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Antibodies targeting the calcium binding skeletal muscle protein calsequestrin are specific markers of ophthalmopathy and sensitive indicators of ocular myopathy in patients with Graves' disease

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Summary

We have identified several eye muscle antigens and studied the significance of the corresponding serum autoantibodies in patients with Graves' disease. Of these antigens, only calsequestrin is expressed more in eye muscle than other skeletal muscles, which could explain at least partly the specific involvement of eye muscle in patients with Graves' disease. Earlier, we found a modest relationship between anti-calsequestrin antibodies and ophthalmopathy, but in that study we used calsequestrin prepared from rabbit heart muscle and measured antibodies by immunoblotting. We have reinvestigated the prevalences of anti-calsequestrin antibodies in larger groups of well-characterized patients with thyroid autoimmunity with and without ophthalmopathy and control patients and healthy subjects, using standard enzyme-linked immunosorbent assay incorporating highly purified rabbit skeletal muscle calsequestrin, which has a 97% homology with human calsequestrin, as antigen. Anti-calsequestrin antibodies were detected in 78% of patients with active congestive ophthalmopathy, in 92% of those with active inflammation and eye muscle involvement, but in only 22% of patients with chronic, 'burnt out' disease. Tests were also positive in 5% of patients with Graves' hyperthyroidism without evident ophthalmopathy (two patients) and one patient with 'watery eyes' but no other clear signs of congestive ophthalmopathy and IgA nephropathy and no known thyroid disease, but in no patient with Hashimoto's thyroiditis, toxic nodular goitre, non-toxic multi-nodular goitre or diabetes, or age- and sex-matched healthy subjects. In serial studies of all 11 patients with Graves' hyperthyroidism who had active ophthalmopathy at the time of the first clinic visit, or developed eye signs during the first 6 months, and positive anti-calsequestrin antibodies in at least one sample, anti-calsequestrin antibodies correlated with the onset of ocular myopathy in six patients. Antibodies targeting calsequestrin appear to be specific markers for ophthalmopathy and sensitive indicators of the ocular myopathy subtype of ophthalmopathy in patients with thyroid autoimmunity. However, these results must be considered preliminary until a large prospective study of patients with newly diagnosed Graves' hyperthyroidism, in which serum levels of calsequestrin antibodies are correlated with clinical changes and orbital eye muscle and connective tissue/fat volumes, has been carried out.

Keywords: autoimmunity, calsequestrin, eye muscle antibodies, Graves' disease, ophthalmopathy.

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Introduction

Peri-orbital swelling, exophthalmos, eyelid lag and impaired vision occur in approximately 35% of patients with Graves' hyperthyroidism [1]. These eye changes, or ophthalmopathy, also occur in patients with Hashimoto's thyroiditis, although much less frequently. Thyroid-associated ophthalmopathy (TAO) may comprise two distinct subtypes, namely,

congestive ophthalmopathy and ocular myopathy [2,3]. Congestive ophthalmopathy is manifest as swelling of the eyelid and surrounding orbital connective tissue, while ocular myopathy results from autoimmune attack of extra-ocular muscle tissue and results in impaired extra-ocular muscle function and diplopia.

The mechanism for the development of the eye changes in patients with Graves' hyperthyroidism is still unknown, although likely to reflect immunological cross-reactivity against a thyroid and orbital tissue antigen [4–6]. Congestive signs may be caused by an autoimmune attack against orbital connective tissue antigens such as the thyroid stimulating hormone receptor (TSH-r) [7,8] or collagen type XIII [9], while ocular myopathy appears to reflect immune-mediated damage of the eye muscle fibre, although the identity of the prime target antigen(s) is unclear. Several serum indicators of immune-mediated damage to eye muscles have been identified including antibodies against the 64 kDa eye muscle protein flavoprotein (Fp) [10,11] and the 55 kDa eye muscle protein G2s, shown recently to be the terminal 141 amino acids of the winged-helix transcription factor FOX P1, which is expressed in the nuclei and cytoplasm of many cell types including the eye muscle fibre [12,13]. While neither of these antigens is expressed specifically in eye muscle, the corresponding serum autoantibodies are good markers of eye muscle damage in patients with thyroid autoimmunity. Collagen type XIII, which is expressed in the membrane fraction of orbital fibroblast [14], and calsequestrin, a calcium binding protein located to the sarcolemmal fraction of the eye muscle fibre [15,16], are other orbital targets of the autoimmune reactions of TAO.

Of these proteins, calsequestrin appears to be the most interesting as it is expressed 4·8 times more in eye muscle than other skeletal muscles [17], which could explain at least partly the orbital specificity of skeletal muscle autoimmunity in Graves' disease. In our earlier studies, we identified calsequestrin as a 63-kDa protein in TAO; the protein was cloned and sequenced and the corresponding serum antibodies measured in enzyme-linked immunosorbent assay (ELISA) and by immunoblotting. In a published study [18], we detected anti-calsequestrin antibodies in 40% of patients with clinically active TAO but in only 4% of those with stable eye disease and 5% of normal subjects by immunoblotting, using a crude preparation of rabbit heart muscle calsequestrin as antigen. Subsequently, when flavoprotein was identified as the so-called 64-kDa protein, we lost interest in calsequestrin as an antigen in TAO. In the present study we have determined the prevalence of anti-calsequestrin antibodies in serum from larger groups of well-characterized patients with Graves' disease with and without ophthalmopathy, and control patients and subjects, using a well-optimized and standardized ELISA incorporating highly purified rabbit skeletal muscle calsequestrin, which has a 97% homology with human calsequestrin as antigen. We show that 92% of patients with active ophthalmopathy and eye muscle involvement, 78% of those with mainly congestive ophthalmopathy but only 22% of patients with long-standing standing chronic ('burnt out') disease had detectable anti-calsequestrin antibodies, while the prevalences in control patients and healthy subjects were very low, making this antibody highly specific for TAO and sensitive for the ocular myopathy subtype.

Materials and methods

Clinical subjects

We studied sera from 68 patients with Graves' hyperthyroidism attending the thyroid clinics at the Geelong Hospital and Nepean Hospital, Australia. Local ethical committee approvals were received for the studies and informed consent of participating subjects was obtained. At the time of their first clinic visit 31 of the patients had eye signs consistent with Graves' ophthalmopathy and 37 had no evident eye signs. The grade, severity and subtype of the eye signs were classified according to recommendations of an international nomenclature committee [19] and as a clinical activity score (CAS) (0–10, where pain = 0–2, swelling = 0–2 and redness = 0–2) as described by Mourits *et al.* [20], which is based on disease activity and eye dysfunction, and a score of 2 or more taken as significant ophthalmopathy. For the purposes of this study we additionally characterize the ocular myopathy subtype of TAO from the presence (1) or absence (0) of double vision and reduced eye movement in the main gazes, scored 0–3, where mild reduction is −1, moderate reduction −2 and severe reduction −3 for each gaze.

At the time of testing the ophthalmopathy was active and of less than 6 months' duration in 22 of the 31 patients, six males and 16 females aged 24–63 (mean age 45 years). Seventeen patients were hyperthyroid and five euthyroid at the time of testing, and stable (for at least 6 months) in the other nine patients. Of all female patients aged 37–65 (mean age 46 years), five were hyperthyroid and four euthyroid, of whom five had residual eye muscle dysfunction and four had 'burnt out' congestive disease. Nine of the 22 patients with active eye inflammation, of whom seven were hyperthyroid and two euthyroid, had mainly congestive signs (congestive ophthalmopathy [2,3]) and 13, 10 of whom were hyperthyroid and three euthyroid, had predominantly eye muscle signs (ocular myopathy). At the time of testing, 11 patients with eye signs, namely, five with active ocular myopathy and six with chronic disease, were being treated with anti-thyroid drugs and 20, namely, all nine patients with active congestive ophthalmopathy, eight with active ocular myopathy and three with chronic eye disease, had received radioiodine.

The other 37 patients with Graves' hyperthyroidism, 10 males and 27 females aged 17–75 (mean age 42 years) of whom 30 were hyperthyroid and seven euthyroid at the time of testing, did not have any eye signs although orbital imaging was not performed in most of them. Of these, 11 were

being treated with anti-thyroid drugs, 25 had received radioiodine (most of whom were being treated with beta blocker) and one had undergone thyroidectomy.

Anti-thyroid peroxidase antibody tests were positive (cut-off titre 100) in 55% of patients with congestive ophthalmopathy, 55% with ocular myopathy, 75% with chronic eye signs, 76% with Graves' hyperthyroidism and in 100% of patients with Hashimoto's thyroiditis. TSH-r antibodies, measured as TSH binding inhibition index (TBII), were positive in 78% of patients with congestive ophthalmopathy, 80% with ocular myopathy, 75% with chronic eye disease and 67% of patients with Graves' hyperthyroidism, and negative in all patients with Hashimoto's thyroiditis.

Of the 68 patients with Graves' hyperthyroidism, repeated testing (three or more serum samples over at least 3 months) was carried out in 23 patients, of whom 11 who had eye signs at the time of the first visit or within the first 6 months were studied further to address the relationship between anti-calsequestrin antibodies and the eye signs, over time.

As controls, we studied:

- seventeen females aged 15–79 (mean age 41 years) with Hashimoto's thyroiditis without evident ophthalmopathy, of whom 10 were euthyroid and seven hypothyroid at testing, 14 of whom were being treated with thyroxine;
- one male and 17 females aged 56–77 (mean age 67 years) with toxic multi-nodular goitre;
- one male and seven females aged 39–75 (mean age 62 years) with non-toxic multi-nodular goitre;
- two euthyroid males and two females aged 25–77 (mean age 47 years) with type 1 diabetes mellitus;
- one male and two females, all euthyroid, aged 47–77 (mean age 55 years) with type 2 diabetes;
- three males and six females aged 32–70 (mean age 56 years) with other disorders, namely, subacute thyroiditis (three patients, all of whom were hyperthyroid and thyroid peroxidase and TSH-r antibody negative), amiodarone-induced hyperthyroidism (one patient), polymyalgia rheumatica (one patient), orbital myositis (one patient), multi-autoimmunity (one patient), hyperparathyroidism (one patient), IgA nephropathy, increased CRP and watery eyes (one patient). All patients, except those with subacute thyroiditis and amiodarone-induced hyperthyroidism, were euthyroid, and no patient was being treated with steroids or immunosuppressive drugs at the time of testing or during the previous 3 months; and
- 15 age- and sex-matched healthy subjects with no personal or family history of thyroid disease, ophthalmopathy or other autoimmunity, five males and 10 females aged 26–58 (mean age 40 years).

Only five patients with Graves' hyperthyroidism, one with Hashimoto's thyroiditis and three with ophthalmopathy, had a past history of other autoimmune disorders or markers.

The diagnoses of the various disorders were based on standard clinical criteria and confirmed by thyroid function testing, thyroid ultrasonography and immunological tests. Patients with Graves' hyperthyroidism did not have ophthalmopathy as determined from routine clinical examination and, in some patients, orbital imaging. Orbital MRI was carried out in 13 patients with Graves' disease and six healthy subjects. The normal ranges for extra-ocular muscle and orbital connective tissue volumes, as well as degree of proptosis and overall globe volumes, were determined from the six normal subjects in a related study. The results of baseline testing of the patients with Graves' disease were as follows: eye muscle volumes were increased in seven of the eight patients with ocular myopathy and in one of the two patients with congestive ophthalmopathy, and normal in the one patient with congestive ophthalmopathy and both patients with Graves' hyperthyroidism studied.

Enzyme-linked immunosorbent assay

The method has been described in previous publications from this laboratory [9–13]. In preliminary studies the assay was optimized in respect to antigen concentration (0·5 μg/ml) and serum dilution (1 : 25). As antigen, we used highly purified rabbit skeletal muscle calsequestrin, which has a 97% homology with human calsequestrin, which was supplied by one of us (N. B.). A second antibody was a goat anti-human IgG at a dilution of 1 in 2000. Results were expressed as optical density (OD) × 1000. To determine a normal range we assayed 30 healthy males aged < 30. We then calculated the mean OD of these healthy subjects + 2 s.d. × 1000, giving a cut-off value of 194.

Other tests

TSH-r antibodies, determined as TBII, were measured at the pathology department of Westmead Hospital using commercial kits according to the manufacturer's instructions, with a cut-off level of +10. Thyroid peroxidase antibodies were measured by the private pathology deliverer, Barratt & Smith Pathology, using a commercial ELISA kit according to the manufacturer's instructions, with a cut-off titre of 100.

Statistical analyses

Prevalences of positive anti-calsequestrin antibody results in the various groups were compared statistically using χ^2 tests and Yeats' correction where appropriate, a P-value of $< 0{\cdot}05$ being taken as significant.

Results

We measured serum antibodies against calsequestrin in patients with TAO, Graves' hyperthyroidism without evident eye disease, Hashimoto's thyroiditis, toxic and non-toxic multi-nodular goitre, subacute thyroiditis, types I and 2 diabetes and age- and sex-matched healthy subjects. We also

measured the antibodies in serial samples from 11 patients with active ophthalmopathy at the time of diagnosis of the associated hyperthyroidism, or who developed eye signs during the first 6 months, and one or more positive antibody results. We correlated positive calsequestrin antibody results with signs or symptoms of eye muscle dysfunction. Antibodies were measured in an ELISA incorporating purified human recombinant calsequestrin, a positive result determined from a reference group of healthy young males aged less than 30.

The results are summarized in Table 1, which shows the prevalence of positive calsequestrin antibody results in the various groups. The statistical significance of the differences in patient groups compared to the age- and sex-matched healthy subjects was determined using χ^2 tests, a P-value of $< 0{\cdot}05$ being taken as significant. Anti-calsequestrin antibodies were detected in 78% of patients with congestive ophthalmopathy and 92% of those with eye muscle involvement or ocular myopathy, but in only 22% of patients with chronic, 'burnt out' disease. The prevalences of positive results in patients with congestive ophthalmopathy and ocular myopathy were not significantly different (χ^2 tests, P = n.s.). Tests were also positive in 5% of patients with Graves' hyperthyroidism (two patients) without evident ophthalmopathy and one patient with 'watery eyes', IgA nephropathy and elevated CRP, but no known thyroid disease. Tests were negative in all patients with Hashimoto's thyroiditis, toxic nodular goitre, non-toxic multi-nodular goitre and diabetes tested, and in all 15 age- and sex-matched healthy subjects tested. The differences, compared to age- and sex-matched normal subjects, were significant for patients with congestive ophthalmopathy ($P < 0{\cdot}001$) and ocular myopathy ($P < 0{\cdot}001$) (Table 1). Thyroid peroxidase antibodies were detected in the great majority of patients with Graves' disease and there were no significant correlations between prevalences of positive tests and (i) ophthalmopathy or (ii) calsequestrin antibody results (results not shown, χ^2 tests, P = n.s.). While the prevalence of positive TSH-r antibodies – measured as TBII index – was increased in patients with ophthalmopathy (81%) compared to those with Graves' hyperthyroidism but no evident ophthalmopathy (67%), the difference was not significant (χ^2 tests, P = n.s.). Considering all patients with Graves disease, there were no close associations between calsequestrin antibody results and (i) treatment of the hyperthyroidism, (ii) thyroid status (i.e. hyperthyroid or euthyroid) or mean (± s.d.) free T4 levels at the time of testing, or (iii) past history of other autoimmunity, for any subgroup of ophthalmopathy (results not shown, P = n.s.).

Table 1. Prevalences of positive anti-calsequestrin antibody results in patients with thyroid autoimmunity, with and without ophthalmopathy, and control patients and subjects, measured in enzyme-linked immunosorbent assay.

Group	Positive tests (%)[1]	P-value[2]
Thyroid-associated ophthalmopathy;		
Congestive ophthalmopathy subtype ($n = 9$)	7 (78%)	$< 0{\cdot}001$
Ocular myopathy subtype ($n = 13$)	12 (92%)	$< 0{\cdot}001$
Chronic, stable ('burnt out') ophthalmopathy ($n = 9$)	2 (22%)	n.s.
Graves' hyperthyroidism ($n = 37$)	2 (5%)	n.s.
Hashimoto's thyroiditis ($n = 17$)	0 (0%)	n.s.
Toxic nodular goitre ($n = 18$)	0 (0%)	n.s.
Non-toxic multi-nodular goitre ($n = 8$)	0 (0%)	n.s.
Diabetes: type 1 ($n = 4$)	0 (0%)	n.s.
Type 2 ($n = 3$)	0 (0%)	n.s.
Other disorders[3] ($n = 9$)		
Age- and sex-matched controls ($n = 15$)	0 (0%)	

[1]Results were expressed as optical density (OD) × 1000. To determine a normal range we assayed 30 healthy males aged < 30. We then calculated the mean OD of these healthy subjects +2 s.d. × 1000, giving a cut-off value of 194. [2]Statistical analyses refer to χ^2 tests comparing prevalences in patient groups to that in age and sex matched normals, a P-value of $< 0{\cdot}05$ being taken as significant, n.s. = not significant. [3]Subacute thyroiditis ($n = 3$), amiodarone-induced hyperthyroidism ($n = 1$), polymyalgia rheumatica ($n = 1$), orbital myositis ($n = 1$), multi-autoimmunity ($n = 1$), hyperparathyroidism ($n = 1$), IgA nephropathy, increased CRP and 'watery eyes' ($n = 1$).

Results of antibody testing on all 11 patients with Graves' hyperthyroidism who had active ophthalmopathy at the time of the first clinic visit or developed eye signs during the first 6 months, and positive anti-calsequestrin tests in one or more serum sample, are summarized in Table 2. Positive antibody tests are shown in italics. Eye signs and symptoms are classified into congestive ophthalmopathy and ocular myopathy subtypes and quantified as described in the Methods. In all patients with eye muscle dysfunction, only upward and upward/outward gazes were affected. Anti-calsequestrin antibodies were first detected at the time of onset of eye muscle dysfunction, or within 3 months, in six of the 11 patients (55%), and with congestive ophthalmopathy in the other five (45%) patients (Table 2).

Discussion

We determined the prevalence of serum antibodies against the calcium binding muscle protein calsequestrin, which is expressed 4·8 times more in eye muscle than in other skeletal muscle [17]. To summarize the main results, calsequestrin antibodies were detected in 92% of patients with active ophthalmopathy and eye muscle involvement and in 78% with active mainly congestive eye signs, but in only 22% of patients with chronic 'burnt out' disease and 5% with Graves' hyperthyroidism without evident ophthalmopathy, while tests were negative in all patients with Hashimoto's thyroiditis, toxic multi-nodular goitre, non-toxic multi-nodular goitre and diabetes and age- and sex-matched healthy subjects tested. In serial studies of all 11 patients with Graves' hyperthyroidism who had active ophthalmopathy at

Table 2. Anti-calsequestrin antibody test results in serial samples from all 11 patients with Graves' hyperthyroidism who had active ophthalmopathy at the time of the first clinic visit, or developed eye signs during the first 6 months, and one or more positive antibody tests, and corresponding eye signs and symptoms.

Patient no.	Time (months)	Anti-calsequestrin antibody levels[1]	Clinical activity score (CAS)[2]	
			Subtype of TAO/severity of signs and symptoms	Ocular myopathy[3]
1.	0	91	3	Nil
	1	161	2	Nil
	2	*225*	2	Nil
	4	183	2	Nil
2.	0	165	0	Nil
	3	144	3	Nil
	4	161	2	Nil
	6	*600*	2	Nil
	9	*273*	2	Nil
	12	125	2	DV, −1/−2
3.	0	*294*	6	Stable (DV, −1/−2)
	2	*285*	6	Better (no DV, −1/−1)
	4	*427*	4	Stable (− 1/−1)
	6	*427*	4	DV, −1/−1
4.	0	*707*	7	Better (no DV, −1/2/−1/2)
	3	*902*	5	Stable (no DV, −1/2/−1/2)
	7	*609*	4	Stable (− 1/2, −1/2)
	10	194	3	DV, − 1/−2
5.	0	*595*	5	Worse (DV, −2/−2)
	1	*300*	6	Better (DV, −1/−1)
	3	*573*	5	Stable (DV, −1/−1)
	4	*872*	5	Stable (DV, −1/−1)
	6	*929*	5	Stable (DV, −1/−1)
	8	*491*	3	Stable (DV, −1/−1)
	10	183	3	Stable (DV, −1/−1)
	12	*302*	3	Worse (DV, −2/−2)
6.	0	*711*	10	Better (DV, −1/−1)
	2	40	6	Stable (DV, −1/−1)
	4	*200*	4	Stable (DV, −1/−1)
	6	176	4	Better (DV, −1/2/−1/2)
	9	172	4	Stable (DV, −1/2/−1/2)
	12	98	2	Nil
7.	0	*912*	6	Nil
	5	*248*	3	Nil
	6	106	3	DV, −1/−1
	8	*320*	3	Nil
	12	*252*	5	Nil
	13	–	6	Nil
8.	0	*1072*	2	Nil
	3	*591*	4	Nil
	6	*804*	3	Nil
	8	*550*	2	DV, −2/−1
9.	0	*526*	7	Worse (DV, −2/−2)
	2	*197*	7	Stable (DV, −2/−2)
	4	188	6	Nil
10.	0	65	0	Nil
	1	168	0	Nil
	3	*335*	0	Nil
	5	*620*	0	Nil
11.	0	59	3	Nil
	3	*530*	5	Nil
	9	165	3	Nil
	21	NT	1	Nil

[1]Measured in enzyme-linked immunosorbent assay. Results were expressed as optical density (OD) × 1000. To determine a normal range we assayed 30 healthy males aged < 30. We then calculated the mean OD of these healthy subjects +2 s.d. × 1000, giving a cut-off value of 194. NT = not tested.

[2]Following classification of Werner [19] as modified by Mourits *et al.* [20]. [3]Eye muscle dysfunction namely, double vision (DV) (scored yes or no) and decreased eye movement, scored −1 to −3 where −1 = mild decrease in upward and upward/outward gaze, −2 = moderate reduction and − 3 = severe reduction (ophthalmoplegia) either being taken as significant ocular myopathy subtype of TAO. Other gazes were normal in all patients.

the time of the first clinic visit, or developed eye signs within the first 6 months, and positive calsequestrin antibody tests on one or more occasions, calsequestrin antibodies were first detected at the time of, or just before, the onset of eye muscle dysfunction in six patients, while positive antibody results correlated with congestive changes in the other five.

The rationale for performing the study was that because calsequestrin is expressed much more in eye muscle than other skeletal muscle, the localization of eye muscle involvement in Graves' disease could be explained by targeting this antigen. In our earlier study, performed several years ago [18], we detected antibodies to a 63 kDa protein by immunoblotting in 40% of patients with active ophthalmopathy and 5% of controls. Now, using a preparation of highly purified rabbit skeletal muscle calsequestrin, which has a 97% homology with human calsequestrin as antigen, the prevalences of calsequestrin antibodies in controls and other control subjects without ophthalmopathy were very low, indeed much lower than reported with the other eye muscle antibodies, suggesting that these antibodies may be the most specific for the ophthalmopathy associated with thyroid autoimmunity.

Antibodies against Fp and G2s are also linked closely to the eye muscle component of ophthalmopathy, but the tests are more often positive in normal subjects and also present in small proportions of patients with Graves' hyperthyroidism and Hashimoto's thyroiditis without ophthalmopathy ([5,10–13]; Gopinath *et al*. submitted). Fp and G2s antibodies are thus unlikely to be implicated in the pathogenesis of the eye muscle disorder and appear to be secondary to antigen release following eye muscle damage, probably through a T cell-mediated mechanism [21]. Although not pathogeneic, flavoprotein antibodies are also good markers of eye muscle involvement in patients with non-specific orbital inflammation [22] and ocular myasthenia gravis [23].

Although the most commonly held theory for the pathogenesis of ophthalmopathy is autoimmunity against a TSH-r-like protein and orbital fibroblast and eye muscle cell [24–26], levels of TSH-r antibodies do not correlate closely with clinical features of eye disease, including signs of eye muscle damage, in seminal case reports [27,28]. In the present study the prevalence of TSH-r antibodies was greater in patients with ophthalmopathy, taken as a group, than Graves' hyperthyroidism without eye involvement, although not significantly so. However, as the former includes nine patients in whom their hyperthyroidism had been treated many months or years earlier, the persistence of TSH-r antibodies does support an association between ophthalmopathy and these antibodies. On balance, TSH-r antibodies may play a role, probably by initiating the orbital inflammatory reaction in patients who develop congestive ophthalmopathy.

Calsequestrin antibodies are linked closely to the eye muscle component of ophthalmopathy being detected in all patients with clinically evident eye muscle disease. Although the antibodies were also detected in 78% of patients with no clinical evidence for eye muscle dysfunction eye muscle damage, and in five patients with congestive ophthalmopathy followed prospectively in our study, orbital imaging was not performed in most of these patients and eye muscle swelling in the absence of eye signs cannot be excluded. However, because eye muscle volumes are increased in as many as 90% of patients with Graves' hyperthyroidism [29] with or without eye signs, orbital imaging may be helpful in the diagnosis of eye muscle damage in patients without clinical signs of the disease. Calsequestrin is primarily localized perinuclearly and in cross-striations flanking Z lines beneath the sarcoplasmic reticulum in the adult eye muscle myofibre, but it is distributed throughout the cell in the myotube stage of differentiation [30], where it could be seen by antibodies and/or T lymphocytes. In conclusion, anti-calsequestrin antibodies appear to be very good markers for TAO, especially the eye muscle component, and their measurement may be helpful in the differential diagnosis of orbital inflammation. The antibodies may also be good markers to assess efficacy of new therapies for ophthalmopathy. However, these results must be considered preliminary until a large prospective study of patients with newly diagnosed Graves' hyperthyroidism, in which serum levels of calsequestrin antibodies are correlated with clinical changes and orbital eye muscle and connective tissue/fat volumes has been carried out. We also need to test patients with other skeletal muscle inflammatory and autoimmune disorders to confirm disease specificity of the antibodies.

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