-point and higher maximal PTH secretion than the controls. One explanation for this may be a higher over-all metabolism in patients with GD and/or a difference in gene expression compared to controls.



*Figure 6.* CiCa clamp of 14 patients with GD, 21 controls and 15 obese patients. Patients with GD have a more left-shifted set-point 1.15 vs 1.20,  $p=0.001$  and also a higher PTH max than the controls. Y-axis presents PTH pmol/L, X-axis Calcium mmol/L.

### Paper III

In the PIVUS cohort, we analysed the allele frequency in three of the most studied SNPs in CaSR, namely rs1801725, rs1042636 and rs1801726 corresponding to A986S, R990G and Q1011E respectively. The SNPs and their haplotypes were analysed according to p-calcium, PTH, creatinine, vitamin D, magnesium and phosphate. Both individuals with biochemically proven, overt pHPT ( $n=18$  p-calcium  $>2.50$  mmol/L and PTH  $> 65$  ng/L) and mild HPT ( $n=40$  p-calcium  $>2.50$  mmol/L and PTH  $>42$  ng/L) had a higher frequency of T alleles than the normocalcaemic individuals. The calcium and PTH levels seems to have a correlation to the frequency of T alleles and also to certain haplotypes.



*Figure 7.* Allele frequency in PIVUS cohort. Individuals with p-calcium >2.50 mmol/L and PTH >42 ng/L had a significantly higher frequency of T allele in rs1801725 corresponding to A986S than the others;  $p = 0.048$ , by chi-square test.

## Paper IV

In this study we compared our own results from PIVUS, of the SNPs in CaSR: rs1801725, rs1042636 and rs1801726 with the sequenced samples of 167 patients with GD. The Allele frequency of the SNPs corresponded well to those data available in dbSNP and to our own analyzed cohort PIVUS. In patients with GD and postoperative hypocalcemia we noticed a lower p-calcium already preoperatively. The same hypocalcaemic patients also had a tendency of lower frequency of T allele in rs1801725. Also this group of patients did not present any individual with the haplotypes; T/G G/A C/C, T/G A/A C/C, or T/G A/A G/C, earlier shown in paper III to be correlated to Higher p-calcium levels.

Table 1. Frequencies of Alleles in patients with Graves' Disease compared to PIVUS cohort

rs1801725		PIVUS	GD Hypo Ca	GD Normo Ca
G allele	number	681	33	108
	frequency	79.1%	89.2%	83.1%
T allele	number	180	4	22
	frequency	20.9%	10.8%	16.9%
Total number		861	37	130

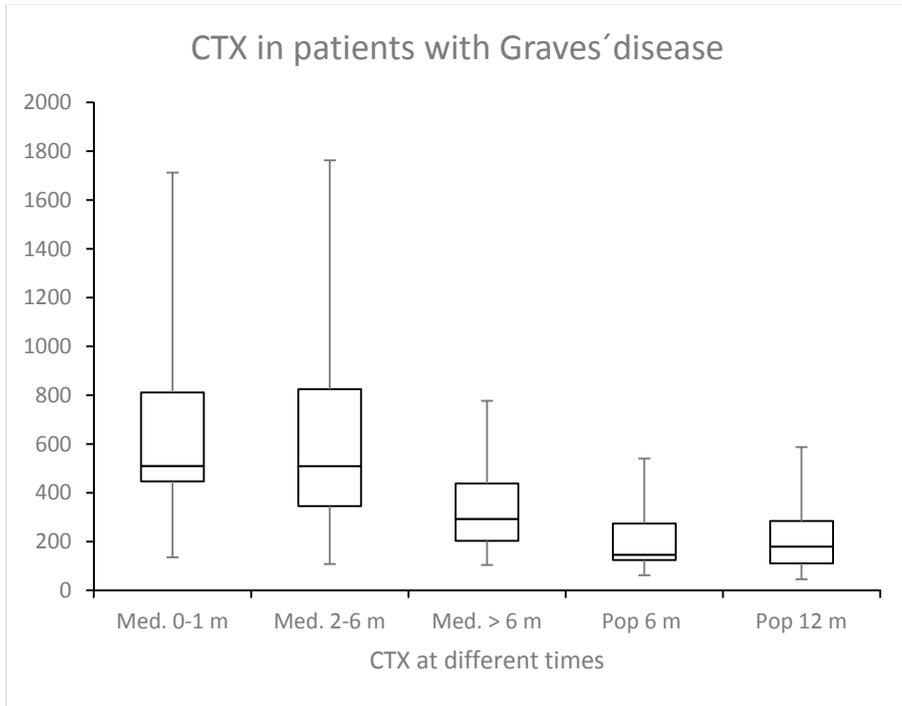
PIVUS = Prospective Investigation of the Vasculature in Uppsala Seniors, a control cohort of 861 individuals. GD Hypo Ca = Patients with Graves' Disease with corrected p-calcium < 2.00mmol/L on postoperative day one. GD Normo Ca = Patients with Graves' Disease with corrected p-calcium > 2.00mmol/L on postoperative day one.

## Paper V

In this study we collected blood samples prospectively in 98 patients. Seventy patients underwent TT, after a period with anti-thyroid treatment of different length (0-48 months). Twenty-eight were newly diagnosed patients at the clinic of internal medicine, treated less than one month with anti-thyroid drugs. Blood samples for plasma calcium, albumin, creatinine, phosphate, PTH, TSH, T4, T3, TRAb and 25-OH vitamin D were collected preoperatively, at day one-two postoperatively and at follow up at one, six and 12 months. Bone metabolism markers used were beta-CTX and P1NP.

Bone metabolism markers did not differ between the groups of patients treated less than one month (i.e. the medicin patients) compared to those treated two-six months. Patients treated more than six months had significantly lower value of CTX compared to patients treated two-six months. After TT the CTX and P1NP was normalized already after six months.

Patients with hypocalcaemia, albumin corrected p-calcium < 2.00 mmol/L on day one postoperatively had higher levels of CTX preoperatively if treated two-six months. This postoperatively hypocalcaemic group also had lower p-calcium preoperatively 2.27 mmol/L vs 2.32 mmol/L  $p = 0.032$  and a higher frequency of patients with low levels of TSH. Also no patients in the group in need of postoperative treatment of calcium and vitamin D had any T allele in rs1801725.



*Figure 8.* Levels in CTX ng/L, Y-axis presented by time of preoperative treatment and postoperatively, X-axis.

# Discussion

Patients with GD have a higher risk of developing more severe and prolonged hypocalcaemia postoperatively than patients operated on due to others diagnoses such as MNG [94-97]. The hypocalcaemia may be related to the surgical procedure used, with some studies proposing a higher complication rate in patients undergoing more radical surgery, such as TT [98-100]. Indeed, the higher rate of hypocalcaemia after TT may be caused by iatrogen damage to the parathyroid glands, and could possibly be reduced if a dedicated endocrine surgeon perform the operation [95]. The ST surgical procedure is related to more recurrences as thyroid tissue is left behind. In cases of GD it is the patients with DO who suffer the most. They are more difficult to treat and cure, and as they suffer not only from swelling, pain and gritty eyes but also from a threat to their eyesight, it is most important to avoid recurrences.

Some studies have indicated that TT can be performed with same complication rates as ST [43, 100, 101]. In Paper I, we were able to contribute to these findings and show that this was also applicable in our own setting. Alas, there are surgical complications that can explain some of the patients with postoperative hypocalcaemia. But even if patients with GD were operated on by endocrine surgeons and had a normal PTH after surgery, they were still at higher risk of hypocalcaemia than the patients who undergo surgery due to MNG [99, 102, 103].

To analyse the function and sensitivity of the parathyroid glands, patients with GD underwent a CiCa clamp (Paper II). We could show that, in comparison to normal individuals, patients with GD had a left-shifted set-point in the calcium/PTH sigmoidal curve and a higher PTH max. This indicates that these patients have more sensitive parathyroid glands. Earlier studies in obese individuals have shown a relationship between low vitamin D levels and a left-shifted set-point [104]. We could not see any such correlation with low vitamin D levels among our GD patients. Therefore, other factors contributing to the left-shifted set-point may be present in these patients, also contributing to the augmented risk of hypocalcaemia. These patients with GD might have an augmented bone metabolism and/or a genetic difference in SNPs related to calcium levels. In large as well as in smaller studies, certain SNPs in CaSR have been associated with the calcium levels [80, 82, 105-107].

The three most commonly described SNPs in CaSR are: rs1801725, rs1042636 and rs1801726 related to codons A986S, R990G and Q1011E respectively. In particular, rs1801725 seems to be related to calcium in Caucasians. There have been no studies on the eventual relationship between those SNPs in CaSR and the hypocalcaemia seen postoperatively in patients with GD.

SNPs and allele frequencies seems to be different in various cohorts according to regions and race [108]. According to this, we wanted to have regional control material for our GD cohort. In Paper III, we analysed the well-defined Swedish cohort, PIVUS [92]. Our results concerning frequencies and haplotypes in this cohort of 881 healthy individuals from Uppsala, are in line with earlier publications and results in databases as dbSNP and Ensemble. The presence of a T allele in rs1801725 seems to be related to higher p-calcium and also a higher PTH level. In individuals with p-calcium above 2.50 mmol/L and PTH >42 ng/L, there was a higher frequency of the haplotype T/G A/A G/C, and fewer individuals with G/G A/A G/C. Taken together, this cluster of SNPs in the intra-cellular tail of CaSR may be of importance in the intercellular signalling concerning calcium regulation.

In Paper IV, when we sequenced the patients with GD according to the above mentioned SNPs, they also corresponded very well to the known frequencies in dbSNP and Ensemble but also with our own controls in PIVUS (Paper III). We could find no significant correlations to calcium levels postoperatively, probably due to a lack of power. But indeed there was a tendency towards lower frequency of the T allele in rs1801725 in those with postoperative calcium levels below 2.00 mmol/L (20.9 % vs. 10.5 %). This may be one reason for the left-shifted set-point of the calcium/PTH curve in patients with GD seen in Paper II and a contributor to the hypocalcaemic phenomenon in patients with GD.

These findings may also be supported by our own findings, namely that the p-calcium levels are already lower before surgery in the group of patients that will become hypocalcaemic on postoperative day one (Paper V). We have also shown, in Paper V, that the patients with permanent need of calcium and/or vitamin D substitution postoperatively are lacking certain haplotypes, and none of them have has a T allele in the rs1801725, A986S codon.

Concerning the matter of bone-hunger and its impact on GD [4, 5, 109, 110] we have reached some findings in Papers II and V that point towards this as a matter of concern, at least in some patients with GD.

In the group of patients with GD who became hypocalcaemic on day one postoperatively, we could show a higher level of bone metabolism markers CTX and P1NP. This was only seen in the group treated two-six months before surgery (Paper V). Anti-thyroid medication was shown to reduce the

bone metabolism markers significantly when patients were treated for more than six months. After TT, the bone metabolism markers were already normalised after six months (Paper V). These hypocalcaemic patients also had lower TSH levels preoperatively. Patients with a temporary need of calcium and/or vitamin D (< 6 months), and patients with a permanent need of treatment (> 12 months), do seem to have a lot in common. When these groups were compared with normocalcaemic patients we noticed that the postoperatively treated patients had the following characteristics before surgery, namely, they were low in TSH and high in T3 (with the highest levels measured in the group requiring permanent treatment). They were postoperatively lower in PTH on day one, and with normalised PTH levels by one and six months (Paper II and V). Unfortunately, it is obvious that there are some patients in whom circulation to the parathyroid glands is disturbed temporarily, by an ending of the earlier higher metabolism, by the surgeon, or even by an additive autoimmune reaction.

Another interesting finding supporting the relation between higher metabolism and postoperative hypocalcaemia was that patients with MNG, also had a negative relation between the preoperatively TSH level and their postoperative calcium level (Paper II). Indeed, patients with MNG and subclinical hyperthyroidism were at higher risk of postoperatively hypocalcaemia.

There are still many questions concerning the autoimmune process. We made an attempt to study this by performing short-time cultures of fresh parathyroid cells (parathyroid adenoma cells) in our laboratory. In order to identify effects of possible autoantibodies we added serum from GD patients with high levels of TRAb as well as from healthy individuals. PTH levels were analysed in the medium after 12 hours, one and two days. We saw an obvious relationship between PTH levels and the amount of sera added, but we could not verify any significant differences in PTH secretion between GD patients and the controls and the results were not replicable.

We also added serum from the same GD patients to bovine parathyroid cells. After adding anti-human secondary antibody we could immunohistochemically verify staining of the bovine parathyroid cells. Unfortunately, this attachment was not specific for the bovine parathyroid cells, and control staining with bovine ovarian cells showed the same results.

There are studies investigating vitamin D and the risk of hypocalcaemia after TT [111-114]. The results indicate that vitamin D deficiency may be a contributing factor to the postoperative hypocalcaemia in GD. In our small cohort we cannot find statistical evidence for vitamin D deficiency in the group of patients with permanent hypocalcaemia but that may be due to technical inter-assay differences.

In our material we have not studied the magnesium levels. Since it is impossible to correct calcium levels as long as magnesium levels are low [49, 53, 54] magnesium may also be a contributing factor to the prolonged hypocalcaemia seen in patients with GD.

To conclude; the postoperative period of hypocalcaemia seen in patients with GD are most likely of multi-factorial origin.

One limitations in this thesis is using rather small cohorts in some of my studies (Papers II, IV and V), rendering less power to the results and their true applicability to our clinical work. Nevertheless, when comparing clinical data from these studies they do not differ from the more extended material in Paper I. The strength of the studies are the long surveillance time and the close follow up.

Another limitation worth mentioning is the use of an arbitrary set cut-off for TSH data (papers II and V). The disease and clinical indications in patients treated for GD give rise to severely right-skewed values for TSH. Moreover, these patients are treated with a block-and-replace method that obscures the time of metabolic stability. In combination with a change in measuring we decided that using a cut-off for TSH at  $<0.05$  mU/L, was the most proper way to separate the hypermetabolic patients from the more normal ones.

Dealing with Vitamin D is difficult, not only due to the presence of both vitamin D2 and vitamin D3 in plasma. There are also several different methods on the market for vitamin D analysis with rather large inter-method differences [88]. In Papers II, III and V we used immuno-competitive assays for 25-OH vitamin D. In Paper II and III all samples were measured at the same clinical laboratory in Uppsala by Liason, DiaSorin. In Paper V, the samples were measured by the same method, but half of them were analysed in Uppsala and half in Falun by Architect, Abbot. By using The Vitamin D External Quality Assessment Scheme (DEQAS) an organized external validation function, it is obvious that samples analysed in Uppsala render lower values. Taken this into account there may be a significant lower level in pre-operative vitamin D values in those patients who after an operation will become hypocalcaemic and need long-lasting treatment of calcium and vitamin D. For the other biochemical parameters in my papers, external and internal regular validations in the clinical chemistry laboratories contribute to the consistency of the results and their comparability.

# Conclusions

The results from this thesis show that the more severe and extended hypocalcaemia seen in patients with Graves' Disease after a total thyroidectomy is multifactorial.

Patients with Graves' Disease can undergo total thyroidectomy surgery without additional complications.

Patients with Graves' Disease have a more left-shifted set-point in the calcium/ PTH balance and a higher PTH max, indicating more sensitive parathyroid glands and/ or certain SNPs in CaSR.

Individuals with p-calcium  $> 2.50$  mmol/L and PTH  $>42$  ng/L have a higher frequency of T alleles in rs1801725 and also a different frequency of haplotypes in CaSR, which may indicate that the T allele and haplotype T/G A/A G/C are of importance in calcium regulation.

Patients with Graves' Disease who become hypocalcaemic on postoperative day one may have lower frequency of T alleles in rs1801725 and are missing certain haplotypes previously shown to be correlated with higher levels of calcium.

Patients with Graves' Disease treated with anti-thyroid drugs for less than six months have a higher bone metabolism than those treated for a longer time. In addition, the hypocalcaemic patients treated less than six months have higher bone metabolism markers than the normocalcaemic patients. The patients with Graves' Disease who became hypocalcaemic on postoperative day one also had a higher level of T3 and lower values of TSH pre-operatively.

# Clinical Implications

According to the results in this thesis, the hypocalcaemia seen in patients with GD stems from several causes. My suggestion include:

When possible, operate on metabolically stable patients, with TSH above 0.05 mU/L and T3 levels as low as possible (<3.5 pmol/L).

Optimize the patient's vitamin D and calcium levels preoperatively, perhaps with medication 3 – 6 months ahead of surgery.

Check magnesium levels postoperatively in patients with long-lasting hypocalcaemia.

When trying to reduce calcium and vitamin D treatment in patients with normal levels of PTH and previously treated on long-term basis, proceed extremely slowly.

And of course, use well-skilled endocrine surgeons.

## Future Directions

The results in this thesis show that GD patients with a high metabolism are more likely to be hypocalcaemic postoperatively. More studies are required to investigate the causality between preoperative hyper-metabolism and the hypocalcaemia seen postoperatively. It would also be of interest to conduct a larger study with a therapeutic approach including calcium and vitamin D supplements preoperatively.

Another interesting finding in this thesis is the left-shifted calcium/ PTH setpoint seen in patients with GD in Paper II. It would be of interest to analyse the GD patients after their TT with a CiCa clamp. Will they have a restored set-point after TT, or will they remain left-shifted, which would indicate the impact of genetic differences possibly in CaSR?

Many questions still remain about the role of immunology in this field. We were not able to draw any conclusions from our parathyroid gland cell cultures. It would be of interest to analyse serum from GD patients and the effect on a stable parathyroid cell line.

# Sammanfattning

Graves' sjukdom (GD) är en av de allra vanligast förekommande autoimmuna sjukdomarna och drabbar sköldkörteln, tyroidea. Sjukdomen leder till en allmänt förhöjd ämnesomsättning i kroppen med höga hormonnivåer av s.k. T4 och T3 samt låga TSH nivåer. Många patienter får även en påverkan på ögonen i form av svullnad och rodnad i och kring ögonen, smärta, ljuskänslighet och till och med dubbelseende. I Sverige brukar GD behandlas med i första hand medicin (Thacapzol eller Tiotil) i ca 18 månader. Ungefär hälften av patienterna blir då friska. Om patienter med GD återinsjuknar försöker man hitta en mer definitiv behandling och väljer ofta radiojod eller kirurgi.

En komplikation som kan uppstå vid kirurgi är skada på stämbandsnerven. Man blir då hes och detta inträffar i ca 1–1.5 % av operationerna. En annan komplikation är låga kalknivåer efter operationen s.k. postoperativ hypokalcemi. Tidigare internationella studier visar en kvarstående hypokalcemi frekvens på 1 %, i vårt svenska tyroidearegister ligger dock siffrorna på 3.9–4.5 %.

Studier har visat att patienter med GD sjunker lättare i kalcium efter en total tyroidektomi (TT) än patienter som opererats för andra diagnoser till exempel vanlig knölstruma, även kallad atoxisk multi-nodös struma (MNG) eller dalastruma. Det finns alltså andra bakomliggande orsaker till hypokalcemin än endast kirurgiskt orsakad skada på kärlförsörjningen till bisköldkörtlarna. Rådande hypoteser är sänkt blodflöde efter operation relaterat till en normaliserad ämnesomsättning efter operationen, möjliga autoimmuna reaktioner kopplade till GD eller en förhöjd benmetabolism s.k. ”bone hunger” med ett ökat sug efter kalcium hos skelettet. Målet med denna avhandling var att försöka finna orsaker till den postoperativa hypokalce och därmed kanske även kunna förebygga denna.

I de studier som ingår i denna avhandling har vi kunnat visa att: Patienter med GD kan genomgå TT, detta är en säker operation att utföra som ger färre återfall i sjukdomen och inte fler komplikationer.

Patienter med GD har en ökad känslighet i sin kalcium/parathormon balans. Detta samspel mellan kalcium och parathormon är centralt i styrningen av kalcium nivåerna i blodet. Den ökade känsligheten kan bero på genetiska skillnader på DNA-nivå i den så kallade *calcium sensing receptor* (CaSR).

Dessa fynd stöds bland annat av skillnader vi kunnat visa i DNA hos CaSR, kopplade till kalcium och parathormonnivåer i blodet.

Hos de patienter med GD som fick låga kalciumvärden efter operationen var bennedbrytningsmarkörer högre före operationen, talande för en ökad benomsättning. Även prover på ämnesomsättningen (TSH och T3) visade på en högre ämnesomsättning före operationen hos dessa patienter. Dessa fynd gällde även de patienter som behövde behandlas med kalcium och vitamin D efter sin operation.

Parathormonnivåerna dag ett efter operationen var övergående låga hos de patienter som fick låga kalciumvärden. Dessa nivåer normaliserades dock redan efter en månad. Detta gällde även för patienter som behövde mer än 12 månaders behandling med kalcium och vitamin D efter operationen.

Sammantaget talar dessa fynd för att perioden med låga kalknivåer efter TT hos patienter med GD beror på flera sammanhängande faktorer. En övergående störning av cirkulationen till bisköldkörtlarna, genetiska skillnader i CaSR hos dessa patienter, samt en högre benomsättning före operationen som möjligen hänger samman med hur länge patienten förbehandlats för sin GD alternativt hur patientens sjukdom har svarat på behandlingen.

Huruvida magnesium, vitamin D och andra ännu okända immunologiska mekanismer spelar någon roll hos dessa patienter får framtida studier utvisa.

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