Calsequestrin is Decreased in the Thyroid Gland of Patients with Graves' Disease – Further Evidence for a Role of Autoimmunity against this Protein in Graves' Ophthalmopathy

Daniele Cultrone[1], Hooshang Lahooti[1], Senarath Edirimanne[2], Leigh Delbridge[3], Bernard Champion[1] and Jack R. Wall[1*]

[1]*Thyroid Research Laboratory, Nepean Clinical School, the University of Sydney and NBMLHD, Kingswood, Australia.*
[2]*University of Sydney, Nepean Clinical School and NBMLHD, Kingswood, Australia.*
[3]*University of Sydney, Royal North Shore Hospital, St Leonards, Australia.*

Authors' contributions

This work was carried out in collaboration between all authors. Author JW designed the study, and wrote the first draft of the manuscript. Author DC collected the data and authors SE and LD provided thyroid tissue specimens at thyroidectomy. Author BC critically read the manuscript and author HL managed the analyses of the study, performed the statistical analysis, wrote the protocol, managed the literature searches and wrote the final draft of manuscript. All authors read and approved the final manuscript.

Original Research Article

ABSTRACT

Background: The pathogenesis of Graves' ophthalmopathy (GO) and the mechanism for its link to thyroid autoimmunity is poorly understood. Our present research focuses on the role of the skeletal muscle calcium binding protein calsequestrin (CASQ1). Earlier studies 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, however, the protein levels remained the same in both groups, 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 the CASQ1 gene polymorphism rs3838216, shown previously to be a risk factor for ophthalmopathy.

*Corresponding author: Email: hooshang.lahooti@sydney.edu.au;

Methods: The CASQ1 protein was measured by quantitative Western blotting. Following electrophoresis, samples were transblotted to polyvinyl difluoride (PVDF) membranes incubated with a 1:1000 dilution of a rabbit anti-CASQ1 antibody and incubated with an horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody, or anti-mouse antibodies for Glyceraldehyde 3-phosphate dehydrogenase (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 respectively. In thyroid tissues, the mean (± SD) (GO 54.9±86.8 pmol/mg) concentration of the CASQ1 protein (GH 37.1±51.8 pmol/mg) was significantly reduced in patients with Graves' disease , with or without ophthalmopathy, compared to normal thyroid tissues from control subjects with multi-nodular goitre or thyroid cancer (144.3±162.5 pmol/mg). The difference between GO and GH was not significant. The decreased CASQ1 protein levels in Graves' thyroid tissues correlated with the homozygous genotype of the rs3838216 CASQ1 polymorphism. A two-fold increase in Levels of CASQ1 protein in toxic nodules compared to Graves' hyperthyroidism was markedly significant.

Conclusions: Decreased CASQ1 in the thyroid tissues of patients with Graves' disease compared to normal thyroid tissues from control subjects may reflect consumption of the protein in the course of an autoimmune reaction against CASQ1 in the thyroid. Comparing CASQ1 protein levels in thyroid tissue from five patients with toxic nodular goitre (74.5±52.8) to controls showed no significance. The relative two fold increase in CASQ1 levels in toxic nodules compared to Graves' disease suggests that this is due to the autoimmune reaction rather than the hyperthyroidism.

ABBREVIATIONS

Calcium binding protein calsequestrin (CASQ), Graves' Ophthalomopathy (GO), Graves' Hyperthyroidism (GH), Myocyte Enhancer Factor-2 (MEF2), Serum Response Factor (SRF) Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Calmodulin Kinase II (CaM Kinase II), enzyme-linked immunosorbent assay (ELISA), polyvinyl difluoride (PVDF), horseradish peroxidase (HRP).

1. INTRODUCTION

The eye disorder associated with Graves' disease, called Graves' ophthalmopathy (GO), or "thyroid-associated ophthalmopathy", greatly reduces the quality of life in affected patients [1,2] and can lead to eye muscles damage and loss of sight. Ophthalmopathy is also present in about 25% of patients with Hashimoto's thyroiditis [3] where it is mainly manifest as eyelid disease. Graves' disease runs in families and is a multi-factorial disorder that probably includes a genetic susceptibility [4-5] and some subsequent external (environmental) stimulus such as infection, stress or trauma. The pathogenesis of the ophthalmopathy and the mechanism for its link with thyroid autoimmunity are poorly understood [6-9]. Our recent research has addressed the possible role of autoimmunity against the calcium binding protein calsequestrin (CASQ1) in the pathogenesis of the eye muscle component of GO.

While we have demonstrated that antibodies against CASQ1 are good markers of the eye muscle component of ophthalmopathy they are not specific to GO, being detected in about 10% of apparently normal subjects and in some patients with other forms of skeletal muscle damage or inflammation [10-13]. CASQ1 is found in abundance in skeletal muscles while its isoform, cardiac calsequestrin (CASQ2), is found in heart muscle and, to a lesser degree, skeletal muscle. CASQ1 is expressed 4.7 times more in fast twitch fibres in the extraocular muscles than in other skeletal muscle [14], providing a possible mechanism for the localisation of a muscle reaction in the obit in Graves' disease. Earlier studies from our laboratory showed that the *CASQ2* gene was the most upregulated in thyroid tissue from patients with Graves' ophthalmopathy compared to those with no eye signs (odds ratio 2.3) [15]. We showed that the *CASQ1* gene was upregulated (odds ratio 4.1) in the thyroid tissues from patients with GO compared to those patients without eye disease [15]. In that study we did not test normal thyroid tissues, we only compared the levels of CASQ1 protein in thyroid tissues with GO patients to the CASQ1 protein in thyroid tissues with GH patients and we did not see any significance difference between the two groups, that is, there was no upregulation of CASQ protein. These surprising findings raised the possibility that the orbital autoimmune reaction may start in the thyroid and spread to the eye muscles. In this study we have quantified the CASQ1 protein in thyroid tissue from patients with Graves' disease with and without ophthalmopathy and in normal thyroid tissues from control subjects with multi-nodular goitre or thyroid cancer and five patients with toxic nodules, by quantitative Western blotting. We showed that CASQ1 protein levels were reduced in the thyroid tissues of both groups of patients with Graves' disease compared to normal thyroid tissues, but not in hyperfunctioning nodules from patients with multi nodular goitre.

2. CLINICAL SUBJECTS AND METHODS

2.1 Clinical Subjects

Thyroid tissue was obtained fresh at thyroidectomy from;

i) 88 patients with Graves' disease, 12 males and 66 females aged 11 to 75 (mean age 37.5 yr) of whom 4 males and 19 females aged 17 to 75 (mean age 37.6 yr), had ophthalmopathy,
ii) 39 patients with multi-nodular goitre or thyroid cancer, 7 males and 32 females aged 35 to 82 (mean age 51.1 yr) and
iii) Five patients, 2 males and 3 females aged 20 and 69 (mean age 55.8 yr) with toxic nodular goitre. The diagnoses of the various disorders were based on standard clinical criteria and confirmed by thyroid function testing, thyroid ultrasonography and immunological tests. Any associated ophthalmopathy was characterised as; Nunery types 1 (without restrictive myopathy) or 2 (with restrictive myopathy) [16], the clinical activity score (CAS) (0-10) of Mourits et al. [17] which is a measure of disease activity and Werner's NOSPECS class [18]. For the purposes of the present study ophthalmopathy was defined as a NOSPECS class of ≥ 2 regardless of the CAS. Isolated upper eyelid retraction and/or lag were not taken as "ophthalmopathy". Local Ethical Committee approval was received for the study and informed consent of participating subjects was obtained.

2.2 Quantitative Western Blotting

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 -70º C until used. Frozen tissues were thawed on ice, minced to small pieces in homogenisation buffer, then homogenised and lysed using a Whole Cell Extract Lysis Buffer. Equal amounts of patients' proteins in thyroid tissue extracts 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 a rabbit anti-CASQ1 antibody and then with an HRP-conjugated goat anti-rabbit antibody, or anti-mouse antibody for GAPDH. Membrane proteins were identified by chemiluminescence 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 from which the CASQ1 protein concentration can be determined as a CSAQ1: GAPDH ratio. Using serial dilutions of highly purified rabbit skeletal muscle CASQ1 protein standards, the CASQ1 is determined as pmol CASQ1/mg total thyroid proteins in the extracts, for both the 50 kDa and 60 kDa forms of the protein.

## 2.3 Enzyme-Linked Immunosorbent Assay

The presence and level of 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 [10-13,19] and 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 > the upper limit of the reference range, which was 194.

2.4 Statistical Analysis

Differences in CASQ1 protein concentrations between patient groups and correlations between levels of the CASQ1 protein and parameters of the eye disease, presence of the genomic polymorphism rs3838216 and CASQ1 antibody titres, in the three groups, were analysed using the Mann-Whitney test, using GraphPad Prism Version 3.03. A p value of <0.05 was taken as significant in all assessments.

3. RESULTS

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. Two forms of CASQ1 in the thyroid were identified, with molecular weights of 50 kDa and 60 kDa respectively (Fig. 1). Quantitative assessment demonstrated variable levels of the CASQ1 protein in Graves' and normal thyroid tissues with 17 patients showing reactivity against the 50kDa protein and 115 patients showing reactivity against the 60 kDa protein. Examples of CASQ1 and GAPDH bands in representative patients and controls are shown in Fig. 2. For quantification CASQ1 and GAPDH levels on each membrane we used the lowest exposure where bands were not saturated to obtain the correct levels of proteins.
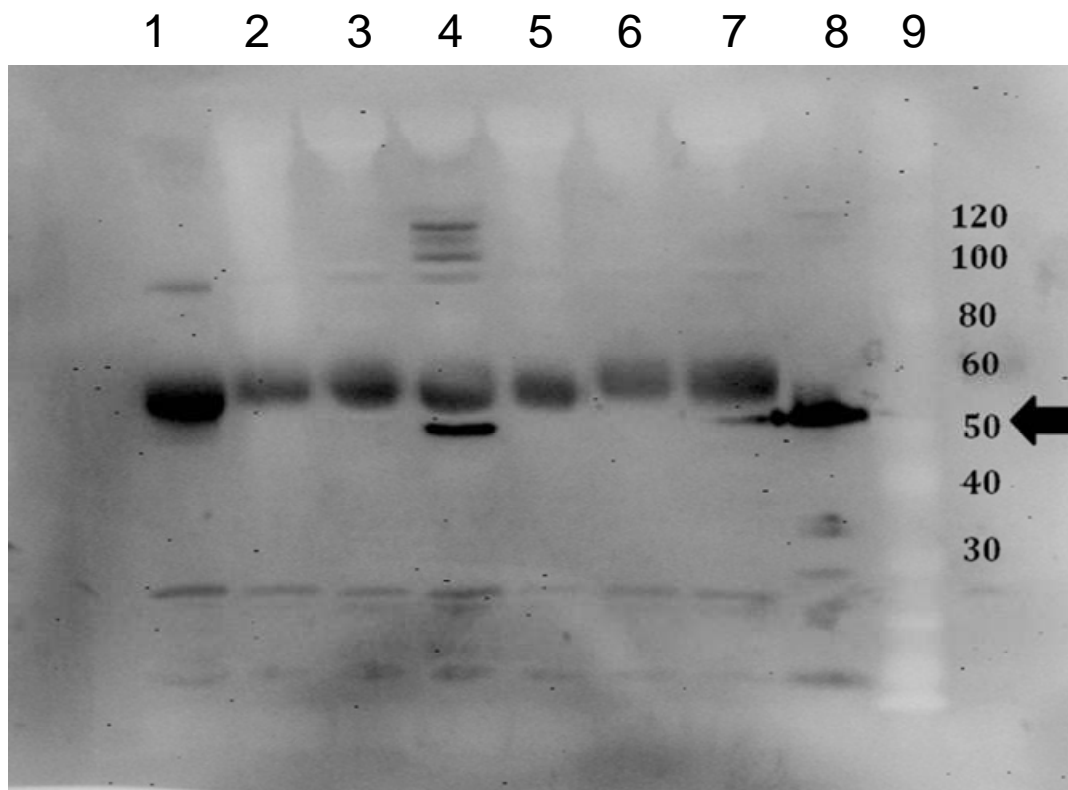
Fig. 1. Western blotting of thyroid extracts from patients with Graves' hyperthyroidism (GH, lane 4) showing bands 50 at 60 kDa. Graves' ophthalmopathy (GO, lanes 3 and 7) and (as normal thyroid) from control patients with multinodular goitre or thyroid cancer (lanes 2, 5 and 6) showing bands at 60 kDa. Lane 1 was uncharacterized thyroid patient's sample and lanes 8 and 9 were positive and negative control respectively. Molecular weight markers are shown to the right of the panel

Next, we measured CASQ1 protein concentrations in thyroid tissues from 65 patients with Graves' hyperthyroidism (GH), 23 with Graves' ophthalmopathy (GO) and in 39 control subjects with nodular goitre or cancer. The results are summarised in Fig. 3, which shows the mean±SD (Control 144.3±162.5; GH 37.1±51.8; GO 54.9±86.8 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, $p < 0.0001$, $p = 0.0009$ respectively) compared to the control subjects. Although mean CASQ1 level in patients with GH was less than that in patients with GO this difference was not significant ($p = 0.441$) (Fig. 3). When we compared the CASQ1 protein level in thyroid tissues from patients having *CASQ1* rs3838216 homozygous genotype, to CASQ1 protein level in normal control thyroid tissues having the *CASQ1* rs3838216 homozygous genotype, we found the reduction in thyroid tissues CASQ1 protein level correlated with the homozygous genotype of the rs3838216 polymorphism in the *CASQ1* gene (Mann-Whitney test, $p = 0.0223$) (Fig. 4) but not with the heterozygous genotype. Next we correlated serum levels of CASQ1 antibodies titres in patients with GH and GO (Fig. 5) against their thyroid tissues CASQ1 concentrations. There was no significant relationship between the two groups (Mann-

Whitney test, p <0.0001 and p = 0.0059 respectively) i.e. positive antibodies generally correlated with lower CASQ1 concentrations.

A
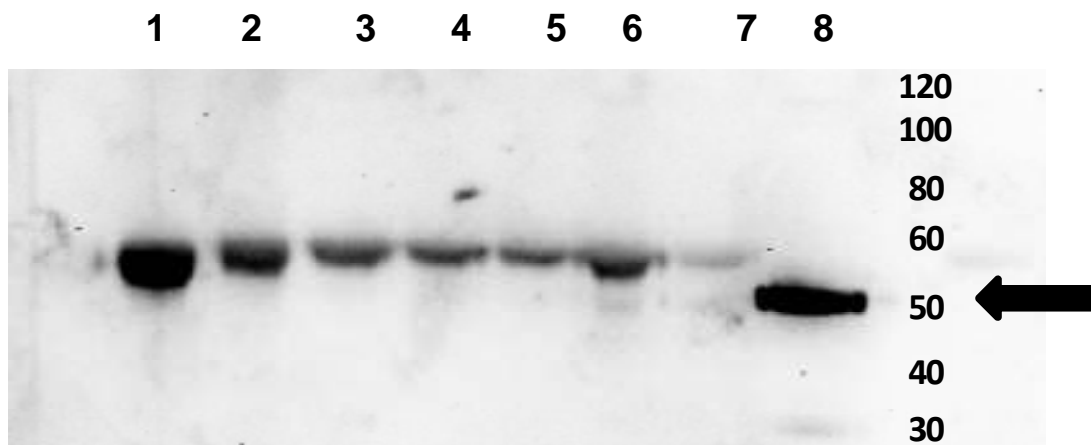
B
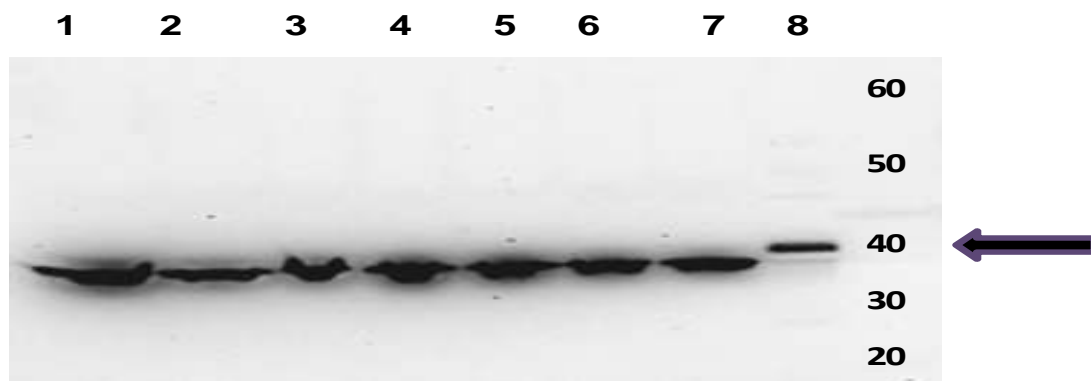
Fig. 2A. PVDF membrane showing different CASQ1 protein concentration in some of the subject analyzed. 2B. PVDF membrane showing reactivity with GAPDH, a very abundant protein present in every cell used to normalize CASQ1 protein levels. Molecular weight markers are shown to the right of the panel

Finally, in order to determine whether the reduced CASQ1 protein levels in Graves' thyroid tissues was due to the autoimmune reaction or the hyperthyroidism itself, we measured the amounts of CASQ1 in toxic nodules obtained by careful sampling of nodular tissue removed at thyroidectomy from 5 patients with toxic nodular goitre. CASQ1 level in toxic nodules (74.5±52.8 pmol/mg), and in normal thyroid tissues (144.3±162.5 pmol/mg), it was significantly greater than that in Graves' disease samples (37.1±51.8) Mann-Whitney Test, p = 0.036) (Fig. 3).

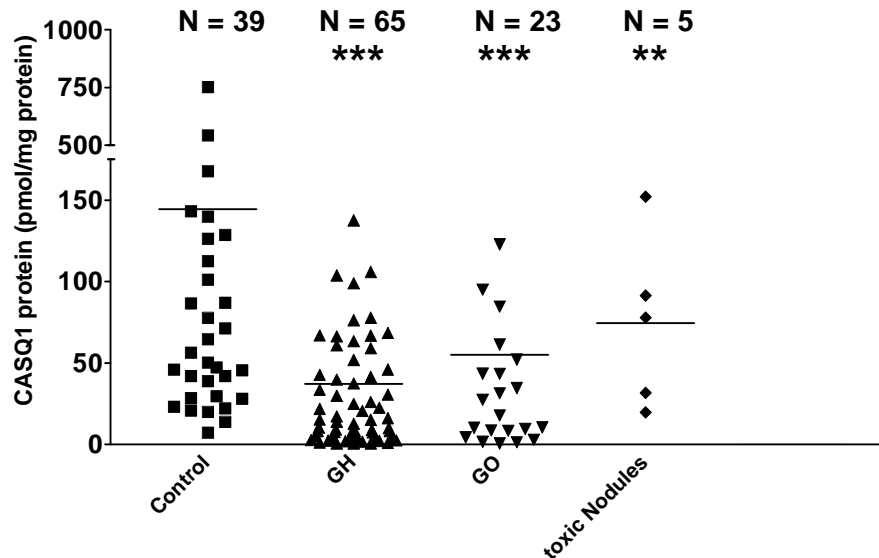**Fig. 3. Mean ± standard deviation (SD), CASQ1 protein concentrations in thyroid tissues from patients with Graves' hyperthyroidism (GH, 37.1±51.8 pmol/mg), Graves' ophthalmopathy (GO, 54.9±86.8 pmol/mg) and as normal thyroid, from control patients with multinodular goitre or thyroid cancer (144.3 ± 162.5 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 and p = 0.0009, respectively). The difference between GH and toxic nodules goitre was significant**
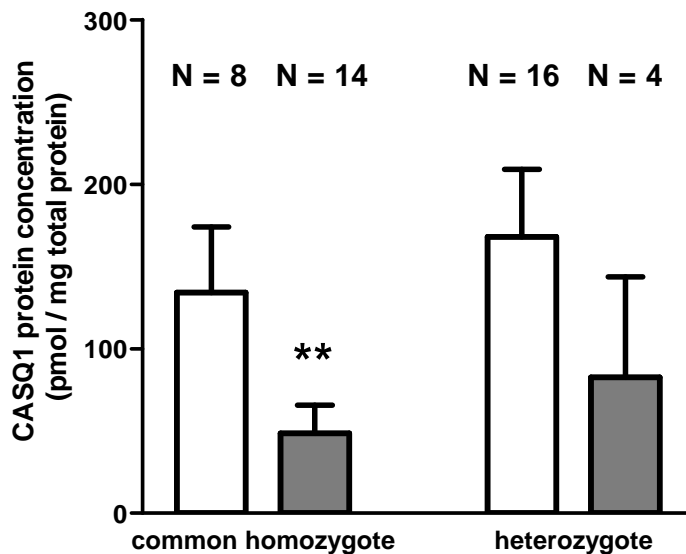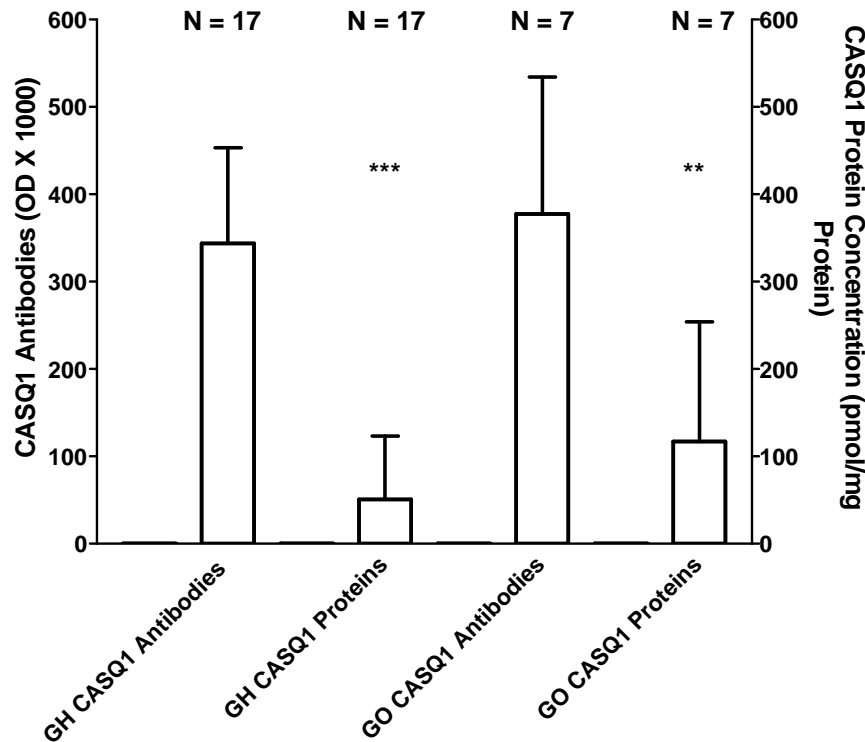*(Mann-Whitney Test p = 0.0360). *** P< 0.0009 – 0.0001, ** P < 0.05 – 0.001, NS = Not Significant*

Fig. 4. Correlation between mean (± SD) 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). ■ GH, □ Control, *** P< 0.0009 – 0.0001, ** P < 0.05 – 0.001, NS = Not Significant

Fig. 5. Correlation between mean (± SD) CASQ1 protein levels and serum CASQ1 antibody levels in patients from whom their thyroid tissue was tested for the CASQ1 protein quantification. There was no significant relationship between the two groups (Mann-Whitney test, p <0.0001 and p = 0.0059 respectively) i.e. positive antibodies generally correlated with lower CASQ1 concentrations.
*** P< 0.0009 – 0.0001, ** P < 0.05 – 0.001, NS = Not Significant*

4. DISCUSSION

Using Western blotting and density quantification, we have demonstrated that the CASQ1 protein is present in the thyroid gland of patients with Graves' disease and in normal thyroid from patients with cancer or multi nodular goitre; we presume that CASQ1 is located in the smooth muscle, but it may also be present in other cells and tissues as well. We demonstrated two bands at 50-kDa and 60-kDa in thyroid from both Graves' patients and controls; the 50kDa and 60kDa forms of the CASQ1 protein do not appear to be products of two different genes or alternative splicing. The 50kDa may represent the unprocessed or post-translationally modified form of the mature protein. The 60kDa form, the more abundant, appears to be the completely post-translationally modified protein. Immunofluorescence can be used to determine the location of the two forms of the CASQ1 protein at the level of cellular organelles in the thyroid follicular cells and surrounding connective tissue.

We showed that levels of the CASQ1 protein were lower in thyroid tissues from Graves' disease compared to control thyroid tissues from subjects with multi nodular goitre and thyroid cancer whereas, in an earlier study, we demonstrated that the *CASQ1* gene was

upregulated in the thyroid tissues from patients with GO compared to those patients without eye disease [15]. In this study we also studied thyroid tissue from a few patients with toxic nodular goitre, in whom levels of CASQ1 protein were two fold higher than those in Graves' disease thyroid tissues, which was markedly significant. These results suggest that the relative increase in CASQ1 protein level in toxic nodular goitre is possibly due to an autoimmune reaction rather than the hyperthyroidism. Genotype analysis of 52 subjects showed a positive correlation between reduced CASQ1 levels and the homozygote genotype for the recently identified *CASQ1* polymorphism rs3838216 (Lahooti, Cultrane, Wall et al, manuscript in preparation) and between the homozygote of this polymorphism and serum tires of CASQ1 antibody.

Because of the now strong evidence that CASQ1 plays a role on the eye muscle component of GO we believe that the presence of CASQ1 in thyroid is not casual but in some way related to the autoimmune reactions in the orbit. For example, it is possible that the upregulation of the two forms of CASQ1 in the thyroid may trigger the development of an autoimmune reaction in the thyroid involving antibodies and sensitized T lymphocytes [20-22], which spreads to the orbit. A feasible explanation for its lower concentration in the thyroid of patients with Graves' disease is that it is consumed in the course of this reaction. This is supported by finding a correlation between lower CASQ1 concentrations with higher CASQ1 antibody titres, although the association is only modest. We speculate that it is also possible that this reduction in CASQ1 in Graves' disease is due to the shutting down of translation of its mRNA in cells damaged by the autoimmune reaction, in order to preserve the integrity of these cells and economise on unnecessary expenditure of regulatory protein production. We have shown that the *CASQ2* gene is upregulated in thyroid tissues but the protein expression for CASQ remained unchanged, that is, there was no upregulation of CASQ protein. It is possible that during the course of the disease this upregulation of the gene may lead to increased expression of the protein transiently with return to basal levels in the latter disease stages. This is reminiscent of the recent reports by Jose Luis Reyes-Juarez et al. [23] which discussed that the transcription factors MEF-2 and SRF play a significant role in the regulation of the *CASQ2* gene and are activated via Ca^{2+}-regulated pathways, like calcineurin for MEF-2 and CaM kinase II for SRF. It is further reported that other studies show no changes in the protein expression of CASQ2 in pathological cardiac states, which are associated with abnormal intracellular Ca^{2+} concentrations, suggesting that non-Ca^{2+} regulated pathways may be involved in the regulation of the expression of *CASQ2* gene. Therefore, it is important that other regulatory pathways affecting the transcription or translation or post-translational modification of a CASQ2 protein be studied. Our future studies will focus on the putative link between the thyroid and orbital reactions and the role of CASQ1 in this. It seems likely that the eye muscle component of thyroid eye disease at least, begins in the thyroid with reaction against CASQ1 there and spreads to the eye muscles in those patients (with Graves' disease) who are genetically predisposed, e.g. lack rs3838216 polymorphism.

4. CONCLUSION

Decreased CASQ1 in the thyroid tissues of patients with Graves' disease compared to normal thyroid tissues from control subjects may reflect consumption of the protein in the course of an autoimmune reaction against CASQ1 in the thyroid. Comparing CASQ1 protein levels in thyroid tissue from five patients with toxic nodular goitre (74.5±52.8) to controls showed no significance. The relative two fold increase in CASQ1 levels in toxic nodules compared to Graves' disease suggests that this is due to the autoimmune reaction rather than the hyperthyroidism.

CONSENT

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

ETHICAL APPROVAL

All authors hereby declare that all experiments have been examined and approved by the Nepean Blue Mountains Local Health District scientific and ethics committees and have therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Rebecca S, Bahn. Graves' Ophthalmopathy .N Engl J Med. 2010;362(8):726–738.
2. Abraham-Nordling M, Wallin G, Träisk F, Berg G, Calissendorff J, Hallengren B, Hedner P, Lantz M, Nyström E, Asman P, Lundell G, Törring O; Thyroid Study Group of TT 96. Thyroid-associated ophthalmopathy; quality of life follow-up of patients randomized to treatment with antithyroid drugs or radioiodine. European Journal of Endocrinology. 2010;163:651–657.
3. Tjiang H, Lahooti H, McCorquodale T, Parmar KR, Wall JR. Eye and eyelid abnormalities are common in patients with Hashimoto's thyroiditis. Thyroid. 2010;20:287-290
4. Khalilzadeh O, Noshad S, Rashidi A, Amirzargar A. Graves' Ophthalmopathy: A Review of Immunogenetics. Current Genomics. 2011;12:564-575.
5. Liao WL, Wan L, Wang TY, Chen CC, Tse SS, Lu CH, Tsai FJ. Association of TLR7 and TSHR copy number variation with Graves' disease and Graves' ophthalmopathy in Chinese population in Taiwan. BMC Ophthalmol. 2014;14:15. doi: 10.1186/1471-2415-14-15.
6. Christian M, Girgis Bernard L. Champion and Jack R. Wall.Current concepts in Graves' disease. Ther Adv Endocrinol Metab. 2011;2(3):135-144.
7. Lahooti H, Parmar KR, Wall JR. Pathogenesis of Thyroid Eye Disease: important role of autoimmunity against calsequestrin and collagen XIII. Pathogenesis of Thyroid Eye Disease: important role of autoimmunity against calsequestrin and collagen XIII. A review. Clin Ophthalmol. 2010;14:417-25.
8. McCorquodale T, Lahooti H, Gopinath B, Wall JR. Long-term follow-up of seven patients with ophthalmopathy not associated with thyroid autoimmunity: heterogeneity of autoimmune ophthalmopathy. Clin Ophthalmol. 2012;6:1063 - 71.
9. Tani J, Gopinath B, Nuygen B, Wall JR. Immunological mechanisms for the eye muscle and orbital connective tissue reactions of thyroid-associated ophthalmopathy. Expert Reviews in Clin Immunol. 2007;3:299-311.
10. Tani J, Wall JR. Analysis - Can the development of thyroid-associated ophthalmopathy be explained by autoimmunity against eye muscle antigens? Canad Med Assoc. 2006;17:239-241.

11. Gopinath B, Musselman R, Beard N, Tani J, Adams C, Wall JR. Antibodies targeting the calcium binding skeletal muscle protein calsequestrin are sensitive and specific markers of ocular myopathy in patients with Graves' disease. Clin Exp Immunol. 2006;145:56-62.

12. Gopinath B, Adams CL, Musselman R, Tani J, Wall JR. Antibodies against calsequestrin and type XIII collagen are good markers for chronic upper eyelid lag and retraction. Ocular Immunol and Inflammation. 2007;15:81–88.

13. de Haan S, Lahooti H, Morris O, Wall JR. Epitopes, immunoglobulin classes and immunoglobulin G subclasses of calsequestrinantibodies in patients with thyroid eye disease. Autoimmunity. 2010;43(8):698-703.

14. Porter J D, Khanna S, Kaminski HJ, Rao J S, Merriam AP, Richmonds CR, Leahy P, Li J, Andrade F H. Extraocular muscle is defined by a fundamentally distinct gene expression profile. PNAS. 2001;98:12062-12067.

15. Wescombe L, Lahooti H, Gopinath B, Wall JR. The cardiac calsequestrin gene (CASQ2) is up-regulated in the thyroid in patients with Graves' ophthalmopathy - support for a role of autoimmunity against calsequestrin as the triggering event. Clin Endocrinol (Oxf). 2010;73:522-8.

16. Nunery WR, Martin RT, Hinz GW, Gavin TJ 1993 The association of cigarette smoking with clinical subtypes of ophthalmic Graves' disease. Ophthalmic Plastic and Reconstructive Surgery. 1993;9:77-82.

17. Mourits MP, Koornneef L, Wiersinga WM, Prummel MF, Berghout A, van der Gaag R. Clinical criteria for the assessment of disease activity in Grave's ophthalmopathy: a novel approach. British Journal of Ophthalmology. 1989;73:639-644.

18. Werner SC. Classification of the eye changes of Graves' disease. Am J Ophthalmol. 1969;68:646–648.

19. Gopinath B, Musselman R, Adams C, Tani J, Beard N, Wall JR. Study of serum antibodies against three eye muscle antigens and the connective tissue antigen collagen XIII in patients with Graves' disease with and without ophthalmopathy – correlation with clinical features. Thyroid. 2006;16:967-974.

20. Nguyen B, Gopinath B, Tani J, Wescombe L, Wall JR. Peripheral blood T lymphocyte sensitization against calsequestrin and flavoprotein in patients with Graves' ophthalmopathy. Autoimmunity. 2008;41:372-6.

21. Gopinath B, Wescombe L, Nguyen B, Wall JR. Can autoimmunity against calsequestrin explain the eye and eyelid muscle inflammation of thyroid eye disease? Orbit. 2009;28(4):256-61.

22. Andrew G Gianoukakis, Nicole Khadavi BS, Terry J. Smith. Cytokines, Graves' disease, and Thyroid-Associated Ophthalmopathy. Thyroid. 2008;18(2):956-958.

23. Reyes-Juárez JL, Juárez-Rubí R, Rodríguez G, Zarain-Herzberg A. Transcriptional analysis of the human cardiac calsequestrin gene in cardiacand skeletal myocytes. J Biol Chem. 2007;282:35554-63.