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Graves' Disease; Aspects on Orbitopathy and Bone Metabolism

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Abstract

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Graves' disease (GD) is an autoimmune disorder that affects the thyroid gland, with an annual incidence of 21 in 100,000 individuals in Sweden. Graves' orbitopathy (GO) is relatively common in GD and affects 50% of all patients to varying degrees of severity, with severe forms affecting 3–5% of patients. Early diagnosis and treatment of GO are crucial to avoid developing complications from the severe forms of GO. Diagnosis and treatment of GO are still challenging in many complicated cases; thus, new biomarkers are required for improved management. Thyroglobulin (Tg) is a glycoprotein produced by the follicular cells in the thyroid gland whose connection to GO has not been clarified. Measurement of thyroid stimulating immunoglobulins (TSI) is described as a more precise method than the traditional third-generation immunoassay of TSH receptor antibodies (TRAb), for improved diagnosis and management of GD. Vitamin D and bone health in GD have been investigated in different studies with conflicting results.

This thesis aimed to explore the role of new biomarkers in the management of GO and to study the impact of GD on bone health.

In Papers I–III, we studied 30 consecutive patients with de novo GD. Our studies show that higher levels of Tg are associated with a higher risk of developing GO. However, TSI was highly correlated to TRAb in GD, and it was not more precise than TRAb in the management of GO. No vitamin D deficiency was observed, but data confirm that hyperthyroidism has a negative effect on bone health. During treatment of GD, bone health improved over a two-year follow-up period.

In Paper IV, a total of 2,134 patients with GD were included in an investigation 16–18 years after inclusion regarding the risk of developing skeletal complications. There were no increased fracture rates in patients with GD compared to up to ten controls from the general population. However, male patients older than 52 years may have an increased risk of vertebral fractures.

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To Shoroq, Amanda and Michelle

‘Live as if you were to die tomorrow. Learn as if you were to live forever’. — **Mahatma Gandhi**

List of Papers

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

I. **Khamisi S**, Lundqvist M, Emadi P, Almby K, Ljunggren Ö, Karlsson FA. Serum thyroglobulin is associated with orbitopathy in Graves' disease. *J Endocrinol Invest*. 2021;44(9):1905-1911.

II. **Khamisi S,** Lundqvist M, Edén Engström B, Larsson A, Karlsson FA, Ljunggren Ö. Comparison between thyroid stimulating immunoglobulin and TSH-receptor antibodies in management of Graves' orbitopathy [published online ahead of print, 2023 Jan 27]. *Exp Clin Endocrinol Diabetes*. 2023;10.1055/a-2021-0596.

III. **Khamisi S**, Lundqvist M, Rasmusson AJ, Engström BE, Karlsson FA, Ljunggren Ö. Vitamin D and bone metabolism in Graves' disease: a prospective study [published online ahead of print, 2022 Sep 27]. *J Endocrinol Invest*. 2022;10.1007/s40618-022-01927-y.

IV. **Khamisi, S.**, Udumyan, R., Sjölin, G., Calissendorff, J., Filipsson Nyström, H., Holmberg, M., Hallengren, B., Lantz, M., Planck, T., Wallin, G. and Ljunggren, Ö. "Fracture incidence in Graves' disease". *Submitted for publication.*

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Abbreviations

GD	Graves' disease
GO	Graves' orbitopathy
TSH	Thyroid stimulating hormone
F-T4	Free thyroxine
F-T3	Free triiodothyronine
TRAb	TSH-receptor antibodies
TSI	Thyroid stimulating immunoglobulin
TPO	Thyroid peroxidase
TPO-ab	Thyroid peroxidase antibodies
Tg	Thyroglobulin
Tg-ab	Thyroglobulin antibodies
25OHD3	25-hyrdroxyvitamin D3
1,25OH2D3	1,25-dihydroxy-vitamin D3
BMD	Bone mineral density
CTX-I	C-terminal telopeptide I
PTH	Parathyroid hormone
DBP	D vitamin binding protein
SHBG	Sex hormone binding globulin

Introduction

Graves' disease

Graves' disease (GD) is an autoimmune disease, with an annual incidence of 21 per 100,000 individuals in Sweden (1), which is the most common cause of hyperthyroidism and more common in women. TSH receptors (TSH-R) are expressed in the thyroid gland and in fibroblasts, adipocytes, bone cells and other sites. GD is caused by autoantibodies to the TSH-R (TRAb) in the thyroid. Activation of TSH-R results in stimulation of thyroid hormone synthesis. There is a genetic and environmental background for GD. However, the exact cause of GD is still unclear (2-4). Symptoms of hyperthyroidism include tachycardia, palpitation, weight loss, increased gastrointestinal motility, tremor, etc. Patients with GD may develop complications such as orbitopathy and osteoporosis.

Graves' orbitopathy (GO)

Also known as endocrine ophthalmopathy or thyroid-associated ophthalmopathy (TAO). GO is relevant in approximately 50% of patients with GD, mainly with mild symptoms but severe symptoms affecting 3–5% of the cases (5). In patients without clinically obvious orbitopathy, radiological signs have been shown in 40% of the cases (6). Smoking (7) and TSH-R stimulation (8-10) are known risk factors for GO. Other risk factors are high TRAb levels (11), low levels of TPO-ab (12-16) and low levels of Tg-ab (13, 16).

The stimulation of TSH-R in the orbita leads to stimulation and proliferation of fibroblasts, an increase in hyaluronic acid production and an increased inflammatory response (9).

GO is classified as mild with symptoms, such as eye irritation and tearing due to dry eyes, redness and/or swelling of the caruncle, and eyelid retraction. In moderate to severe GO, more intensive symptoms such as more severe redness and/or swelling of the eyelids, pressure or pain in the eyes, chemosis, exophthalmos, double vision, or optic nerve compression may appear.

Fig. 1 Graves' orbitopathy

Adapted from Jonathan Trobe, M.D. - University of Michigan Kellogg Eye Center

Early diagnosis and treatment are important to protect against the development of complications due to severe GO. Steroids are still considered as the drug of choice for severe forms of GO (17). Steroids inhibit inflammation and reduce the synthesis and secretion of glycosaminoglycans, which are produced by orbital fibroblasts. Nowadays, there are also promising new treatments such as interleukin-6 inhibitors and human monoclonal anti-IGF-1R blocking antibodies. However, in many cases, GO is still a challenge for clinicians in both diagnosis and treatment.

Thyroglobulin (Tg)

Thyroglobulin is a protein produced by the follicular cells in the thyroid gland and used in the synthesis of thyroid hormones (18). Smaller quantities of Tg may be secreted from the thyroid into the blood circulation under physiological and pathological conditions. A study showed serum Tg in normal individuals to be 9.5 ± 0.9 ug/L, while higher levels were observed in untreated (424 ± 101 ug/L) and treated (328 ± 222 ug/L) subjects with diffuse toxic goiter (19). In another study, Izumi et al. found higher Tg levels in untreated GD patients compared to healthy subjects and observed elevations in serum Tg within 24–48 h after subtotal thyroidectomy or radioactive iodine therapy (RAI) for GD (20). A study of 12 patients with subacute thyroiditis demonstrated elevated Tg levels of 300 ± 204 ug/L, which declined to 43 ± 21 ug/L within 20 days of steroid therapy and gradually eventually returned to the normal range (21). In another study, it was shown that Tg released in subacute thyroiditis is iodinated due to a destruction of thyroid follicles, while non-iodinated Tg was found in GD (22).

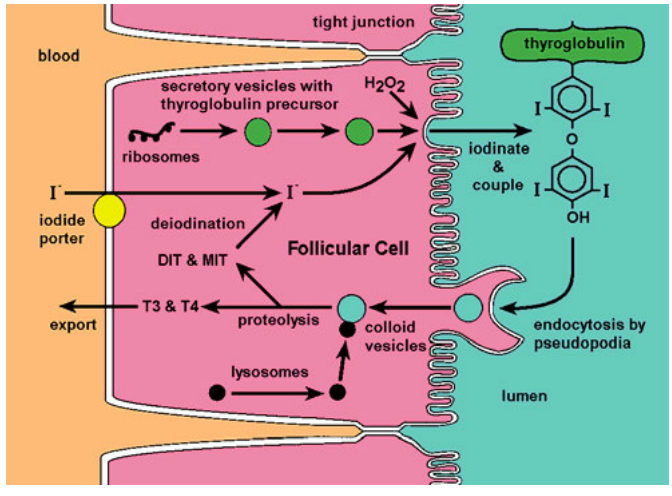


Fig. 2 The role of thyroglobulin in thyroid hormone synthesis

Adapted from Teaching – Biochemistry and Microbiology (leeds.ac.uk)

Elevated serum Tg levels that tend to decrease have been observed in patients with GD during anti-thyroid drug (ATD) treatment. High Tg level has also been associated with an increased risk of relapse. Uller et al. studied 34 GD cases (23), and elevated Tg levels were observed both before and after ATD in patients whose GD relapsed compared to those who went into remission. Gong ST et al. have studied the changes that occur in Tg levels in 65 patients who received ATD and were able to demonstrate that Tg was significantly higher, both before and after treatment, in patients who relapsed (24). Gonzàlez-Jimènz et al. examined 51 patients with GD and noted that changes in serum Tg and antithyroid antibodies after 6 months of ATD in relapses versus early remissions were different. S-Tg > 75 ug/L and TRAb > 30 U/l (ref < 15 U/L) at the time of discontinuation of ATD were considered as markers predicting GD recurrence (25).

At present, Tg is not used as a biomarker in predicting relapse risk or in the assessment of GO. We have now studied the differences in Tg levels at diagnosis and during treatment of patients with newly diagnosed GD.

Thyroid stimulating immunoglobulin (TSI)

Autoantibodies against TSH-R are found in approximately 95% of patients with GD. TRAb usually stimulates thyroid hormone production via activation of TSH-R (2, 26). However, other types of antibodies block the action, or they

are neutral without any functional effect on the TSH-R (27, 28). TRAb is a serological hallmark in GD, and the measurement of TRAb is a standard method for diagnosis, monitoring of treatment, and also prediction of remission or relapse in patients with GD (29-32). The third generation of competitive binding TSH-R autoantibody tests are widely used for measurement of TRAb. A potential pitfall of using TRAb is that the method may also measure, if present, blocking and neutral antibodies.

The IMMULITE 2000/2000 XPi TSI assay uses recombinant TSH-R chimeras constructed from the N-terminal domain of the TSH-R, which contains the Luteinizing Hormone receptor from which the blocking epitopes are absent and used as capture antigens. This assay is supposed to be more specific for measurement of stimulating TSH-R antibodies and less affected by blocking antibodies (33). However, there is a need for more evidence that demonstrate clear benefits of using this method compared to the traditional third generation assays in GD, particularly in the assessment and management of GO.

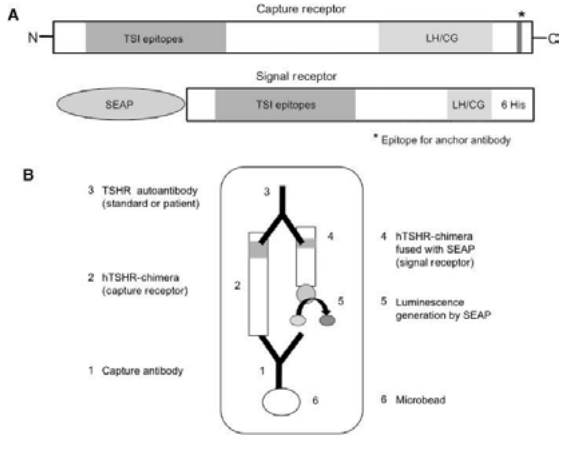


Fig. 3 TSI assay. (A) Linear structure of the capture and the signal receptor. (B) Principle of the assay.

Adapted from Clin Chem Lab Med 2017 Jan 1;55(1):58-64.

Osteoporosis

Osteoporosis is a chronic condition with progressive weakening of the bones. As a result, patients suffer from multiple fractures that often lead to considerable reduction in the quality of life, and also to premature death (34). Osteoporotic fractures constitute an enormous health problem throughout the world, with a constantly increasing challenge to health economy (35).

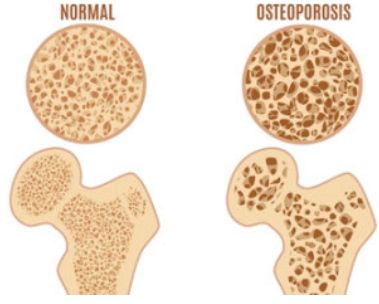


Fig. 4 Osteoporosis

Bone fragility is caused by several underlying pathophysiological mechanisms. In primary osteoporosis, postmenopausal and senile osteoporosis, the balance in bone remodelling is shifted towards a net loss in each remodelling cycle, causing progressive bone loss (36-38). Secondary osteoporosis is also due to a disturbance in the remodelling cycle. The most common cause is glucocorticoid use (39). In addition, several diseases, such as hyperparathyroidism, myeloma and renal failure, cause osteoporosis (40-43). Hyperthyroidism may also cause secondary osteoporosis by acceleration of bone remodelling (44-46). It is also well known that the treatment of hyperthyroidism leads to improvement in bone density and decreases the fracture risk (47).

Vitamin D

Vitamin D plays an essential role in the body's calcium homeostasis, bone mineralisation and skeletal metabolism. Vitamin D exists in two forms: cholecalciferol (D3) and ergocalciferol (D2). Vitamin D3 is formed from 7-dehydrocholesterol in the skin with the help of ultraviolet B light. We get smaller amounts of vitamin D through our diet, mainly from fatty fish, vitamin-enriched dairy products, or vitamin D supplements.

Vitamin D is converted in the liver by hydroxylation to calcidiol (25OHD3), which in turn, is converted in the kidneys to the metabolically active hormone calcitriol (1,25OH2D3). Calcidiol and calcitriol are transported in serum bound to vitamin D-binding protein (DBP). 1,25OHD3 affects the vitamin D receptor (VDR). Vitamin D receptors are found in all cells and can influence a range of functions, including cell division and cell differentiation.

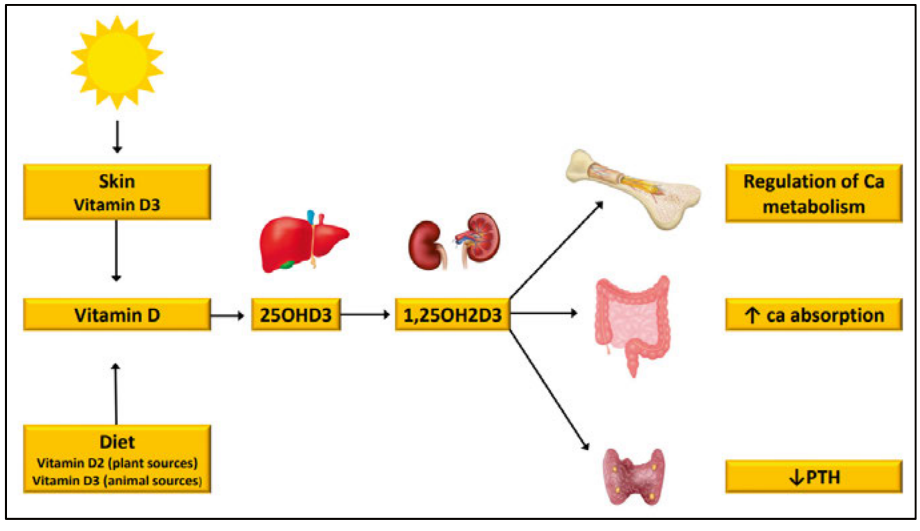


Fig. 5 Vitamin D metabolism

Vitamin D and bone metabolism in Graves' disease

Epidemiological studies have shown a connection between vitamin D deficiency and the risk of several diseases, such as cancer, diabetes, cardiovascular and infectious diseases (48-54). Deficiency in vitamin D has also been observed in autoimmune diseases, such as multiple sclerosis (55-58). However, there is a lack of solid evidence for an association between the incidence of GD and vitamin D deficiency. In a Japanese study, 40% of women and 18% of men with GD had vitamin D deficiency (59). In another study from Japan, 35% of the cases with thyrotoxicosis had vitamin D deficiency (60). In a study of thirty patients with hyperthyroidism in India, 26% of the cases had vitamin D deficiency (25OHD3 less than 25 nmol/L) (61). In a Swedish study, lower 25OHD3 levels were noticed in GD patients compared to the general population (55.0 ± 23.2 vs 87.2 ± 27.6 nmol/L). However, the levels of vitamin D in that study were not associated with laboratory or clinical parameters of GD (62). Other studies have also shown a significant effect of vitamin D deficiency on skeletal metabolism (63-65).

The studies on vitamin D in GD summarised above were cross-sectional and reported levels of 25OHD3. No levels of the active vitamin D, 1,25OH2D3, have been described in GD.

Fracture incidence in Graves' disease

Hyperthyroidism may result in secondary osteoporosis due to the acceleration of bone resorption (44-47, 66). Reduced bone mineral density (BMD), increased bone turnover and increased risk of fracture have been shown in previous studies, even in subclinical hyperthyroidism, especially in postmenopausal women (67, 68). The bone loss in hyperthyroidism is caused by stimulation of the osteoclastic activity (69). However, many other factors are affected by elevated thyroid hormones, such as calcium metabolism, vitamin D, parathyroid hormone (PTH) and sex hormones, making the effect on the skeleton more complicated and multifactorial in hyperthyroidism.

Studies show that markers of bone formation and resorption are elevated and correlate with the severity of hyperthyroidism in pre- and postmenopausal women in thyrotoxicosis (70-72). Similar findings have been observed in men (73). Some studies show an association between hyperthyroidism and an increased fracture risk. In a study by Bauer et al., women older than 65 with low TSH levels had an increased risk of new hip and vertebral fractures (74). In another study, hyperthyroidism was found to be associated with a transient increased risk of fracture that reverses upon normalisation of thyroid hormones by using ATD (75). However, a Swedish study by Hallengren et al. showed no increased incidence of fractures in patients treated for hyperthyroidism (76).

Fig. 6 Vertebral compression fracture (Adapted from Scott Camazine/science photo library)

Treatment of hyperthyroidism has a positive impact on BMD. In a study regarding treatment of pre-menopausal women, there was a 4% increase in BMD within 1 year (77).

Various studies, mostly retrospective and cross-sectional, on the impact of thyroid diseases on bone turnover and fracture risk, have been reported. However, interpretation of the literature is complex, not least because of heterogeneous cohorts. In addition, only few studies have been prospective (66).

Aims

The purpose of this thesis is to explore the role of Tg and TSI in the management of GO. Another aim was to study the role of vitamin D in GD and the impact of GD on bone health.

Objectives

Paper I To investigate the association of thyroglobulin to GO

Paper II To evaluate the role of TSI in the management of GO

Paper III To prospectively study the vitamin D levels in patients with newly diagnosed GD and the effect of treatment of GD on bone turnover marker and bone mineral density.

Paper IV To investigate fracture rates in GD.

Subjects and Methods

Papers I–III

Thirty patients with newly diagnosed GD were recruited at Uppsala University Hospital from February to November 2017. Diagnosis of GD was based on low levels of TSH and positive TRAbs. The median age of subjects was 55, with a range of 35–72 years old; Out of the 30, 29 were women. Only two subjects were smokers.

The study comprised six visits over two years of follow-up. At baseline, visit 1, all patients underwent an examination including recording of family and medical history, smoking habits and current medication, including questions about medications without any prescription, such as vitamin D or calcium. Blood samples were taken, and BMD with Dual-energy X-ray absorptiometry (DXA) was performed during the study as shown in Figure 7.

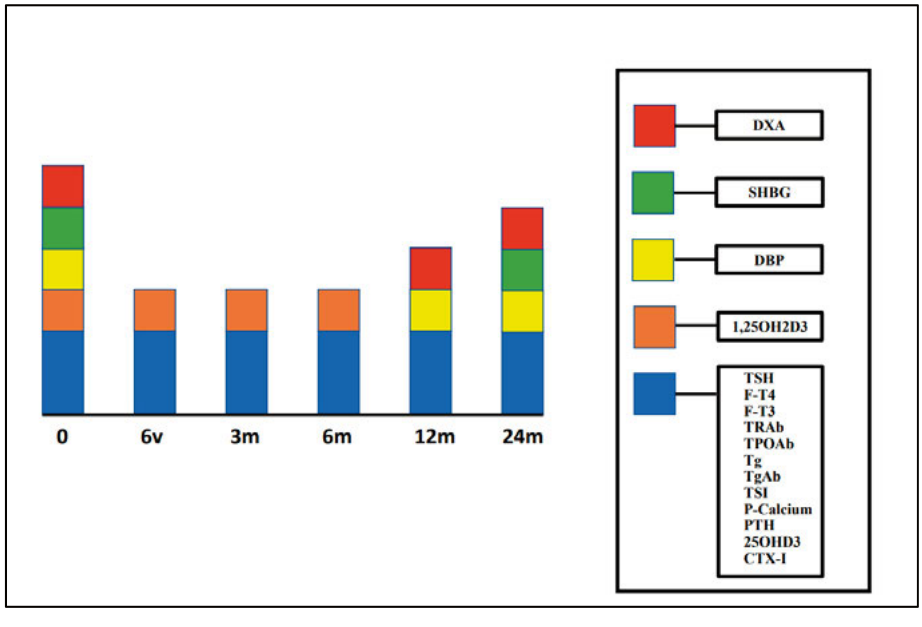


Fig. 7 Flow chart for the blood samples and DXA during the study period

To assess the severity of GO, we used the European Group on Graves' orbitopathy (EUGOGO) classification (17). All eye symptoms and signs of GD were continuously documented in the medical record by both the treating endocrinologist and the research nurse at each visit.

For all patients, methimazole (10–20mg) or propylthiouracil (150–300mg), was initiated at visit 1 and given in a block and replace regimen in combination with thyroxine 50–100 μg daily, which was added when thyroid hormone levels decreased to the normal range.

One patient had a spontaneous recovery before the start of treatment and thus did not receive ATD. The medical treatment was changed in eight cases, where the patients instead received radioiodine treatment (RAI) (n = 5) or underwent surgery (n = 3). Four cases of those receiving RAI were due to continued high TRAb after 10–15 months of ATD therapy. One patient was treated with RAI after 5 months, according to the patient's request. Three cases underwent surgical treatment in the form of total thyroidectomy. Two patients underwent surgery due to neutropenia related to ATD, and one female patient underwent surgery after 5 months due to pregnancy desire. Of the remaining patients, 21 patients received full medical treatment with ATD for 18–24 months until a negative TRAb result. The patient with spontaneous recovery did not develop GO and is included in the ATD group [all patients who did not undergo surgery or RAI (n = 22)].

Paper IV

All patients with newly diagnosed GD (n=2204) were included in seven cities in Sweden from 2003 to 2005, with a cover area of about 40% of the Swedish population. The diagnosis of GD was confirmed by low TSH and elevated concentrations of more than one of the following thyroid hormones: fT4, total T3 and/or fT3, in combination with elevated TRAb and/or an increased diffuse isotope uptake on thyroid technetium scintigraphy (1).

Now, 16–18 years after diagnosis, 2,134 patients were included in an investigation to study the risk of developing skeletal complications by studying the incidence of fractures and the use of anti-osteoporosis medication. The drug, patient and population registries belonging to the National Board of Health and Welfare and Statistics Sweden´s databases have been used for this reason. By using the drug register, we obtained data on medications against osteoporosis and treatments with steroids. Anti-resorptive treatments against osteoporosis and systemic corticosteroids are prescribed in Sweden and are available in the drug register. The inclusion of GD patients in this study began in 2003, and there is no information on medications until July 2005 because the registry

was started in Sweden at that time. We have now studied the drug register for the included subjects until 31st of December 2019.

For the diagnoses, we have used access to the ICD-10-SE diagnosis codes in the patient register. The population register has been used to identify immigration and date of death.

To compare the fracture rates between patients with GD and the general population, we used a random reference group of up to ten individuals for each patient from the National Register, who were matched for sex, year of birth and county of residence.

The primary outcome was fragility fractures, for which the information was obtained from the patient register by searching for all hip, vertebral, humeral and forearm fractures.

The secondary outcomes were anti-osteoporosis and corticosteroid treatments. Regarding anti-osteoporosis treatment, antiresorptive treatments in the form of bisphosphonates, both oral and intravenous and denosumab, which covers the majority of those who received treatment for their osteoporosis, were included in this study.

Regarding steroids only systemic treatments obtained from the register, both oral and intravenous, were included, but not local treatments such as ointments.

Survival time was calculated from the date of GD diagnosis (index date) to fracture date and was censored at the date of emigration, death or end of follow-up (31 December 2019), whichever occurred first. For the matched controls, follow-up started from the date the matched patient received a GD diagnosis.

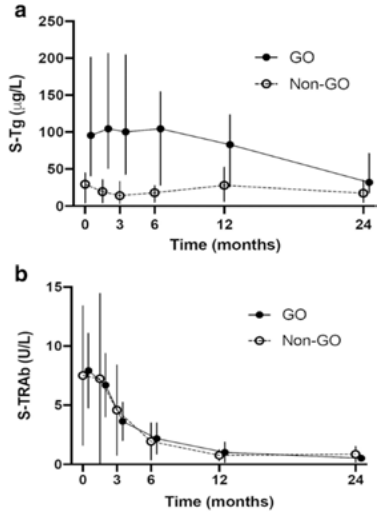
In the analyses comparing the rates of anti-osteoporosis treatment or steroids among GD patients and reference population, survival time was computed from 1 July 2005 until date of medication collection and was censored at the date of emigration, death or end of follow-up (31 December 2019), whichever occurred first. Individuals who had emigrated or died before 1 July 2006 are excluded from these analyses.

Results

Paper I

There was a significant difference in the Tg levels between patients with GO (n = 11) and those without GO (n = 19) at the baseline visit (visit 1), with higher levels of Tg in the case of GO ($p = 0.042$) (Paper I, Fig. 1a). Six patients were diagnosed with GO after visit 1 during the follow-up period. After adding these 6 patients (All GO group n = 17), compared to non-GO (n = 13), who never experienced GO either at diagnosis or during the follow-up period, the difference in Tg levels becomes more significant ($p = 0.001$) (Paper I, Fig. 1b). In all GO group, the fT3 and fT4 were higher and TPO-ab was lower with a tendency towards higher TRAb and lower Tg-ab. Between Tg levels and TRAb, fT3 or fT4, there were no significant correlations. After surgical treatment with total thyroidectomy, Tg dropped to very low levels, as expected.

Twenty-two subjects completed the ATD regimen (GO n = 12, non-GO n = 10). The AUC of Tg was significantly higher in GO vs non-GO. However, the AUC of TRAb did not differ between the GO and non-GO (Paper I, Fig. 2).



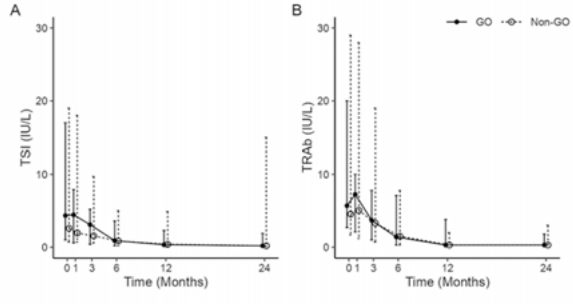
Paper I, Fig. 2 Tg and TRAb levels during the whole study for the ATD group ($n = 22$) divided into GO ($n = 12$) and non-GO ($n = 10$).

Paper II

At baseline (n=30), fT3 correlated to both TSI (rs=0.44, p=0.01) and TRAb (rs=0.4 p=0.02) (Paper II, Fig. 1). However, during the rest of the follow-up period, there were no significant correlations between fT3 and TSI or TRAb. TSI was highly correlated with TRAb at baseline (rs=0.64, p<0.001) and during all other visits and treatments (rs>0.60, p<0.001) for all visits.

A total of 17 patients had GO during the study period, of whom 11 patients were at diagnosis and 6 patients who experienced GO during the follow-up period. At baseline, TSI and TRAb did not differ between GO and non-GO patients.

In the ATD group, in which the subjects received a complete medical treatment for 18–24 months (n=22), TSI was higher during the first three visits in GO (n=12) compared to non-GO (n=10) (Paper II, Fig. 2). However, this difference was not significant when comparing AUC over the entire follow-up period (p=0.49). TRAb, on the other hand, was similarly reduced and normalised in both groups.



Paper II, Fig. 2 TSI and TRAb levels presented as median and range in all visits for the ATD group (*n* = 22), GO (*n* = 12) and non-GO (*n* = 10).

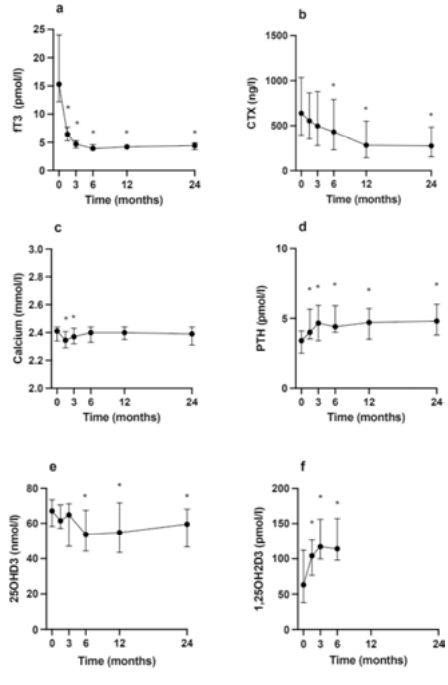
Paper III

All thirty patients are included in the baseline data. Follow-up analyses included twenty-seven patients, after excluding those who started vitamin D supplementation after baseline.

25OHD3 and 1,25OH2D3 at baseline and during treatment

At baseline, the level of 25OHD3 was 66.8 (37.3–130) nmol/L; four patients had values below 50 nmol/L, but as minimum 36.8 nmol/L. There were no correlations between 25OHD3 and thyroid hormones fT3 and fT4, TSH or

TRAb. Ater treatment start with ATD, values of fT3 became normalised, and the levels of 25OHD3 declined significantly (Paper III, Fig. 2a, e). Regarding 1,25OH2D3, the level at baseline was 68 (17–155) pmol/L; 14 patients had levels below the lower reference range. After treatment start, there was a significant increase in the levels of 1,25OH2D3 (Paper III, Fig. 2f). There were no correlations between 1,25OH2D3 and fT3, fT4, TSH or TRAb, apart from a negative correlation between 1,25OH2D3 and fT4 at visit 4 (Rs -0.394, $p = 0.042$).



Paper III, Fig. 2 Levels of fT3 (a) CTX (b), calcium (c), PTH (d), 25OHD3 (e), 1,25OH2D3 (f) during the follow-up period of 24 months.

Measures of calcium homeostasis

There was initially a transient decrease in s-Ca (Paper III, Fig. 2c) and an increase in PTH (Paper III, Fig. 2d) after the start of treatment. Moreover, s-phosphate decreased from 1.13 (0.79–1.63) at baseline to 1.0 (0.73–1.33) at visit 6. CTX-I, which is a marker of bone resorption, decreased significantly during the follow-up period (Paper III, Fig. 2b).

DBP did not change during treatment ($p = 0.77$), while SHBG, as expected, decreased from 172 (65–634) at baseline to 69.5 (14–299) at visit 6 ($p < 0.001$).

Bone mineral density

Bone mineral density (BMD) was analysed in all patients with DXA at baseline and after 12 and 24 months, respectively. The mean level of BMD at baseline in the spine was 1.12 (+/- 0.17) g/cm2. In the left total hip, BMD was 0.95 (+/- 0.14) g/cm2 and 0.91 (+/- 0.13) g/cm2 in the left femoral neck (Paper III, Fig. 3). Regarding the T-score, only one patient had a T-value lower than -2.5 in the spine.

The mean BMD increased significantly at all locations during the 2-year follow-up; in the spine, 5.3% (± 7.5) ($p = 0.002$); in the left total hip, 7.5% (± 7.9) ($p < 0.001$); and in the left femoral neck, 4.4% (± 7.0) ($p = 0.005$) (Paper III, Fig. 3).

Paper IV

In total, 2,134 GD patients and 21,261 age-, sex- and county-matched population controls were included in the analysis. Median follow-up was 15.2 years (range: 5 days to 17.1 years). The median age at study entry (corresponds to the date of GD diagnosis in individuals with GD) was 46 years (IQR: 35–58, range: 6–96). The median age at first recorded diagnosis of fragility fractures after the index date was 68 years (IQR: 58.0–80.0, range: 11–102). GD patients were more likely to have a diagnosis of diabetes than the reference population. Otherwise, there were no differences in the other matching variables.

Fracture incidence in GD compared to controls

During follow-up, 2,114 (9.0%) individuals had fragility fractures (194 GD patients and 1,920 population controls), 918 (3.9%) emigrated, 2,484 (10.6) died and 17,879 (76.4%) were followed until the end of the study.

After adjustment for age, sex, county, and co-morbidities, as well as fractures and falls before the index date, the data did not show significant differences between GD patients and controls, but only a significant difference in vertebral fracture rates in male GD patients aged >52 compared to their controls, HR=2.83 (1.05–7.64) (Paper IV, Table 3).

Treatment against osteoporosis and steroids use

Compared to the reference population, the rates of treatment against osteoporosis as well as treatment with steroids were higher in patients with GD.

Paper IV, table 3 Cox proportional hazards regression analyses comparing fracture rates among 2,134 patients diagnosed with Graves´ disease in 2003 to 2005 in Sweden and matched controls from the general population

	All fragility fractures			Hip fractures			Vertebral fractures			Arm fractures		
	N of events[c]	N of events	HR (95% CI) [a]	N of events[c]	N of events	HR (95% CI) [a]	N of events[c]	N of events	HR (95% CI) [a]	N of events[c]	N of events	HR (95% CI) [a]
Overall	194	2,114	0.99 [0.86,1.15]	59	674	0.96 [0.73,1.25]	16	161	1.07 [0.64,1.80]	132	1,480	0.96 [0.81,1.15]
Among women	167	1,862	0.96 [0.82,1.13]	51	576	0.94 [0.71,1.26]	9	122	0.75 [0.38,1.47]	119	1341	0.95 [0.79,1.15]
Aged ≤52 yrs[b]	46	589	0.84 [0.62,1.14]	2	31	0.59 [0.14,2.53]	2	40	0.52 [0.13,2.17]	42	522	0.87 [0.64,1.20]
Aged >52 yrs[b]	121	1,273	1.03 [0.85,1.24]	49	545	0.96 [0.71,1.28]	7	82	0.90 [0.41,1.95]	77	819	1.02 [0.80,1.29]
Among men	27	252	1.22 [0.82,1.83]	8	98	0.98 [0.48,2.04]	7	39	2.24 [0.98,5.14]	13	139	1.05 [0.59,1.86]
Aged ≤52 yrs[b]	7	87	0.82 [0.37,1.79]	0	8	N/A	2	14	2.22 [0.47,10.41]	5	67	0.71 [0.28,1.79]
Aged >52 yrs[b]	20	165	1.53 [0.96,2.45]	8	90	1.11 [0.54,2.31]	5	25	**2.83 [1.05,7.64]**	8	72	1.37 [0.66,2.87]

[a] Adjusted for age, sex, county, comorbidity (connective tissue diseases, diabetes mellitus, end stage kidney disease, congestive heart failure, COPD, cerebrovascular disease, cancer) as well as fractures and fall accidents before the index date
[b] age at index date, which is the date of Graves' disease (GD) diagnosis for the patients and the date on which the matched patient was given a GD diagnosis for population controls
[c] number of events in GD patients

Discussion

In Papers I–III, thirty patients with *de novo* GD were included to study the role of thyroglobulin and TSI in the management of GO. Another aim was to study vitamin D and bone metabolism in GD prospectively.

Serum thyroglobulin is associated with orbitopathy in Graves' disease

The study shows differences among patients with and without eye involvement in GD. Compared to patients without GO, the levels of Tg were significantly higher in patients with GO, both before and during treatment with ATD, and this difference was more marked than the levels of thyroid hormones and TRAb. In both groups, TRAb is normalised in the same way and there was no significant difference during treatment, although it tended to be higher in the GO-group at baseline. Thus, the differences in Tg should not be related to higher disease activity, which is usually associated with TRAb levels.

In a previous study, Tg was elevated in 56% of patients who had GO compared to 28% of patients without GO. The same study has referred to the so-called Kriss hypothesis, which speculates about the accumulation of Tg, probably through the lymph flow, which leads to the initiation of the inflammatory process in the orbital tissue (78). However, other recent studies investigating the pathophysiology of GO have been able to demonstrate other mechanisms that better explain the occurrence of GO (79).

An interesting difference in GD compared to other conditions leading to cell destruction that occurs in, for example, subacute thyroiditis is that the Tg secreted into the blood circulation is non-iodinated in the case of GD, while iodinated in the case of subacute thyroiditis. This probably suggests that a newly formed Tg, which is non-iodinated, is transported to the extra-follicular space through a mechanism controlled by TRAb or TSH (80). In healthy subjects, stimulation with recombinant human thyrotropin (rhTSH) increased the median serum TSH to more than 200 mU/L after 4 h, and Tg increased 13- and 3-fold after 48 h and 28 days, respectively (81). However, Tg was still elevated

in our study despite a normalisation of TRAb during the follow-up period. This suggests that other mechanisms than TSH-R stimulation is a cause of the Tg elevation in GD.

In previously reported studies, Tg is elevated in hyperthyroidism (19, 20, 23). However, in our study, 11 patients had a normal Tg level at baseline. Despite normalised thyroid hormones and TRAb, Tg continued to decrease, making it possible to use Tg as a marker for monitoring the disease activity. To investigate this aspect, larger studies with a longer follow-up period are needed. Our data show that Tg is associated with GO and could help in identification of patients with increased risk of developing GO.

The role of TSI in management of GO

Measurement of TSH-R antibodies is necessary in GD. There are three different types of antibodies: stimulating, blocking and neutral, based on their functional effect on TSH-R. The stimulating TSH-R antibodies are the cause of hyperthyroidism in GD. The methods of TRAb measurement have a high sensitivity and specificity, and its use is essential in the management of GD according to different guidelines. Third-generation immunoassays are used widely to discover all types of TSH-R antibodies by analysing the ability to compete with a labelled ligand. These assays are automated and cost-effective, unlike bioassays. However, it is of interest in certain clinical situations to differentiate between different types of antibodies, such as in some complicated cases of unexplained changes in thyroid function, and also during or after pregnancy. Assessment of stimulatory antibodies in the management of GO is still understudied.

Bioassays detect purely stimulatory antibodies. Methods such as Thyretain by Quidel use engineered CHO cells with chimeric TSH-R (MC4) to measure luciferace deletion gene expression due to activation of the cyclic adenosine-3',5'-monophosphate (cAMP) signal transduction pathway. A study showed that clinical sensitivity and specificity of Mc4-TSI was greater than TRAb in GO (27). Another study showed that both a third-generation assay and the Mc4-TSI test were clinically relevant as predictors of a severe course of GO, and the predictive power of the third-generation assay was as strong as that of the Mc4-TSI bioassay (28).

The IMMULITE 2000 TSI assay by Siemens is a bridge-binding immunoassay by which TSH-R antibodies bind to a pair of recombinant TSH-R constructs: a capture and a signal receptor by measuring of secreted alkaline phosphatase (SEAP). This method is supposed to be more sensitive to measure

stimulating antibodies compared to the routinely used third-generation competitive-binding assay. However, our data could not show any significant difference compared to the third-generation assay among GD patients at diagnosis or follow-up. Morover there were no difference between patients with and without GO. Similar results were reported by a newly published study (82), in which 140 participants, 75 cases (53.6%) had GO. Although the Immulite TSI level was higher in the case of GO in that study, it showed poor diagnostic accuracy and no correlation with clinical markers of GO severity or activity. To our knowledge there is only one other study to investigate TSI in GO in which TSH-R antibodies were measured with Immulite TSI assay and with Elecsys IMA in patients with moderate to severe GO (83). In that study, both methods showed a comparable correlation with GO activity during intravenous therapy with glucosteroides. The data from our prospective study confirm and add to the findings from these studies showing similar performance of the two assays in clinical routine.

Vitamin D and bone metabolism in GD

In this study that examined vitamin D levels, bone turnover and BMD in GD, no deficiency in vitamin D could be demonstrated at diagnosis. In addition, there were no differences in either 25OHD3 or 1,25OH2D3 that would be associated with the development of GO. Therefore, vitamin D deficiency has no association with the development of GD or subsequent orbitopathy. Our data show that GD had a significant negative effect on bone metabolism and BMD, which was reversible after treatment of hyperthyroidism. CTX-I, which is a marker of bone resorption, decreased continuously during the observation period and suggests that there is a marked effect on bone metabolism.

Different studies have shown an association between vitamin D deficiency and various autoimmune diseases, including autoimmune thyroid diseases (84). However, according to a recently published study, treatment with vitamin D supplementation did not significantly reduce relapse of GD (85). In our study, 25OHD3 was significantly lower at follow-up than before starting with ATD therapy. The data show that there are no correlations between vitamin D levels and thyroid hormone levels and that deficiency in vitamin D is not prevalent in GD.

In this study, a hitherto unshown shift in vitamin D metabolism during treatment of GD has been observed. 25OHD3 decreased and 1,25OH2D3 increased after starting treatment with ATD. The levels of DBP did not differ before and after treatment, suggesting that the changes in vitamin D were not related to the levels of the binding protein. This suggests that changes in the metabolism of vitamin D were not related to GD but to the treatment of the

hyperthyroidism and reduction of thyroid hormones, leading to a reduction of bone resorption. This, in turn, causes an elevation of PTH, which stimulates the conversion of 25OHD3 to 1,25OH2D3 which is the active form of vitamin D. It is noteworthy that the half-life of 25OHD3 is 15 days (86), while the half-life of circulating 1,25OH2D is only 4–6 hours (87).

BMD, as expected, has gradually increased after the start of treatment in all measured areas, but most prominently in the spine and the total hip. This was in connection with a significant decrease in CTX-I, which suggests that the impact on bone density in hyperthyroidism may be of a greater extent than previously thought. The significant increase in BMD occurs despite an increase in PTH, which usually, in other pathological conditions, leads to increased bone resorption. Data on BMD in this study are in line with a study in which 15 female patients with GD underwent DXA at diagnosis and after 12 months of ATD. BMD increased significantly in the lumbar spine and femoral neck (88). Two further studies (89, 90) have shown similar results.

There was no seasonal effect which could influence the study's results, given that the recruitment of the study patients took place from February to November, and blood samplings were collected 6 times during the follow-up period of 2 years. Similar vitamin D levels were measured in a cohort of 870 healthy individuals in Uppsala, of whom 52% were females (91). Furthermore, the levels of 25OHD3 were higher than those reported in another study of 102 patients in the primary care (92). Thus, we believe that the vitamin D levels in our cohort do not differ from the rest of the population.

In this study, we observed an increase in BMD concomitant with decreasing levels of 25OHD3. The exact role of 25OHD3 as a predictor or a therapeutic agent in skeletal involvement due to GD therefore needs to be further investigated. The data suggest that the increased conversion of 25OHD3 to 1,25OH2D3 may be of more interest than the serum levels of 25OHD3.

Fracture incidence in GD (Paper IV)

A total of 2,134 patients with newly developed GD were included in an investigation, 16–18 years after diagnosis, regarding the risk of developing skeletal complications. Each patient was compared with up to ten controls from a general population using patient, medical and population registries belonging to the National Board of Health and Welfare and Statistics Sweden´s databases in Sweden. In general, no difference in fracture incidence between GD patients and controls could be found. However, the data show a higher risk for vertebral fractures in GD males older than 52 years. Patients with GD had

significantly more treatments against osteoporosis, except GD males older than 52, and more steroids, except females and males older than 52 years.

Many studies and meta-analyses for the assessment of skeletal complications as a result of hyperthyroidism could not show a decisive conclusion, not least that some studies have shown no association at all to hyperthyroidism. This is the case, even though hyperthyroidism is perceived as one of the causes of secondary osteoporosis. A significant weakness in most studies is the heterogeneous cohort, which includes various types of hyperthyroidism, including GD, TNG, overtreatment with levothyroxine, etc. In this study, however, we have only studied patients with verified GD.

An interesting finding in this study is that men older than 52 have a higher vertebral fracture risk compared to the general population. This is despite the fact that previous studies have shown that skeletal effects as a result of GD are reversible after treatment (77). However, the data in our study was difficult to assess, not least because of the low number of events, probably due to underdiagnosis of vertebral fractures because of limited radiological examinations for the investigation of back pain, and also it is relatively common with asymptomatic vertebral fractures. The results may thus be a random finding, which makes it difficult to establish a solid conclusion based on these findings. In Paper III, we have shown a significant improvement in bone density for 2 years after initiation of treatment against hyperthyroidism in GD. However, the bone density measurement only shows mineralisation and not the bone quality. There was no increased fracture risk in the women included in the current study. This can probably be explained by the fact that women possibly receive more osteoporosis prophylaxis in the case of steroid treatment against GO. In addition, more bone density measurements are commonly performed on women than men. A relative hypogonadism in men associated with increasing age may have a negative effect on the skeleton. This may differ compared to women who receive more sex hormones than men at increasing age after menopause, and it is well known that long-term hormone therapy for postmenopausal women is effective for prevention of postmenopausal osteoporosis (93). In men, hypogonadism with testosterone concentrations of <12nmol/l is estimated to occur in 7% of men aged 40–60 years, increasing to 21% in 60–80-year-old men, and 35% for men aged >80 years (94). Several studies have demonstrated a gradual decline in testosterone levels with increasing age, which varies in different individuals and is affected by medications, chronic diseases and adiposity (95-98). However, the diagnosis of hypogonadism is often neglected, and the opportunity to replace testosterone in older men is highly debated (99). Furthermore, heavy smoking is more prevalent in men compared to women. However, these possible risk factors should also affect the controls in the same way and do not fully explain the finding of the increased risk of vertebral fractures in this group.

GD patients included in this study have significantly more anti-osteoporosis treatments and treatments with steroids, but not for men older than 52. This means that the increased risk of vertebral fractures in this group is not due to steroid treatments. It may be that steroid treatment against GO contributes to the fact that the GD patients generally had more steroids compared to the control group. In addition, patients with GD are at increased risk of other autoimmune diseases that require steroid treatment. Otherwise, it is not entirely surprising that a well-controlled group of patients who have regular contact with the healthcare system receive more prescribed drugs, including steroids.

Conclusions

Papers I and II

Measurement of thyroglobulin at diagnosis and follow-up of GD can act as a marker for prediction of patients with increased risk of developing eye complications. Increased release of Tg at GD could possibly cause a disorder affecting the retroorbital tissue at GO.

On the other hand, TSI was highly correlated with TRAb, and the tests showed no significant difference between GO and non-GO patients before treatment of GD. After treatment with ATD, both TSI and TRAb levels similarly decreased and normalised. Data show that TSI is equivalent but not better than the third-generation assay of TSH-R antibodies in management of GO.

Paper III

In Graves' disease, no vitamin D deficiency could be demonstrated at diagnosis, and the levels of 25OHD3 decreased significantly during the follow-up period after correction of hyperthyroidism. Lower levels of 25OHD3 occur after treatment initiation, secondary to a higher conversion to 1,25OH2D3 due to increased PTH levels. GD has a significant negative effect on the skeleton, and treatment of hyperthyroidism leads to improved BMD at a follow-up period of 2 years after the start of treatment.

Paper IV

Patients with GD do not have an increased incidence of fractures compared to the general population. However, a subgroup, men older than 52 with GD may have an increased risk of vertebral fractures but the data is difficult to interpret and should be confirmed by prospective studies focused on this patient group. The rates of treatment against osteoporosis as well as treatment with steroids were higher in patients with GD.

In summary, Graves' disease is associated with eye and skeletal complications. Our data show that Tg might be a new diagnostic tool, and that TSI measures

are not superior to the traditional third generation assays for measurement of TRAb. These findings can be implemented in the clinical care of patients with Graves' orbitopathy. Regarding bone turnover, our data show that vitamin D metabolism is of less importance than hyperthyroidism per se. Furthermore, there is no increased risk of fracture after undergoing GD, but older men may likely have an increased risk of vertebral fractures.

Svensk sammanfattning (summary in Swedish)

Graves sjukdom (GD) är en autoimmun sjukdom med en årlig incidens på 21 av 100000 individer. Graves orbitopati (GO) är kliniskt relevant hos cirka 50 % av patienterna med GD, med svåra former som drabbar 3–5 % av patienterna. Tidig diagnos och behandling av GO kan i de flesta fall skydda mot utveckling av komplikationer vid svår GO. Tyreoglobulin (Tg) produceras av follikulära celler i sköldkörteln. Dess relation till GO har inte klarlagts. Sköldkörtelstimulerande immunglobuliner (TSI) är en relativt ny metod för att bedöma TSH-receptorantikroppar (TRAb) som mäts rutinmässigt för diagnostik och uppföljning av GD.

Vitamin D och osteoporos vid GD har undersökts i tvärsnittsstudier med divergerande resultat. Här studerade vi prospektivt vitamin D-metabolism och benhälsa hos patienter med nydiagnostiserad GD och studerade även en stor svensk patientkohort angående frakturrisk i GD.

Artikel I visar att högre nivåer av Tg är associerade med högre risk att utveckla GO.

Artikel II visar att TSI hos patienter med nydiagnostiserad GD var starkt korrelerad till TRAb i GD vid diagnos och under 2 års uppföljning. Ingen skillnad i TRAb- eller TSI-nivåer mellan patienter med och utan ögonsymptom. I denna studie var TSI inte mer specifik än TRAb i hanteringen av GO.

Artikel III visar ingen D-vitaminbrist vid diagnos av GD. Övriga resultat understryker att tyreotoxikos har en negativ inverkan på benhälsan. Vid behandling förbättrades benhälsan under en uppföljningsperiod på 24 månader.

I artikel IV, totalt 2134 patienter med nyutvecklad GD inkluderades i en undersökning 16–18 år efter inklusion avseende förekomsten av skelettkomplikationer. Ingen ökad förekomst av frakturer kunnat påvisas vid GD. Dock män äldre än 52 med GD kan ha en ökad risk för kotfrakturer. Dessutom uppskattas behandlingsfrekvensen mot osteoporos vara högre hos patienter med GD.

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ORIGINAL ARTICLE

Serum thyroglobulin is associated with orbitopathy in Graves' disease

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Abstract

Purpose Serum thyroglobulin levels are often elevated in Graves' disease (GD) and in most cases decrease during treatment. Its relation to Graves' orbitopathy (GO) has not been clarified. Previously, a risk of GO has been linked to smoking, TSH receptor stimulation, high TSH-receptor antibodies (TRAb), low thyroid peroxidase and thyroglobulin antibodies (TPOAb, TgAb).

Methods We examined Tg levels in 30 consecutive patients with GD were given drug therapy (methimazole + thyroxine) for up to 24 months. GO was identified by clinical signs and symptoms. 17 patients had GO, 11 of whom had it at diagnosis while 6 developed GO during treatment. During the study, 5 subjects were referred to radioiodine treatment, 3 to surgery. The remaining 22 subjects (GO $n = 12$, non-GO $n = 10$) completed the drug regimen.

Results At diagnosis, Tg levels in GO patients ($n = 11$) were higher (84, 30–555 µg/L, median, range) than in non-GO patients ($n = 19$) (38, 3.5–287 µg/L), $p = 0.042$. Adding the 6 subjects who developed eye symptoms during treatment to the GO group ($n = 17$), yielded $p = 0.001$ vs. non-GO ($n = 13$). TRAb tended to be higher, while TPOAb and TgAb tended to be lower in the GO group. For the 22 patients who completed the drug regimen, Tg levels were higher in GO ($n = 12$) vs. non-GO ($n = 10$), $p = 0.004$, whereas TRAb levels did not differ.

Conclusion The data may suggest that evaluation of thyroglobulin levels in GD could contribute to identify patients at increased risk of developing GO. Possibly, thyroidal release of Tg in GD reflects a disturbance that also impacts retroorbital tissues.

Keywords Thyroglobulin · Graves' disease · Graves' orbitopathy · Graves' ophthalmopathy

Introduction

Thyroglobulin (Tg) is produced by the follicular cells of the thyroid gland and secreted into the apical lumen of the follicles where it is concentrated and used as a substrate for the synthesis of thyroid hormone [1]. Small amounts of Tg are released from the thyroid under physiological as well as pathological conditions. For example, Izumi and Larsen [2] found elevated serum Tg levels in untreated Graves' disease (GD) patients, 132 ± 124 µg/L (mean ± SD) as opposed to 11 ± 6.4 ug/L in healthy subjects and observed elevations in serum Tg up to 7000 µg/L within 24–48 h after subtotal thyroidectomy or 131I treatment of GD. Pacini et al. [3] reported serum Tg in normal individuals to be 9.5 ± 0.9 µg/L (mean ± SE), in untreated and treated subjects with diffuse toxic goiter 424 ± 101 and 328 ± 222 ug/L, respectively, in nontoxic goiter 61.4 ± 15 µg/L, in untreated differentiated thyroid carcinoma 89.5 ± 19 µg/L and in subacute thyroiditis 138 ± 67 ug/L. Madeddu et al. [4] studied 12 patients with subacute thyroiditis over 3–4 months, ten of whom demonstrated similar patterns with initially elevated Tg values 300 ± 204 ug/L (mean ± SD, a decline to 43 ± 21 ug/L within 20 days of steroid therapy, a further decline to normal values for 20 days and then a rise above normal for approximately 40 days, eventually returning to the normal range. Interestingly, Druetta et al. [5] in a skillful study showed that Tg molecules released in subacute thyroiditis are iodinated, i.e. contain thyroid hormones and likely reflect a destruction of thyroid follicles. In contrast, non-iodinated Tg molecules

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were found in the circulation of seven out of eight untreated GD patients. The mechanism behind the release of non-iodinated Tg has not been clarified.

Increased serum levels of Tg that tend to fall during antithyroid drug (ATD) treatment have been observed in patients with Graves' disease with no simple relationship to the extent of thyrotoxicosis. Uller et al. [6] studied 34 GD cases. Patients whose GD relapsed had higher Tg levels both before and after ATD, while the levels were lower and dropped in patients that went into remission. Aizawa et al. [7] examined 113 patients with hyperthyroidism who received ATD for 10 months and subsequently a T3 suppression test. Cases with a positive suppression test had significantly lower Tg at the time of the test. Gonzàlez -Jimènz et al. [8] studied 51 patients with GD and noted that in relapses vs early remissions the changes in antithyroid antibodies and serum Tg after 6 months of methimazole therapy were different. At the termination of ATD, serum levels of Tg > 75 ug/L and TRAb > 30 U/l (ref < 15 U/L) were considered markers predicting GD recurrence. Gong ST et al. [9] studied changes in Tg during the course of ATD in 65 patients and found that the Tg levels were significantly higher in the relapse group than in the remission group both before and after ATD. Werner et al. [10] in a cohort of 42 patients with GD reported a non-significant difference in Tg concentrations between patients who were in remission or relapse, 132 ± 242 (mean ± SD) vs. 214 ± 235 ug/L, respectively.

Graves' orbitopathy is clinically relevant in approximately 50% of patients with GD, with severe forms affecting 3–5% of patients [11]. In patients without clinically apparent ophthalmopathy, radiological signs of muscle enlargement have been found in 40% [12]. Several studies have been conducted to predict the risk of recurrence of GD. Smoking [13] and TSH receptor stimulation [14–16] are known risk factors for GO. Furthermore, high TRAb levels have been associated with an increased risk of GO [17] as have low levels of TPOAb [18–22] and lower TgAb [19] [22]. In one study, higher TgAb [20] levels were linked to risk of GO. For evaluation of Tg in relation to GO, Lahooti et al. examined a group of 54 patients with GD, out of whom 23 patients had GO. 52% of patients with GO had elevated levels of Tg compared to 26% in the group with no GO [23].

Early diagnosis and treatment of GO may in most cases protect against the development of complications and sequelae from severe GO. At present, Tg is not used as a marker to predict either recurrence risk or risk of GO. In this paper, we prospectively studied patients with newly diagnosed GD to investigate differences in Tg levels at diagnosis and during treatment depending on GO status.

Materials and methods

Study subjects

Thirty patients with de novo Graves' disease, diagnosed with decreased levels of TSH and positive TRAbs, were recruited at the Uppsala University Hospital during February through November 2017. Their median age was 55 years (range 35–72 years), 2 were smokers, 29 were women. Four patients were on betablockers, three on selective serotonin reuptake inhibitors and one on thyroxine. Ultrasound was performed in one case with a palpable thyroid nodule and showed benign findings. One patient had a TRAb below the reference range (1.7 IE/L, reference < 1.75). This patient otherwise had symptoms and laboratory findings typical for GD, which was in line with a homogeneous uptake at scintigraphy.

The study consisted of 6 visits. During the first visit (v1) all patients underwent an examination including the recording of demographic characteristics, medical history, family history and concomitant medication. Blood sampling to measure TSH, fT4, fT3, TRAb, TPOAb, TgAb and thyroglobulin was performed at baseline (v1), and 6 weeks (v2), 12 weeks (v3), 6 months (v4), 12 months (v5) and 24 months (v6) after treatment start. In all 30 subjects, anti-thyroid drugs, methimazole or propylthiouracil, were initiated in conjunction with visit 1 and given in a block-replace regimen, beginning with 10–20 mg methimazole doses or 150–300 mg propylthiouracil. When thyroid hormone levels fell into the normal range, thyroxine 50–100 µg daily was added and TSH levels kept in the low normal range. Throughout, care was taken to avoid treatment-induced episodes of hypothyroidism.

The medical regimen was altered in eight subjects, who were referred to radioiodine treatment ($n = 5$) or surgery ($n = 3$). Four patients received radioiodine (RAI) because of persistently elevated TRAb after 10–15 months ATD treatment. One patient received RAI after 5 months at the request of the patient. Post-RAI, one patient experienced mild GO, not requiring steroid medication. Three patients underwent total thyroidectomy. For two patients, this was due to neutropenia, which developed directly after start of methimazole in one case and after 10 months on methimazole in the other. One female patient was operated after 5 months because of pregnancy desire. Out of the remaining 22 subjects, 21 patients received ATD for 18–24 months until negative TRAb. One non-GO patient had a spontaneous recovery before treatment initiation.

Evaluation and classification of GO

All patients received clear information about GD and they were examined at each contact by a doctor and a nurse with experience in managing GD regarding the presence of eye symptoms and signs and were repeatedly told to contact the

clinic in the case of eye symptoms. GO was defined as eye symptoms and signs related to GD and classified as 'mild' GO with symptoms such as gritty sensation and tearing due to dry eyes, caruncle swelling and/or redness, upper eyelid retraction, or as 'moderate to severe' in the instance of redness and/or swelling of the eyelids, chemosis, pressure or pain in the eyes, exophthalmos, diplopia or signs of optic nerve compression. The eye signs and symptoms were detailed in the medical records by the attending endocrinologist as well as by the research nurse at every visit. To assess the severity of GO we used the EUGOGO classification [24]. Three patients with moderate to severe GO were referred to an ophthalmologist, who also examined and advised on 6 mild cases. The mild cases had mostly mild tissue involvement and corneal exposure responsive to lubricants. In the moderate to severe GO group, one patient presented signs and symptoms already at visit 1. This patient had a unilateral exophthalmos with eyelid retraction, redness and diplopia. One patient had increasing eyelid swelling at visit 2 and one patient developed a unilateral exophthalmos at visit 5. Subjects were divided into an "all GO" group ($n=17$), including patients who at diagnosis presented ($n=11$) or during treatment (n = 6; v2, v3, v3, v4, v5 and v5) developed eye signs and symptoms typical of GO and a "non-GO" group of patients without any signs or symptoms of eye disease ($n=13$) throughout the study period. Out of the 22 subjects who were not referred for surgery or RAI during the study period, 12 were classified as GO (at diagnosis or during treatment) and ten as non-GO.

Assays

Plasma TSH (reference interval 0.4–4.0 mIU/L, free T4 (reference interval 12–22 pmol/L, free T3 (reference interval 3.1–6.8 pmol/L, Tg (reference interval 3.5–77 µg/L), TRAb (reference < 1.75 IE/L), TPOAb (reference < 34 KIE/L), TgAb (reference < 115kIE/L) were measured with methods in routine use at the Department of Clinical Chemistry of the Uppsala University Hospital. The method used for measuring Tg in serum was the immunometric Elecsys Tg II assay by Roche on the Cobas instrument (Roche Cobas8000, e602). Tg concentrations in subjects without hyperthyroidism fall within the reference range 3.7–77 ug/L, based on measurements of 478 healthy Caucasian people (224 women and 254 men).

Statistical analysis

All statistical analyses were performed using SPSS version 25. Graphs were constructed using Graph Pad Prism version 8.3.0. Data are presented as median (range) unless otherwise indicated. *P* values < 0.05 were considered significant. Since the main endpoint of the study was Tg levels at baseline and during follow-up, and the other thyroidal biomarkers were only analyzed to put possible Tg findings into perspective, no correction for multiple testing was undertaken. Baseline data at the first visit were compared between GO and non-GO groups using Mann–Whitney *U* Tests. For all subjects who did not undergo surgery or RAI ($n=22$), the area under the curves of Tg and TRAb for the 24-month follow-up period were calculated with the trapezoid method and compared between the groups GO ($n=12$) and non-GO ($n=10$) using Mann–Whitney *U* tests. Missing values (1 for Tg, 11 for TRAb) were interpolated or extrapolated based on linear regression of adjacent values.

Results

Thyroglobulin Tg levels differed significantly between subjects who presented with GO at the baseline visit ($n=11$) and those who did not ($n=19$) (Fig. 1a), while there were no differences in other thyroidal biomarkers (Table 1). Adding the 6 subjects who developed eye signs during treatment to create an "all GO" group ($n=17$) and comparing them to non-GO ($n=13$) enhanced the group difference for Tg (Fig. 1b). In addition, fT3 and fT4 were higher, TPOAb was lower and there were tendencies towards higher TRAb and lower TgAb in the all GO group vs. the non-GO group (Table 2). In the three patients who underwent total thyroidectomy, Tg dropped as expected post-surgery to very low levels, between 0.04 and 0.18 ug/L at visit 6. We found no significant correlations between Tg levels and TRAb, fT3, or fT4.

Twenty-two subjects (GO $n=12$, non-GO $n=10$) completed the ATD regimen. Table 3 displays hormone, Tg and thyroid autoantibody levels determined on 6 occasions during up to 98 weeks. As can be seen, hormone and

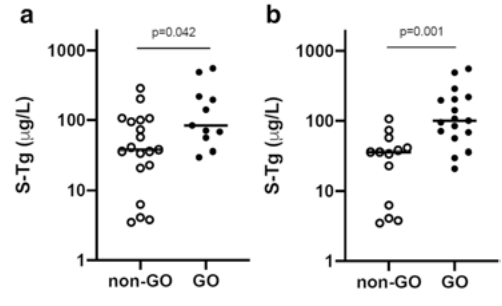

Fig. 1 At baseline, Tg levels in GO patients ($n=11$) were higher than in non-GO ($n=19$) (**a**), $p=0.042$. Adding the 6 subjects who developed eye signs during treatment into GO ($n=17$) vs. non-GO ($n=13$) (**b**), yielded $p=0.001$

autoantibody levels were numerically similar and changed similarly in both groups. The AUC of Tg was significantly higher in group GO *vs*. non-GO [2049 (144–5285) vs. 583 (29–1936) μg/L × months, $p = 0.004$], whereas the AUC of TRAb did not differ between the groups [41.8 (14.6–120.6) *vs*. 36.0 (10.5–171.6) U/L × months, $p = 0.674$].

Fourteen patients presented with or developed mild eye signs/symptoms and three patients had moderate to severe GO. The three patients with moderate to severe orbitopathy received steroids, tapered over time according to clinical response. Two were treated with oral steroids, one after 11 months (i.e. one month prior to v5) received 30 mg prednisolone, tapered over 6 weeks (serum Tg at v1, v2, v3, v4, v5, v6 was 107, 144, 223, 279, 26, 71 μg/L, respectively). The other received 20 mg prednisolone tapered over 1 month (v2 to v3, serum Tg at v1, v2, v3, v4, v5, v6 was 21, 13, 4.2, 8.7, 3.5, 4.7 μg/L, respectively). One patient was given intravenous corticosteroids between v3 and v4 (500 mg and 250 mg methylprednisolone once a week for 6 plus 6 weeks) because of persistent GO despite prior oral steroids between v1 and v3, 20 mg prednisolone tapered to 7.5 mg over 3 months, compliance not documented (serum Tg at v1, v2, v3, v4, v5, v6, was 30, 51, 64, 25, 27, 100 ug/L, respectively).

Table 1 Thyroid hormone, thyroglobulin and autoantibody levels (median and range) of 30 patients with de novo GD divided into GO and non-GO groups on the basis of GO present at diagnosis

	TSH	F-T4	F-T3	Tg	TRAb	TPO-Ab	Tg-Ab
All GD ($n = 30$)	0.005 (0.005–0.040)	41.0 (17.6–100.0)	15.6 (5.6–34.0)	62.5 (3.5–555)	5 (1.7–78)	28 (1.88–600)	14.5 (10–1354)
GO ($n = 11$)	0.005 (0.005)	48 (25–100)	18.5 (9–34)	84 (30–555)	9 (2.7–78)	22 (1.88–216)	11 (10–1354)
Non-GO ($n = 19$)	0.005 (0.005–0.040)	39 (17.6–85)	13.7 (5.6–30)	38 (3.5–287)	4.9 (1.7–29)	57 (7.6–600)	40 (10–1281)
p	0.350	0.232	0.158	0.042	0.216	0.372	0.445

Statistical differences between the two groups are given in the bottom line

Table 2 Thyroid hormone, thyroglobulin and autoantibody levels (median and range) at diagnosis of 30 patients with de novo GD divided into an all GO group, including 6 patients who developed GO after visit 1, and a non-GO group

	TSH	F-T4	F-T3	Tg	TRAb	TPO-Ab	Tg-Ab
All GD ($n = 30$)	0.005 (0.005–0.040)	41.0 (17.6–100.0)	15.6 (5.6–34.0)	62.5 (3.5–555)	5 (1.7–78)	28 (1.88–600)	14.5 (10–1354)
GO ($n = 17$)	0.005 (0.005)	48 (25–100)	18.3 (9–34)	100 (21–555)	6.3 (2.7–78)	12.1 (1.88–285)	10 (10–1354)
Non-GO ($n = 13$)	0.005 (0.005–0.040)	35 (17.6–60)	12.4 (5.6–28)	36 (3.5–106)	4.9 (1.7–29)	98 (8.2–600)	154 (10–637)
p	0.157	0.007	0.025	0.001	0.053	0.048	0.157

Statistical differences between the two groups are given in the bottom line

Table 3 Longitudinal thyroid hormone, thyroglobulin and autoantibody levels determined in connection with 6 visits from 0 to 24 months in 22 patients with de novo Graves' disease

Visit	GO	TSH	F-T4	F-T3	Tg	TRAb	TPO-ab	Tg-ab
0	GO	0.005 (0.005)	47.5 (25–85)	17.7 (9–34)	95.5 (21–287)	5.7 (2.7–20)	16.8 (7.6–285)	10 (10–1354)
	non GO	0.005 (0.005–0.04)	34 (17.6–60)	12.3 (5.6–28)	29.5 (3.5–73)	4.55 (1.7–29)	132 (12.3–600)	97 (10–413)
6 w	GO	0.005 (0.005–0.007)	2 (14.2–36)	6.5 (4.5–8.1)	104.5 (13–240)	7.25 (2.1–10)	15.6 (9.3–246)	10 (10–1314)
	non GO	0.005 (0.005–0.9)	21 (13.9–26)	5.2 (3–7.8)	19.5 (2.5–48)	5.05 (1.2–28)	115 (9–600)	86 (10–396)
12 w	GO	0.005 (0.005–5.68)	22 (11.7–33)	4.95 (3.4–12.6)	100.5 (4.2–274)	3.7 (1–7.8)	12.5 (7.5–158)	10 (10–1185)
	non GO	0.019 (0.005–3.56)	17.9 (12.5–29)	4.7 (3.4–6.4)	14 (1.8–50)	3.3 (0.7–19)	84.5 (11.4–600)	59 (10–375)
6 m	GO	0.202 (0.005–10.4)	17.2 (12.2–31)	4.1 (3.6–5.6)	104.5 (8.7–531)	1.4 (0.3–7.1)	13.1 (7.4–87)	10 (10–1128)
	non GO	0.86 (0.05–4.2)	17.35 (11–22)	3.85 (3.2–4.8)	18 (0.67–147)	1.5 (0.3–7.8)	62.5 (11.6–258)	40.5 (10–290)
12 m	GO	0.755 (0.04–4.67)	17 (12.8–25)	4.2 (3.4–4.6)	83 (3.5–186)	0.35 (0.3–3.8)	11.9 (9–103)	10 (10–1129)
	non GO	1.41 (0.3–4.4)	18.3 (14.7–24)	4.2 (3.8–6.1)	28 (0.9–79)	0.3 (0.3–2)	39.5 (10.8–159)	22.5 (10–252)
24 m	GO	2 (0.07–4.47)	16 (10–19.7)	4.6 (3.7–5.1)	32 (4.7–167)	0.3 (0.3–1.8)	14.1 (5–93)	10 (10–1091)
	non GO	1.79 (0.2–2.6)	15.6 (12.8–25)	4.6 (3.4–6.9)	17.5 (1.3–56)	0.3 (0.3–3)	45 (12.3–218)	31.5 (10–325)

The data (median and range) are presented in GO ($n = 10$) and non-GO ($n = 12$) groups. RAI and thyroidectomy were excluded

Discussion

In this study, we found that Tg levels were significantly higher both before and during treatment with ATD in patients with GO as compared to patients who did not develop GO. The difference in Tg levels was more marked than that of other thyroidal biomarkers. While TRAb levels tended to be higher at baseline, they normalized similarly in both groups and did not differ during treatment. Thus, it is unlikely that the observed difference in Tg levels was mediated by higher disease activity, as reflected by TRAb levels. We have no experience with the new assay of stimulating thyrotropin-receptor antibodies [27].

Most studies performed to evaluate the role of Tg in the assessment of disease activity in GD have shown that Tg may have a role in predicting the risk of recurrence [6–9]. We are aware of only one study that has investigated serum Tg in relation to GO [23]. Serum Tg levels were measured in 54 single samples from patients with GD. Increased levels were determined in 56% of sera from GO patients vs. 28% in samples from patients without GO. The same study also referred to the so-called Kriss hypothesis from the early 1970s [25] which stipulates that the reason for the development of GO is due to an accumulation of Tg in the orbital tissues, possibly through the lymph flow, leading to an autoimmune reaction against Tg bound to the extraocular muscles. However, later studies have suggested other pathological mechanisms to better explain the development of GO, making the Kriss theory controversial [26].

According to previous observations, thyroglobulin is elevated in thyrotoxicosis [2, 3, 6]. However, in our cohort, 11 of 30 patients with GD had a normal s-Tg at inclusion. Most patients displayed declining Tg levels months after discontinuing ATD (data not shown), which may allow Tg to be used as a marker for assessing disease activity even at normalized thyroid hormone levels and TRAb. To examine this aspect, larger studies with longer follow-up are needed.

In normal conditions, only small amounts of Tg leaks into the circulation, but Tg rises in some thyroid diseases such as GD. Tg might be released by a destruction of the follicular epithelium secondary to an inflammatory process. However, in the case of GD, Tg seems to be non-iodinated, in contrast to subacute thyroiditis which is characterized by cell destruction and causes release and elevation of iodinated Tg [5]. Possibly, newly formed non-iodinated Tg is secreted from transport vesicles in thyrocytes into the extrafollicular space by a mechanism that is controlled by TSH or TRAb [28]. In healthy individuals with an intact thyroid gland, rhTSH stimulation increased the median of serum TSH to more than 200 mU/L after 4 h and the circulating Tg increased 13- and threefold after 48 h and 28 days, respectively [29]. In the present study, Tg was still elevated despite a normalization of TRAb during the course of the disease (cf. Fig. 2b), suggesting mechanisms other than TSH-receptor stimulation behind the Tg elevation in GD. Another possibility which has been discussed is that proteins passes by a modification of the epithelium through the follicular cell wall via an intercellular route [30]. In addition, Tg may move from the follicular lumen through pinocytosis [31].

Steroids are still considered first-line treatment for active GO. The effect of steroids on GO involves inhibition of inflammation and a reduction in synthesis and secretion of glycosaminoglycans by orbital fibroblasts [32]. Three patients received steroid treatment, after which Tg levels decreased. In one of these cases, the oral treatment appeared insufficient, Tg levels decreased only following the administration of iv steroids. The majority of the patients in the all GO group (14/17) had merely mild symptoms and did not receive steroids. The clinical phenotype of GO nowadays is milder than in the past [33], likely due to reduced smoking habits and improved management of GD.

The small sample size of the present study should be considered when interpreting the findings. There is a need for confirmatory studies in larger cohorts, which should be powered

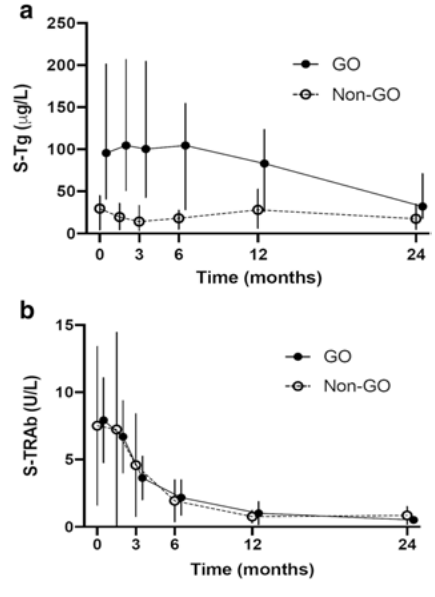

Fig. 2 Tg and TRAb levels in all visits for the ATD group ($n=22$) divided into GO ($n=12$) and non-GO ($n=10$), showing a protracted course for Tg (**a**) in GO vs non-GO group, whereas TRAb levels (**b**) were similarly reduced and normalized in both groups. Data presented as medians and interquartile ranges

for more advanced statistical methods such as regression analyses to investigate the independence of the association between Tg levels and GO status when adjusting for other thyroidal biomarkers. A larger cohort might also enable the development of a clinical scoring system to assess the risk of GO for the individual patient. The strength of the study is that it is prospective and that the included patients underwent continuous follow-up by both endocrinologist and nurse with experience of GD. Furthermore, ophthalmologists were consulted for assessment, characterization and follow-up for 9 of 19 patients with GO. All patients with newly diagnosed GD and were referred to our department during the period February-November 2017 were asked for participation in this study and all have agreed and are included in the study except 1 patient. Thus, there has not been any selection of patients to this study.

In conclusion, the data indicate that evaluation of thyroglobulin levels at diagnosis of GD could help identify patients at increased risk of developing GO and thereby impact the choice of therapy. Possibly, the thyroidal release of Tg in GD reflects a disturbance that also influences the retroorbital tissue in Graves' orbitopathy. Further studies with larger cohorts are warranted.

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Author contributions SK study design, data collection and analysis and wrote the manuscript. ML data collection and analysis, manuscript revision. PE data collection, manuscript revision. KA data collection, manuscript revision. ÖL study design, data analysis, manuscript revision. FAK study design, data analysis, manuscript revision. All authors contributed to the manuscript, have reviewed and approved the final version.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Ethical approval The study complies with the Declaration of Helsinki. It was approved by the Regional Ethics Committee in Uppsala (Dnr 2015/469).

Informed consent Written consent was obtained from each patient after full explanation of the purpose and nature of the study.

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Paper II

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Comparison Between Thyroid Stimulating Immunoglobulin and TSH-Receptor Antibodies in the Management of Graves' Orbitopathy

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ABSTRACT

Objectives TSH-receptor antibodies (TRAb) targeting the TSH receptor (TSH-R) induce hyperthyroidism in Graves´ disease (GD). Graves´ orbitopathy (GO) is influenced by stimulation of the TSH-R in the orbita. GO has been, among other factors, linked to high TRAb levels. Thyroid stimulating immunoglobulins (TSI) is a relatively new method for assessing TSH-receptor antibodies. The aim of this study was to investigate the role of TSI in the management of GO.

Methods Patients with newly diagnosed GD (n = 30, median age 55 years (range 35–72), 29 women) received pharmacological therapy (methimazole + thyroxine) for up to 24 months. GO was identified by clinical signs and symptoms. Eleven patients had GO at diagnosis, and another six developed GO during treatment. Blood samples for TSI and other thyroidal biomarkers were obtained at baseline and on five occasions during the 24-month follow-up. Twenty-two subjects completed the drug regimen without surgery or radioiodine treatment.

Results At baseline, TSI was highly correlated with TRAb ($r_s = 0.64$, $p < 0.001$), and both assays similarly correlated to fT3 values. TSI and TRAb did not differ significantly between GO and non-GO patients for visit v1 (n = 30, 17 GO during the whole study) or at follow-up (n = 22, 12 GO during the whole study). During follow-up, levels of TSI and TRAb decreased and normalized in both groups.

Conclusion The present study does not support any added benefit of TSI compared to TRAb for the prediction and management of GO.

Introduction

Graves´ disease (GD) is an autoimmune disease accompanied by the production of thyroid stimulating hormone (TSH)-receptor antibodies (TRAb) that bind to the TSH-receptor (TSH-R) and activate the cyclic adenosine monophosphate (cAMP) signal transduction pathway [1, 2]. TSH-receptor-stimulating immunoglobulin (TSI) is a subtype of TRAb that causes the stimulation of thyroid hormone production [3, 4]. Other types of antibodies block the action of TSH-R and TSH-R-binding inhibitory immunoglobulins, yet others are neutral without any functional effect [5, 6]. Graves´ orbitopa-

thy (GO) is clinically relevant in approximately 50 % of patients with GD, with severe forms affecting 3–5 % of patients with GD [7]. The risk of GO has been linked to TSH receptor stimulation [8–10], high TRAb levels [11], smoking [12], low levels of thyroid peroxidase antibodies [13–17], and thyroglobulin antibodies [14, 17]. Measurement of TRAb is a standard method for confirmation of diagnosis, monitoring of therapy, and prediction of remission or relapse in patients with GD [18–21]. A potential pitfall of using TRAb is that the method will also measure blocking and neutral antibodies, if present.

At present, there are two methods for analyzing antibodies against TSH-R. The third-generation thyrotropin-binding inhibitor immunoglobulin (TBII) assay and the functional thyroid-stimulating immunoglobulin bioassay. The TBII assay uses the ability of TRAb to inhibit the binding of radiolabeled TSH to TSH receptors. In contrast, the bioassay measures cAMP production after TSH-R antibodies bind to the TSH receptor. This method makes it possible to identify the functional properties of TSH-R antibodies.

The IMMULITE 2000/2000 XPi TSI assay uses recombinant thyroid stimulating hormone receptor (TSHR) chimeras constructed from the N-terminal domain of the TSHR, which contains the TSI epitopes, and the luteinizing hormone (LH) receptor from which the blocking epitopes are absent and used as capture antigens. This assay is suggested to be more specific for activating antibodies that cause hyperthyroidism and is less influenced by blocking antibodies [22]. However, there is a lack of clinical studies that demonstrate a clear benefit of using TSI versus TRAb.

Tozzoli et al. examined a cohort of 72 patients with untreated GD. The diagnostic performance of the Immulite TSI assay in GD patients was comparable to that of current TRAb assays [23]. In a study including 166 patients with GD, the TSI method showed perfect sensitivity according to the clinical diagnoses of GD [24].

GO is caused by the stimulation of TSH-R in the orbita [9]. Severe forms of GO may cause irreversible eye complications and, in some severe cases, can even threaten vision [25]. A delay in the diagnosis is associated with a worse prognosis of GO. Therefore, there is an increased need for a scoring system that includes different markers for the prediction and assessment of GO.

In a retrospective study from Korea, a total of 112 patients were investigated whether serum TSH-R antibodies in newly diagnosed, untreated GO patients were predictive of disease course beyond 1 year after initial GO diagnosis. The predictive power of the third-generation TBII assay and Thyretain TSI Stimulating Reporter BioAssay were similarly strong [26].

In a recent prospective study, we investigated a cohort of 30 patients with newly diagnosed GD and presented data that may suggest that evaluation of thyroglobulin levels in GD could contribute to identifying patients at increased risk of developing GO [27]. The TRAb levels were not significantly correlated to GO in that study. To investigate whether TSI is a more sensitive biomarker than TRAb for the evaluation of GO, we have now analyzed TSI with the IMMULITE 2000 Systems Analyzers using stored blood samples from the same cohort of 30 patients that participated in the previously reported study.

Materials and Methods

Study subjects

Thirty patients with *de novo* GD were recruited at the Uppsala University Hospital. GD diagnosis was based on decreased levels of TSH and sero-positivity for TRAbs. In one patient, the TRAb levels were below the reference range (1.7 IE/L, reference < 1.75). This patient otherwise had symptoms, laboratory findings, and homogenously increased uptake on thyroid scintigraphy in line with GD. The median age of the study cohort was 55 years (range 35–72 years). Two were smokers and 29 were women.

The study consisted of six visits: at baseline, 6 weeks, 12 weeks, 6 months, 12 months, and 24 months from diagnosis. At the first visit (baseline), all patients underwent an examination, including a recording of demographic characteristics (sex and age) and medical and family history. At each visit, blood was sampled to measure TSH, fT4, fT3, TRAb, and TSI.

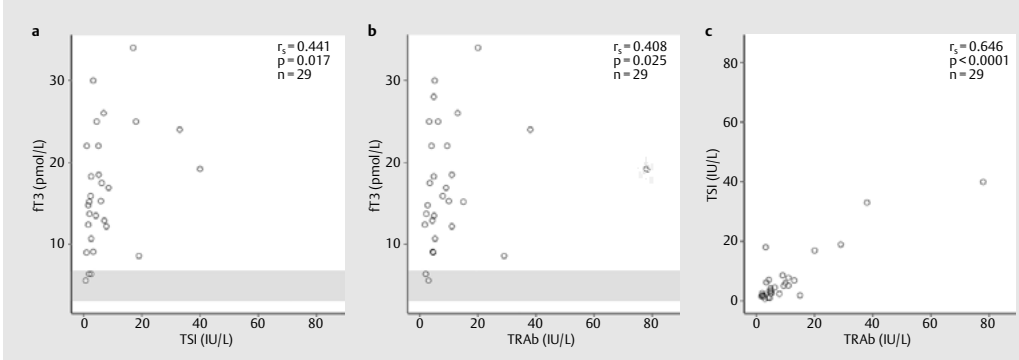
All 30 patients received the conventional block and replace treatment (tiamazol and levothyroxine). Three patients underwent total thyroidectomy, and five received radioiodine treatment during the study period. These patients were not included in the longitudinal analyses described below. Out of the remaining 22 subjects, 21 received antithyroid drug (ATD) for 18–24 months until negative TRAb. One patient had a spontaneous recovery before treatment initiation but was included in the longitudinal analyses. GO was identified by clinical signs and symptoms, and the severity of GO was assessed according to The European Group on Graves´ Orbitopathy guidelines [28]. All patients were examined by a doctor and a nurse at each visit, and the patients received clear information about eye symptoms. GO was classified as 'mild' with symptoms such as gritty sensation and tearing due to dry eyes, caruncle swelling and/or redness, upper eyelid retraction, or as 'moderate to severe' in the instance of redness and/or swelling of the eyelids, chemosis, pressure or pain in the eyes, exophthalmos, diplopia or signs of optic nerve compression. The eye signs and symptoms were detailed in the medical records by the attending endocrinologist as well as by the research nurse at every visit [27].

Assays

Plasma TSH (reference interval 0.4–4.0 mIU/L), free T4 (reference interval 12–22 pmol/L), free T3 (reference interval 3.1–6.8 pmol/L), TRAb (reference < 1.75 IE/L), were all measured on Cobas immunoanalyzers (e 801, Roche Diagnostics, Rotkreuz, Switzerland) at the Department of Clinical Chemistry of the Uppsala University Hospital. The laboratory is accredited according to SS-EN ISO 15189:2012 by Swedac (Borås, Sweden). The total coefficient of variation (CV) for the methods was TSH: 5 % at 0.1 mIU/L, free T4: 4 % at 16 pmol/L, free T3: 4 % at 15 pmol/L, Tg: 4 % at 23 µg/L, and TRAb: 6 % at 17.4 IE/L. TSI assay was conducted on the IMMULITE 2000 and IMMULITE 2000 XPi Immunoassay Systems (Siemens Healthineers, Erlangen, Germany). The total CV was 5 % at 0.7 IU/L. All laboratory testing was performed blinded to clinical information.

Statistical analysis

All statistical analyses were performed using R 4.0.2. Data are presented as median (range) unless otherwise indicated. *P* values < 0.05 were considered significant. Spearman's Rank correla-



▶ **Fig. 1** Correlation between fT3 vs. TSI (**a**), fT3 vs. TRAb (**b**), and TSI vs. TRAb (**c**) at baseline. Missing TSI and TRAb value for one subject. r_s = Spearman's Rho coefficient. Grey areas in A and B represent the normal range of fT3. TSI: thyroid stimulating immunoglobulins; TRAb: TSH-receptor antibodies.

tions between TSI, TRAb, and fT3 were performed at baseline and every follow-up visit. At v1, TSI and TRAb values for GO (n = 17) and non-GO (n = 13) patients were tested for the significant difference using the Mann-Whitney-U test.

For all subjects who received ATD and did not undergo surgery or radioiodine treatment (*n* = 22), the area under the curves (AUC) of TSI and TRAb after treatment (v2–6) were calculated with the trapezoid method and compared between GO (n = 12) and non-GO (n = 10) with Mann-Whitney-U tests.

Results

For all subjects, fT3 but not fT4 correlated to both TSI (r_s = 0.44, p = 0.017, ▶ **Fig. 1a**) and TRAb (r_s = 0.41 p = 0.025; ▶ **Fig. 1b**) at baseline (n = 30). During the remainder of the visits (n = 22), no significant correlations were observed between fT3 and TSI or TRAb (data not shown), whereas fT4 correlated to TRAb at v6 only (r_s = 0.53, p = 0.012) and did not correlate to TSI at any visit. TSI was highly correlated with TRAb at baseline (r_s = 0.64, p < 0.001; ▶ **Fig 1c**) and across all visits and treatments (r_s = 0.60, p < 0.001 for all visits, data not shown). There were no correlations between the levels of TSI or TRAb in relation to the Tg levels (data not shown).

In total, 17 patients were diagnosed with GO at baseline (n = 11) or during the study follow-up period (n = 6). TSI and TRAb did not differ significantly between GO and non-GO patients at baseline (GO:non-GO = 17:13), while fT3 and fT4 were higher in the GO group than the non-GO group at baseline (▶ **Table 1**). In the ATD group (n = 22), TSI levels appeared to be higher in GO (n = 12) vs. non-GO (n = 10) during the first three visits, but this difference was not significant when comparing the AUC during the whole follow-up period (p = 0.49) (▶ **Fig. 2a**), and TRAb levels (▶ **Fig. 2b**) were similarly reduced and normalized in both groups.

▶ **Table 1** TSI, TRAb, fT3, and fT4 at baseline presented as median and range in GO (during the whole study) vs non-GO, compared using Mann-Whitney-*U*-test.

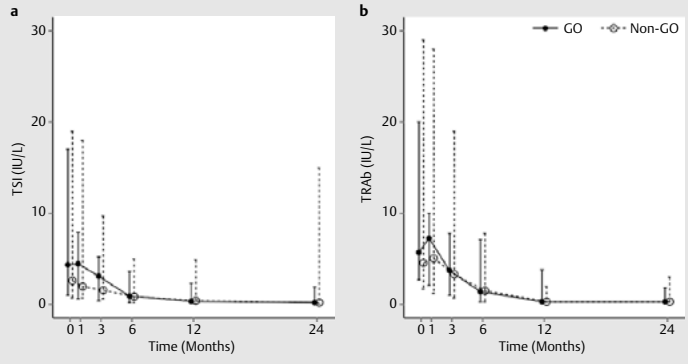
	GO (n = 17)	Non-GO (n = 13)	*p*-value
TSI	4.5 (1–40)	2.6 (0.7–19)	0.535
TRAb	6.3 (2.7–78)	4.3 (1.7–29)	0.053
fT3	18.3 (9–34)	12.4 (5.6–28)	0.026
fT4	48 (25–100)	35 (17.6–60)	0.008

GO: Graves´ orbitopathy; TSI: thyroid stimulating immunoglobulins; TRAb: TSH-receptor antibodies; fT3: Free triiodothyronine; fT4: free thyroxine.

Discussion

GO is a common complication that is clinically relevant in about 50 % of patients with GD. The symptoms are often mild, but severe forms of GO occur in 3–5 % of patients [7]. GO may begin early in the course of the disease, but it is not uncommon that GO occurs later, during the follow-up period, despite ongoing treatment with ATD [9]. There are difficulties in identifying patients with a risk of developing GO and those who will develop severe forms of eye complications. There is, therefore, a need for better markers that can be useful for the management of GO. Previous studies have suggested the benefits of using TSH receptor-stimulating antibodies for the management of GO [29]. In this study, we made a comparison between TSI and TRAb in the management of GO but could not detect any superiority of TSI compared to TRAb in the prediction of GO.

TSH-R is well-documented to be expressed in the orbita [30]. Stimulation of TSH-R in the orbita leads to stimulation of fibroblasts resulting in fibroblast proliferation, an increase in hyaluronic acid production, and an increased inflammatory response [9]. Higher levels of TRAb have been associated with an increase in the prevalence of GO [13], which we also noted in the present study. A Swedish report found that a TRAb level of 6.3 IU/L at diagnosis of GD was associated with an elevated risk of developing GO [16]. However,



▶ **Fig. 2** TSI and TRAb levels presented as median and range in all visits for the ATD group (n = 22) divided into GO (n = 12) and non-GO (n = 10), showing a higher but not significant course for TSI during the first 3 months (**a**) in GO vs. non-GO group, whereas TRAb levels (**b**) were similarly reduced a ATD: antithyroid drug; GO: Graves´ orbitopathy; TSI: thyroid stimulating immunoglobulins; TRAb: TSH-receptor antibodies.

current standard methods, which are widely routinely used, measure all antibodies, i. e., if present, also blocking and neutralizing antibodies. Techniques that measure only the stimulating antibodies would possibly increase the precision of the diagnosis and follow-up of patients with GD, including those suffering from eye complications. Currently, only bioassays appear to be able to detect purely stimulating antibodies. Thyretain by Quidel is one such method that uses engineered CHO cells with chimeric TSH-R (MC4) to measure luciferase gene expression due to activation of the cAMP signal transduction pathway [5]. Lytton et al. showed that the clinical sensitivity and specificity of the Mc4/TSI were greater than TRAb in GO [5]. An interesting new technique, "aequorin TSAb assay," has been recently developed [31]; it uses a cell line CHO transfected with human TSH receptor, cyclic nucleotide-directed calcium channel, and aequorin. These bioassays appear to be precise and might add to the prediction of the course of disease and risk of GO, but it is unlikely that they will be developed into clinical routine. However, the TBII assay has satisfactory clinical sensitivity and specificity (97.4 % and 99.2 %, respectively). In this setting, the bioassays should be reserved for fine and complex diagnoses and for particular clinical conditions [32, 33].

The IMMULITE 2000 TSI assay by Siemens is a bridge-binding immunoassay by which TSH-R antibodies bind to a pair of recombinant TSH-R constructs; one is a capture, and the other is signal receptor for measuring secreted alkaline phosphatase. This method is supposed to provide a more sensitive measurement of stimulating antibodies compared to the routinely used competitive-binding assay. In this prospective study of patients with newly diagnosed GD, we could, however, not show any significant difference between TSI and TRAb, neither among GD patients at diagnosis or follow-up nor between those with and without GO. The correlation between fT3 and TSI and fT3 and TRAb in this study is statistically significant but with low coefficients. This may be related to the bioactivity of the antibodies and their effect on the degree of hyperthyroidism. Typically, high antibody titers lead to more hyperthyroidism, but pronounced hyperthyroidism may also occur at lower antibody levels and vice versa. The correlation between TSI and TRAb at baseline and in the subsequent visits was good throughout, indicating that TSI is not more specific than TRAb in assessing the severity of hyperthyroidism.

Our results are in line with a newly published study from Australia [34], in which 140 participants were recruited, of which 75 (53.6 %) had GO. Although Immulite TSI level was higher in the presence of GO, it showed poor diagnostic accuracy and no correlation with clinical markers of GO severity or activity. To our knowledge, only one more study has been reported to investigate TSI in GO [35]. In that study, TSH-R antibodies were measured with Immulite TSI assay and with Elecsys IMA in patients with moderate to severe GO. Both methods showed a comparable correlation with GO activity during ivGCs therapy. The data from our prospective study confirm and add to the findings from these studies showing similar performance of the two assays in clinical routine.

The weakness of our study is the relatively small sample size. However, the prospective design with continuous follow-up of the clinical and laboratory status for two years gives the present study sufficient statistical strength to rule out significant differences regarding the parameters we have measured.

In conclusion, TSI is highly correlated with TRAb in patients with GD. The tests did not differ significantly between GO and non-GO patients before the treatment of GD. After treatment, both TSI and TRAb levels were reduced and normalized in both groups. The present study does not support any added benefit of TSI compared to TRAb for the prediction and management of GO.

Patient Consent

Consent was obtained from each patient after a full explanation of the purpose and nature of all procedures used.

Ethical Approval

The study complied with the Declaration of Helsinki and was approved by the Regional Ethics Committee in Uppsala (Dnr 2015/469).

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection, and analysis were performed by SK, ML, and ÖL. The first draft of the manuscript was written by SK and ÖL, and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Conflicts of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Paper III

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ORIGINAL ARTICLE

Vitamin D and bone metabolism in Graves' disease: a prospective study

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Abstract

Purpose Vitamin D and osteoporosis in Graves' disease (GD) have been examined in cross-sectional studies with divergent results. Here, we prospectively studied vitamin D metabolism and bone health in patients with newly diagnosed GD.

Methods Thirty consecutive patients with de novo overt thyrotoxicosis diagnosed with GD were included. At diagnosis, none of the patients were treated with vitamin D or anti-osteoporotic drugs. All patients were initially treated with antithyroid drugs. Blood samplings were taken at baseline and at 6 weeks, 3, 6, 12 and 24 months after treatment start. Serum levels of 25OHD3, 1,25OH2D3, calcium, parathyroid hormone (PTH), and C-terminal telopeptides of Type I collagen (CTX-I) were analysed. Bone mineral density (BMD) was measured at baseline, and 1 and 2 years after treatment initiation.

Results At diagnosis, patients with GD did not have vitamin D deficiency. There were no significant correlations between levels of 25OHD3 and thyrotoxicosis. Upon treatment of the thyrotoxicosis, serum calcium fell transiently, and PTH and 1,25OH2D3 increased. 25OHD3 fell within the normal range and stabilised at 6 months. CTX-I fell over 12 months, BMD increased significantly up to 2 years, $p = 0.002$, < 0.001 and 0.005 in the spine, left total hip and left femoral neck, respectively.

Conclusions The present data underline that thyrotoxicosis has a negative impact on bone health and demonstrate fine-tuned dynamics in bone and vitamin D metabolism. Upon treatment, bone health improved over a follow-up period of 24 months despite rising PTH. Increased conversion of 25OHD3 to 1,25OH2D3 occurs during treatment of GD.

Keywords Thyrotoxicosis · 25-Hydroxyvitamin D3 (25OHD3) · 1,25-Dihydroxyvitamin D3 (1,25OH2D3) · Vitamin D binding protein · Osteoporosis

Introduction

Graves' disease (GD) is an autoimmune form of hyperthyroidism with an annual incidence of 21 in 100,000 individuals [1]. There is a genetic background that makes the disease associated with other autoimmune diseases and is more common in women, but the cause of the disease is unknown [2–4]. Hyperthyroidism often results in secondary osteoporosis by acceleration of bone resorption, causing loss of bone mineral density [5–9]. Even subclinical hyperthyroidism is a risk factor for osteoporosis [10]. It is generally believed that T3 stimulates osteoclastic activity and that an elevated thyroid hormone level is the primary cause of bone loss [11]. However, bone remodelling is influenced by many factors, such as calcium homeostasis, parathyroid hormone (PTH), vitamin D status and sex hormones. All of these are potential factors that might be influenced by thyrotoxicosis, making the exact mechanism causing bone loss in GD unknown.

Epidemiological studies have reported associations between vitamin D (25OHD3) deficiency and the risk of developing a variety of diseases, such as cardiovascular diseases, cancer, diabetes and infectious diseases [12–19]. In addition, in autoimmune diseases, such as multiple sclerosis, vitamin D deficiency has been reported as a risk factor [20–23]. With GD, the literature is not conclusive regarding vitamin D deficiency as a risk factor. In a study with thirty hyperthyroid patients in India, 26% of the study subjects had vitamin D deficiency, i.e., levels of 25OHD3 less than 25 nmol/L [24]. Yamashita et al. have also reported vitamin D deficiency in 40% of women and in 18% of men having GD from Japan [25]. In another study from Japan, Doi

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et al. showed that 35% of patients with thyrotoxicosis had vitamin D deficiency [26]. Other studies have also shown a significant impact of 25OHD3 deficiency on bone structure and bone metabolism [27–29]. However, in a Swedish study, patients with GD had lower 25OHD3 levels compared to the general population (55.0 ± 23.2 vs 87.2 ± 27.6 nmol/L). However, vitamin D levels were not associated with laboratory or clinical parameters of GD [30]. All studies of vitamin D and GD mentioned above were cross-sectional and focused only on 25OHD3. Levels of the active metabolite, 1,25OH2D3, in GD have not been described.

In the present study, we have prospectively monitored vitamin D metabolism and calcium homeostasis in patients with newly diagnosed GD, taking into consideration the severity of thyrotoxicosis and changes in bone mineral density.

Materials and methods

Study subjects

Patients with de novo GD, diagnosed by decreased levels of thyroid stimulating hormone (TSH) and positive autoimmune antibodies against TSH-receptors (TRAb), were recruited at the Uppsala University Hospital from February through November 2017. Inclusion criteria: males and females, aged 30–80 years, with newly diagnosed GD. Exclusion criteria**:** pregnant or planning to be pregnant during the study, secondary osteoporosis, corticosteroids treatment in the last 2 years as well as vitamin D and/or calcium supplementation. TRAb was below the reference range (1.7 IE/L, reference < 1.75) in one patient. This patient otherwise had laboratory findings and symptoms typical of GD, with a high and homogeneous uptake at scintigraphy and was included in the study. Two patients started with vitamin D supplementation without prescription after the baseline visit. One patient had osteoporosis at time of diagnosis and started treatment with bisphosphonate and calcium with vitamin D. These three patients were excluded from the follow-up analyses.

The study comprised six visits. During the first visit (baseline), all patients underwent an examination, including recording of demographic characteristics (sex and age), medical history, family history of fracture, concomitant medication, weight and height, and a physical examination. Blood samplings were taken to measure TSH, freeT4, freeT3, TRAb, CTX-I, 25OHD3, calcium, phosphate and PTH at baseline and at 6 weeks, 3, 6, 12 and 24 months after diagnosis and treatment start. We also measured 1.25OH2D3 at the first four visits. Vitamin D-binding protein (DBP) was measured at visits 1, 5 and 6, and sexual hormone binding globulin (SHBG) was measured at visits 1 and 6. During visit 1, the patients completed a questionnaire regarding risk factors for osteoporosis. Dual-energy X-ray absorptiometry (DXA) was performed on the hip and lumbar spine within 2 weeks after visit 1 and then 1 and 2 years after study inclusion. All 30 patients included in the study received conventional block and replace treatment. Anti-thyroid drugs, methimazole or propylthiouracil, were initiated in conjunction with visit 1, started with 10–20 mg methimazole doses or 150–300 mg propylthiouracil daily. Thyroxine 50–100 µg was added daily when thyroid hormone levels fell into the normal range, and TSH levels were kept in the low normal range. During follow-up, four patients received radioiodine (RAI) because of persistently elevated TRAb after 10–15 month treatment. One patient received RAI after 5 months at the request of the patient. Three patients underwent total thyroidectomy, two patients due to neutropenia, which developed directly after the start of methimazole in one case and after 10 months on methimazole in the other. One female patient underwent surgery after 5 months because of desire to become pregnant. Out of the remaining 22 subjects, 21 patients received ATD for 18–24 months until negative TRAb. One patient had a spontaneous recovery before treatment initiation.

Graves` orbitopathy (GO) was defined as signs and eye symptoms related to GD and classified as 'mild' GO with symptoms, such as gritty sensation and tearing due to dry eyes, caruncle swelling and/or redness, upper eyelid retraction, or as 'moderate to severe' in the instance of redness and/or swelling of the eyelids, pressure or pain in the eyes, exophthalmos, chemosis, diplopia or signs of optic nerve compression. The eye signs and symptoms were detailed in the medical records by the attending endocrinologist as well as by the nurse at all visits.

Assays

Plasma TSH (reference interval 0.4–4.0 mIU/L), free T4 (reference interval 12–22 pmol/L), free T3 (reference interval 3.1–6.8 pmol/L), TRAb (reference < 1.75 IE/L), and CrossLaps® (CTX-I) was analyzed using Cobas® pro instruments and reagents (Roche Diagnostics, Rotkreuz, Switzerland). An ELISA was used for the quantification of C-terminal telopeptides degradation products of Type I collagen (reference pre-menopause < 580 ng/L, post-menopause < 1000 ng/L), according to manufacturer (BioVendor). Plasma calcium (reference 2.15–2.5 mmol/L); serum PTH (reference interval 1.6–6.9 pmol/L); serum 25OHD3 (deficiency < 25 nmol/L and insufficiency < 50 nmol/L); serum 1,25OH2D3 (reference interval 60–210 pmol/L); plasma phosphate (reference interval 0.8–1.5 mmol/L); SHBG levels (reference interval for women 16–50 years: 26–110 nmol/L, > 50 years: 14–70 nmol/L and for men 14–48 nmol/L) were all measured per routine methods used at the Department of Clinical Chemistry at the Uppsala

University Hospital. Vitamin D binding protein was measured by colorimetric sandwich-ELISA (Quantikine ELISA Human Vitamin D BP Immunoassay, catalogue number DVDBP0B; Lot P252667, R&D Systems).

Statistical analysis

Data are presented as median (range) unless otherwise indicated. Changes in plasma levels of bone turnover markers and bone mineral density (BMD) were analysed using repeated-measures ANOVA with Greenhouse–Geisser correction. We performed post-hoc paired *t* tests compared with visit 1 using Bonferroni-adjusted alpha levels. For each analysis, normal distribution was assessed by Shapiro–Wilkes test at each timepoint. If the data were not normally distributed, they were log transformed (base 10), after which the assessment was reiterated. No extreme outliers were found after visual inspection of the data and the residuals. Due to violation of normality even after log transformation, changes in free T3 and DBP were analysed using Friedman's test with post-hoc Bonferroni-adjusted Wilcoxon signed rank tests. For the same reason, changes in SHBG levels were analysed using Wilcoxon signed rank test. P values < the Bonferroni-adjusted alpha level (0.05/n, where n is number of hypotheses tested) were considered significant. SPSS version 27 (IBM Corp, Armonk, NY, USA) was used for the analyses.

Table 1 Patient characteristics and concomitant medications at diagnosis

	N
Gender	29 females, 1 male
Age	55.5 (30–89) median and range
Menopause (> 52 years)	19
Smoking	2
Steroids	0
Estrogen	0
Biphosphonates	0
Vitamin D supplementation	0

Results

Thirty subjects with overt hyperthyroidism without vitamin D supplementation or treatment against osteoporosis at diagnosis of GD were included in the study. Median age was 55 years (range 35–72 years), all but one was of Scandinavian origin, two were smokers, 29 were women. The baseline data present all 30 included patients (Table 1). Twenty-seven patients were included in the follow-up analyses after exclusion of those who started with vitamin D supplementation after baseline. Menopause was estimated as being at age 52 or above; no FSH data were available.

25OHD3 and 1,25OH2D3 at baseline and during treatment

At baseline, levels of 25OHD3 were 66.8 (37.3–130) nmol/L (Table 2); moreover, four patients had values below 50 nmol/L (Fig. 1a). There were no correlations between 25OHD3 and free T3 values (Fig. 1a), nor to TSH, TRAb or free T4 (data not shown). During treatment, values of free T3 became normalised, and the levels of 25OHD3 declined significantly (Figs. 1b, 2e, Table 2). At baseline, levels of 1,25OH2D3 were 68 (17–155) pmol/L (Table 2); moreover, 14 patients had 1,25OH2D3 levels below the lower reference range (Fig. 1c). During treatment, there was a significant increase in the levels of 1,25OH2D3 (Figs. 1d, 2f, Table 2). There was a negative correlation between 1,25OH2D3 and free T4 at visit 4 (Rs − 0.394, $p = 0.042$). Otherwise, there

Table 2 Thyroid hormone, Trab, Crosslaps, calcium, PTH, 25OHD3, 1,25OH2D3 and phosphate, levels (median and range) at diagnosis of and follow-up during 24 months of 30 patients with de novo GD

	Baseline	6 w	3 m	6 m	12 m	24 m
S-TSH	0.005 (0.005–0.04)	0.005 (0.005–0.911)	0.01 (0.005–5.68)	0.76 (0.005–10.4)	0.91 (0.005–4.67)	1.26 (0.07–4.47)
S-fT4	41 (17.6–100)	23 (13.9–49)	19.4 (11.2–33)	17.4 (11–31)	18.4 (12.8–30)	16.7 (10–27)
S-fT3	15.6 (5.6–34)	6.2 (3–12.1)	4.6 (3.4–12.6)	3.9 (3–5.6)	4.2 (3.3–6.1)	4.5 (3–6.9)
S-TRAb	5 (1.7–78)	5.8 (1.2–83)	3.8 (0.3–60)	1.5 (0.3–39)	0.4 (0.3–38)	0.3 (0.3–8.3)
S-Crosslaps	644.5 (161–1829)	542 (253–1566)	508 (147–2052)	477 (141–1748)	302 (87.5–970)	280 (97.1–895)
P-calcium	2.4 (2.3–2.6)	2.34 (2.1–2.5)	2.38 (2.2–2.5)	2.4 (2.1–2.5)	2.4 (2.2–2.6)	2.39 (2.2–2.6)
P-PTH	3.4 (2–6.1)	4 (2.5–9.7)	4.85 (2.4–8.1)	4.4 (2.2–10)	4.9 (2.4–9.2)	4.8 (2.4–10.6)
S-25OHD3	66.8 (37.3–130)	60.8 (30.2–108)	60.3 (27.4–102)	52 (28.8–90.1)	52.2 (28.2–93.8)	59.4 (29.1–88.8)
S-1,25OH2D3	68 (17–155)	104 (22–224)	117 (57–236)	114 (72–221)		
P-Phosphate	1.1 (0.79–1.63)	1.0 (0.61–1.36)	1.1 (0.77–1.36)	1.1 (0.75–1.46)	1.1 (0.77–1.33)	1.0 (0.73–1.33)

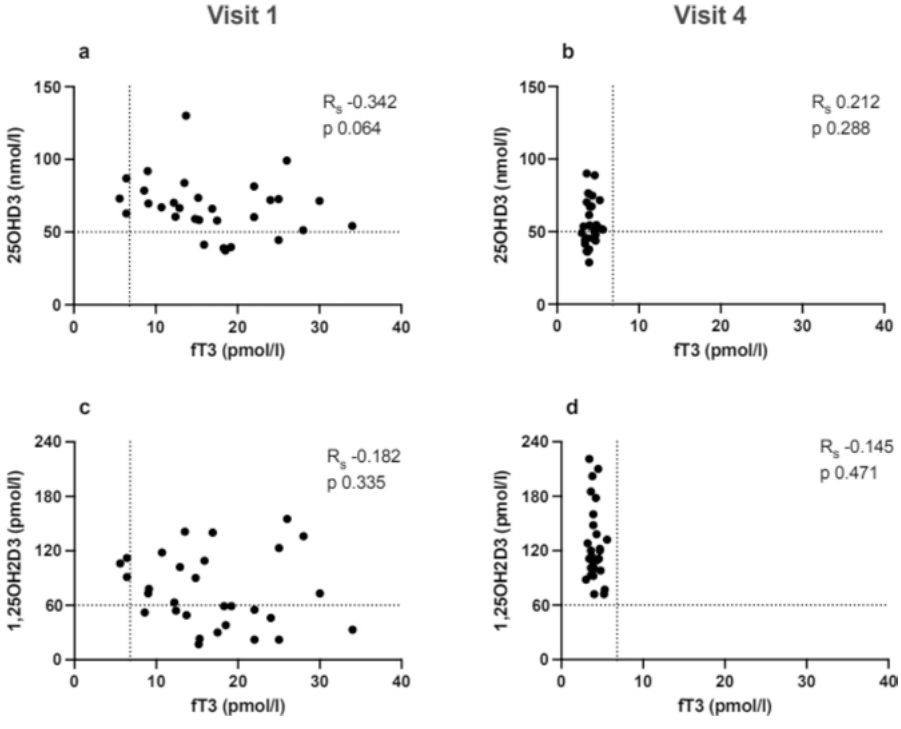


Fig. 1 25OHD3 and 1,25OH2D3 vs free-T3 at visit 1 ($n=30$) (**a**, **c**) and 4 ($n=27$) (**b**, **d**). Vertical dashed line represents 6.8 pmol/l, the upper reference limit for free-T3 with the laboratory method that was utilised. R_s = Spearman Coefficient. Median and range for 25OHD3 were 66.8 (37.3–130) and 52.8 (28.8–90.1) at visit 1 and visit 4, respectively. Median and range for 1,25OH2D3 were 68 (17–155) and 119.5 (72–221) at visit 1 and visit 4, respectively

were no correlations between 1,25OH2D3 and free T3, TSH, TRAb or free T4 (data not shown).

Vitamin D binding protein and SHBG

During treatment, the levels of DBP did not change ($p=0.77$), whereas SHBG declined from 172 (65–634) at baseline to 69.5 (14–299) at visit 6 ($p<0.001$) (data not shown).

Measures of calcium homeostasis

During the treatment, the levels of 25OHD3 significantly declined, whereas the levels of 1,25OH2D3 increased (Fig. 2e, f, Table 2). There was an initial and transient decrease in s-Ca and an increase in PTH (Fig. 2d, Table 2), and there was also a decrease in s-phosphate from 1.13 (0.79–1.63) at baseline to 1.0 (0.73–1.33) at visit 6 (Table 2). Regarding marker of bone resorption, there was a marked decrease in CTX-I (Fig. 2b, Table 2).

When calculating calcium values regarding fT4 at baseline using Spearman's Rho the value of $r_s=0.20257$, p (2-tailed) = 0.28. Although a positive trend but the relationship between the variables is not significant.

Bone mineral density

Bone mineral density was analysed by DXA at baseline and after 12 and 24 months, respectively. At baseline, the mean levels of BMD were 1.12 (± 0.17) g/cm^2 in the spine, 0.95 (± 0.14) g/cm^2 in the left total hip and 0.91 (± 0.13) g/cm^2 in the left femoral neck (Fig. 3). The mean values regarding z scores were: 0.13 (± 1.3), 0.05 (± 1.07) and 0.006 (± 0.7) and T-score − 0.48 (± 1.4), − 0.5 (± 1.17) and − 0.5 (± 1.17), respectively (data not shown). There was only one patient with a T-score lower than − 2.5 in the spine.

Two years after treatment, the mean BMD increased significantly at all locations, in the spine 5.3% (± 7.5) ($p = 0.002$), in the left total hip 7.5% (± 7.9) ($p < 0.001$) and in the left femoral neck 4.4% (± 7.0) ($p = 0.005$) (Fig. 3). Explorative exclusion of the only male subject did not alter these results.

Vitamin D and risk for GO

During the study 17 patients developed GO. Three patients with moderate to severe orbitopathy received steroids, two were treated with oral steroids and one with iv steroids. At baseline, there was no significant difference regarding the levels of 25OHD3 during the trial and risk of GO, 66.1 (37.3–99.2) and 67.1 (41.3–130) for GO and non-GO group, respectively ($p = 0.5$). Nor was there any difference of 1,25OH2D3, 59 (17–155) and 102 (22–136), respectively ($p = 0.17$).

Discussion

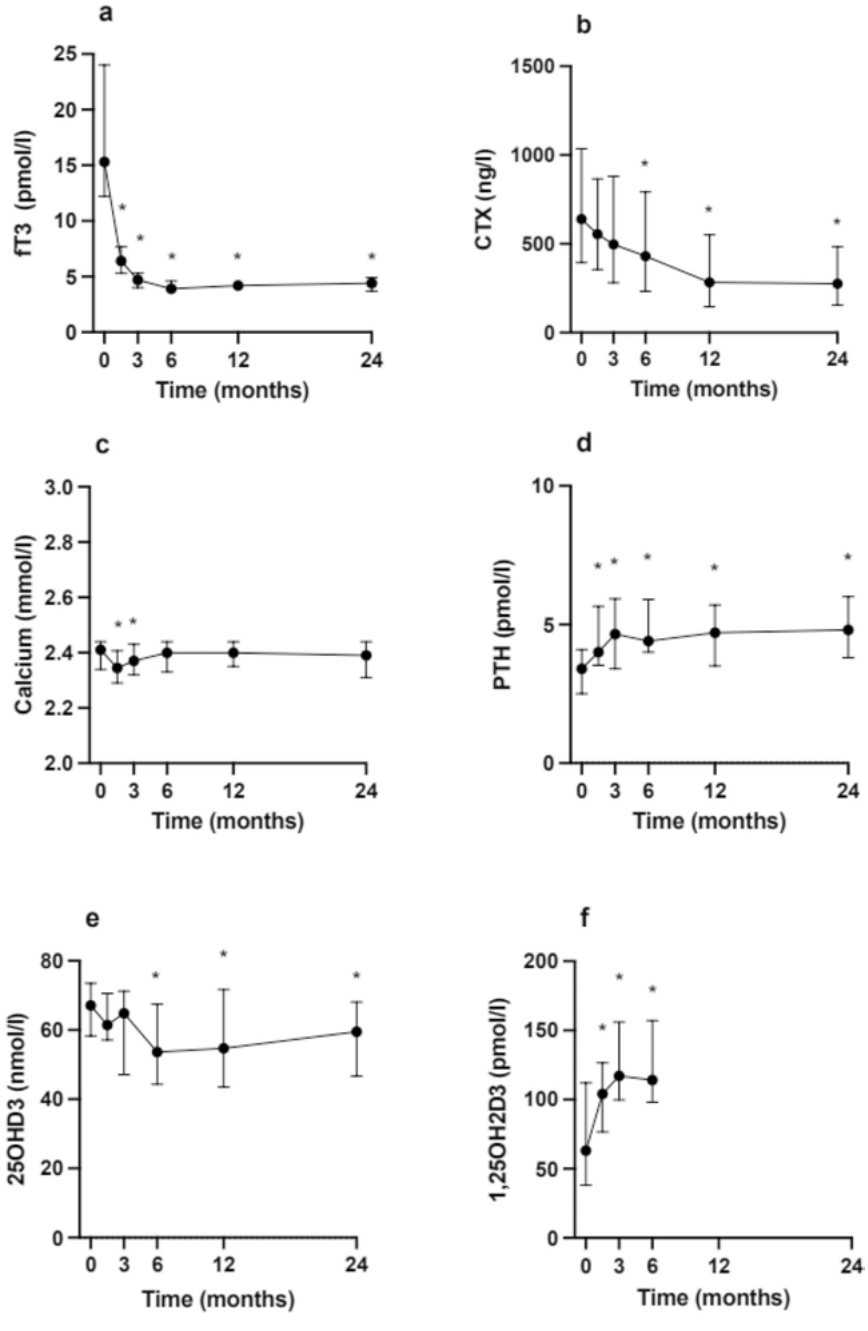
In this prospective study on vitamin D, bone turnover and BMD in GD, there was no vitamin D deficiency at diagnosis. Furthermore, we could not detect any differences regarding the levels of 25OHD3 or 1.25OH2D3 at baseline and risk of developing GO. These data indicate that vitamin D deficiency is not associated with risk for developing GD or subsequent eye complications. GD had a significant impact on bone turnover and BMD, which was reversible after treatment of thyrotoxicosis. The data concerning the biochemical marker of bone metabolism CTX-I suggest that there should be a marked effect on bone density. This was demonstrated in this study.

Many studies have reported a correlation between vitamin D deficiency and different autoimmune diseases, including thyroid autoimmune diseases [31]. Vitamin D supplementation did not markedly reduce the recurrence of Graves' disease, according to a newly published study [32]. In our study, 25OHD3 was significantly lower at follow-up than before treatment start with ATD. The data indicate that vitamin D deficiency is not prevalent in GD, nor are there any correlations between levels of vitamin D and levels of thyroid hormones. Regarding the correlation between free T4 and calcium at baseline, although a positive trend, the relationship between the variables was not significant. We believe that this might be related to the limited sample size. We found a negative correlation with free T4 at visit 4. However, this correlation was not very strong and especially when considering the multiple tests that were performed without formal correction could very well represent chance findings. We observed, however, a hitherto unshown shift in vitamin D metabolism during treatment, in that the levels of 25OHD3 decreased and the levels of 1,25OH2D3 increased. The levels of DBP did not differ from the levels at baseline, suggesting that the observed shift in 25OHD3 levels was unrelated to levels of vitamin D binding protein. Taken together, these data suggest that the shift in vitamin D metabolism was not related to GD but to the treatment, causing a decline in thyroid hormones. The reduction in thyroid hormones, thereby decreasing bone resorption and causing an increase in PTH, which, in turn, stimulates the conversion of 25OHD3 into its active form 1,25OH2D3, explains the lower levels of 25OHD3 after start of treatment. To be considered the half-life of 25OHD3 is 15 days [33], while the half-life of circulating 1,25OH2D is only 4–6 h [34].

As expected, BMD gradually increased after starting treatment, most prominent in the spine and total hip. These data are in line with the decreased CTX-I levels and suggest that the influence of thyrotoxicosis on bone density might be of a larger magnitude than previously believed, especially since the significant increase in BMD occurs despite an increase in PTH. The data on BMD in this study are congruent with a study from Australia, in which 15 female patients with active GD underwent BMD analysis by Dual-energy X-ray absorptiometry (DXA) at baseline and after 12 months with ATD. The bone mineral density increased significantly in the lumbar spine and femoral neck [35]. Two more studies [36, 37] have shown similar results.

In this study, we observe an increase in BMD concomitant with declining levels of 25OHD3. There are, however, results from randomised clinical studies, finding that calcium and vitamin D can be used in the treatment of osteoporosis [38]. Furthermore, patients with GD and low levels of 25OHD3 had the greatest improvement in the bone microarchitecture following treatment of hyperthyroidism [39]. Thus, the exact role of 25OHD3 as a predictor or therapeutic agent in osteoporosis, secondary to GD, needs further investigation. Data from this study imply that the increased conversion of 25OHD3 to 1,25OH2D3 might be a more relevant factor than the actual serum levels of 25OHD3.

Regarding the levels of vitamin D, the inclusion of patients was from February through November and blood test collected six times during the follow-up period for 2 years. The vitamin D levels in this study were similar to those measured in a cohort of 870 healthy Uppsala residents (52% females) [40] and higher than those reported in a study of 102 patients in the primary care [41]. We, therefore, believe that our cohort in this study does not deviate from the rest of the population regarding vitamin D levels.


a
fT3 (pmol/l)
25
20
15
10
5
0
0 3 6 12 24
Time (months)
b
CTX (ng/l)
1500
1000
500
0
0 3 6 12 24
Time (months)
c
Calcium (mmol/l)
3.0
2.8
2.6
2.4
2.2
2.0
0 3 6 12 24
Time (months)
d
PTH (pmol/l)
10
5
0
0 3 6 12 24
Time (months)
e
25OHD3 (nmol/l)
80
60
40
20
0
0 3 6 12 24
Time (months)
f
1,25OH2D3 (pmol/l)
200
150
100
50
0
0 3 6 12 24
Time (months)

◂**Fig. 2** Levels of free-T3 (**a**) CTX (**b**), calcium (**c**), PTH (**d**), 25(OH) D3 (**e**), 1,25(OH)D3 (**f**) during the follow-up period of 24 months. Data are presented as median and interquartile range. Repeated measures analyses generated $p < 0.01$ for **b**–**f** (RMANOVA) and $p < 0.001$ for a (Friedman's test). *$p <$ Bonferroni-adjusted a-level (0.010 for all panels, except d where 0.013) for post-hoc Wilcoxon signed rank test (**a**) or paired *t* test (**b**–**f**) compared to visit 1 (at 0 months). CTX and PTH have been log transformed prior to analysis. Missing data points: 1/162 (**a**, **b**), 2/162 (**c**, **e**), 3/162 (**d**), 15/108 (**f**). Complete cases were 26/27 (**a**, b) 25/27 (**c**, **e**), 24/27 (**d**) and 17/27 (**f**, all had samples at baseline and visit 4). Only complete cases were included in the repeated measures analyses

The limitation of this study is the relatively small sample size. The strength, however, is the prospective design and the fact that not only 25OHD3 was analysed but also other parts of the calcium homeostasis. This includes the active form of vitamin D, 1,25OH2D3, which hitherto is less investigated in GD. Considering this, and that the reported findings reach statistical significance, we believe that the study offers sufficient strength for the conclusions presented.

In summary, no vitamin D deficiency was observed at time of diagnosis of Graves' disease, and levels of 25OHD3 were significantly decreased at follow-up. Lower levels of 25OHD3 are likely to occur secondary to a higher conversion to 1,25(OH)D3 due to increased PTH levels after treatment of thyrotoxicosis.GD has a significant impact on the skeleton, and treatment of thyrotoxicosis leads to improved BMD, which continues to rise for 2 years after treatment start.

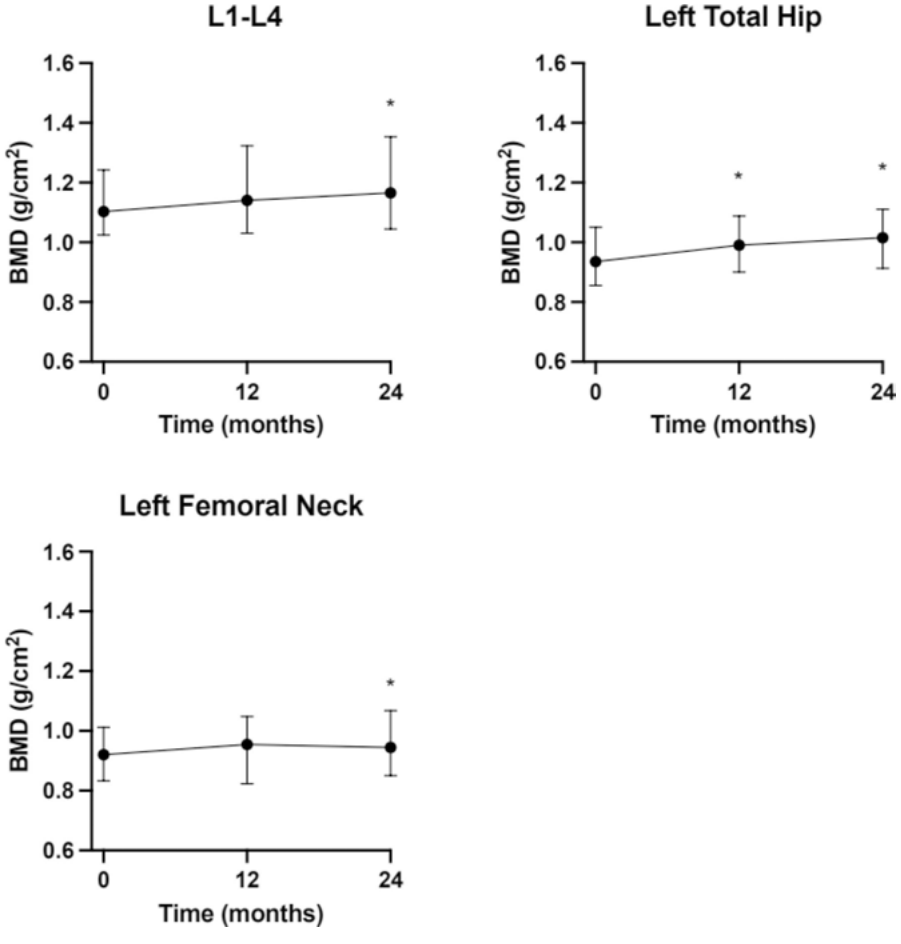


Fig. 3 Bone mineral density measured by DXA during the follow-up period of 24 months. Data are presented as median and interquartile range. RMANOVA generated $p = 0.008$ for L1–L4, $p < 0.001$ for the left total hip and $p = 0.010$ for the left femoral neck

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Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by SK, ML and ÖL. The first draft of the manuscript was written by SK and ÖL and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Ethical approval The study complied with the Declaration of Helsinki and was approved by the Regional Ethics Committee in Uppsala (Dnr 2015/469).

Informed consent Written consent was obtained from each patient after full explanation of the purpose and nature of the study.

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Paper IV

Fracture incidence in Graves' disease

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Short title

Fractures in Graves´ disease

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Keywords

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Abstract

Background

Population-based studies have suggested an increase in bone turnover in hyperthyroidism with a subsequent decrease in bone mineral density and increased risk of fractures, especially in postmenopausal women. However, heterogeneity between studies prevents a definite conclusion. Graves' disease (GD) is an autoimmune disease, and it is the most common cause of hyperthyroidism. The aim of this study was to investigate fracture risk in patients with GD.

Methods

A total of 2134 atients with incident GD and 21261 age, sex- and county-matched controls were included in a retrospective cohort study 16-18 years after diagnosis regarding the risk of developing skeletal complications. For this aim, drug and patient national registries in Sweden have been used. Up to ten age, sex- and county-matched controls were selected from The National Board of Health and Welfare´s and Statistics Sweden´s databases. Cox proportional hazards models were fitted to estimate hazard ratios (HR) and 95% confidence intervals.

Results

The analysis is based on 2134 GD patients and 21261 controls. There were no significant differences in fracture rates between GD and age, sex- and county-matched controls but after further adjustment for co-morbidities, the data showed higher vertebral fracture rates in male GD patients aged >52 years compared to male controls, HR=2.83 (1.05-7.64). The rates of treatment against osteoporosis as well as treatment with steroids were higher in patients with GD. However, hazard ratios for the association between GD and fractures remained largely unchanged after adjustment for treatments against osteoporosis and treatments with steroids.

Conclusions

There are no significant differences in fracture rates between GD and the general population. Men older than 52 years have possibly higher vertebral fractures, but the data is difficult to

interpret, and a definitive conclusion cannot be established. However, this observation implies that more studies should be performed to investigate the skeletal complications in males who experienced GD. This study also shows that patients with treated GD receive more anti-osteoporosis treatment compared to the general population.

Introduction

Graves' disease (GD) is an autoimmune form of hyperthyroidism with an annual incidence of 21 in 100,000 individuals [1] and it is the most common cause of hyperthyroidism in Sweden [2,3]. Other causes like multinodular goiter and toxic adenoma, which can be merged to the concept toxic nodular goiter (TNG), usually cause milder hyperthyroidism, but often with significantly longer duration to diagnosis and treatment [4].

Bone fragility is caused by several underlying pathophysiological mechanisms. In primary osteoporosis reasons are postmenopausal and senile osteoporosis [5-7], where the balance in bone remodeling is shifted towards a net loss in each remodeling cycle causing progressive bone loss. This is the cause of the huge fracture burden seen in the elderly. Secondary osteoporosis is also due to a disturbance in the remodeling cycle. Glucocorticoid-induced osteoporosis is the most common form of secondary osteoporosis [8]. In addition to this several diseases, such as myeloma [9], hyperparathyroidism [10] and renal failure [11,12] cause bone loss. Hyperthyroidism may cause secondary osteoporosis by acceleration of bone remodeling [13-15] It is also well known that the treatment of hyperthyroidism leads to improvement in bone turn-over and decreases the fracture risk [16].

In thyrotoxicosis markers of bone formation and resorption are elevated and correlated with the severity of diseases in pre- and postmenopausal women [17-19]. Same findings have been found in men [20]. According to some studies, there is an increased risk of fractures in patients with hyperthyroidism. Women older than 65 years of age with subclinical hyperthyroidismare are at increased risk for new hip and vertebral fractures [21]. Hyperthyroidism is associated with a temporary increase in fracture risk that is reversed upon normalization of the thyroid disorder and anti-thyroid drugs (ATD) may lower fracture risk through reversal of the hyperthyroidism [22]. However, a 20-year population-based follow-up of in patients treated for thyrotoxicosis showed no increased incidence of fractures in patients treated for hyperthyroidism [23].

Reversal of hyperthyroidism is likely to affect bone mineral density (BMD). Treatment of pre-menopausal women with thyrotoxicosis resulted in a 4% increase in BMD within 1 year [24]. In subclinical hyperthyroidism, studies have shown increased bone turnover, reduced BMD and increased risk of fracture, especially in postmenopausal women [25,26].

Interpretation of the literature is difficult and complex, not least because of heterogeneous cohorts. Although many retrospective and cross-sectional studies on the effects of thyroid dysfunction on bone turnover and bone mass have been reported, only a small number of studies have been prospective, whereas even fewer have investigated fracture susceptibility [27].

In this study 2,134 cases with GD diagnosed between 2003 and 2005 have now evaluated through national registries to investigate the incidence rate of skeletal complications compared to 21,261 controls.

Materials and Methods

Study population and data sources

The study design in details has previously been published [1]. In brief, all patients with incident GD (n=2,204) diagnosed between 2003 and 2005 in 7 cities in Sweden covering about 40% of the Swedish population, were included in a study [1]. GD was diagnosed by suppressed TSH and elevated concentrations of more than one of the following thyroid hormones: fT4, total T3, and/or fT3 in combination with elevated TRAb and/or an increased diffuse isotope uptake on thyroid technetium scintigraphy. Of these patients, 2,134 were included in this cohort study on the risk of developing skeletal complications. For this study, we used data from the drug-, patient- and population-registries belonging to the National Board of Health and Welfare in Sweden. All persons who are registered in Sweden are given a personal identity number which is used in these registries. From the prescribed drug register [28,29], we identified patients and controls who collected from the pharmacy prescribed steroids and medicines against osteoporosis like bisphosphonate and anabolic treatment against osteoporosis since July 2005. Information on prescriptions before July 2005 are not available due to the start of the register on 1 July 2005. We did not identify prescriptions for calcium and vitamin D since those may be available without a prescription and be used by other thyroid related reasons such as after thyroid surgery and different other conditions not related to thyroid diseases.

Anabolic treatment such as teriparatide is not included in this investigation as there are also drugs such as cinacalcet under the same diagnosis code. In addition, anabolic treatment is relatively new and is only used sporadically, usually after treatment failure with other osteoporosis treatments. Hormone replacement therapy was also difficult to be investigated because under the same diagnosis code there are also drugs that are not used for treatment against osteoporosis such as progestogens. In addition, under the same diagnosis code there is a large variety of different drugs such as estrogen, testosterone, antiandrogens, dehydroepiandrosterone (DHEA) etc. For the same reason, Raloxifene has not been reported in this study. From the Inpatient and outpatient registers [30], we have identified patients

diagnosed with hip, spine, humerus or forearm fractures using ICD-10-SE diagnosis codes (Supplementary Table S1). The population register [31] has been used to identify emigration date and date of death.

Controls

To compare fracture rates between patients with GD and the general population, a reference group of up to ten individuals for each patient was randomly selected from the Swedish Population Register and matched on sex, birth year and county of residence. Similar information as for the patients were extracted from the drug- and patient-registries.

Outcome's assessment

The primary outcome was fragility fractures, which have been obtained from the patient registry by searching for the following diagnoses: all hip, vertebral, humeral and forearm fractures.

The secondary outcomes were antiosteoporotic and corticosteroid treatments identified from the Prescribed Drug Register using Anatomical Therapeutic Chemical (ATC) codes (Supplementary Table S2). Regarding antiosteoporosis treatment, a single treatment with bone-specific treatment was sufficient to report as a treatment. Bone-specific treatment included bisphosphonates, both oral and intravenous, and denosumab.

Regarding steroid treatment, only systemic treatments (oral and intravenous), which were obtained from the drug registry, was included, but not local treatment such as ointments. The patient must have collected the medicine from the pharmacy on at least two occasions to be considered as treatment, this to be able to minimize the risk to include patients who receive steroids against seasonal allergies.

Survival time was computed from the GD diagnosis date (index date) until fracture date and was censored at the date of emigration, death, or end of follow up (December 31, 2019), whichever happened first. For the matched controls, follow-up started from the date on which

the matched patient was given a GD diagnosis.

In the analyses comparing rates of antiosteporotic treatments or treatment with steroids among patients diagnosed with GD and the reference population, survival time was computed from July 1, 2005 (start of the Prescribed Drugs Register) until medication collection date and was censored at the date of emigration, death, or end of follow up (December 31, 2019), whichever occurred first. Individuals who had emigrated or died before 1 July 2006 were excluded from these analyses.

Statistical analysis

The distribution of baseline characteristics was tabulated by GD and population controls and compared using the conditional logistic regression.

We estimated the incidence rates of fractures per 1000 person-years among GD patients and age-, sex- and county-matched controls. Cox proportional hazards models with time since index date in years as the underlying time scale were fitted to estimate hazard ratios (HR) and 95% confidence intervals (CI). Test and plots of Schoenfeld residuals were used to evaluate the assumption of proportional hazards, which was satisfied for the variable indicating case status. The adjusted Cox model included the variables used for matching (age at GD diagnosis; sex, county of residence) [32]; comorbidity (connective tissue diseases, diabetes mellitus, end stage kidney disease, congestive heart failure, chronic obstructive pulmonary disease, cerebrovascular disease, cancer) as well as fractures and fall accidents (Supplementary Table S3) before the index date. The analyses were performed in the entire study cohort as well as stratified by sex and 52 years of age (likely age of menopause) at index date. In analysis restricted to GD patients, we compared fracture rates in cases with different initial treatments with fracture rates in ATD treated GD patients (this was the most common treatment).

Sensitivity analyses were performed where patients and their controls of less than 20 years of age at index date were excluded. We also performed analysis adjusted for treatment against osteoporosis and steroids where these treatments were modeled as time-varying covariates.

Statistical analyses were performed using STATA software, version 14/SE for Windows (StataCorp). Tests were two sided, and statistical significance was defined as $P<0.05$.

Results

The analysis is based on 2,134 GD patients and 21,261 age-, sex- and county-matched population controls. The median age at study entry (corresponds to the date of GD diagnosis in individuals with GD) was 46 years (IQR: 35-58, range: 6-96). The median age at first recorded diagnosis of fragility fractures after index date was 68 years (IQR: 58-80, range: 11-102). The corresponding age was 70 (IQR: 59-82, range: 13-99) among GD patients and 68 (IQR: 58-80, range: 11-102) in the reference population.

Matching ensured an equal distribution of the matching variables (birth year, sex, county) among GD patients and the reference population, but GD patients were more likely to have diagnosis of diabetes than reference population (Table 1).

Incidence rate of fractures

Median follow-up time, estimated from observed follow-up times, was 15.2 years (range: 5 days to 17.1 years). Over about 318,396 person-years of follow-up, 2,114 (9.0%) individuals had fragility fractures (194 GD patients and 1,920 population controls), 918 (3.9%) emigrated, 2,484 (10.6) died and 17,879 (76.4%) were followed to the study end.The incidence rates of all fragility fractures (per 1000 person-years) were similar among GD patients and age-, sex- and county-matched controls with a corresponding statistically insignificant HR of 1.00 (95% CI: 0.86-1.16) (Table 2). Nor there were any significant differences in hip, vertebral or arm

fracture rates, HR= 0.95 (0.73-1.24), 1.09 (0.65-1.83) and 0.97 (0.81-1.16) respectively (Table 2). Furthermore, there was no statistically significant difference in the incidence rates of fragility fractures in patients aged ≤52 [HR=0.84 (0.63-1.11)] or aged >52 [HR=1.09 (0.91-1.29)] at time of diagnosis compared to matched controls (Table 2). There were no differences between female and male GD patients compared to respective sex in controls (data not shown). Furthermore, there were no significant differences in fall accidents between GD patients and controls (data not shown).

Adjustment for co-morbidities

After further adjustment for co-morbidities, as well as fractures and falls before the index date, the data showed a statistically significant difference in vertebral fracture rates in male GD patients aged >52 compared to their controls, HR=2.83 (1.05-7.64) (Table 3). Otherwise, there were no statistically significant differences in other types of fractures, nor any difference among women older or younger than 52. Sensitivity analyses excluding patients and their controls of less than 20 years of age at index date, produced similar results.

Treatment against osteoporosis and steroids use

The rates of anti-osteoporotic treatments as well as treatment with steroids were higher in patients with GD compared to the reference population (Table 4a, 4b). However, hazard ratios for the association between GD and fractures remained largely unchanged after adjustment for treatments against osteoporosis and steroids (data not shown).

Discussion

In this study, a total of 2134 patients with newly developed GD were included in a -retrospective cohort 16-18 years after the diagnosis, regarding the risk of developing skeletal complications.

Each patient was matched to up to ten controls from the general population. The data in general shows no differences in fracture rates between patients with GD and the general population. However, the data shows a higher risk for vertebral fractures in GD males older than 52 years compared with matched controls. Patients with GD were more likely to get osteoporotic treatment, except GD males older than 52, and more steroids, except females and males older than 52 years.

Although hyperthyroidism is perceived as one of the causes of secondary osteoporosis, studies and meta-analyses examining skeletal complications as a result of hyperthyroidism were inconclusive [27], and some studies have shown no association at all between hyperthyroidism and skeletal complications [23,33]. A significant weakness in the majority of studies is the heterogeneity of study populations as they included various types of thyrotoxicosis such as GD, TNG, overtreatment with levothyroxine, etc. In this study, however, we have only studied patients with verified GD.

Previous studies have shown that skeletal effects as a result of GD are reversible after treatment [24]. However, an interesting finding in this study is that men older than 52 have a higher vertebral fracture risk compared to the general population.In a previous study, we have shown a significant improvement in bone density for 2 years after initiation of treatment against hyperthyroidism in GD [34]. However, the bone density measurement only shows mineralization and not the bone quality. There was no increased fracture risk in women included in the current study. This can be explained by the fact that women possibly receive more osteoporosis prophylaxis in case of steroid treatment against GO. In addition, bone density measurements are performed on women more than men. Probably a relative hypogonadism in men associated with increasing age may have a negative effect on the skeleton. This may differ compared to women who receive more sex hormones than men at increasing age after menopause for control of menopausal symptoms [35]. In men, hypogonadism with testosterone concentrations of <12nmol/l is estimated to occur in 7% of men aged 40-60 years, increasing to 21% of 60-80 year old, and 35% of men aged >80 years

[36]. Several studies have demonstrated a gradual decline in testosterone levels with increasing age, which varies in different individuals and affected by medications, chronic diseases and adiposity [37-40]. However, the diagnosis of hypogonadism is often neglected and the opportunity to substitute testosterone in older men highly debated [41]. In addition, smoking may have a role in osteoporosis in men, not least because the prevalence of heavy smokers is more pronounced in men than in women. Whether hypogonadism or smoking is the cause of the increased fracture risk, this should also affect the control group in the same way. However, it is common for patients who go for regular check-ups regarding a certain disease to be subject to more intensive investigations, including radiological examinations that can reveal silent vertebral fractures. In addition, the low number of patients and only few numbers of events. Taking into account what was discussed above, this finding may just be a coincidence, but it should be of interest to be studied and verified with prospective studies focusing on this patient group.

On the other hand, men generally tend to experience more severe GO and therefore should have received at least as much steroid treatment, if not more. However, our data show that men older than 52 years received less steroid treatment compared to the other individuals with GD included in the study, i. e., steroids had no impact on the higher incidence of vertebral fractures in these men. However, the fact that men older than 52 did not receive more anti-osteoporosis treatment or steroids still raises the suspicion that hyperthyroidism per se may possibly have an impact on the increased incidence of vertebral fractures.

The weakness of this study is the lack of data on weight, BMI and smoking. In addition, there is no comparison between those who have and do not have sex hormone treatment such as estrogen treatment. The strength, however, is a large cohort with only verified GD as a cause of hyperthyroidism and to compare with up to 10 controls without hyperthyroidism. In addition, a long follow-up period for 16-18 years.

In conclusion, long-term observation of a large cohort of patients with previous GD shows no significant differences in fracture rates compared to up to ten controls from the general

population. A sub-analysis shows that older men have a likely increased risk of vertebral fractures but due to the limited number of events, no conclusion can be established. Further studies focusing on older males who experience GD should be performed to verify this finding. Antiresorptive treatment for osteoporosis is used to agreater extent in patients with previous GD except for elderly male patients.

Authors' Contributions

All authors treated these patients, collected data, and discussed the form for presentation.

Author Disclosure Statement

No competing financial interests exist.

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Ethical approval

The study complies with the Declaration of Helsinki. It was approved by the Regional Ethics Committee in Uppsala (Dnr 2015/253).

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Table 1. Baseline characteristics and comorbidity among patients diagnosed with GD in the years 2003-2005 and age-, sex-, and county-matched controls

	GD (**n =** 2,134)		**Controls** (**n =** 21,261)		**P-value**[a]
	N	Col %	N	Col %	
Age at index (GD diagnosis) date, yrs, mean (SD)	47.2	(16.8)	47.2	(16.8)	N/A
Male sex	430	20.1	4296	20.2	N/A
Menopause (age at diagnosis >52 years)	795	37.3	7909	37.2	N/A
Initial treatment of GD patients					
ATD	1,167	54.7			
RAI	431	20.2			
Surgery	65	3.1			
Conservative	59	2.8			
Unknown	412	19.3			
Comorbidity before index date					
Diabetes mellitus (all types)	78	3.7	470	2.2	<0.001
Chronic obstructive pulmonary disease	18	0.8	131	0.6	0.204
Cerebrovascular accident	46	2.2	344	1.6	0.058
Connective tissue disease	28	1.3	207	1.0	0.137
End stage renal disease	1	0.0	19	0.1	0.528
Cancer	44	2.1	503	2.4	0.363
Fractures	46	2.2	398	1.9	0.354
Accidental falls	131	6.1	1342	6.3	0.742

[a] p-value is from conditional logistic regression. Col% is column percentage -used for categorical variables. Standard deviation was used for age under that column.

Table 2. The incidence rate of fractures and fall accidents per 1000 person-years among GD patients and age-, sex- and county-matched controls who were followed up to 31 December 2019 and corresponding hazard ratios

	Incidence rates per 1000 person-years		**Hazard ratio (95% CI)** [a]
	All GD **N=**2,134	**All controls** **N=**21,261	
All fragility fractures (n=2,114)	6.7 (5.8; 7.7)	6.6 (6.3; 7.0)	1.00 (0.86,1.16)
Hip fractures (n=674)	2.0 (1.5; 2.5)	2.1 (1.9; 2.2)	0.95 (0.73,1.24)
Vertebral fractures (n=161)	0.5 (0.3; 0.9)	0.5 (0.4; 0.6)	1.09 (0.65,1.83)
Arm fractures (n=1,480)	4.5 (3.8; 5.3)	4.6 (4.4; 4.9)	0.97 (0.81,1.16)
Fractures [b] **in aged ≤52 at diagnosis (n=676)**	2.7 (2.1; 3.5)	3.2 (3; 3.5)	0.84 (0.63,1.11)
Fractures [b] **in aged >52 at diagnosis (n=1,438)**	14.8 (12.5; 17.4)	13.9 (12.9; 4.3)	1.09 (0.91,1.29)

[a] **adjusted for age, sex and county**

[b] all fragility fractures

Table 3 Cox proportional hazards regression analyses comparing fracture rates among 2,134 patients diagnosed with Graves´ disease in 2003 to 2005 in Sweden and matched controls from the general population

	All fragility fractures			Hip fractures			Vertebral fractures			Arm fractures		
	N of events[c]	N of events	HR (95% CI) [a]	N of events[c]	N of events	HR (95% CI) [a]	N of events[c]	N of events	HR (95% CI) [a]	N of events[c]	N of events	HR (95% CI) [a]
Overall	194	2,114	0.99 [0.86,1.15]	59	674	0.96 [0.73,1.25]	16	161	1.07 [0.64,1.80]	132	1,480	0.96 [0.81,1.15]
Among women	167	1,862	0.96 [0.82,1.13]	51	576	0.94 [0.71,1.26]	9	122	0.75 [0.38,1.47]	119	1341	0.95 [0.79,1.15]
Aged ≤52 yrs[b]	46	589	0.84 [0.62,1.14]	2	31	0.59 [0.14,2.53]	2	40	0.52 [0.13,2.17]	42	522	0.87 [0.64,1.20]
Aged >52 yrs[b]	121	1,273	1.03 [0.85,1.24]	49	545	0.96 [0.71,1.28]	7	82	0.90 [0.41,1.95]	77	819	1.02 [0.80,1.29]
Among men	27	252	1.22 [0.82,1.83]	8	98	0.98 [0.48,2.04]	7	39	2.24 [0.98,5.14]	13	139	1.05 [0.59,1.86]
Aged ≤52 yrs[b]	7	87	0.82 [0.37,1.79]	0	8	N/A	2	14	2.22 [0.47,10.41]	5	67	0.71 [0.28,1.79]
Aged >52 yrs[b]	20	165	1.53 [0.96,2.45]	8	90	1.11 [0.54,2.31]	5	25	**2.83 [1.05,7.64]**	8	72	1.37 [0.66,2.87]

[a] Adjusted for age, sex, county, comorbidity (connective tissue diseases, diabetes mellitus, end stage kidney disease, congestive heart failure, COPD, cerebrovascular disease, cancer) as well as fractures and fall accidents before the index date
[b] age at index date, which is the date of Graves' disease (GD) diagnosis for the patients and the date on which the matched patient was given a GD diagnosis for population controls
[c] number of events in GD patients

Table 4a. Cox proportional hazards regression analyses comparing rates of treatment against osteoporosis among patients diagnosed with Graves' disease and matched controls from the general population

	In the overall study population (n=22,888) [b]		Among GD patients and population controls with index date on or after 1 July 2005 (n= 3,234)	
	N of events [d]	HR (95% CI) [a]	N of events [d]	HR (95% CI) [a]
Overall	185	1.50 [1.28,1.75]	32	2.74 [1.85,4.03]
Among women	170	1.47 [1.25,1.72]	30	2.82 [1.89,4.22]
Aged ≤52 yrs [c]	47	1.90 [1.38,2.61]	11	5.78 [2.75,12.12]
Aged >52 yrs [c]	123	1.33 [1.10,1.61]	19	2.17 [1.32,3.55]
Among men	15	2.22 [1.27,3.87]	2	2.44 [0.50,11.92]
Aged ≤52 yrs [c]	5	4.13 [1.46,11.64]	1	N/A
Aged >52 yrs [c]	10	1.85 [0.94,3.62]	1	1.18 [0.14,9.94]

[a] Adjusted for age, sex, county, comorbidity (connective tissue diseases, diabetes mellitus, end stage kidney disease, congestive heart failure, COPD, cerebrovascular disease, cancer) as well as fractures and fall accidents before the index date

[b] Individuals who had emigrated or died before 1 July 2006 are excluded from this analysis. Individuals included in this analysis, came under observation on 1 July 2005 (start of the Prescribed Drugs Register).

[c] age at index date, which is the date of Graves' disease (GD) diagnosis for the patients and the date on which the matched patient was given a GD diagnosis for population controls

[d] number of GD patients treated with M05B

Table 4b. Cox proportional hazards regression analyses comparing rates of treatment with steroids among patients diagnosed with Graves' disease and matched controls from the general population

	In the overall study population (n= 22,892)[b]		**Among GD patients and population controls with index date on or after 1 July 2005 (n= 3,225)**	
	N of events [d]	**HR (95% CI) [a]**	**N of events [d]**	**HR (95% CI) [a]**
Overall	381	1.50 [1.35,1.67]	57	1.76 [1.33,2.34]
Among women	318	1.48 [1.32,1.67]	49	1.83 [1.35,2.49]
Aged ≤52 yrs [c]	193	1.82 [1.56,2.13]	32	2.41 [1.64,3.53]
Aged >52 yrs [c]	125	1.15 [0.95,1.38]	17	1.21 [0.72,2.04]
Among men	63	1.62 [1.24,2.12]	8	1.58 [0.74,3.35]
Aged ≤52 yrs [c]	32	2.24 [1.53,3.29]	4	2.27 [0.77,6.74]
Aged >52 yrs [c]	31	1.31 [0.90,1.90]	4	1.20 [0.42,3.44]

[a] Adjusted for age, sex, county, comorbidity (connective tissue diseases, diabetes mellitus, end-stage kidney disease, congestive heart failure, COPD, cerebrovascular disease, cancer) as well as fractures and fall accidents before the index date

[b] Individuals who had emigrated or died before 1 July 2006 are excluded from this analysis. Individuals included in this analysis, came under observation on 1 July 2005 (start of the Prescribed Drugs Register).

[c] age at index date, which is the date of Graves´ disease (GD) diagnosis for the patients and the date on which the matched patient was given a GD diagnosis for population controls

[d] number of GD patients treated with steroids

Suppelemtary Table S1. Fracture´s codes according to ICD-10

Fracture proximal humerus	S42.2
Fracture distal radius	S52.5
Fracture thoracic spine	S22.0
Fracture lumbar spine	S32.0
Hip fracture	S72.0
Femur pertrochanteric fracture	S72.1
Subtrochanteric fracture	S72.2

Suppelemtary Table S2. Comorbidities and accidental falls according to ICD-10

Diabetes mellitus (all types)	E10-E14
Chronic obstructive pulmonary disease	J43-44
Cerebrovascular accident	I60-69, G45-46, H34.0
Connective tissue disease	D86, M05, M06, M08, M09
End stage renal disease	Z94.0, Z99.2, Z491, Z49,1, Z492, N18.5, Z49.0, KAS.10, KAS20, DR014, DR024, DR012, SP312, SP322, SP332, SP342, SP352, SP392, DK001, TJA33, JAK10, DJ008, DR013
Cancer	C00-D489
Fall accidents in the same plane	W00-W19

Suppelemtary Table S3. Medication´s codes

Bisphosphonates oral and intravenous Denosumab	M05B
Steroids for systemic use excluding sex hormones	H02