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Calsequestrin as a risk factor in Graves' hyperthyroidism and Graves' ophthalmopathy patients

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Disclaimer

I hereby declare that the work in this thesis is that of the candidate alone, except where indicated in the text, and as described below.

Dr. Hooshang Lahooti contributed and helped with the design of the research experiments, acquisition of the knowledge in the field of this thesis and troubleshooting with the experiments, not to mention part of the corrections of this thesis.

Professor Jack R. Wall contributed to help in the acquisition of the knowledge in the field, weekly discussions of the progresses, experiments and corrections of this thesis.

This thesis has gone under review and intensive rewriting changes in order to comply with the suggestion of the reviewers that have read this document. All the examiners' suggestions have been included in this thesis (from structure planning to general incongruences sprung from research inexperience). Much more additional research work has been carried on over the course of this long examination period and it is now a much stronger piece of research and the paper that will be written in the context of the thesis is also much stronger.

Acknowledgment

I would like to express my sincere gratitude to my supervisors Professor. Jack Wall and Dr. Hooshang Lahooti for their continuous support during the course of this research, their patience, motivation, enthusiasm, and great knowledge. Their guidance helped me to complete my research on time and writing this thesis. I thank the postgraduate coordinator Professor. Ralph Nanan for helping with the completion of this Master thesis

My sincere thanks goes to Dr. Christopher Vidal, Mrs Sandra Bermeo, and Mrs Krishanthi Gunaratnam, for the “problem solving”, helpful advices and valuable insightful disussion relevant to this study.

I thank Robyn Lewis and Sue Dowd for their kindness and thank all members of Nepean Clinical School for allowing me to work in a friendly and stimulating environment.

Last but not the least, I would like to thank my family and my friends, those that are here with me in Australia and those back in Italy, for the continuous support I received during this long journey.

Abstract

Background: The pathogenesis of Graves' ophthalmopathy (GO) and the mechanisms for its link to thyroid autoimmunity are poorly understood. Our present research focuses on the role of the skeletal muscle calcium binding protein calsequestrin (CASQ1). Earlier studies from our group (1) showed that the CASQ1 gene was up regulated in thyroid tissue from patients with GO compared to those with Graves' hyperthyroidism (GH) without eye signs, raising the possibility that the orbital autoimmune reaction begins in the thyroid gland. Here, we measured the concentration of the CASQ1 protein in normal and Graves' thyroid tissue, correlating levels with parameters of the eye signs, CASQ1 antibody levels and CASQ1 gene polymorphism rs3838284.

Methods: The CASQ1 protein was measured by quantitative Western Blotting. Following electrophoresis, samples were transblotted to PVDF membranes, incubated with a 1:1000 dilution of a rabbit anti-CASQ1 antibody and incubated with an HRP-conjugated goat anti-rabbit antibody, or anti-mouse antibodies for GAPDH. The protein concentrations were determined from density quantification using the Quantity One 4.4.0 ChemiDoc program and expressed as pmol/mg total protein by reference to CASQ1 standards.

Results: Western blot analysis showed the presence of two forms of CASQ1 in the thyroid of 50 and 60 kDa molecular weight. The mean (156.71 ± 30.14 pmol/mg) concentration of the CASQ1 protein was significantly reduced in patients with Graves' disease, with and without ophthalmopathy, compared to normal thyroid from control subjects with multi-nodular goitre or thyroid cancer. Although the mean was (74.5 ± 23.63 pmol/mg) of CASQ1 concentrations, in patients with GO it was lower than that and in compared with patients with GH this difference was not significant. Reduced CASQ1 in Graves' thyroid correlated with the homozygous genotype of the rs3838284 CASQ1 polymorphism.

Conclusions: Decreased CASQ1 in the thyroid of patients with Graves' disease compared to normal thyroid from control subjects is not explained but may reflect consumption of the protein in the course of an autoimmune reaction against CASQ1 in the thyroid. Normal CASQ1 protein levels in thyroid tissue from patients with toxic nodular goitre may suggest that this is due to the autoimmune reaction rather than the hyperthyroidism.

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Abbreviations

A: Adenine

Abs: antibodies

AGRF: Australian Genome Research Facility

C: Cytosine

canFam2: dog

CASQ: calsequestrin protein

casq1: calsequestrin 1 gene

CASQ1: calsequestrin-1 protein

CASQ2: calsequestrin-2 protein

Col.XIII: collagen XIII protein

DEL: deletion

DEL-INS: deletion-insertion

ELISA: Enzyme Linked Immunosorbent Assay

EOM: Extraocular Muscle

ER: Endoplasmatic Reticulum

fr2: fugu

G: Guanine

galGal3: chicken

GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase

GH: Graves' Hyperthyroidism

GO: Graves' Ophthalmopathy

hg19: human

IgG: Immunoglobulin G

INS: insertion

mm9: mouse

momDom4: opossum

p: p-value

PCR: Polymerase Chain Reaction

PVDF: Polyvinylidene difluoride

rheMac2: rhesus macaque

SNPs: Single Nucleotide Polymorphisms

SR: Sarcoplasmic Reticulum

T: Thymine

T3: triiodothyronine

T4: thyroxine

TAO: Thyroid Associated Ophthalmopathy

TED: Thyroid Eye Disease

TRH: Thyroid-Releasing Hormone

TSH: Thyroid-Stimulating Hormone

TSHr: Thyroid Stimulating Hormone receptor

UTR: Untranslated Region

X^2 : Chi-square test

xenTro2: xenopus

Introduction

Graves' disease (GD) is a heterogeneous autoimmune disorder affecting, with varying degree of severity, the thyroid, eyes, and skin. Graves' ophthalmopathy (GO) is considered to be a chronic, autoimmune inflammatory disorder that affects all orbital tissue sections and results in various eye features (2). Ophthalmopathy greatly reduces the quality of life in affected patients and rapid worsening of GO can occur at any time. Graves' disease can be seen in both sporadic and familial forms, it is well known that Graves' disease runs in families and is a polygenetic and multi-factorial disorder. The sequence of events that leads to the development of Graves' hyperthyroidism include: a genetic susceptibility and some subsequent external (environmental) stimulus such as infection, stress, or trauma. It is important to note that no gene has been identified that shows the genetic cause of the Graves' ophthalmopathy (3).

The pathogenesis of the ophthalmopathy and, in particular, the mechanism for its link to thyroid autoimmunity (Graves' hyperthyroidism and the more common Hashimoto's thyroiditis) is poorly understood. Our detailed studies over several years have shown that Abs against the "64 kDa proteins", later showed to comprise flavoprotein (Fp), the ID protein, and the calcium binding protein calsequestrin (CASQ), were frequently detected in patients with ophthalmopathy. Calcium binding protein calsequestrin is responsible for muscle contraction-relaxation, respiration and heartbeat. Calsequestrin is located in the sarcoplasmic membrane of the muscle fibers where it binds and releases calcium ions during either processes. CASQ is an acid glycoprotein located in the luminal space of the terminal cisternae of the sarcoplasmic reticulum, binds the calcium ion with the low affinity but high capacity and is believed to function as a storage protein for calcium. It is found in cardiac, skeletal and smooth muscle and has been isolated in several non-muscle tissues including in the

cerebellum and plant cells. The “skeletal” isoform of CASQ1 is found in fast-twitch skeletal muscle, while the cardiac isoform CASQ2 is expressed in the heart and in slow-twitch skeletal muscle. Two different genes encode the isoform, the human skeletal muscle CASQ gene CASQ1, located to band 1q21, and the cardiac CSQ gene CASQ2, located to bands 1p11-p13.3, share 64% amino acid homology, however, recent studies showed that antibodies against CASQ1 and CASQ2 do not share epitopes as previously thought.

Previous studies have shown that antibodies against calsequestrin-1 protein are typically associated with eye muscle damage in patients with Graves’ ophthalmopathy (GO) (1, 3-6). Calsequestrin gene has been found upregulated in the thyroid of patients with Graves’ disease (7), patients with thyroid autoimmunity, especially those with ophthalmopathy. These findings suggest that calsequestrin genetic upregulation may begin in the thyroid tissues, then, spreads to the eye muscles, which in some cases results in damage and loss of sight.

A possible genetic role of calsequestrin has been raised by the results from several studies; Single Nucleotide Polymorphisms (SNPs) on the calsequestrin gene, for example, have been associated with type 2 diabetes (8-10), and noncoding SNPs in CASQ1 gene found to alter diabetes susceptibility (9). In this thesis study, 8 informative SNPs in calsequestrin gene were found.

We also showed by quantitative western blotting that CASQ1 protein levels were reduced in thyroid of patients with Graves’ disease with and without ophthalmopathy compared to normal thyroid.

Graves’ ophthalmopathy is also present in about 5-10% of patients with Hashimoto’s thyroiditis (11) and in patients with more subtle cases of subacute and silent thyroiditis (12). Ophthalmopathy is a disorder that occurs in families and it has different causes that might include genetic factors, although no gene has been clearly identified.

Although antibodies against CASQ1 are good markers they are not specific to GO, in fact, they have been detected in small portions of apparently normal subjects and patients with skeletal muscle damage or inflammation (13).

Calsequestrin 1 gene (*CASQ1*) was expressed 4.7 times more in the extra ocular muscle fibers than in other skeletal muscle (14) and upregulated 4.1 fold in thyroid tissues of patients with GO but this increase was not significant. CASQ2 gene was the most upregulated in thyroid tissue from patients with GO compared to those without ophthalmopathy (7). Interestingly, Zeiger et al., 2010 reported that calsequestrin-1 was decreased in extra-ocular muscle, and in this study our results shows CASQ1 is decreased in thyroid tissue.

A possible explanation for the reduction of CASQ1 protein in thyroid tissues could be due to autoimmune reaction or hyperthyroidism. To distinguish between these two possibilities, it is necessary to measure the levels of CASQ1 in isolated multi-nodules of thyroid tissue, if the levels of CASQ1 are reduced in the nodules, this indicates CASQ1 protein expression is influenced by hyperthyroidism rather than autoimmune reaction.

The specific interactions of calsequestrin with thyroid autoimmunity are not well understood, however, this study and others (13, 15), are supporting a role for CASQ1 as a molecular marker in Graves' disease.

Chapter I: Literature Review

- The role of the thyroid in eye disease -

Thyroid

The thyroid is one of largest endocrine glands in the human body. It has a butterfly shape and it is located in the neck, shielded by the cartilage that also forms the so called Adam's apple. This gland consists in two endocrine components. The first produces thyroid hormones (T3 and T4), the second is involved in the production of calcitonin.

Thyroid is a follicular gland that is constituted by follicular structures where the produced hormones are stored. Calcitonin, a small peptide of 32 amino acids, instead is produced by C cells, or parafollicular cells, located outside the follicles.

The main role of the thyroid is the production of the above-mentioned hormones and calcitonin. The function of the gland is to control how quickly the body uses energy, makes proteins and how sensitive the body has to be towards other hormones. T3 and T4, triiodothyronine and thyroxine respectively, regulate the growth and the function of many other systems in the body. On the other hand, calcitonin plays a role in calcium homeostasis.

The upstream regulation of the hormonal output from the thyroid is supervised by yet another hormone, the Thyroid-Stimulating Hormone (TSH), produced by the anterior pituitary, which in turns is regulated by Thyrotropin-Releasing Hormone (TRH) produced by the hypothalamus. There is therefore a continuous communication between thyroid and hypothalamus which in unfortunate circumstances misregulates the results in development of thyroid disorders.

Thyroid Gland

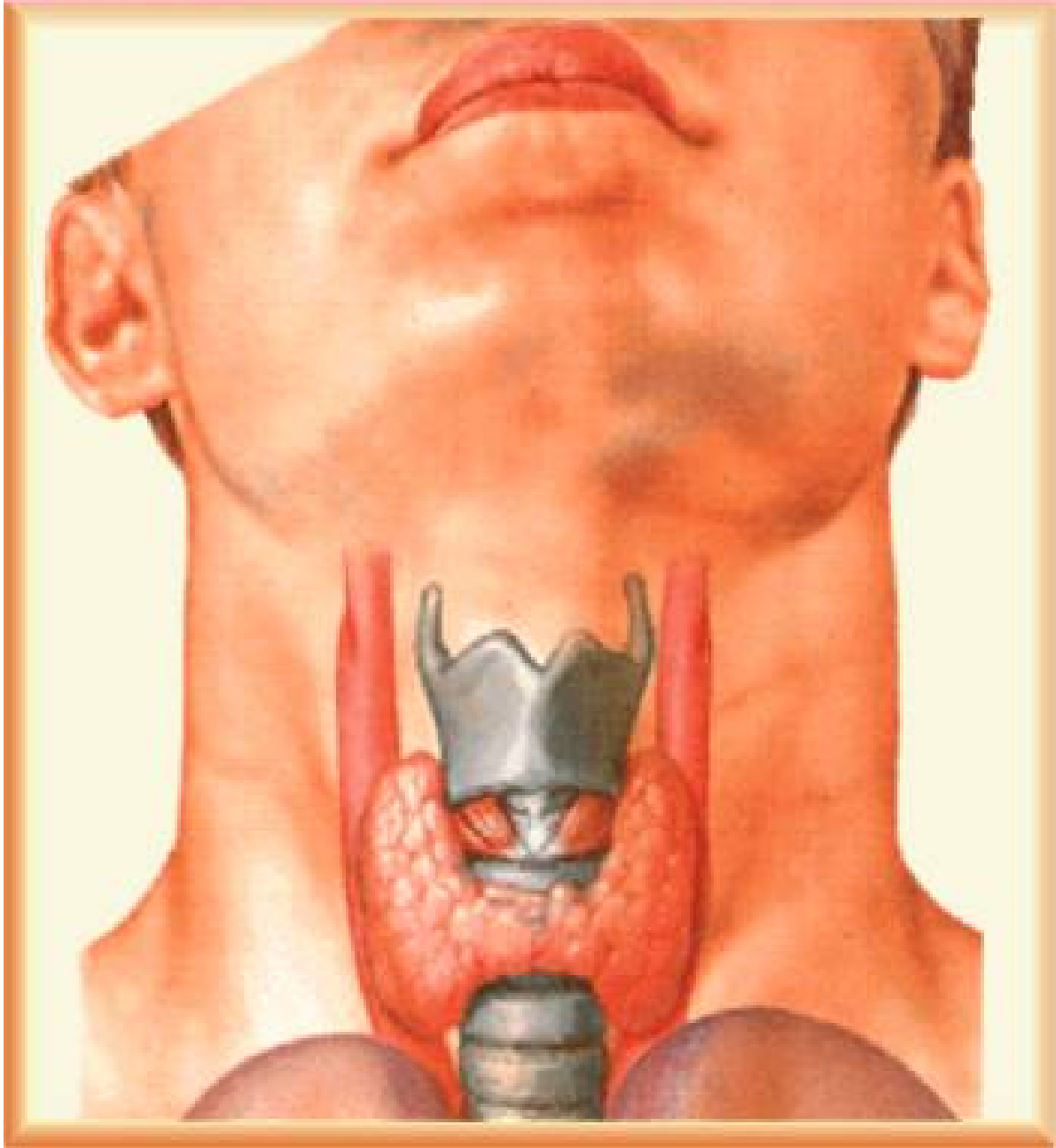


Figure 1. Thyroid gland is butterfly shaped, located next to the trachea.

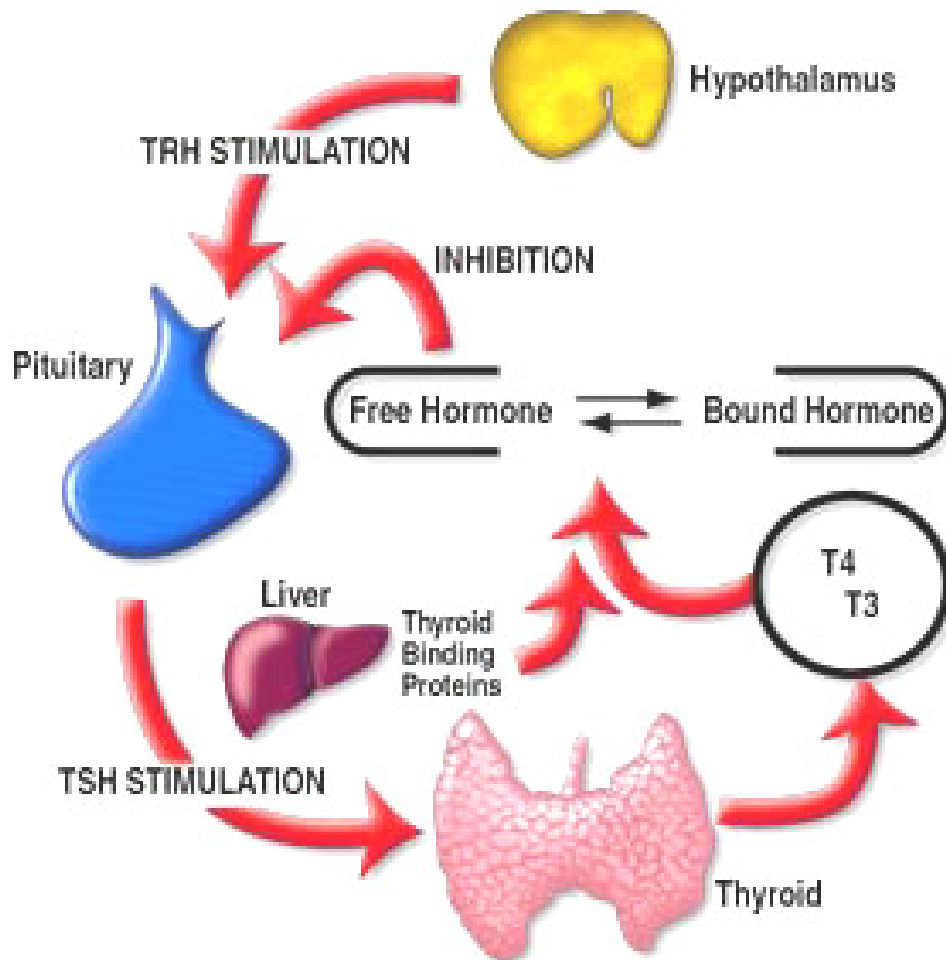


Figure 2. The hypothalamus stimulates the pituitary gland to secrete thyroid stimulating hormone, and the TSH stimulates the thyroid to produce thyroxin. Thyroxin has two forms, T3 and T4. T3 is the active form of the hormone.

Thyroid Diseases description: background

The Persian physician and philosopher, Avicenna first described the relationship between thyroid related eye disease and the classic swollen neck feature of this disease, called goiter, around AD 1000 (16). Current terminology refers to the ophthalmopathy as associated with Graves' disease or Graves' ophthalmology (GO) (17). A detailed classification of this disease subtypes can be defined by three groups, namely:

Graves' hyperthyroidism (GH), initially results from the activation of the TSH receptors by TSH and TSH-receptor stimulating antibodies. However, recent hypothesis explains further argumentation of hyperthyroidism and thyroid ophthalmopathy as the results from increased levels of CASQ1 and Col.XIII antibodies in patients serum(1).

Thyroid-associated ophthalmopathy (TAO), which is a more general term referring to the swelling and inflammatory process against the extraocular muscles and orbital connective tissue (18), and it divides in three subgroups: **ocular myopathy, congestive ophthalmopathy and mixed congestive and myopathic ophthalmopathy**. The trigger that causes Graves' disease appear to be a genetic predisposition, which plays a modest role, but also well known and proven environmental and endogenous factors (stress, smoking and gender) (2).

Euthyroid Graves' disease, which occurs in apparent absence of thyroid autoimmunity features and Thyroid-Stimulating Hormone receptor (TSHr) antibodies. Ophthalmopathy is also present in 5-10% of patients with Hashimoto's thyroiditis (11), and it is characterized by hypothyroidism. However the mechanism of autoimmunity that characterizes Graves' hyperthyroidism and Hashimoto's thyroiditis, are not well understood. Prominent characteristics features of Thyroid eye disease (TAO) can be seen in the following figures (Fig. 3 and Fig. 4).

TAO



Figure 3. Three pictures of patients with GO (from left to right):

- Patient with inflammatory Graves' ophthalmopathy, showing redness of the eyelids and diffuse redness of the conjunctiva. Edema of the eyelids is also clearly visible;
- Patient with inflammatory Graves ophthalmopathy showing grossly enlarged caruncle and edema of the eyelids;
- Patient with burnt out GO. The swelling of the eyelids is not caused by edema;

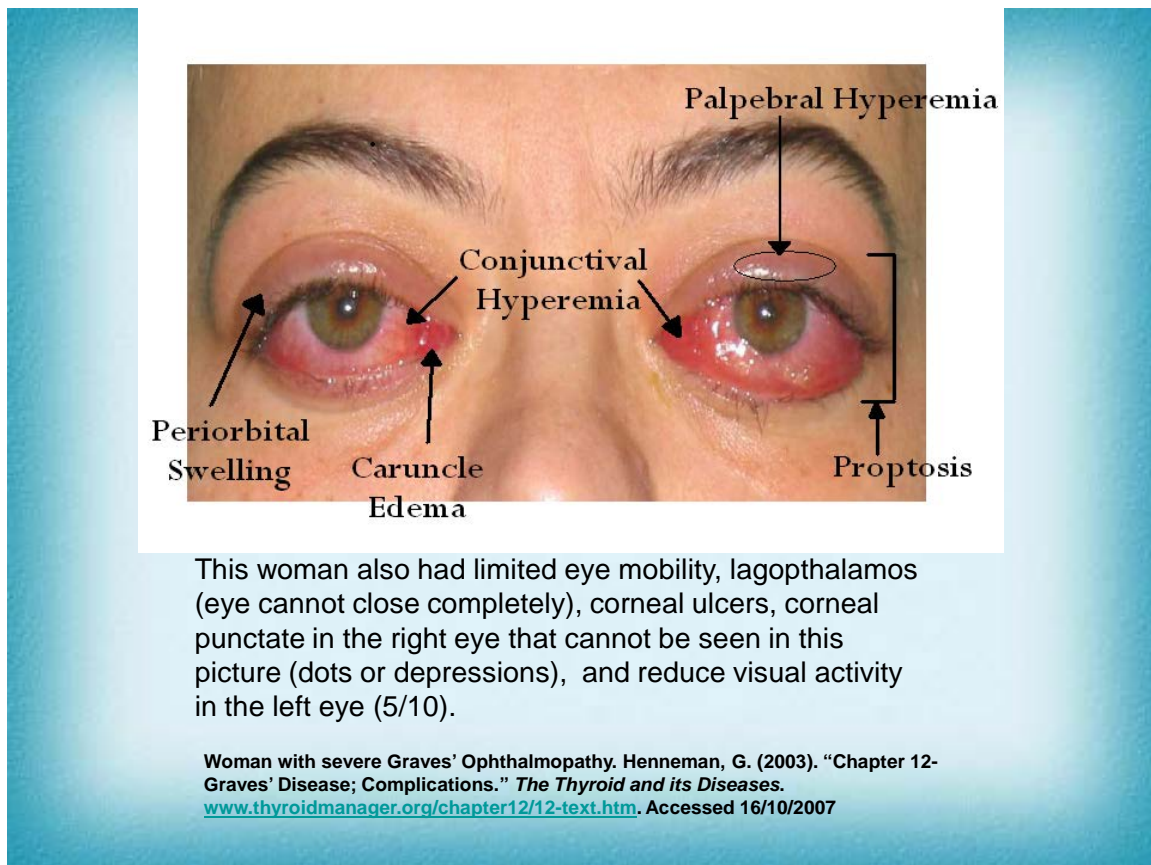


Figure 4. Graves' disease complications in a subject.

Subgroups and clinical features of TAO

The three main subgroups of TAO are:

- **ocular myopathy** is the most sever form of TAO. The extraocular muscle is functionally and structurally damaged through eye muscle autoimmune reaction, clinical features include diplopia, eye orbital muscle dysfunction and exophthalmos.
- **congestive ophthalmopathy** leaves the eye in minimal swelling, periorbital edema, chemosis and/or exophthalmos state during an inflammatory process against the eyelid and orbital tissue.
- **mixed congestive and myopathic ophthalmopathy** shows characteristics from the both previous subgroups (17, 18).

TAO affects both genders with a skewedness towards the females of 5.5 to 1 (16 women and 9 men per year over a population of 100,000 people) (17). It is an autoimmune disease; however, its pathogenesis is still poorly understood. GO is often mild and self-limiting, but sometimes symptoms can be severe.

To classify various forms of TAO initially NOSPECS classification was used to look at the severity of symptoms; however, it was not informative enough to tell about the activity of TAO, therefore, it is no longer in use. Mourits et al. (19) derived a Clinical activity score. Which looks at the symptoms of inflammation, but does not distinguish between different types of TAO and it is very subjective. Nunery distinguishes between TAO without restrictive myopathy (type 1) and with restrictive myopathy. Ocular myopathy score, the Congestive ophthalmopathy score, Upper eyelid retraction score, and eye assessment, looks at different types of TAO and severity and activity. Various features of TAO are summarized in Table 1.

TAO subtype	Main signs and symptoms	Autoantigens
Ocular myopathy	<ul style="list-style-type: none"> • Diplopia • Eye muscle dysfunction • Exophthalmos 	<ul style="list-style-type: none"> • Calsequestrin • G2s • Flavoprotein
Congestive ophthalmopathy	<ul style="list-style-type: none"> • Watery, gritty eyes • Periorbital edema • Conjunctival injection, chemosis • Exophthalmos 	<ul style="list-style-type: none"> • TSH-r • Collagen XIII
Chronic eyelid lag	<ul style="list-style-type: none"> • Eyelid lag and retraction • Inability to completely appose eyelids • Corneal ulceration 	<ul style="list-style-type: none"> • Flavoprotein • Calsequestrin

Note: TSH-r = thyroid-stimulating hormone receptor.

*Mixed disease, the subtype most commonly seen, is not listed separately from its components.

Table 1. The most common eye signs and symptoms of the main subtypes* of thyroid-associated ophthalmopathy (TAO) and the serum autoantibodies detected most often.

Pathogenesis

There are several different hypotheses for the cause of TAO. Thyroid-Stimulating Hormone receptor, calsequestrin-1 and collagen XIII proteins are candidate markers that shown to be associated with TAO. TSH-r is the most accepted marker by most investigators, however, in recent years, the last two markers have also shown good potential in several studies.

The most accepted hypothesis for the development of TAO is the so called “TSH-r hypothesis”. This model is widely accepted, however, it still remains controversial for the following reasons: the uncertainty is due to the presence of TSH-r in other cell types (fibroblasts and adipocytes), in different locations of the body, like the abdominal wall, which are not affected during the development of Graves’ disease (1). This controversial role of TSH-r as marker led to search for alternative markers associated with TAO. Calcium binding protein calsequestrin-1, an internal protein found over expressed in extraocular muscle in patients with ophthalmopathy and collagen XIII (Col.XIII), an orbital fibroblasts’ membrane protein have shown to be additional markers associated with TAO.

Studies from our research group have demonstrated significant prevalence of antibodies against CASQ1 and Col.XIII both in patients with Graves’ thyroiditis (20). Euthyroid graves’ patients are ambiguous cases where serum antibodies against CASQ1 and Col.XIII are shown to be positive and TSH-r antibodies are negative for the patients tested (21). These findings strengthen the role of genetics in the development of the ophthalmopathy. In addition, CASQ1 antibodies have also been detected in patients with Hashimoto’s thyroiditis and patients with transient thyroiditis (1). Hashimoto’s thyroiditis often leads to changes to the eyes and these changes have been associated with antibodies against calsequestrin-1 and collagen XIII. The ophthalmopathy associated with Hashimoto’s thyroiditis cannot be explained by the TSH-r hypothesis as antibodies against TSH-r generally not detected in these patients serum (11).

Ophthalmopathy is an autoimmune disease that has poorly understood mechanisms and a relation between proper markers failed to be found. Nonetheless calsequestrin-1 and collagen XIII have continually demonstrated to be more exhaustive markers. TSH-r hypothesis is summarized in Fig. 5

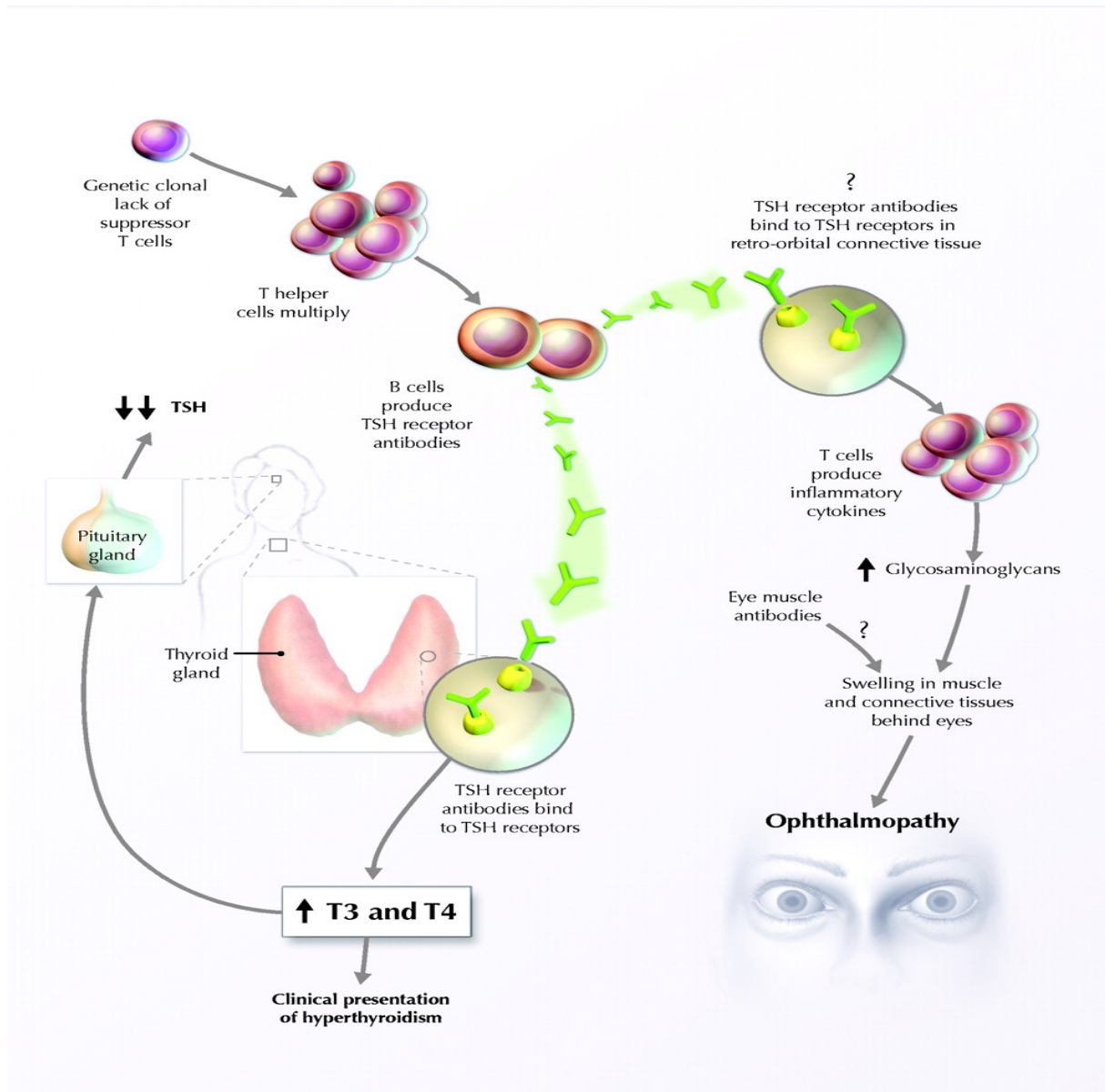


Figure 5. Pathogenesis of Graves' Disease and Ophthalmopathy. Ginsberg, J. (2003) 'Diagnosis and management of Graves' Disease.' Canadian Medical Association Journal. V. 168; 575-585.

Causes

While the causes of the TAO remain unclear some of the processes involved have been identified. The autoimmune reaction is the result of T-lymphocytes that induce B-cells to secrete autoantibodies against thyroid tissue and/or (it depends on how much the disorder develops in the patient) common orbital tissue antigens. This mechanism does not explain the whole process for development of ophthalmopathy because unequivocal antigens have not yet been associated with it. A common antigen is, as previously mentioned, TSH-r, but others have been identified over the past decade suggesting an important role in the autoimmunity reaction: G2s, fragment of FOXP1 transcription factor; the flavoprotein subunit of the mitochondrial succinate dehydrogenase (18); collagen XIII, found on the cell membranes of the orbital fibroblasts, which may be related to the periorbital inflammation of the ophthalmopathy, and the skeletal calcium binding protein calsequestrin, an internal protein attached to the sarcolemmal membrane.

The markers listed above are shown in figure 6, which shows the reorganization of autoantigens in an extraocular fiber and in orbital fibroblasts.

Calsequestrin-1 positive lymphocyte in particular have been found prevalent in all patients with Graves' ophthalmopathy, in the majority of those where the disease process was ongoing and a portion of patients with Graves' hyperthyroidism without ophthalmopathy (6). Detailed studies from our research group over many years have shown that the above markers were frequently detected in patients with ophthalmopathy. Collagen XIII and calsequestrin-1 antibodies have proven to be a promising path to follow for characterization of Graves' disease (6), although, this thesis will be focusing mostly on the research carried on the role of calsequestrin-1 protein in reference to these specific pathologies.

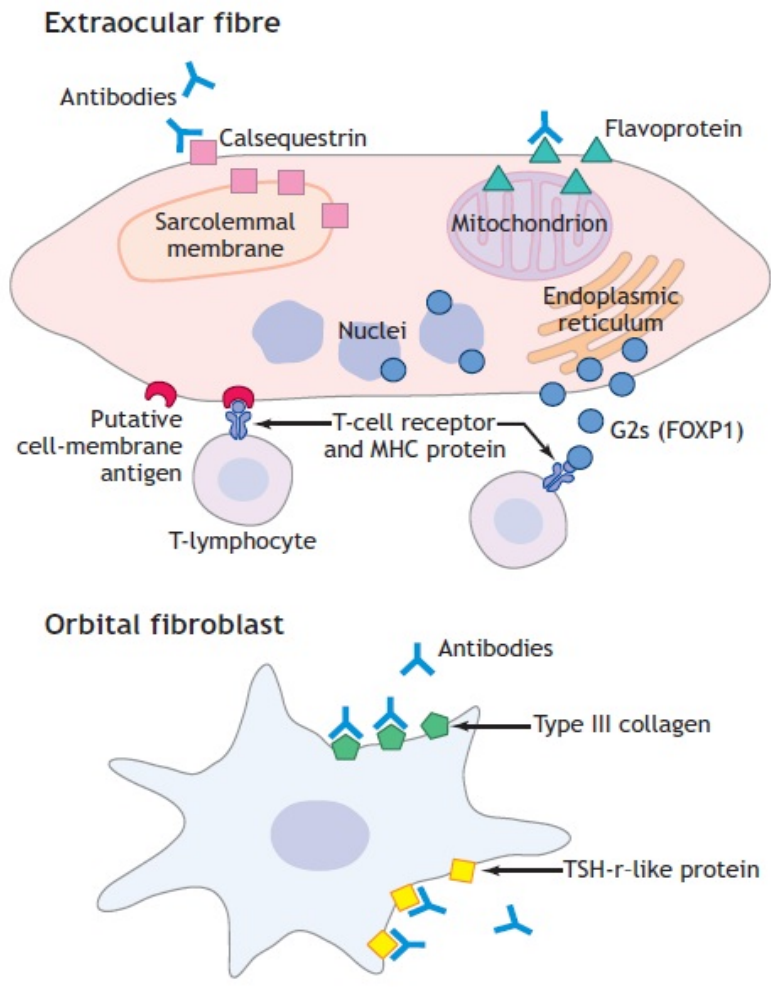


Figure 6. Eye muscle and orbital fibroblast autoantigens recognized by T-lymphocytes or antibodies in TAO. The working hypothesis is that the ocular myopathy subtype of TAO is initiated by T-lymphocyte-mediated targeting of calsequestrin or a yet unidentified eye muscle cell membrane antigen. Serum antibodies against flavoprotein and G2s are likely to be secondary to release of the proteins following muscle fiber necrosis. Chronic eyelid disease, which is a feature of TAO or a dominant sign in patients with Hashimoto’s thyroiditis, may be the result of T-lymphocyte-mediated targeting of calsequestrin in the upper eyelid levator palpebrae superioris muscle. The congestive ophthalmopathy subtype of TAO is likely to result from a reaction against the TSH-r or collagen XIII in the fibroblast cell membrane, which leads to fibroblast stimulation and excess production of collagen and glycosaminoglycans. The different reactions shown in the figure may occur alone or in combination. Reproduced with the permission from: Bednarczuk T, Gopinath B, Ploski R, Wall JR. Susceptibility genes in Graves' ophthalmopathy: searching for a needle in a haystack? Clin Endocrinol (Oxf). 2007;67(1):3-19. Epub 2007/05/25.(22). Abbreviations: MCH, major histocompatibility complex; TSH-r, thyroid-stimulating hormone receptor.

- Role and function of calsequestrin-1 -

Overview: role, function and localisation of calsequestrin protein

Calsequestrin is a major Ca^{2+} binding protein that is present in two isoforms: calsequestrin-1 found in fast-twitch, skeletal muscle tissues and calsequestrin-2 specifically in cardiac muscle but the two isoforms can be found in several other non-muscle tissues. Its role is to provide necessary levels of calcium for muscle contraction-relaxation, respiration and heartbeat (23, 24).

Calsequestrin is found in cardiac, skeletal and smooth muscle and has been isolated in several non-muscle tissues including in the cerebellum and plant cells. It stores calcium ions in sufficient quantities (up to 20 mM) to allow repetitive contractions and is essential to maintain movement, respiration and heartbeat. Calsequestrin is a highly acidic protein with up to 50 calcium binding sites formed simply by the clustering of two or more acidic residues. Calsequestrin is 367 amino acids glycoprotein with and molecular weight of approximately 40 kDa, however, on SDS-PAGE gel electrophoresis it appears as two bands with ~ 60, and 50 kDa. The monomer contains three redox inactive TRX-fold domains (Thioredoxin domain). When calcium binds there is a structural change whereby the alpha-helical content of the protein increases from 3 to 11% (25). Both forms of calsequestrin are phosphorylated by casein kinase II, but the cardiac form is phosphorylated more rapidly and to a higher degree. The human skeletal muscle CASQ gene CASQ1 has 11 exons, located to chromosom 1 band q21, and the cardiac CSQ gene CASQ2, located to chromosom 1 bands 1p11-p13.3, share 68.4% amino acid homology (7), however, Abs against CASQ1 and CASQ2 do not share epitopes.

The synthesis and maturation of the protein takes place in the endoplasmic reticulum (ER) where, once completely formed, it is packed and protected by a complex of chaperonin proteins (26) to the mitochondria and some of the CASQ protein moves and incorporated in the cell membrane during the assembly of the protein (27). Calsequestrin protein that migrates to the cell membrane interacts mainly with some abundant transmembrane protein such as: triadin, a 26 KDa protein, and ryanodine receptors, a class of intracellular calcium channels. These interactions allow calcium-induced calcium-release processes in cell tissues like muscle and neurons. These processes are triggered like shown in Fig. 7, by the recognition of CASQ by two receptors on the SR membrane, namely Ryanodine receptor and Triadin receptor. Calsequestrin protein carrying calcium builds up in the Lumen side of the SR membrane. The presence of calcium (or magnesium) on the Lumen side of the SR membrane activates the epitopes on the RyR2 on the membrane, this allows the calcium to be transferred from the Lumen side of the SR membrane to the Cytosolic side. Once the calcium crosses the RyR2 it inactivates its binding on a particular site of the receptor. This receptor will be reactivated when the empty calsequestrin protein binds to the Triadin receptor allowing it to shift to a site of the RyR2 giving the signal to the later receptor to open or close in relation to the needs for calcium ions (28).

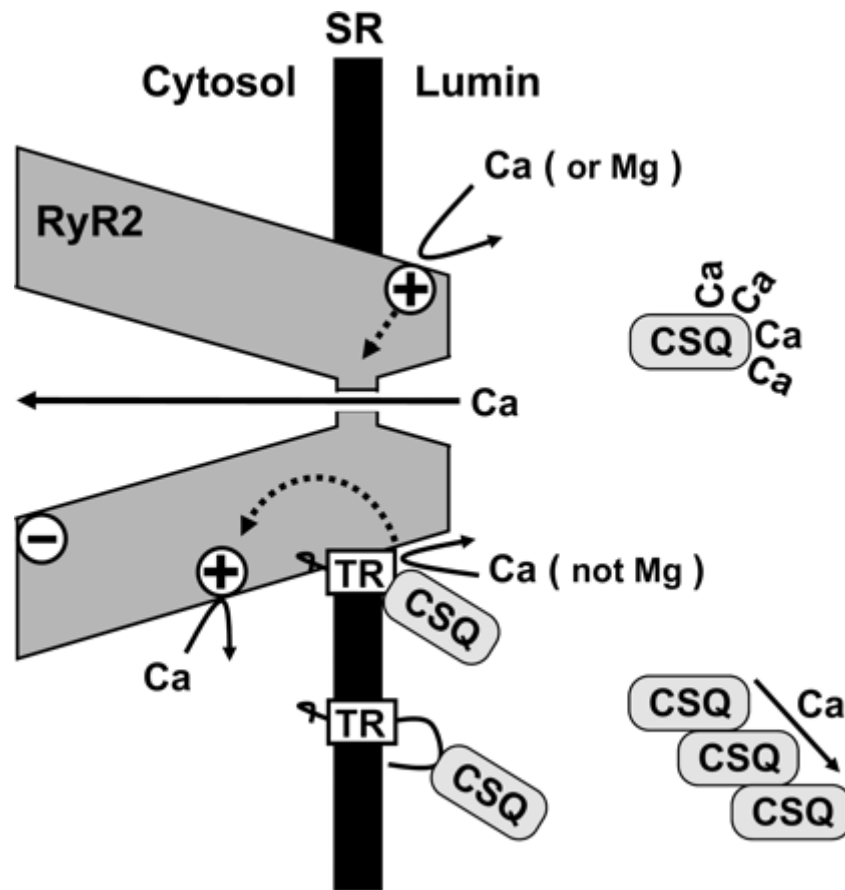


Figure 7. Summary cartoon of RyR2 luminal Ca^{2+} regulation. Triadin is labeled TR. Calcium binding sites that activate channel are indicated with a plus. The cytosolic Ca^{2+} inhibitory site is marked with a minus. Reproduced with the permission from: Qin J, Valle G, Nani A, Nori A, Rizzi N, Priori SG, et al. Luminal Ca^{2+} regulation of single cardiac ryanodine receptors: insights provided by calsequestrin and its mutants. *J Gen Physiol.* 2008;131(4):325-34. Epub 2008/03/19.

The two forms of calsequestrin, cardiac and skeletal are both located in the ER and in the lumen of the sarcoplasmic reticulum (SR) however, the two isoforms polymerize in different compartments of the ER. Cardiac calsequestrin is found in the most proximal ER exits sites whereas the skeletal calsequestrin is on the opposite side, concentrating on the distal part of the ER (29). Recent reports show the presence of CASQ1 protein in thyroid tissues (30). It is conceivable to think that this research may open the way to challenging questions about the role(s) of calsequestrin in calcium homeostasis and its interactions with other intracellular proteins in the thyroid tissues of patients suffering from thyroid diseases

The genetic factor in TED

It was reported by Porter *et al* (14) that CASQ1 gene was expressed in the extraocular muscle **4.7** times more than in other skeletal muscle while others (7) showed that CASQ2 was expressed **2.7** times more. Our team was the **first** to show the presence of CASQ protein in the thyroid tissue. Recently, a comprehensive detailed molecular study of TED using micro array technology showed that 295 genes were differentially expressed in thyroid tissues from patients with Graves' hyperthyroidism with and without ophthalmopathy. Of these, the cardiac calsequestrin gene (CASQ2) was the most highly expressed gene in GO (2.2-fold increase, $p < 0.05$) (7).

These and other recent studies confirm the potential importance of the CASQ protein in the pathogenesis of TED. A critical role for CASQ2 in maintaining cardiac muscle function has been confirmed by the recently identified missense mutation, producing a single amino acid substitution (D307H), in a family suffering from an autosomal recessive catecholamine-induced polymorphic ventricular tachycardia (31). These emerging reports raise the possibility that skeletal CASQ1 polymorphisms may be associated with skeletal muscle myopathies.

The complexity of GO indicates that GO is a genetically heterogeneous disorder with different genes, gene-gene, and gene-environmental factor interactions causing the same phenotype (22, 32-35). Although a large number of association studies with candidate genes in GO have been published, the results are so far inconclusive. It should be noted that the vast majority of studies are underpowered and the GO groups poorly characterized (reviewed in (36, 37)).

In this study, initially, 8 pathogenic SNPs identified in a cohort of 86 patients and the normal control by High through put IPLEX technology using MassARRAY System through single

base primer extension to identify the informative SNP(s). In subsequent studies, when the number of patients in the cohort increased to 440, 4 of these 8 identified pathogenic SNPs were present in all the patients and showed to be informative. Therefore, it was considered to carry out a case control association study investigating these 4 SNPs in CASQ1 gene in patients with various manifestations of TED.

CASQ antibodies have shown to have good specificity as markers for early eye muscle inflammation process in patients with GO (38). The eye muscle antibodies such as CASQ1 and COL XIII antibodies are also present in those cases where disease symptoms and/or TSH-r antibodies are absent.

Recent studies investigating the genetics behind calsequestrin demonstrated that CASQ1 gene has been involved also in heat strokes and hyperthermia in mice (25, 39) due to its knockout from their genotype. Similarly, a critical role of CASQ2 protein exacerbates cardiac muscle functions through a missense mutation in a family with associated autosomal recessive catecholamine-Induced polymorphic ventricular tachycardia (31).

It remains to determine whether the identified polymorphisms may play significant role(s) in the pathogenesis of TED.

Polymorphisms in *casq1* gene

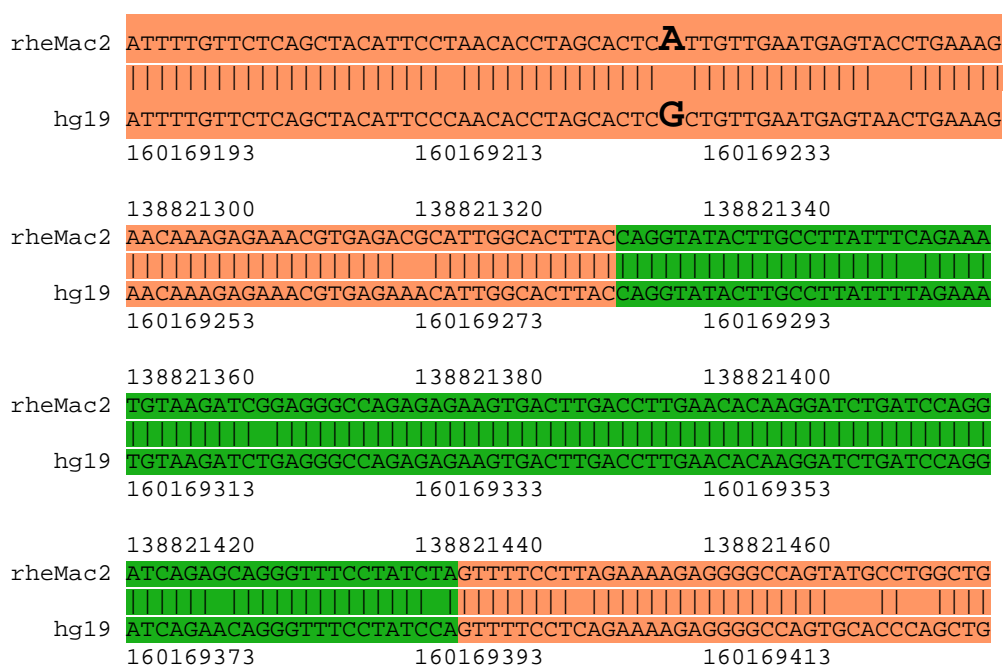
Polymorphisms in calcium binding protein CASQ1 have been mainly associated with type 2 diabetes in many studies (9). In silico studies of CASQ1 gene (see the methods chapter for detail) showed the presence of 33 out of 122 reported polymorphisms to be conserved. These 33 SNPs were studied by IPLEX MASS-ARRAY technologies. Four polymorphisms showed to be informative and significant. They are as follow:

rs74123279 SNP found in the promoter area of CASQ1 gene might play a modulatory role in transcription of CASQ1 gene. In fact its change from G to A causes a loss of transcription factors such as CTCF (40) and BORIS (41) gene family transcriptional regulators with 11 highly conserved zinc finger domains (*unpublished work*).

rs3838216 SNP in Intron 1 has been evolutionary conserved and recent preliminary studies show an association to thyroid disorders (Graves' ophthalmopathy) (42).

rs74123289 SNP located in Exon 11 3'UTR shows a change from C to T. Such alteration indicated that this region may be a control region for the translational activity of CASQ1 gene through a binding site for MicroRNA (mir-224) (*unpublished work*). MicroRNAs are small molecules of RNA that are not codified, usually of a length between 20-22 nucleotides. These molecules are part of a net of regulatory genes and have various functions, among which, the most known is the post-translational regulation (inhibiting the messenger RNAs).

rs17368853 SNP in Intron 9 is located in a highly evolutionary conserved region containing a transposon element. Preliminary studies conducted on this SNP have indicated that the region of the intron 9 may be deleted or absent in CASQ1 gene of patients suffering from severe ophthalmopathy (*unpublished work* and <http://ecrbrowser.dcode.org/>).



The two sequences aligned above belong to the human (hg19) and the rhesus macaque (rheMac2) CASQ1 protein. This particular region in green, flanked by the intronic regions in dark pink, of the CASQ1 gene could be a transposon site. Transposons are notorious elements of the genome that have the ability to move from a gene to another. This process, in which they are involved, is known as “transposition” and require the presence of recombination’s sites on the DNA, located both on the transposon and on the target chromosome. These elements, flanked with particular sequences of insertions, may have been removed, or located elsewhere, in patients with severe ophthalmopathy not allowing those individuals to express calsequestrin-1 in a correct way or sufficient quantity.

Chapter II: Methodology

- SNPs study -

Introduction

The complex modulation of the human genome is subject to many kinds of alterations, along with insertions, deletions, repetitions and, of course, polymorphisms. Single nucleotide polymorphisms in calsequestrin gene are potential genetic modifiers. Previous studies, targeting *casq1* gene polymorphisms, have suggested an association between calsequestrin-1 protein and type 2 diabetes (8, 9). In other studies, the upregulation of the cardiac calsequestrin gene has been reported in patients with Graves' ophthalmopathy (7), supporting its role as trigger for these pathology. Furthermore, examples of specific and sensitive affinity between antibodies targeting CASQ1 protein and ophthalmopathy have suggested being an alternative to TSH-r as future screening marker (13). The identification of 4 informative SNPs in calsequestrin-1 gene has given strength to the hypothesis that sees calsequestrin-1 to have a genetic role in this particular disease context.

Previous studies from our laboratory

Microarray results from previous studies carried by our research group identified 295 genes that were differently expressed in patients with and without ophthalmopathy. Specifically, it showed that the CASQ1 and CASQ2 genes were upregulated in thyroid tissue in patients with Graves' disease after thyroidectomy. The cardiac calsequestrin gene was found to be upregulated in patients with Graves' disease and TAO. The skeletal calsequestrin gene was upregulated as well, but not significantly. These findings have raised the question of whether the binding of the antibodies occurs on the shared epitopes. The latter hypothesis was lately

disproved by the same research group (43) through the measurement of antibodies against CASQ1 and CASQ2 using enzyme-linked immunosorbent assay (ELISA) from patients with and without ophthalmopathy. Following studies then narrowed to CASQ1 because of the over expression in the extraocular muscle (EOM), 4.7 times more than in the jaw muscle, whereas cardiac calsequestrin was only 2.7 times more in EOM (14)

fetal brain	whole brain	temporal lobe	parietal lobe	occipital lobe	prefrontal cortex	cingulate cortex	cerebellum
cerebellar peduncle	amygdala	hypothalamus	thalamus	subthalamic nucleus	caudate nucleus	globus pallidus	olfactory bulb
pons	medulla oblongata	spinal cord	ciliary ganglion	trigeminal ganglia	superior cervical ganglion	dorsal root ganglia	thymus
tonsil	lymph node	bone marrow	BM-CD71+ early erythroid	BM-CD33+ myeloid	BM-CD105+ endothelial	BM-CD34+	whole blood
PB-BDCA4+ dendrite	PB-CD14+ monocyte	PB-CD56+ NKCells	PB-CD4+ Tcells	PB-CD8+ Tcells	PB-CD19+ Bcells	leukemia lymphocyte	721 B lymphoblast
lymphoma Burkitt	leukemia promyelocytic	lymphoma Burkitt...	leukemia chroni...	colorectal adenoma	Appendix	skin	Adipocyte
fetal thyroid	thyroid	pituitary gland	adrenal gland	adrenal cortex	Prostate	salivary gland	Pancreas
pancreatic islets	atrioventricular...	heart	cardiac myocytes	skeletal muscle	Tongue	smooth muscle	uterus
uterus corpus	trachea	bronchial epithelium	fetal lung	lung	Kidney	fetal liver	liver
placenta	testis	testis Leydig cell	testis germ cell	testis interstitial cell	testis seminiferous	ovary	

Figure 8. Organs distribution of CASQ1 proteins; deep red colour indicates high levels of expression of protein. Skeletal muscles have high levels of CASQ1 protein, other tissues such as tongue, thyroid, Heart and uterus corpus have relatively high concentration of CASQ1 protein (source: www.ecrbrowser.dcode.org).

Present studies, results and findings

Analysis through ECR browser (<http://ecrbrowser.dcode.org/>) aligned CASQ1 gene of several species (Fig. 9), such as lampreys, fugu, xenopus, dog, marsupial, mouse, and rhesus monkey with the human sequence. The result of this analysis showed the conserved elements between the different genomes. The yellow regions are untranslated 5'UTR where the gene promoter is located and 3'UTR where the stop signal is located. Blue sections are exons, of which CASQ1 gene has 11; dark pink regions between the exons are introns. CASQ1 has 10 introns, within its intronic regions there are highly conserved intergenic subregions, indicated by the green colour, these are transposons and repetitive elements.

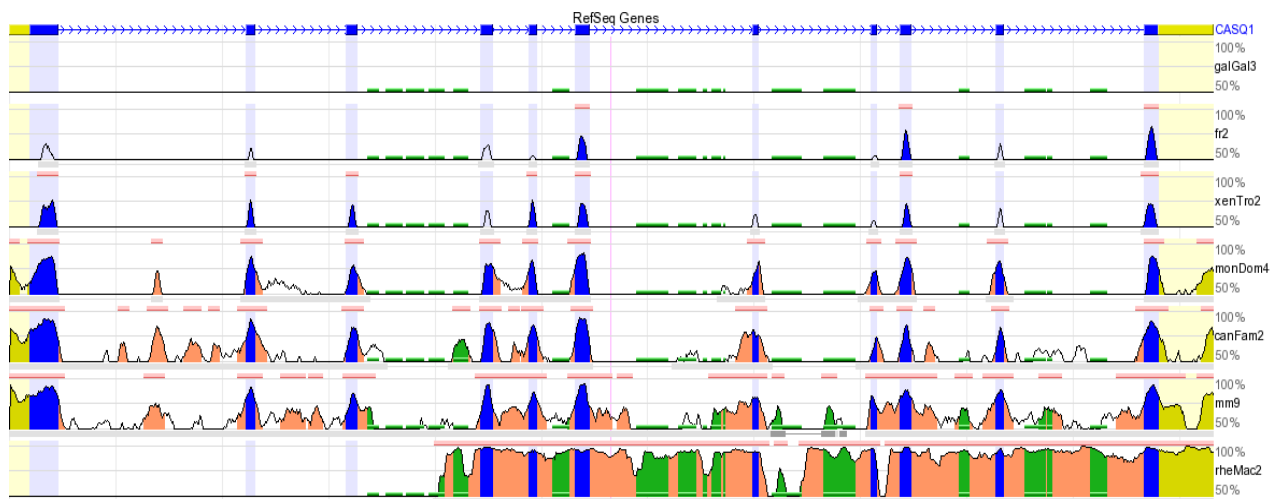


Figure 9. Whole genome alignment of different species showing, in green, highly conserved regions of calsequestrin-1 gene, the baseline represents the human genomic sequence. Yellow regions are untranslated 5'UTR (left side of the figure) where the gene promoter is located and 3'UTR (right side of the figure) where the stop signal is located. Blue blocks are exons, and CASQ1 gene has 11, whereas the dark pink regions are introns. CASQ1 has 10 introns, within these regions there are highly conserved intragenic regions indicated by the colour green. These green coloured regions are transposons and repetitive elements. Abbreviations on the figure are the followings: galGal3 (chicken), fr2 (fugu), xenTro2 (xenopus), momDom4 (opossum), canFam2 (dog), mm9 (mouse), rheMac2 (rhesus macaque)

The comparison of the 11 Kb human genomic region on chromosome 1, including non-coding parts of the gene and the ~5 kb downstream and ~5 kb upstream intergenic regions, with the corresponding mouse sequence, resulted in the identification of 27 conserved DNA sequences that met the criteria of at least 100 bases in length and at least 70% identity. The intragenic regions of the gene contain 13 conserved non-coding sequences, mostly with 90% identity or more. Further 8 sequences are located downstream and other 6 upstream of CASQ1 gene.

The whole calsequestrin-1 sequence was then processed using ECR browser to extrapolate the list of the SNPs for the designing of the primers that would cover the entire gene. The conserved regions showed 122 polymorphisms in CASQ1 gene, 33 of which were highly conserved and 22 were in the human sequence (Fig. 10).

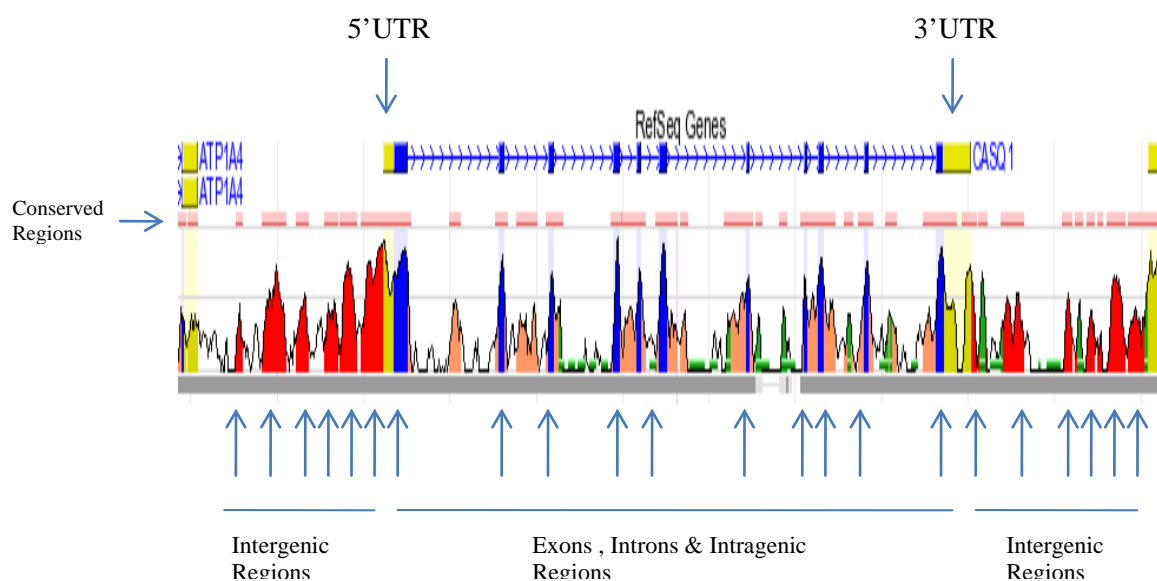


Figure 10. CASQ1 gene sense strand, or + strand, indicated by the arrow to the right. The arrows at the bottom of the figure indicate the 22 conserved SNPs in human calsequestrin-1 gene.

Genomic DNA purification from white blood cells

Genomic DNA was isolated from patients' blood with thyroid disease (Graves). Promega genomic DNA purification kit was used for this purpose. The blood samples were sorted in 4x300 µl clean, DNase, RNase, pyrogen free 1.5 ml tubes, 900 µl of cell lysis solution have been added to each tube and inverted 5-6 times. Incubated at RT for 10 min the tubes have

been inverted 2-3 times to lyse the remaining red blood cells and then centrifuged at 18000g for 1 min for each 300 µl sample. The supernatant from the blood was aspirated and 300 µl of nuclei lysis solution were added to the pellet to completely lyse the white blood cells and then incubated for 1 hour at 37°C. RNase solution, 1.5 µl in volume, was added to each tube and gently mixed by inverting them before incubation at 37°C for 15 minutes. After 100 µl of protein precipitation solution was added to the tubes they were vortexed for a few seconds and then centrifuged again at 18000g per 10 min at RT. The supernatant was then transferred to a new, clean set of 1.5 ml tubes containing 300 µl of isopropanol and then gently mixed until a white thread of DNA became visible. Centrifuged at 18000g for 5 min at RT the samples' supernatant was then gently decanted and a 100 µl volume of 70% ethanol (stored at -20°C) was added and gently washed by several rotations. A further centrifugation at 18000g for 5 min was completed and the ethanol aspirated, letting the solution to air-dry for 10-15 min. DNA rehydrating solution, 100 µl per tube, was added before incubating the tubes at 65°C for 1 hour and then stored at 2-8°C until it was used. To measure the purity of the DNA extracted each tube has been measured with the spectrophotometer at length between 260 and 280 nm. The DNA samples isolated and stored in 4 different tubes each have been added together and diluted 1:10 into a 0.5ml tube. The concentration of the DNA was then normalised to 50 µg/ml of DNA.

Primers

The next step in the research was to design primers that would cover the entire gene. Invitrogen customer primer design tool was used to select the correct forward and reverse primers that would respect the following criteria:

- Melting temperature of the primers;
- % of GC, between 50 and 60%;

- Avoiding terminations in T or A, because of the possible dimerization with other primers that would cause the PCR product to be an underestimation of the results.

Once the 15 forward and reverse primers (Table 2) arrived they were reconstituted using TE (10mM Tris-HCl, pH 8.0, 1mM EDTA) added at proper volume according to the manufacturer's instructions. The reconstituted primers were then stored at -20°C until use.

Promoter rs7412379 forward	AGC CCC AGC TTC ATT TAC CT
Promoter rs77412379 reverse	CCC ACC TGG TCC TGT CTA AA
5UTR Exon 1 rs74123280 forward	ATT CCT CCA CCT GAC CCT TT
5UTR Exon 1 rs74123280 reverse	CCC CAT CCT GTC TGT AGC AC
Exon 1 rs35923530 forward	GCT AGG GAC ACC CAA GTC AG
Exon 1 rs35923530 reverse	CTC ACC TCC AGG ATC AGC TC
Exon 3 rs3747623 forward	GAT TAT CAA ACG GGC TGT GG
Exon 3 rs3747623 reverse	CCA GGG GAG TCC TTA CAT CA
Exon 4 rs34415466 forward	CAC ACA CCC ACA CAC TGT CA
Exon 4 rs34415466 reverse	GTG AGA TGG GGA TCC AGA GA
Intron 1 rs3838216 forward	TAT GGT TGC AGG AGG TGT CA
Intron 1 rs3838216 reverse	AGG GGT GCA CAA AAT GAG AG
Intron 3 rs67278792 forward	CTA CCA ACC CCA ACC TGA CA
Intron 3 rs67278792 reverse	CAT GTG GTG TGT GTG TGC AT
Intron 4 rs2275703 forward	TCT CTG GAT CCC CAT CTC AC
Intron 4 rs2275703 reverse	GTC AGT CTC TGG GGA AAC CA
Intron 5 rs3818952 forward	CAA CCC CAG GCA AGT TAA GA
Intron 5 rs3818952 reverse	CCA TGA AGG CCT CGT AGA AA
Intron 6 rs2275704 forward	ACC TTC GTG GAG GAG CAC AG
Intron 6 rs2275704 reverse	CAC TGG TGC CCA AAG TGT ATT
Intron 9 rs12132035 forward	GGC CTT GGA CTG TGA CAT CT
Intron 9 rs12132035 reverse	TGT GCC TCA CAA CAT TGG TT
Intron 10 rs12133035 forward	TGG TCC CAT ACT GGG AGA AG
Intron 10 rs12133035 reverse	GAA TTT GAA CTG GGC CCT GA
Exon 11 rs13375800 forward	ACC AGG CCA GCT CTC TCT TA
Exon 11 rs13375800 reverse	GCC AAG GAC AGG ACA GTC AG
Exon 11 rs74123288 forward	CCC ACT GGT CTT TTC ATG CT
Exon 11 rs74123288 reverse	GAG AGC TGG CCT GGT GTC TA
Exon 11 rs74123289 forward	ATA TGC TGG GTG CTG AGA CA
Exon 11 rs74123289 reverse	TGC ATA GAT GGA GGG AAC AG

Table 2. List of CASQ1 primers (forward and reverse) designed to cover the whole CASQ1 gene and ordered through Invitrogen.

Polymerase Chain Reaction (PCR)

Use of PCR technique was involved to provide evidence on the correct amplification of the gene. The first PCR screening was carried on 21 samples only and the patients' amplifications (Fig. 13) frequently lack of the gene region amplified by the 11th primer (Fig. 14a-14b), the region amplified by the 11th primer covered intron 9 region of the gene, previously mentioned to be site where one of the SNPs of our interest is located (rs17368853).

```
ctgctatccttaaggtggggccttggactgtgacatcttcttagtgagggc
tttgtttcagaggtcccatccccacctcctagctggggggccttggagG Intron 9
gtatgcgtgttgcattgcttaagggcactggtggcatggagggcatcctga G -> T
gcaaggggaaacaggacggacatcacacattcccagagcagaggctgg rs12132035
atgggtagctcaggtgctagggctgactcctaaagaaaccaatgttgtg
aggcacaatagtcctgcacatgggtatacacactcaatcatagaatcata
gcacagagattcctgtttcaggagatgattttgttctcagctacattcc
caacacctagcactcGctgttgaatgagtaactgaaagaacaagagaaa rs17368853
```

```
cgtgagaaacattggcacttaccaggtatacttgccctatttttagaaatg G -> A
taagatctgagggccagagagaagtgacttgaccttgaacacaaggatct
gatccaggatcagaacaggggttccctatccagttttcctcagaaaagagg
ggccagtgcaccagctgaccctcccgatctctccttttaggatttctc
ctcttttgttgttagttctacccttgcccttgatgcctcacagggtggac
ctgtccccatccactgccagttcgactgtcccctctccatccctgggct
gaccctcacactcatcctcctggattcatgtgctccctagtatctattaa
gttgctgtattttgatctctctacagCTGGTCCCATACTGGGAGAAG
```

The sequence above belongs to the human CASQ1 protein, in particular the region where intron 9 sits. The sequences highlighted in dark red are the primers designed to replicate the region in between. The section highlighted in pale blue is the one that contains the polymorphism. It might be that in patients the transposon region, normally inactive and non-movable, becomes active and in the moving it brings with it flanking regions causing the non-amplification of the region.

The amplification of each patient's CASQ1 gene was done using a polymerase chain reaction (PCR) technique. The PCR master mix (MM) included the following buffer: 166 mM (NH₄)₂SO₄, 670 mM Tris-HCl, 37 mM MgCl₂ working into a pH range between 8.0 and 8.3. The reagents added to the MM were the following: 14.2 µl dH₂O, 3 µl 10x buffer, 4.5 µl

dNTP (5 mM), 1.5 µl DMSO (1 M), 3 µl Betaine (5 M), 0.3 µl Life Technologies Platinum Taq DNA Polymerase, 1 µl primers (5 picmol/µl), 2.5 µl DNA (20 ng/µl). The reactions were performed using an MJ Research PTC-200 Peltier Thermal Cycler machine set on the following protocol:

- **Denaturation 95°C for 7 min**

- **Amplification's cycles:**

95°C for 1 min	95°C for 1 min	95°C for 1 min	95°C for 1 min	95°C for 1 min	95°C for 1 min
65°C for 1 min	63°C for 1 min	61°C for 1 min	59°C for 1 min	57°C for 1 min	55°C for 1 min
72°C for 1 min	72°C for 1 min	72°C for 1 min	72°C for 1 min	72°C for 1 min	72°C for 1 min
}	}	}	}	}	}
2x	2x	2x	2x	2x	30x

- **72°C for 5 min** (stored at 4°C O/N)

The amplified samples were run on the gel prepared on the day with 3g of agarose type I in 100 ml stained TEA 1x (SYBR Save DNA gel stain 10,000x concentrate in DMSO, 40 µl per 1L of TEA 1x) and microwaved until boiling before the DNA was added to the wells, then poured into the assembled chamber with an 18 well comb. The chamber was filled with TEA 1x and 10 µl of each sample was mixed with 1 µl of Bio-Rad Nucleic Acid Sample Loading Buffer 5x. The marker used was: of Bio-Rad EZ Load 100bp Molecular Ruler and 5 µl were added as reference to our PCR products. The gels were run at 100 V per ~1 hour for a good bands' separation. Sequence of Primers and the regions for amplification)

- Western Blot study -

Introduction

Early results from our laboratory showed that the calsequestrin-2 gene was up-regulated in thyroid tissue from patients with Graves' ophthalmopathy (GO) compared to those with no eye signs.

The aim of this study was to measure the CASQ1 protein levels in thyroid tissue extracts from patients with Graves' disease with and without ophthalmopathy and control subjects, which were patients with thyroid carcinoma, by quantitative Western blotting.

We hypothesize that CASQ1 concentration in thyroid tissue from patients with Graves' hyperthyroidism (GH) or GO are different compared to the ones found in normal thyroid tissue and that these differences may reflect either autoimmune reaction or hyperthyroidism.

Samples size and subjects' clinical description

Thyroid tissue was obtained fresh at thyroidectomy from 28 patients with Graves' disease, 6 males and 22 females aged 11 to 75 (mean age 36.29 yr) of whom, 2 males and 5 females aged 17 to 75 (mean age 43.71 yr) had obvious ophthalmopathy, 22 patients, as control thyroid with multi-nodular goitre, thyroid cancer or toxic nodular goitre, 4 males and 19 females aged 35 to 82 (mean age 43.92 yr) (Table 3). The diagnoses of the various disorders were based on standard clinical criteria and confirmed by thyroid function testing, thyroid ultrasonography and immunological tests. The grade, severity and activity of any associated ophthalmopathy were classified as: I) Nunery types 1 (without restrictive myopathy) or 2 (with restrictive myopathy) (3) II) the clinical activity score (CAS) (0-10) of Mourits *et al.* (19) which is a measure of disease activity and III) Werner's NOSPECS class (44). For the purposes of the present study "obvious" ophthalmopathy was defined as a CAS of 3 or more plus a NOSPECS class 2 or more. Isolated upper eyelid retraction and/or lag were not taken

as “ophthalmopathy”. All patients, GH and GO, are treated with anti-thyroid drugs before surgery and a total thyroidectomy was performed as an alternative to radio iodine therapy, the effect of these drugs on calsequestrin-1 protein expression is unknown.

Local Ethical Committee approval was received for the study and informed consent of participating subjects was obtained.

Group	Number	Mean age (years)
GRAVES' DISEASE	28	36.29
Males	6	
Females	22	
GRAVES' OPTHALMOPATHY	7	43.71
Males	2	
Females	5	
CONTROL	22	43.92
Males	4	
Females	18	

Table 3. Demographics and clinical details in population of patients with thyroid autoimmunity and control subject without autoimmune disease.

Quantitative Western Blotting methodology

A quantitative western blot method was used to measure concentrations of the CASQ1 protein in cellular extracts of thyroid tissue. Test and control (normal) thyroid tissue specimens were collected at thyroidectomy and stored at -70°C until used. Frozen tissues were thawed on ice, minced to small pieces in homogenisation buffer.

Every tissue sample was left in 1 ml Whole Cell Extract Lysis Buffer for the whole process of protein extraction. This buffer was made of:

- 400 µl of 400mM KCl;
- 50 µl of 50 mM Hepes pH 7.4;
- 3 µl of 1.5 mM EDTA ph 8.0;
- 1 µl 1 mM DTT;
- 200 µl 100% Glycerol;

- 100 µl of 10 mM Na₂MoO₄ (Sodium Molybdate);
- 100 µl of 20 mM NaF (Sodium Fluoride);
- 10 µl of 100 µM Na₃VOH (Sodium Orthovanadate);
- 5 µl of 0.5% Tergitol-type NP-40 (IPGEL);
- 100 µl of β-glycerol P 20 mM;
- 10 µl of Proteases inhibitors (100 x 1ml);
- 2.3 µl of PMSF (added at the end because of its high sensibility);
- 18.7 µl of dH₂O to adjust the total volume of the solution;

The whole process of lysis of the tissue cells had been done on ice to preserve the stability of the proteins within the tissue and to avoid the proteases to start degrading the rest of the cells.

After being homogenized the samples have been centrifuged for ~1 hour at 18,000 g in the cold room and the supernatant collected. Total protein concentrations from each patient were measured by performing an ELISA test, BCA Protein Assay Kit from Thermo Scientific according to manufacturer's instructions.

Equal amounts of patients' thyroid tissue extracts, 50 µg/ml, were loaded onto NuPAGE® Novex 4-12% Tris-Bis gels. Following electrophoresis, samples were transblotted to PVDF membranes, incubated with a 1:1000 dilution of calsequestrin 1 antibody (Santa Cruz Biotech c#:H-60), a rabbit polyclonal IgG (Santa Cruz Biotech c#:sc-28274). The secondary antibody was diluted 1:10,000 and it was an HRP-conjugated goat anti-rabbit antibody (Santa Cruz Biotech c#:sc-2004), or anti-mouse antibody for GAPDH (Millipore c#:MAB374). Membrane proteins were identified by chemiluminescence using SuperSignal Western Blot Kit and scanned in a Universal gel documentation Hood using Quantity One 4.4.0 ChemiDoc software which enumerates the pixels in matching CASQ1 and GAPDH bands (Fig. 11) from which the CASQ1 protein concentration can be determined as a CASQ1:GAPDH ratio.

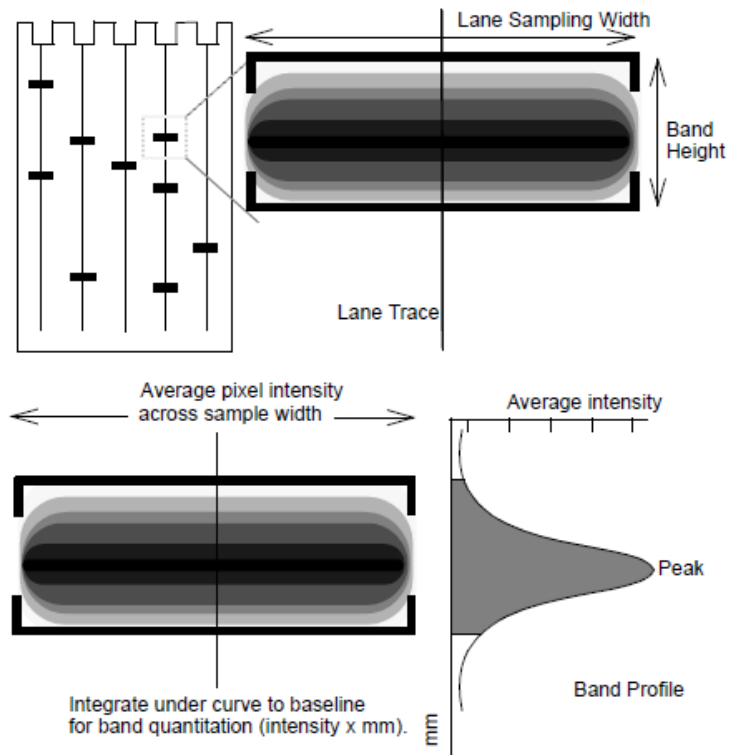


Figure 11. Illustration of bracket quantitation used by the software to determine the density of the band and from there calculating the concentration of protein with extreme precision (45).

Using CASQ1 protein standards (Fig. 12), the protein levels are determined as pmol CASQ1/mg total thyroid proteins in the extracts, for both the 50 kDa and 60 kDa forms of the protein.

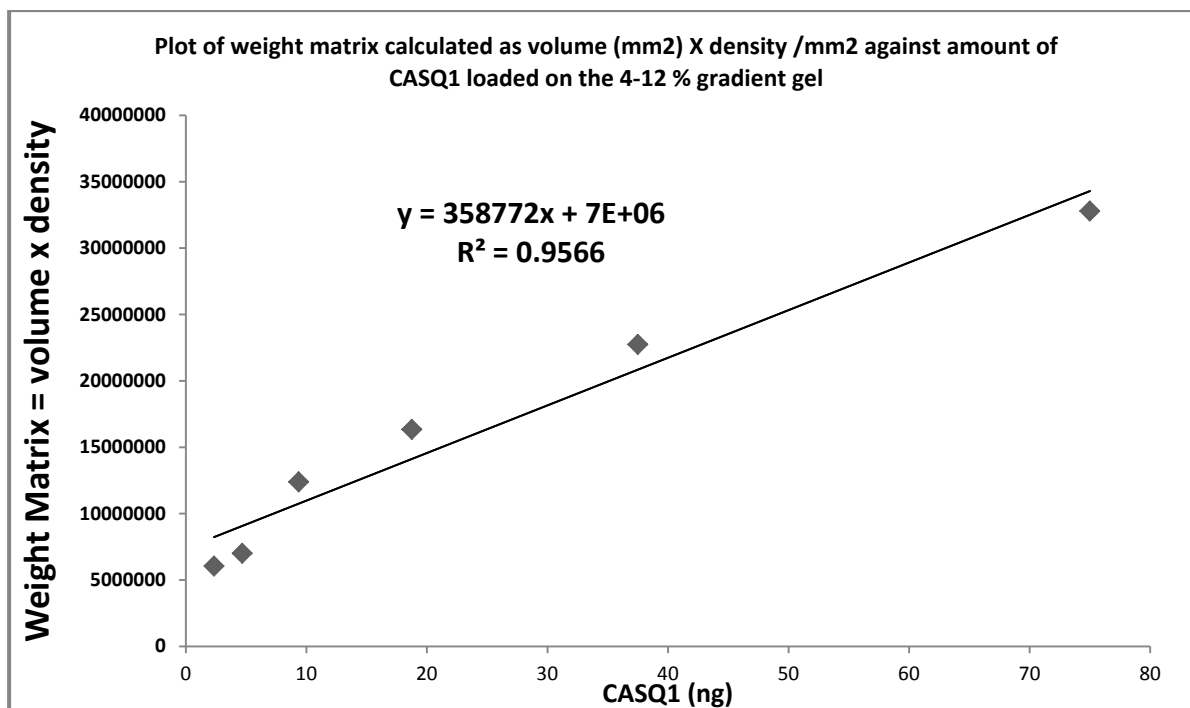


Figure 12. Calsequestrin-1 standards read with Quantity One 4.4.0 ChemiDoc software and plotted to generate the equation then used to calculate the amount of CASQ1 protein per patients.

Quantity One 4.4.0 ChemiDoc identifies the bands by quantitating the average intensity of the bands scanned and selected by the user. The intensity profile of each band is then processed to have a result in INTENSITY x mm, expressed in Table 4.

Gel name : GEL 1 56-62 9-5-2012 2 (Raw 1-D Image)						
Index	Name	Volume	Concentration	Area	Density	
		INT*mm ²		mm ²	INT/mm ²	
1	U1	27273.7640612447	N/A	129.8014372432	3098.3324109616	
2	U2	15953.9801007835	N/A	80.5664093234	2919.9638158850	
3	U3	14868.5715307326	N/A	89.8573167958	2439.9348636031	

Table 4. Program output of three selected bands from a scanned gel. Volume and Density values of each band are then used to calculate the ‘weight matrix’, which represents the ‘y’ in the linear equation extrapolated from the CASQ1 plotted standards.

Once we have the values in nanograms of CASQ1 protein we can determine its ‘weight matrix’ (Table 5) we then determine the weight matrix for GAPDH (Table 6), relative to the

same subjects. The ratio of the two matrixes is used to correct the nanograms values for CASQ1 protein. The protein concentrations, nanomoles of CASQ1 over milligrams of extract (Table 7), is then obtained.

Subject #	Gel 1 Upper band Volume	Gel 1 Upper band Density	Gel 1 Upper band (weight matrix) (Vol * Density) <u>The 'y' in the CASQ1 standards'</u> <u>equation</u>	ng of CASQ1 protein <u>The value of 'x' in the</u> <u>equation</u>
56	27274	3098	84494852	216
57	15954	2919	46569726	110.292
58	14868	2439	36263052	81.5645

Table 5. Weight Matrix for CASQ1 bands and ng of protein on each band on the gel.

Volume of GAPDH	Density of GAPDH	Weigh matrix for GAPDH	Weight Matrix of CASQ1/ Weight matrix of GAPDH <u>(ratio of changes for loading to gel)</u>
9091	1797	16336527	5.172142892
6188	1649	10204012	4.563864292
5062	1541	7800542	4.6487862

Table 6. Weight Matrix for GAPDH, ratios of the weight matrixes used to adjust CASQ1 protein ng values.

Corrected ng of CASQ1 (ratio * nanograms)	nmol of CASQ1 = ng/60000 dalton (weight of the upper band)	pmol of CASQ1 = nmol*1000	pmol CASQ1/mg total protein extract = (pmol*1000 /0.05 mg protein loaded on the gel)
1117.184306	0.018619738	18.61973844	372.4
503.3582875	0.008389305	8.389304792	167.8
379.175834	0.006319597	6.319597233	126.4

Table 7. Corrections according to the weight of the band (50kDa or 60kDa, respectively, lower and upper band). Final values for CASQ1 protein expressed as picomoles over milligrams of total protein from each extract.

This method of obtaining CASQ1 protein levels can be achieved thanks to the great precision of the software used. The reason for these calculations is that having a concentration, instead of a ratio of CASQ1 and GAPDH, allows our results to be compared to others with a great level of precision.

Chapter III: Results and discussion from SNPs study

Introduction

The results from this first part of the study might change a little, maybe expand, the way *casq1* gene roles are seen within the Graves' disease context. This study identifies 4 informative SNPs in calsequestrin-1 gene, each of them with their entourage of speculations over the effect and function implicated in different aspects of the protein's roles.

Materials and methods

Use of bioinformatics tools, such as <http://ecrbrowser.dcode.org/>, has been extensively employed to circle the 'critical' SNPs in *casq1*. Subsequently a set of primers have been designed to specifically cover the entire length of the gene. Ultimately, PCR technique was involved to amplify the entire *casq1* gene.

Results from SNPs study

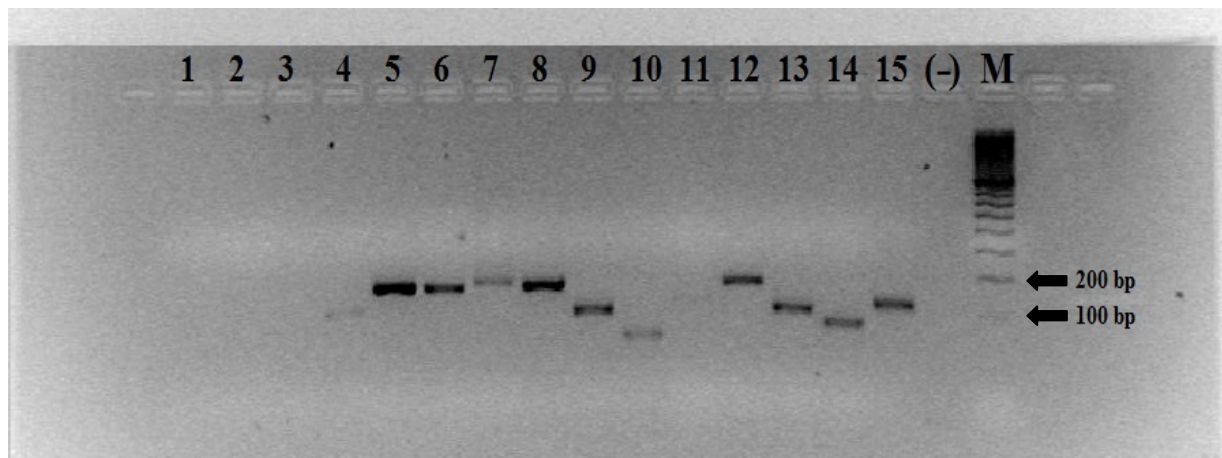


Figure 13. Typical gel image of PCR product lacking the amplification of the gene covered by the 11th primer. The latter primer covers the region of the calsequestrin-1 gene where Intron 9 is present, spot of a highly conserved polymorphism. Lines 1, 2 and 3 ran the primers that covered the first part (promoter area) of CASQ1 gene, the not amplification of these regions is maybe due to the fact that the promoter region was rich in GC content and the polymerase enzyme couldn't continue the transcription. These regions of the gene also included SNPs.

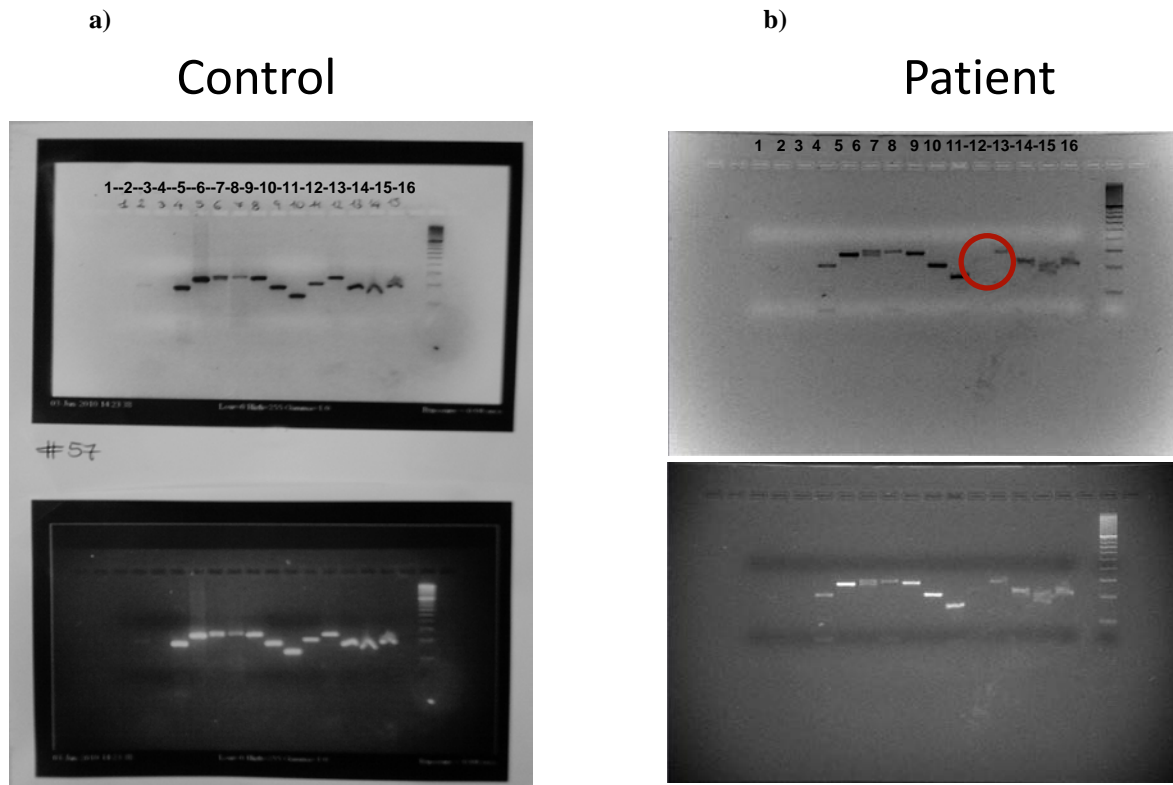


Figure 14a. Gel amplifications of a control patient **14b.** On the gel, circled, absence of amplification of section 11 that covers intron 9 region on CASQ1 gene. This part of the gene also contains transposons that are normally inactive and not moveable.

Subsequently, CASQ1 polymorphisms were genotyped from a bigger cohort of 183 subjects (Table 9) that included patients with Graves' disease or Hashimoto's thyroiditis and normal subjects used as controls. DNA samples were extracted from patients and controls white blood cells and purity of the DNA was assessed. The samples were then normalized to 400 ng of DNA in a 96-well plate template as requested by AGRF (Australian Genome Research Facility) and sent for sequencing using MassARRAY SNP analysis (a technology used for SNP genotyping, methylation detection and quantitative gene expression analysis) using iPLEX technology of SEQUENOM. iPLEX is another type of technology developed by SEQUENOM, it allows to genotype polymorphisms in genes associated with drug absorption, distribution, metabolism, and excretion.

The study identified the presence or absence of the 22 SNPs and the data generated narrowed down the initial selection from 22 to only 8 SNPs (Table 8) through selection criteria such as Chi-square and p-value (Table 10). Although at the beginning of the study the cohort wasn't big enough it showed a tendency to the significance of some of the SNPs. During the analysis the equilibrium of the alleles' frequencies was respected (Hardy-Weinberg equilibrium). Other statistical analysis like "Linkage disequilibrium" would have been a useful tool to identify small chromosomal regions such as the one discussed in this thesis, but at that stage the sample size wasn't big enough.

SNP position, name and modification	SNP sequence
Promoter rs74123279 G → A	Gagggaaggagcccctgggagcctg gggtagaagtgtaggaggtgggaggat
Exon 1 UTR before ATG start, rs74123280 A → G	Ccctt GGCTCTGTCTGGCAGTTTCTCCAAGGACCCAGCAGTGCC CTCTGTCCAC
Intron 1 rs3838216 [-/ggcattcagataggcct] Insertion	agctcct gtggccccttctctggcct cagctgtccagagcttgagcatt
Intron 2 rs3747623 C → T	gaagcctgggatcatggctcactaccacc ctgcgccggctcactcct
Intron 4 rs2275703 A → C	tgtactctgggggttcccagacaagcacctccagtattccacctc Ccctca
Intron 9 rs17368853 G → A	agctacattcc caacacctagcactcgtgttgaatgagtaactgaaagaac
Intron 10 rs55887151 A → G	Ggaagttcagtggtgcaagagcacgggagacagattgaaggg agaaggtcta
Exon 11 rs74123289 C → T	TTTTCCTAGACACCAGGCC AGCTCTCTCTTATCTGACTTCTGTTTCTTATC

Table 8. Sequence of the 8 highly informative SNPs identified in CASQ1 human gene, in red the promoter, dark yellow for the exons and turquoise for the intronic sequences.

Assay	Common Homozygote	Heterozygote	Rare Homozygote	Fails	Assay Pass Rate	Population Size	Major Allele Frequency	Minor Allele Frequency
rs17368853 Intron 9	169	11	0	3	98%	180	0.969	0.031
rs2275703 Intron 4	62	80	32	9	95%	174	0.586	0.414
rs3747623 Intron 2	65	79	36	3	98%	180	0.581	0.419
rs3838216 Intron 1	97	72	9	5	97%	178	0.747	0.253
rs55887151 Intron 10	177	4	0	2	99%	181	0.989	0.011
rs74123280 Exon 1	179	2	0	2	98%	181	0.994	0.006
rs74123289 Exon 11	180	2	0	1	99%	182	0.995	0.005
rs74123279 Promoter	111	63	6	3	98%	180	0.792	0.208

Table 9. Genetic parameters used to identify informative SNPs in the cohort of 183 DNA samples from patients with thyroid autoimmune disease and normal controls. Chi Square and P values tests were performed to confer statistical relevance to the observations. Hardy-Weinberg equilibrium respected (major allele + minor allele = frequency of 1).

Assay	Expected Common Homozygote	Expected Heterozygote	Expected Rare Homozygote	Chi Square	P value (1 df)	Significance
rs17368853 Intron 9	169.168	10.664	0.168	0.179	0.672	
rs2275703 Intron 4	59.793	84.414	29.793	0.476	0.490	0.5<P<0.3
rs3747623 Intron 2	60.668	87.664	31.668	1.758	0.185	0.2<P<0.1
rs3838216 Intron 1	99.376	67.247	11.376	0.889	0.346	0.5<P<0.3
rs55887151 Intron 10	177.022	3.956	0.022	0.023	0.881	
rs74123280 Exon 1	179.006	1.989	0.006	0.006	0.940	
rs74123289 Exon 11	180.005	1.989	0.005	0.006	0.941	
rs74123279 Promoter	112.813	59.375	7.812	0.671	0.413	0.5<P<0.3

Table 10. Statistical selection criteria used to determine which SNPs to choose. P-values indicated in the “Significance” column indicate a tendency towards the significance.

Assay	SNP position	Alleles	Ancestral Allele	Gene(s)	Role	Amino acid change	Amino acid position	SNP relative to Chromosome
rs17368853 Intron 9	chr1:160169230	A/G	G	CASQ1	Intron	-	-	+
rs2275703 Intron 4	chr1:160165095	A/C	A	CASQ1	Intron	-	-	+
rs3747623 Intron 2	chr1:160163509	C/T	C	CASQ1	Intron (boundary)	-	-	+
rs3838216 Intron 1	chr1:160161633; 160161634	*/GGCATT CAGATAG GCCT	Not Available	CASQ1	Intron	-	-	+
rs55887151 Intron 10	chr1:160170799	A/G	A	CASQ1	Intron	-	-	+
rs74123280 Exon 1	chr1:160160386	A/G	A	CASQ1	5' UTR	-	-	+
rs74123289 Exon 11	chr1:160171371	C/T	C	CASQ1	3' UTR	-	-	+
rs74123279 promoter	chr1:151389828	G/A	G	CASQ1	Promoter	-	-	+

Table 11. Genetic parameters used to identify informative SNPs in the cohort of 183 DNA samples from patients with thyroid autoimmune disease and normal controls. The table shows: the SNPs tested (Assay column), the position on the gene (SNP position), the alleles changes of base (Alleles), the ancestral allele (Ancestral Allele), the gene on which the SNPs are analysed (Gene(s)), the region those SNPs are in the CASQ1 gene (Role), and the effect those changes in base have on “Amino acid change”, “Amino acid position” and “SNP relative to Chromosome” (minus ‘-’ if they don’t have an effect, plus ‘+’ if those changes have an effect).

In summary, the 8 SNPs were associated with different degrees of the diseases (Table 12):

- a) Introns 9, 4, 2, 1, 10, exon 11 and promoter SNPs in Graves’ disease without ophthalmopathy
- b) Introns 4, 2, 1 and promoter SNPs in Graves’ disease with Ophthalmopathy
- c) Introns 4 and 2 SNPs in Hashimoto’s Thyroiditis without ophthalmopathy
- d) Introns 4, 2 and 1 SNPs in Hashimoto’s Thyroiditis with ophthalmopathy
- e) Introns 4, 2 and promoter SNPs in Silent Thyroiditis

f) Introns 4, 2 1, exon 1 and promoter SNPs in Pregnant women with Graves' hyperthyroidism without ophthalmopathy

Disease Status	rs17368853 Intron 9- Alleles A/G	rs2275703 Intron 4- Alleles A/C	rs3747623 Intron 2- Alleles C/T	rs3838216 Intron 1 - Allels - /GGCATTCA GATAGGCCT	rs55887151 Intron 10 - Alleles A/G	rs74123280 Exon 1 - Alleles A/G	rs74123289 Exon 11 - Alleles C/T	rs74123279 Promoter - Alleles G/A
Graves' Hyperthyroidisms	2	8	9	9	2		2	5
Graves' Hyperthyroidism + Ophthalmopathy		20	16	11				13
Hashimoto's thyroiditis		1	1					
Hashimoto + ophthalmopathy		2	1	2				
Euthyroid		1						
Silent Thyroiditis		4	1					1
Graves, Pregnant		2	1	3		1		2
Unclassified Graves	3	17	15	8				9
Normal controls		2	2	1				

Table 12. Classification of the disease associated with informative SNPs in the first cohort of 183 subjects. The total number of patients tested reaches 177 because of the ~96% success rate of the sequencing machinery involved. The numbers in the cells of this table represent the number of patients from the cohort that fall into the reported categories on the axis of the table (vertical-right and horizontal-top).

Use of MassARRAY SNP analysis was involved again to sequence a bigger cohort of patients and controls; the increase in the cohort was used to narrow down our selection to just 4 SNPs on which we have concentrated our study:

1. Promoter (*rs74123279*);
2. Intron 1 (*rs3838216*) SNP which shows a strong association to thyroid disorders (Graves' ophthalmopathy) (42);

3. Exon 11 (*rs74123289*) SNP:

```
ATATGCTGGGTGCTGAGACACTGA rs74123288
TCCCCCTCATTGATGAGCAAATGAGCTACTTTTCCCTAGACACCAGGCC
AGCTCTCTCTTATCTGACTTCTGTTTCTTATCCATAACTTACTTGTATC C→T
TATTATGTGTCTCTTCCATCACTCTCCATACTCTTTCTTGTGATTCTCCT Exon11
CTAGCCATATATATGGGCCCATCTCTGTTCTGTTCCCTCCATCTATGCA rs74123289
```

The sequence above consists of primers (dark red sequences), transposon (green sequence), sequence where the SNP is located (pale blue) and exon sequence (dark pink).

(source: www.ecrbrowser.dcode.org)

4. Intron 9 (*rs17368853*) SNP in Intron 9 which has been found absent in many patients suffering from severe ophthalmopathy, unfortunately this type of study hasn't been performed on controls (cancer patients) because of their absence at the beginning of the study, but it would be a good point to expand later on when more controls will be collected.

The second SNP of the list was then identified in DNA samples from 441 subjects (99 males and 342 females) from different groups: GO (n = 75), GH (n = 131), Hashimoto thyroiditis (HT) (n = 96) and control subjects (n = 108) (total of 440 due to assay failure for 1 subject) (Table 13).

Group	Number (%)	Years \pm SD
Male	99 (22)	41.5 \pm 16.6
Female	342 (78)	49.2 \pm 15.8
Graves Ophthalmopathy (GO)	75 (17)	52.4 \pm 14.6
Graves Hyperthyroidism (GH)	131 (30)	48.7 \pm 15.8
Hashimoto's (HT)	96 (22)	51.8 \pm 15.3
Hashimoto's with Ophthalmopathy	4 (1)	46.5 \pm 19.3
Control	108 (24)	35.7 \pm 12.8
Common Homozygote	246 (56)	46.6 \pm 15.8
Heterozygote	171 (39)	49.4 \pm 16.8
Rare Homozygote	24 (5)	43.1 \pm 16.2
Total Population Size	441 (100)	
Major allele	663 (75)	47.7 \pm 16.2
Minor allele	219 (25)	48.6 \pm 16.8

Table 13. Demography, clinical details, genotypes and alleles of CASQ1 SNP rs3838216 in population of patients with thyroid autoimmunity and control subject without autoimmune disease.

Its evolutionary preservation supports the idea that CASQ1 gene SNP rs3838216 is a genetic marker for ophthalmopathy in patients with GH. Potentially, after an extended study on a larger population with different ethnic backgrounds, rs3838216 can become a detection parameter for ophthalmopathy.

The outcome of this study not only presents new ground in Graves' disease research but also points to other new directions such as calsequestrin functional roles and the link to Graves' disease. Such knowledge might improve screening and detection of this disease.

Discussion from SNPs study

This study set the basis for another point of view from where to look at the role of calsequestrin-1 in the Graves' disease context.

The results from this study presented a genetic perspective from where to speculate on its 2 main findings: the 4 informative polymorphisms, regulating different features of the gene, and the frequent lack of the region where intron 9 stands in patients with severe TED.

The set of 4 informative SNPs located on calsequestrin-1 gene are implicated in different aspects of the protein's tasks. Functions and interactions might be affected by changes in the sequence of these regions.

The first SNP, **rs74123279**, located in the promoter area, might play a reductive modulatory effect in the transcription of CASQ1 gene. CASQ1 protein is already present in the cell in very small concentrations (in the order of picomoles), a reduction in the final product could disrupt the cascade of fine regulation in which this protein is involved. This could be verified with further studies on the promoter area, designing a specific promoter with a luciferase flag attached to see whether there is a change in CASQ1 protein production subsequent to a change on concentration in thyroid hormone.

Intron 1 contains a highly conserved SNP, **rs3636216**, which has shown strong association to Graves' ophthalmopathy (*unpublished work*), later correlated to the CASQ1 protein levels in the second part of this study.

Another SNP, **rs74123289** located in exon 11 3' UTR has a translational activity control role through the binding of a MicroRNA. The alteration of this function might alter the level of expression of CASQ1 protein.

Finally, one of the most absent region in our PCR products happened to be the one where SNP **rs17368853** is. This polymorphism is located in intron 9 and its absence has been observed in many patients with severe ophthalmopathy.

The last SNP of this study was observed in concomitance with the absence of amplification of the intron 9 region in the PCR product of the gene. This missing region is observed both in subjects suffering from the disease and in subjects with history of thyroid disease in their families.

This region contains a highly conserved transposable element of 159 bp which is passed, or not passed, through the subject's germ line to the next generation.

Normal subjects, that don't present any symptoms of thyroid disease, will then still have this missing region in the amplified calsequestrin-1 gene product through PCR.

However, even if these normal subjects are not affected from Graves' disease, the absence of this region in calsequestrin gene could affect the production of CASQ1 protein.

The defective gene can give imperfect products and this may perhaps lead to its instability in patients with severe TED.

With these premises is reasonable to think that CASQ1 protein product might even be involved in processes the protein accomplish on several levels, such as:

- Function in terms of affinity of CASQ1 with calcium molecules;
- Structural changes and affected capacity to store calcium;
- Interactions with other protein partners;
- Localisation of CASQ1 protein in sub-cellular compartmentalisation and membranes;
- Productions of CASQ1 protein in normal levels;
- MicroRNA levels controlling expression of CASQ1 protein;

Chapter IV: Results and discussion

from Western Blot study

Introduction

The results from the second part of this study aimed to find a correlation between CASQ1 protein concentration and analysed groups (Controls, GH, and GO). The underlying hypothesis was that the concentration of CASQ1 protein in patients affected by Graves' disease, are different compared to those found in thyroids which are not engaged in autoimmune reactions or hyperthyroidism.

Materials and methods

This part of the study consisted in the collection of samples that respected the selected criteria reported in Chapter II: Methodology – Western Blot study. The latter were then subject to total protein extraction process, followed by protein quantification through Western Blot technique. The results were finally read and quantified with a Universal gel documentation Hood and Quantity One 4.4.0 ChemiDoc software.

Results from Western Blot study

Western blot analysis and quantitative densitometry were used to determine the concentration of the CASQ1 protein in extracts of thyroid tissue from patients with Graves' disease and, as control, normal thyroid tissue from patients with goitre or thyroid cancer. CASQ1 protein concentrations on 4-12% gradient gels were determined as a CASQ1: GAPDH ratio which was adjusted to CASQ1 concentration namely, pmol CASQ1/mg total protein, by reference to a CASQ1 protein standard. Two forms of CASQ1 in the thyroid were identified, with

molecular weights of 50 kDa and 60 kDa respectively (Fig. 15). Quantitative assessment demonstrated variable levels of the CASQ1 protein in Graves' and normal thyroid tissues with 17 patients showing reactivity against the 50 kDa protein and 85 patients showing reactivity against the 60 kDa protein. Examples of CASQ1 and GAPDH bands in representative patients and controls are shown in Fig. 16a-16b.

Next, we measured CASQ1 protein concentrations in thyroid tissues from 50 patients with Graves' hyperthyroidism (GH), 23 with Graves' ophthalmopathy (GO) and in 22 control subjects with nodular goitre or cancer. The results are summarised in figure 17, which shows the mean (Control 156.71 ± 30.14 ; GH 41.01 ± 8.09 ; GO 57.08 ± 17.87 pmol/mg) concentrations of the CASQ1 protein in the three groups of patients. The mean concentration of CASQ1 was significantly reduced in patients with both GH and GO (Mann-Whitney test, $p < 0.0001$, $p = 0.0012$ respectively) compared to the control subjects. Although mean CASQ1 level in patients with GO was greater than that in patients with GH this difference was not significant ($p = 0.193$) (Fig. 17). Reduced thyroid CASQ1 was also correlated with the homozygous genotype of the rs3838216 polymorphism in the CASQ1 gene (Mann-Whitney test, $p = 0.0223$) (Fig. 18). Next we correlated serum CASQ1 antibody titres in patients with GH and GO (Fig. 19) with constant concentration of the CASQ1 protein in thyroid and thyroid gland. The aim of this experiment was to show how the immune response was related to the production of Abs against CASQ1 and how much CASQ1 protein was still present in the titres of patients with GH and GO.

In order to determine whether the reduced CASQ1 levels in Graves' thyroid was due to the autoimmune reaction or the hyperthyroidism itself, we measured the amounts of the CASQ1 protein in toxic nodules obtained by careful sampling nodular tissues removed at thyroidectomy from 5 patients with toxic nodular goitre. The mean for the CASQ1 protein in toxic nodules was 74.5 ± 23.63 pmol/mg.

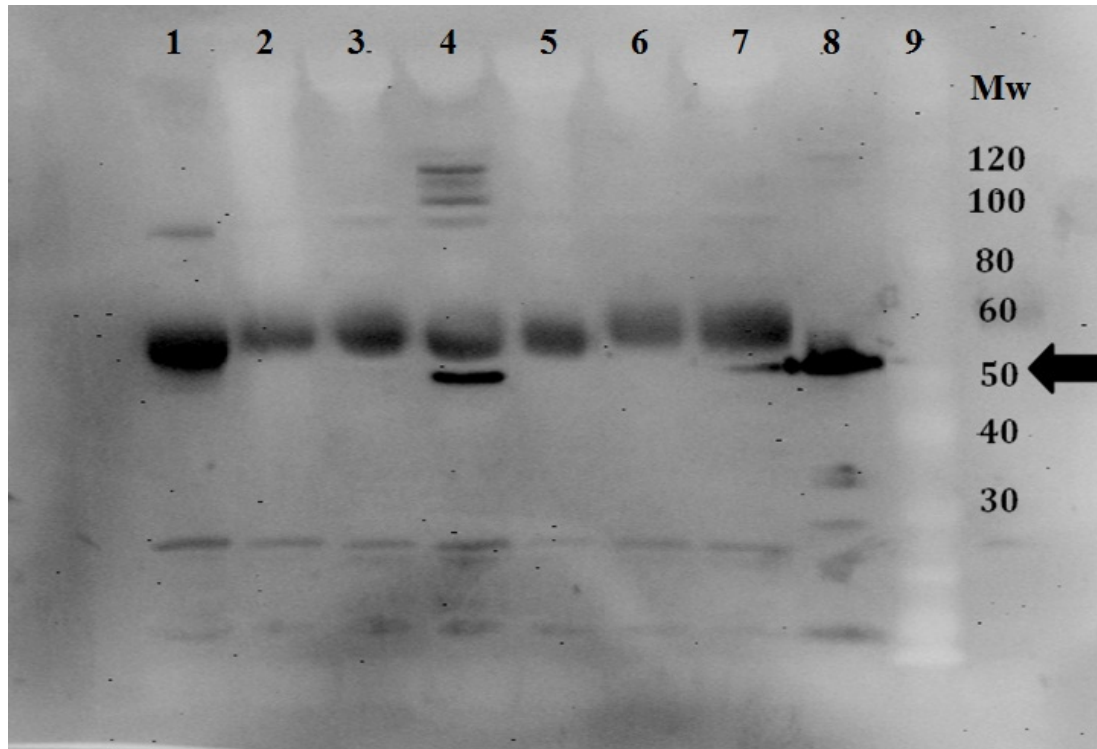


Figure 15. Western blotting of thyroid extracts from patients with Graves' hyperthyroidism (lane 4), Graves' ophthalmopathy (lanes 3 and 7) and (as normal thyroid) from control patients with multinodular goitre or thyroid cancer (lanes 2, 5 and 6), showing bands at 50 and 60 kDa. Lane 1 was an unknown thyroid patient's sample, lanes 8 and 9 were, respectively, positive and negative controls.

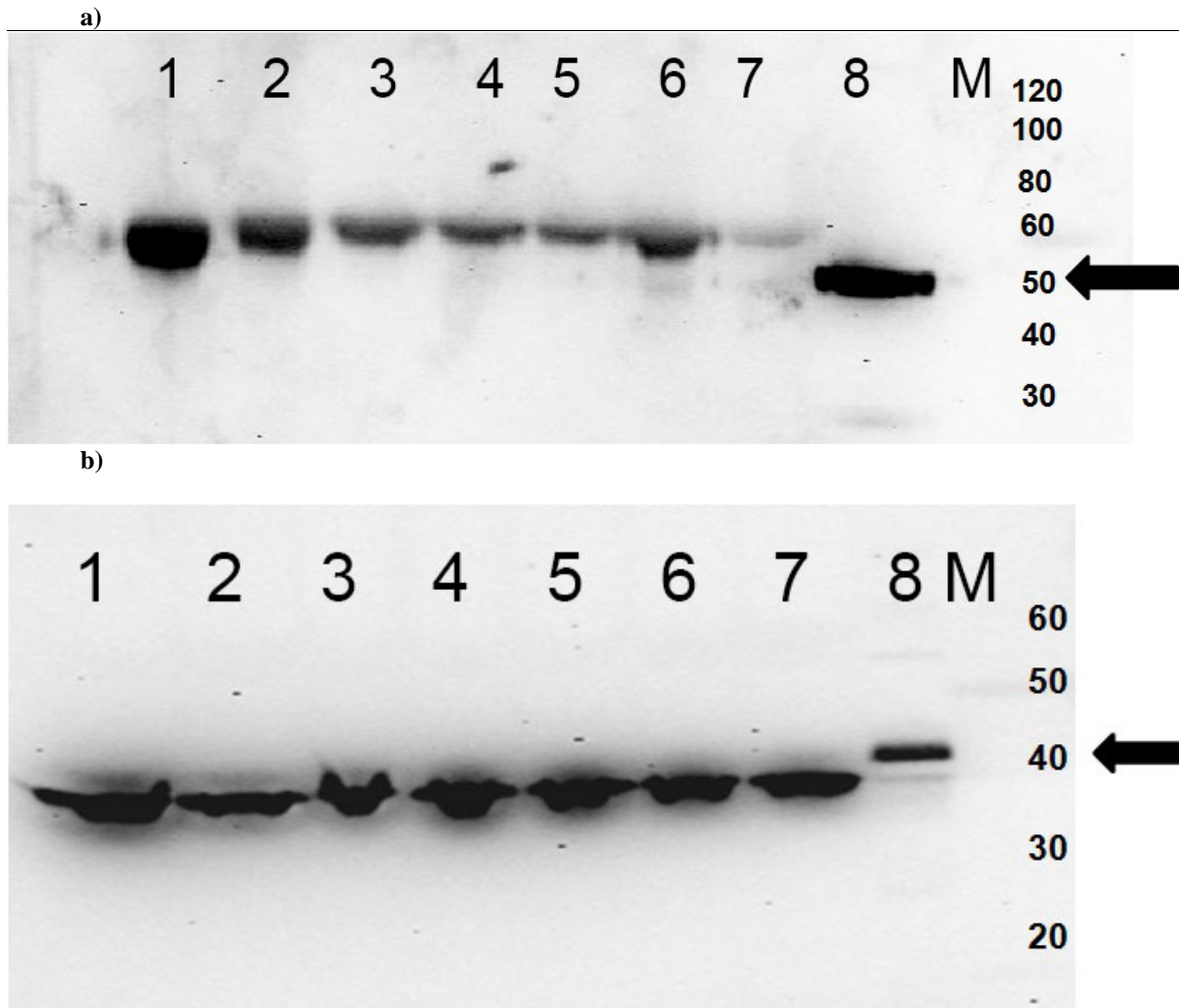


Figure 16a. PVDF membrane from western blotting of thyroid extract of protein from patients with Graves' disease, thyroid cancer or multinodular goitre showing various CASQ1 protein concentrations **16b.** PVDF membrane showing reactivity with GAPDH, a very abundant protein present in every cell used to normalize CASQ1 protein levels in thyroid extracts. Lines 1 and 2 were Controls, lines 3 to 5 and 7 were GH, line 6 was GO, line 8 was the standard and M as marker.

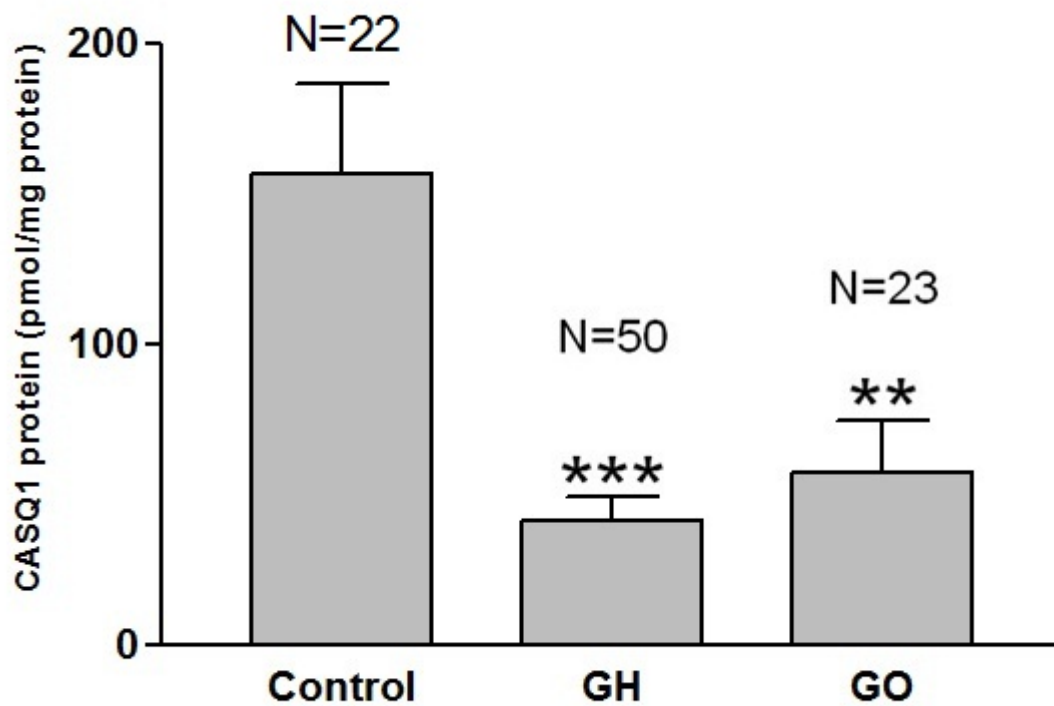


Figure 17. Mean (\pm standard error) of CASQ1 protein concentrations in thyroid tissues from patients with Graves' hyperthyroidism (GH, 41.01 ± 8.09 pmol/mg), Graves' ophthalmopathy (GO, 57.08 ± 17.87 pmol/mg) and, as normal thyroid, from control patients with multinodular goitre or thyroid cancer (156.71 ± 30.14 pmol/mg) determined from quantitative Western blotting. The difference between GH and controls and GO and controls was highly significant (Mann-Whitney test $p < 0.0001$, $p = 0.0012$, respectively).

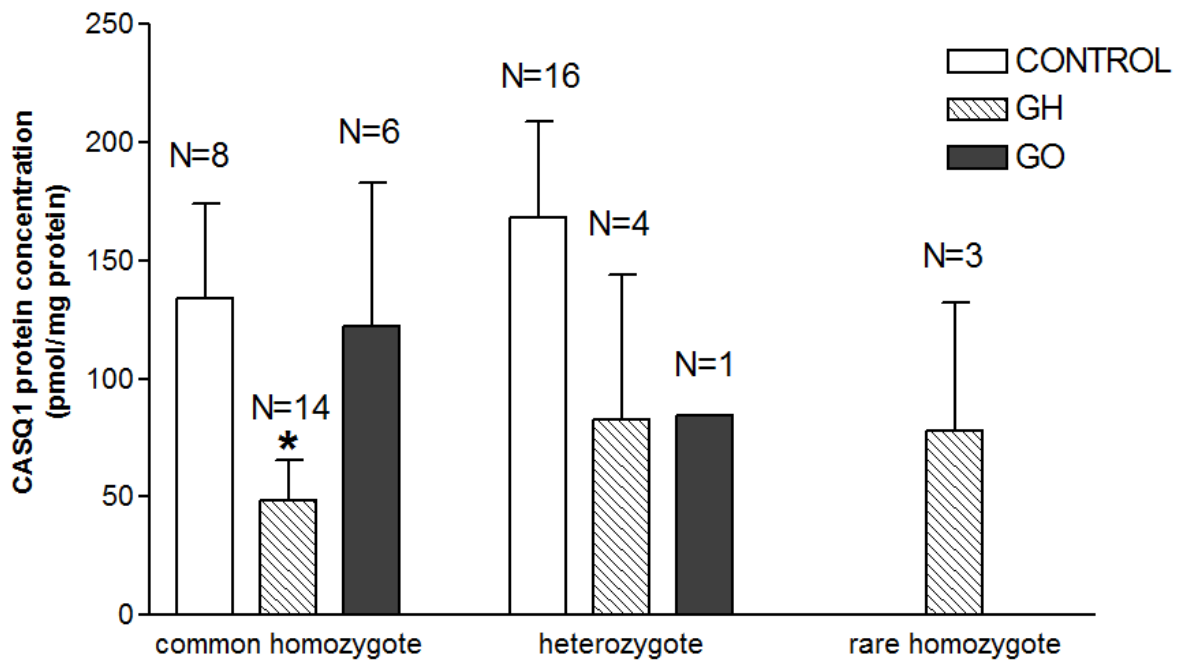


Figure 18. Correlation between mean (\pm standard error) CASQ1 protein levels and SNP rs3838216 genotype of the CASQ1 gene in patients with Graves' hyperthyroidism (GH), which was significant (Mann-Whitney test, $p = 0.0223$) for the homozygote genotype but not for the heterozygote genotype (Mann-Whitney test, $p = ns$).

Enzyme-linked immunosorbent assay (ELISA)

The presence and level of serum orbital antibodies in serum from patients whose thyroid tissue was used in the studies were determined using an enzyme-linked immunosorbent assay (ELISA). This procedure has been described in previous publications by this laboratory (5, 12, 38) and it is standard. The antigen used was highly purified rabbit skeletal muscle CASQ1 which shares 97% homology with human calsequestrin. Results were expressed as optical density (OD) at 405 nM. A positive test was taken as an OD over the upper limit of the reference range, which was 194.

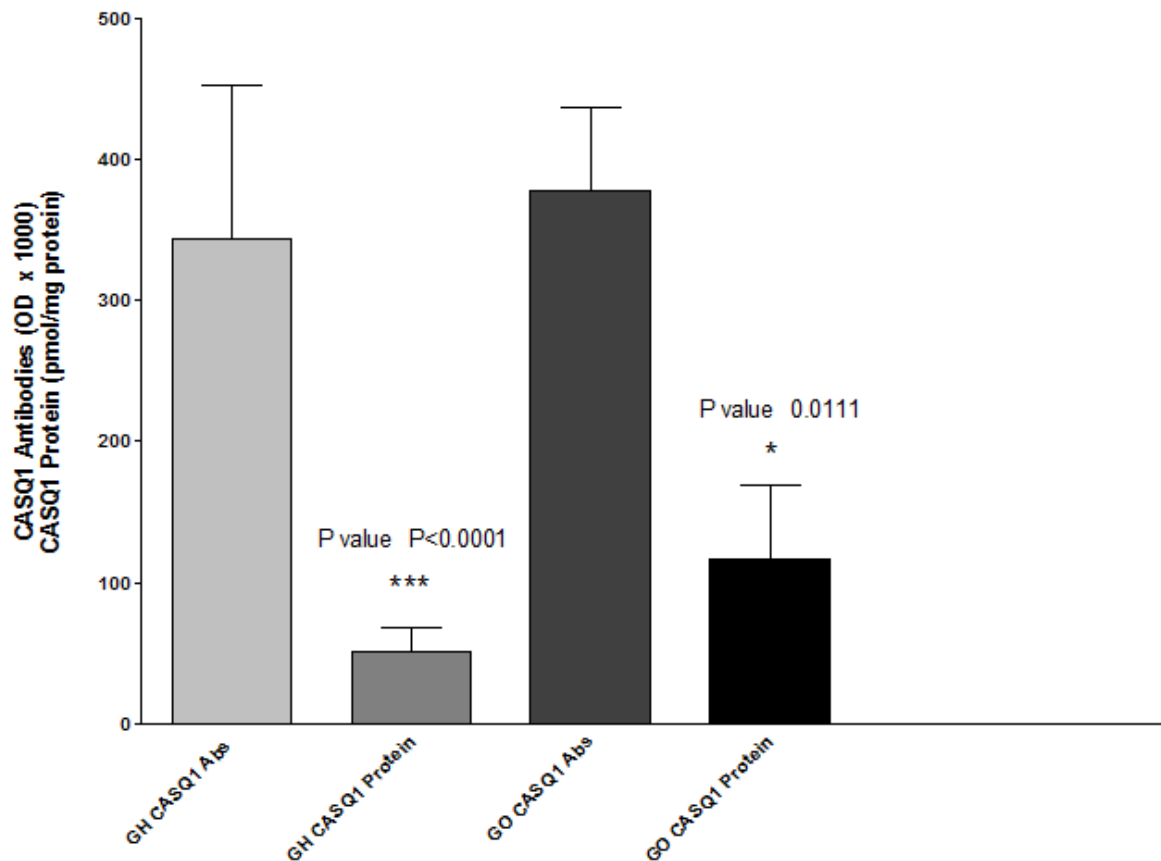


Figure 19. Levels of CASQ1 antibodies and protein antigen in GH and GO. There is clear significant differences between the antibody levels and protein antigen levels. Correlation between CASQ1 antibody levels determined by ELISA and CASQ1 protein concentration measured in quantitative western blot in patients with Graves' hyperthyroidism and Graves' ophthalmopathy.

Interestingly the presence of CASQ1 in thyroid is greater in controls than in patients with Graves' disease. This suggests that the presence of the protein there in some way relates to the underlying autoimmune reaction, which, we believe precedes the orbit inflammatory process associated with Graves' ophthalmopathy.

Genotype analyses of 52 subjects have shown a correlation between the Control group and the patients with Graves' Hyperthyroidism, linking the homozygote genotype to the disease.

The 50kDa and 60kDa forms of the CASQ1 protein do not appear to be products of two different genes or alternative splicing. The 50 kDa may represent the unprocessed or post-

translationally modified form of the mature protein. The 60 kDa form, the most abundant, appears to be the completely post-translationally modified protein.

Discussion from Western Blot study

The second part of the study involved the use of western blotting and density quantification allowing us to demonstrate that CASQ1 protein is present in the thyroid gland of patients with Graves' disease and in controls' thyroids, obtained from patients with cancer or multi nodular goitre.

CASQ1 protein is present in smooth muscle cells as well as in other tissues.

We showed the presence of two bands on the gel, at 50 kDa and at 60 kDa, in thyroid extract from both Graves' patients and controls; both protein bands are the product of CASQ1 gene and do not appear to be the results of two different genes or alternate splicing.

The two different molecular weights forms of CASQ1 protein may represent unprocessed and a mature form of the same protein; the 50 kDa may correspond to a CASQ1 protein that hasn't undergone post-translational modifications. The 60 kDa form, the more abundant, appears to be the post-translationally modified form of the protein.

Future use of immunofluorescence can determine where these two forms of CASQ1 protein are located, reaching the level of cellular organelles in the thyroid follicular cells and surrounding connective tissue.

The results from this study showed that levels of the CASQ1 proteins were lower in Graves' hyperthyroidism and Graves' ophthalmopathy compared to controls.

Further analyses from this study also took into account levels of CASQ1 protein in 5 patients with toxic nodular goitre and found that they weren't significantly lower compared to those in control thyroid ($p = 0.2663$).

Genotype analyses of 52 subjects from this study were also linked to one polymorphism newly found in calsequestrin-1 gene.

The results of this association showed a positive correlation between the reduced CASQ1 levels and the homozygote genotype for the recently identified CASQ1 polymorphism rs3838216 (*Lahooti, Cultrone, Wall et al, unpublished observations*) (previously shown in Fig. 18) and between the absence of this polymorphism and serum titres of CASQ1 antibody.

CASQ1 has been proven many times to be part of an ongoing process on the eye muscle component of Graves' ophthalmopathy patient (4, 13, 46).

The way CASQ1 works is not yet clear but speculations can be put together from our findings to try to elucidate and give shape to CASQ1's role. A possible explanation to the reactions happening in the thyroid could be that the up regulation of the two forms of CASQ1 in the thyroid works as a trigger.

This reaction, involving antibodies against CASQ1 protein and sensitized T-lymphocytes (6), then might spread to the orbit. It follows that, with the productions of the latter molecular actors targeting CASQ1, the concentration of the protein in thyroid will decrease, as seen in patients with Graves' disease.

Further studies will have to quantify CASQ1 protein concentration in many more toxic nodules in order to determine if there really is an autoimmune reaction or if it is due to Graves' hyperthyroidism. If the toxic nodules show low levels of CASQ1 protein, like in the two cases analysed before the submission of this thesis, then it means that the reduction is due to hyperthyroidism. On the contrary, if the following analyses on more toxic nodules show the CASQ1 levels to be the same or higher, like in the one toxic nodule analysed after the submission of this thesis, then it means that an autoimmune process is going on.

Another possible explanation to the reduction of CASQ1 protein in Graves' disease is the shutting down of the translation of its mRNA. This mechanism could be a way for the

damaged cells to economize on unnecessary expenditure and to preserve the integrity of the cells caused by autoimmune reaction, although this is purely speculative.

It seems likely that, in thyroid eye disease at least, the attack against the eye muscle component begins in the thyroid, with reaction against CASQ1, it then spreads to the eye muscles.

Further studies from our research group unveiled an association between SNP rs3838216 and patients with severe eye disease. Patients with homozygous genotype (i.e. lacking the polymorphism on both alleles of the gene (DEL)) seem to have reduced CASQ1 protein levels as compared to control. Analysed data show high levels of CASQ1 antibody in Graves' ophthalmopathy patients that have homozygous genotype (GO DEL) as compared to control group (CONTROL DEL). This increase in antibody levels, measured by ELISA technique, was not significant ($p = 0.075$). No statistical significance was found between CONTROL DEL and Graves' hyperthyroidism homozygous group (GH DEL).

In summary, results indicates that patients suffering from GO and GH with a homozygous genotype (absence of rs3838216 polymorphism), have reduced levels of CASQ1 protein antigen measured by quantitative Western blot. However, in contrast to the reduction in protein levels, CASQ1 antibody levels increased in both GO and GH groups with homozygous genotype. Therefore, our results indicate that people who carry a homozygous genotype may have a propensity towards the development of the two forms of Graves' disease, hyperthyroidism or ophthalmopathy. This particular genotype can be used as a risk factor marker aiming to the development of genetic test for GH and GO to confirm clinical observations in regards to particular subjects.

Chapter V: Conclusions and future directions

Conclusions and future directions

While there are other possibilities to explain this apparently contradictory finding our future studies will focus on the presumed link between the thyroid and orbital reactions and the role of CASQ1 in this context.

The outcomes of this research studies adds more knowledge to the biological processes involved, outlines more paths to follow, not only in terms of management and prevention of thyroid disease but also in therapeutic directions.

Larger populations of patients and controls of different ethnic backgrounds might confirm the findings of SNP rs3838216 as genetic marker for Graves' disease.

Molecular genetic testing in larger samples, combined with autoantibody testing, could allow for the detection of other subjects 'at risk' of developing Graves' disease, in its different forms and degrees.

The results might also find other regions of the gene involved in the disease process, making possible the determination of phenotype-genotype correlations of CASQ1 to Graves' disease.

Testing on larger cohorts may add even more candidates within the pathways of Graves' ophthalmopathy, which could give a better understanding of the paths from where to tackle the disorder.

This will result in a better direction of preventive therapy, if available in the future, or avoidance of negative environmental stimuli such as smoking (proven to be high risk factor also for this typology of disease (3)).

At this stage there aren't many clinical tests for ophthalmopathy other than the measurement of antibodies against calsequestrin and collagen XIII. This test has been found useful by ophthalmologists and endocrinologist to deal with various eye and thyroid disorders in their patients.

Continuing on this research path might present new future management techniques for ophthalmopathy that are based on a simple genetic test capable of identify with what propensity a patient might develop ophthalmopathy.

In regards to these speculations the future strategy might focus on creating a vector to be used in template cells and generate mutations in regions of interest on CASQ1 gene. Exploring the consequences of these controlled aberrations on CASQ1 protein structure and function will give us an insight into the processes involved, eventually re-addressing the focus of the research. In the longer term we may be able to prevent this distressful, frequently cosmetically disable, eye disorder.

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- Regulation of endoplasmic reticulum-Ca-ATPase by glycolysis in eukaryotic cells

Pathogenesis of thyroid-associated ophthalmopathy: does autoimmunity against calsequestrin and collagen XIII play a role?

Cardiac response to doxorubicin and dexrazoxane in intact and ovariectomized young female rats at rest and after swim training

Structure and expression of the mouse cardiac calsequestrin gene

Effect of thyroid hormone on the expression of mRNA encoding sarcoplasmic reticulum proteins

Epitopes, immunoglobulin classes and immunoglobulin G subclasses of calsequestrin antibodies in patients with thyroid eye disease

Eye Muscle Antibodies in Patients with Ocular Myasthenia Gravis: Possible Mechanism for Eye Muscle Inflammation in Acetylcholine-Receptor Antibody-Negative Patients

[Pathogenesis of thyroid eye disease - does autoimmunity against the TSH receptor explain all cases?]

Pathogenesis of thyroid eye disease--does autoimmunity against the TSH receptor explain all cases?

Eye findings and immunological markers in probands and their euthyroid relatives from a single family with multiple cases of thyroid autoimmunity

Calsequestrin distribution, structure and function, its role in normal and pathological situations and the effect of thyroid hormones

Can autoimmunity against calsequestrin explain the eye and eyelid muscle inflammation of thyroid eye disease?

A 63 kDa skeletal muscle protein associated with eye muscle inflammation in Graves' disease is identified as the calcium binding protein calsequestrin

Reevaluation of the prevalences of serum autoantibodies reactive with 64-kd eye muscle proteins in patients with thyroid-associated ophthalmopathy

Peripheral membrane proteins of sarcoplasmic and endoplasmic reticulum. Comparison of carboxyl-terminal amino acid sequences

Type-2 iodothyronine 5'deiodinase in skeletal muscle of C57BL/6 mice. I. Identity, subcellular localization, and characterization

Eye signs and serum eye muscle and collagen XIII antibodies in patients with transient and progressive thyroiditis

Extraocular muscle autoimmunity and orbital fat inflammation in thyroid-associated ophthalmopathy

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Rituximab treatment in patients with active Graves' orbitopathy: effects on proinflammatory and humoral immune reactions

Antibodies against calsequestrin and type XIII collagen are good markers for chronic upper eyelid retraction

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