Genetic Study of Thyroid Eye Disease

Dr Jwu Jin KHONG

Student Number 33622

ORCID Number 0000-0002-2660-4668

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1 Abstract
Thyroid-associated orbitopathy (TO) is an autoimmune-mediated orbital inflammation that affects 25% of patients with Graves’ disease. The close temporal relationship between onset of Graves’ disease and TO suggests they share a common aetiology. While the complex inheritance of Graves’ disease is better characterized, little is known of the genetic susceptibility in TO. Multiple environmental factors such as smoking, male, older age are known risk factors for development of TO, however the extent of gene-environmental interaction remains largely unknown. The molecular mechanisms driving the development of TO is incompletely understood, hence targeted treatment options for TO remained limited.

This thesis is undertaken to test the hypothesis that there is genetic susceptibility that predispose to development of thyroid-associated orbitopathy. The research project initially examined exogenous risk factors associated with TO in a large Australian cohort with Graves’ disease, in order to identify environmental factors important for subsequent covariates adjustment when analyzing genetic findings. The risk factors association study found smoking, older age and longer duration of Graves’ disease correlated positively with TO, and secondarily there was relative selenium deficiency in TO cases compared to Graves’ disease patients without eye involvement.

A genome-wide association study using deoxyribonucleic acid pooling approach and high-throughput array platform were used to discover gene variants associated with thyroid-associated orbitopathy in a case-control study design. The genetic findings were followed by a second stage individual genotyping targeting fewer markers to validate the genetics variants identified through genome-wide association study in the discovery cohort and independent replication study cohort. MACROD2, a novel gene that encodes an eraser of mono-ADP-ribosylation, possibly has a role in nuclear factor κβ signaling, showed evidence of association with TO in genome-wide association study and also in validation genotyping.

A secondary aim of the thesis is to determine differentially expressed genes 
*a priori* in active thyroid-associated orbitopathy orbital adipose tissue using microarray to explore molecular mechanisms of TO and to correlate gene
expression findings with the genetic study. The study found \textit{TIMD4, DEFA1, DEFA1B,} and \textit{DEFA 3} were over-expressed in active TO compared with inactive TO suggesting a pathogenic role of the innate immune response in TO. Active TO was marked by up-regulation of multiple genes involved in cell-mediated, innate and inflammatory responses with concurrent enhancement of orbital adipogenesis. For the first time, epigenetic factors was implicated in the pathogenesis of TO. However \textit{MACROD2} were not differentially expressed in active TO when compared with either inactive TO or normal control.

Overall the findings from this thesis further our understanding on the genetic and environmental risk factors involved in thyroid-associated orbitopathy and give new insights into the underlying complex molecular mechanisms. The novel insights into candidate molecules and pathways can be explored to develop alternative treatment strategies for TO.
2 Declaration
This thesis comprises solely my original work towards the Ph. D. except the introductory chapters which comprises review of the body of literatures pertaining to the risk factors and pathogenesis of thyroid-associated orbitopathy, and where indicated in the preface.

Due acknowledgement has been made in the text to all other copyrighted material used.

The thesis is fewer than 100 000 word limit in length, exclusive of tables, figures, bibliographies and appendices, as approved by the Research Higher Degrees Committee.
3 Publications

Peer-reviewed articles:


4 Preface

Multi-disciplinary collaborations were carried out for the research work. The contribution of others involve statistical support, supervision on data analysis using statistical softwares, technical support on DNA extraction and pooling of DNA for running of the microarrays, and manuscripts language editing by co-authors, comprising an overall contribution of 20% of the research work.

None of the research work presented in the thesis has been submitted for other qualifications, and none was carried out prior to enrolment in the degree. No third party editorial assistance was requested in preparation of the thesis.

For the multi-authored published paper “Baseline characteristics and risk factors in thyroid-associated orbitopathy in Australian Thyroid-associated orbitopathy Research (ATOR)”, Dr Sue Finch provided statistical strategy to group measured variables, choosing the best-fit logistic regression model and advised on tables and figure presentation. Mr Chamika De Silva assisted in recruiting 3% of ATOR study population. Ms Stacey Rylander recruited 8% of ATOR study population. Professors Jamie Craig, Dinesh Selva, Peter Ebeling and Dr Sue Finch provided editorial assistance.

For the multi-authored published paper “Serum selenium status in Graves’ disease with and without orbitopathy: a case-control study”, Dr Rebecca Goldstein compiled thyrotropin receptor antibody levels and thyroid function tests for the studied subjects and recruited 1% of ATOR study population. Professor Kerrie Sanders provided statistical support in multivariate analysis, Dr Hans Schneider provided laboratory service for measuring selenium level using atomic absorption spectrophotometer and Mr Jeffrey Pope conducted the selenium level measurement. Professors Kathryn Burdon and Jamie Craig provided editorial assistance. Professor Peter Ebeling was involved in the conception of this research project and supervised the overall conduct of the experiment.

For the multi-authored published papers “Pooled genome wide associated study is effective in detecting genomic loci associated with Graves’ disease” and “Association of polymorphisms in MACRO domain containing 2 with thyroid-associated orbitopathy”, Associate professor Kathryn Burdon coordinated DNA pooling constructions, taught Plink analysis for individual genotyping and
contributed intellectually towards the analysis of the data. Professor Grant Montgomery coordinated running of the microarray experiments and Dr Yi Lu performed statistical analysis for the pooled genome wide associations study (GWAS) genotyping under supervision of Professor Stuart Macgregor. Ms Kate Laurie and Ms Lefta Leonardos conducted DNA extractions. Drs John Walsh, Adam Gajdatsy, Paul Baird and Srujana Sahebjada contributed study samples for the study cohorts. Drs Peter Ebeling, Peter Hamblin, Rosemary Wong, Simon Forehan, Mark Stein, Spiros Fourlanos, Peter Colman, Anthony Roberts and Matthew Doogue (endocrinologists), Drs Alan McNab, Thomas Hardy, Richard Stawell, Dinesh Selva and Garry Davis (ophthalmologists) referred patients for recruitment to the ATOR study population. Prof Jamie Craig and Prof Stuart Macgregor validated the pooled GWAS methodology, Dr Angelo Tsirbas provided conception of idea for the genetic study of thyroid-associated orbitopathy. Prof Jamie Craig has oversight of the pooled GWAS project.

For the multi-author published paper “Differential gene expression profiling or orbital adipose tissue in thyroid-associated orbitopathy”, Ms Lynn Wang performed biostatistics under the supervision of Prof Gordon Smyth. Prof Gordon Smyth performed additional analysis following peer-review feedback. Drs Alan McNab and Thomas Hardy procured orbital adipose tissues for the study. Dr Bastien Llamas taught RNA extraction technique. Dr Chol-Hee Jung performed preliminary analysis of microarray gene expression profiles and taught me basics of biostatistics. Drs Kathryn Burdon provided intellectual input and Shiwani Sharma assisted in selecting genes for validation. Prof Dinesh Selva, Peter Ebeling and Jamie Craig provided editorial assistance and facilitated conduct of the experiment.

Ophthalmic Research Institute of Australia funded research titled “A national registry of thyroid eye disease for genomic and transcriptomic studies” in 2011 in support of this PhD research.

The National Health and Medical Research Council of Australia (NHMRC 1031362) funded project titled “Genome-wide associations studies to identify major genetic determinants of 5 blinding eye diseases using pooled DNA” between 2012-2014.
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8 Introduction
Thyroid-associated orbitopathy (TO) is an autoimmune inflammatory disorder involving the orbit and is known by various names: thyroid eye disease, Graves’ ophthalmopathy (GO), endocrine ophthalmopathy, dysthyroid ophthalmopathy and euthyroid ophthalmopathy. TO is a clinical feature of autoimmune thyroid disease (AITD). In some patients typical eye findings occur in the absence of thyroid dysfunction. The first reported hallmark correlation of exophthalmos with tachycardia and enlargement of the thyroid gland was described within a short period of time by three physicians from 1802 to 1840 namely Flajani in Italy, Graves in Ireland and Basedow in Germany; Parry from England described tachycardia, enlargement of the thyroid gland and heart, weight loss but did not mention exophthalmos in his eight patients series. The four physicians provided data on 18 patients, in combination give a fairly accurate description of AITD as it is known today: predominates in female patients, the presence of goiter in a majority, exophthalmos in a third with more severe course in males, presence of pretibial myxoeedema and acryopachy.

8.1 Epidemiology
90% of patients with TO have Graves' disease and are hyperthyroid, 5% of patients are hypothyroid and another 5% are euthyroid. TO occurs in 25-50% of patients with AITD, 3-5% presenting severely with optic nerve compression or exposure keratopathy. In the setting of non tertiary referral centre, 73.7% newly diagnosed Graves’ hyperthyroid patients had no ocular involvement, 20.2% had mild and inactive TO, 5.8% had moderate-to-severe and active TO and 0.3% had compressive optic neuropathy. When the diagnostic criteria for TO was inclusive of periocular soft tissue involvement (NOSPECS class 2 or higher), close to 50% of Graves’ disease patients were reported to have TO. The prevalence of TO had been approximated from incidence of Graves’ disease by Lazarus. Given the proportion estimate of TO at 50% of Graves’ disease and TO duration of 25 years, the prevalence estimates were between 0.1 to 0.3% across Europe, United States, China and India, suggesting that the expression of TO phenotype in Graves’ disease was similar across diverse ethnic groups.
TO is an uncommon disease. Bartley studied the incidence rates of TO in Olmsted County in Minnesota between 1976 through to 1990. The overall age-adjusted incidence rate for females was 16.0 cases per 100,000 population per year, whereas the rate for males was 2.9 cases per 100,000 population per year. Of note was the bimodal peak incidence rates in the age groups 40-44 years and 60-64 years in females and older again in males in the age groups 45-49 years and 65-59 years. The average age at the time of diagnosis of TO was 44.7 +/- 17.4 years (median 43.4, range 8.2-88.7). More recently a Danish study comparing incidence of moderate to severe TO before and after iodization of salt between 1992-2009 found over the 18 years period, the incidence of moderate to severe TO was 16.1 per million per year with no difference in incidence rates before (15.9 per million per year) and after (16.3 per million per year) iodization of salt. The Danish population incidence rate is 1/10 that of Bartley's Olmsted county incidence rate, perhaps because the criteria for diagnosing GO was different, being moderate to severe in Laurberg's paper and mild to severe in Bartley's paper.

8.2 Diagnostic criteria for TO
The diagnostic criteria and severity classification for TO remains a work in progress as there is not a gold standard definition universally used by the research workers. The clinical diagnosis of TO is perhaps most precisely defined by the following characteristics, as proposed by Frueh in his thesis: 1) A personal history of thyroid disease; 2) a positive computed tomography (CT) scan defined by the presence of at least two enlarged extraocular muscles in one orbit, or at least one enlarged in the other 3) exophthalmos, defined as exophthalmometer reading of 20mm or greater 4) lid retraction defined as either palpebral aperture greater than 11mm or at least one lid is 7mm or more from the centre of the pupil. 5) extra-ocular muscle involvement, defined as restriction of at least one extraocular muscle. At least two of the following clinical features—exophthalmos, lid retraction or extraocular muscle involvement and bilaterality on CT scan or bilaterality of the above clinical features were proposed to be diagnostic.
However more simplistic criteria has been widely used. The severity grading of TO was first proposed by Werner in 1969, the NOSPECS classification was further modified in 1977 to include class 0 for no signs of TO, class 1 for presence of lid retraction, eyelid lag only, class 2 periocular soft tissue swelling, class 3 proptosis 23mm or greater, class 4 for extraocular muscles involvement, class 5 corneal involvement with exposure keratopathy, class 6 compressive optic neuropathy.\textsuperscript{10,11} NOSPECS classification has been recommended by the American thyroid association for many years\textsuperscript{12} and used in numerous research papers as way to classify TO. However NOSPECS classification is fraud by several limitations; the progression of TO is not sequential through each of the 6 classes, proptosis as defined does not take into consideration of ethnicity factor where upper limits of normal exophthalmometer for Japanese and Chinese are 18mm and for Black American is 22mm.\textsuperscript{13} Disease activity of TO was not accounted for and patients have a combination of clinical findings stranding across the classes proposed. Using the modified NOSPECS classification, significant proptosis was underestimated in 12% of TO cases compared to 1969 NOSPECS when proptosis was defined as 21mm and above.\textsuperscript{13} The European Group of Grave’s orbitopathy (EUGOGO) prospectively recorded in 90 consecutive untreated TO cases 90% with soft tissue involvement, 30% with proptosis at 23mm or more and eye muscle involvement in 60% of TO patients. Orbital CT showed enlargement of inferior rectus in 60%, medial rectus in 50%, superior rectus in 40% and lateral rectus in 22% of TO cases; unilateral eye involvement was present in 14% of patients using the revised NOSPECS classification.\textsuperscript{13} 1992 International Working group including American thyroid association, European thyroid association, Asia-Oceania thyroid association and Latin America thyroid association considered the application of NOSPECS as useful mnemonics and reached consensus on new guidelines for the assessment of TO, emphasizing the objective quantitative measurement of changes in eyelids, cornea, extraocular muscles, proptosis, optic nerve function, indexation of TO activity score and patient self assessment.\textsuperscript{14}

Since then, the European group on Graves’ Orbitopathy's (EUGOGO) severity scale, the Clinical Activity Score (CAS) and the Vision, Inflammation, Strabismus and Appearance (VISA) classification have evolved to characterize rather than
categorize TO for the disease severity and activity.\textsuperscript{15-17} The grading of severity of TO is still somewhat subjective and imprecise, a study is currently underway by International thyroid eye disease society to assess the reliability of grading schemes by clinical and photographic measurements.\textsuperscript{18}

In the key landmark papers on clinical presentation of TO, Bartley reported that bilateral upper lids retraction were present in 90.8\% of patients at some point of the clinical course of 120 incident TO; proptosis, where exophthalmometer reading was 20mm or above was present in 62.4\% of patients. Compressive optic neuropathy was present in 7 patients (5.8\%) attributable to TO.\textsuperscript{2} In Kendler’s cohort of 557 consecutive TO patients, the most common reported symptoms were soft tissue symptoms (40\%), bulging eyes (35\%) and diplopia (20\%); Loss of vision is less common at 6\%.\textsuperscript{19} In Kendler’s cohort of TO patients, soft tissue signs were most common being noted in 72.2\% of all eyes, exophthalmos >20mm was recorded in 49.2\% where unilateral proptosis was present in 13.1\% of all patients.\textsuperscript{19} Based on these papers, upper lid retraction is the most common finding in TO, with a characteristic lateral flare and should be considered as a minimum diagnostic criteria for TO; proptosis is the second most common clinical finding in TO, followed closely by periocular soft tissue swelling.\textsuperscript{18}

8.3 Natural History of TO
The natural progression of TO without treatment was initially described by Rundle and Wilson in 1945, who did serial measurement of exophthalmos in 2 affected patients over 30 months. Their study revealed TO progressed rapidly in the early dynamic phase, reaching a maximal point of severity, then plateau over time to a static phase.\textsuperscript{20} The curve of the principal ocular changes based on eye protrusion, range of eye elevation and upper lid retraction during the dynamic (active) and static (inactive) phase of TO over months is famously known as the Rundle’s curve, and depicts the natural history of TO.\textsuperscript{21, 22}

Many patients with TO developed ocular symptoms within the first year of diagnosis of Graves’ disease. In 196 patients with newly diagnosed Graves’ disease in Switzerland, 70\% of patients developed TO in the same year when hyperthyroidism was diagnosed, the remaining 11 patients (14\%) developed TO in the year following the diagnosis of hyperthyroidism and 10 patients (13\%)
with TO presented over 2 years after the occurrence of hyperthyroidism; a further 2 patients (3%) developed TO preceeding the manifestation of hyperthyroidism.\textsuperscript{6} In Kendler's cohort of 557 patients with TO, 368 (66%) of patients presented with TO within 18 months of diagnosis of thyroid disease, the mean absolute interval between thyroid disease and orbital disease was 3.3 years in men and 3.6 years in women, with a significantly longer interval of eye disease presentation in patients older than 50 years (4.24 versus 1.78 years, p<0.001).\textsuperscript{19}

The progression and outcome of new-onset TO were studied by a few groups of researchers. Perros followed the natural history of a group of 59 untreated mild to moderate TO patients with a median duration of follow up for 12 months, spontaneous improvement in the severity of eye disease occurred in 22% substantially, 42.4% showed minor improvement%, 22% eye disease remained stable and 13.5% deteriorated progressively to the extend where immunosuppressive treatment was considered necessary.\textsuperscript{23} In Noth's paper looking at TO as a whole, including treated and untreated patients (n=83), 34 patients (42%) TO course remained stationary, 26 (32%) improved partially, 18 (22%) improved to full remission, and 3 (4%) progressively deteriorated over a mean observation period of 2.85 years.\textsuperscript{6} Of the 53 TO patients without intervention, 25 (47%) improved substantially, 26 (49%) remained stable and 2 (4%) deteriorated progressively, where TO was defined as improved or worsen by the following criteria: regression or progression of exophthalmos by 2mm or more, changes in inflammatory symptoms or signs, regression or progression of diplopia, improvement or deterioration of vision.\textsuperscript{6} The outcome of TO in a more recent cohort of Graves' disease patients in Tanda et al appeared better where progression from mild TO or no TO to moderate-to-severe TO rarely develop during anti-thyroid drug treatment, bearing in mind patients needing total thyroidectomy or radioactive iodine treatment were excluded from the study.\textsuperscript{5} Tanda had full follow up over 18 months for 237 patients who had anti-thyroid medication treatment for Graves' hyperthyroidism over 18 months; of the 43 (18.1%) patients with mild and inactive TO at baseline, one (2.4%) progressed to moderate-to-severe TO and 25 (58.1%) experienced complete remission. Of the
194 (81.9%) Graves’ patients without TO at baseline, 5 (2.6%) developed TO that progressed to moderate-to-severe TO.5

Menconi and associates retrospectively studied variation of exophthalmometry, eyelid aperture, clinical activity score (CAS), diplopia, visual acuity, NOSPECS score and overall TO outcome in 65 patients with untreated TO over two periods of assessment at median follow up of 7 months and 40 months, and found significant improvement in CAS and NOSPECS status over time. Plotting of CAS and NOSPECS status resembled Rundle’s curve with CAS peaked between 13 and 24 months after appearance of TO symptoms and NOSPECS curve slightly delayed and peaked between 19 and 24 months then decreased between 25 and 36 months, and remained stable thereafter.24 In Menconi’s cohort of patients 50.8% of patients had improved, 33.8% remained stable and 15.4% had worsen moderate to substantially at last follow up.24

In summary according to the natural history of at least the mild TO group, about half improve spontaneously while the remainder of TO cases remained non-progressive and a small proportion of mild TO cases deteriorate to moderately severe TO in 3-5% of patients.
9 Thyroid-associated orbitopathy: an update on pathogenesis and a review on the genetic and exogenous risk factors

Publication arising from this chapter:


Orbital changes in thyroid-associated orbitopathy (TO) result from de novo adipogenesis, hyaluronon synthesis, interstitial oedema and enlargement of extra-ocular muscles. Recent advances in transcriptomics and proteomics have brought new insights into the molecular basis of TO. These discoveries have lead to the emerging use of monoclonal antibodies and will undoubtedly eventually lead to more specific therapies for this challenging condition. This review explores the underlying molecular mechanisms of TO, highlighting the basis for emergent prevention and treatment options; and provides an overview of the current understanding of the environmental and genetic risk factors for TO in Graves’ disease (GD) patients.

9.1 Current understanding of pathogenesis of thyroid-associated orbitopathy

9.1.1 Anatomical and pathological changes in TO

The early active phase of TO is dominated by orbital inflammation, manifest clinically as periocular swelling, exophthalmos, retro-orbital pain, red eyes and double vision.25, 26 Pathological changes of TO in the orbit appears to involve both the extraocular muscles and the orbital fat compartments, with computed tomography (CT) indicating most patients have a mixture of both extraocular muscle enlargement and orbital fat expansion.27 Proptosis is due to expansion of orbital tissue within the unyielding confines of the bony orbit. The consequent increase in orbital pressure can also lead to venous outflow congestion and chronic periorbital oedema.28

Histological examination of affected extraocular muscles shows extraocular muscles enlargement due to deposition of glycosaminoglycan (GAG),
predominantly hyaluronan (HA) within the muscles' endomysial space.\textsuperscript{29} GAG are long un-branched polysaccharides consisting of repeating disaccharide units. GAG in connective tissues consists of chondroitin sulfate, dermatan sulfate and HA; heparan sulfate is found usually in basement membrane or associated with cells. HA is the only non-sulfated GAG that has varying molecular weights depending on tissue types.\textsuperscript{29-31} Hansen and Kahaly discovered total orbital GAG in TO is markedly elevated with significant increase in chondroitin sulfate and HA; correspondingly 24 hour urinary total GAG, dermatan sulfate and HA are also elevated in TO compared with normal control.\textsuperscript{32, 33} Transmission electron microscopy of extraocular muscle section in inactive TO cases showed expansion of the endomysial space in between the muscle fibres are filled with fibrous collagen fibres interspersed with amorphous material, which on immunogold staining was confirmed to be HA.\textsuperscript{29} HA from orbital cells is primarily >500 000 daltons high molecular weight polymers.\textsuperscript{31} As HA is highly anionic, intense water binding leads to pronounced orbital interstitial oedema and extraocular muscle expansion without disruption of muscle fibres.\textsuperscript{29, 31, 32}

Furthermore, immunostaining of extraocular muscles in TO shows diffuse and focal lymphocytic infiltrates, interstitial oedema and fibrosis, whereas orbital fat and connective tissue contained few infiltrating cells. The majority of mononuclear cells are T cells, along with a few B cells, macrophages and mast cells in the intercellular space.\textsuperscript{34, 35} Macrophages, monocytes and mast cells are also located in the perivascular interstitial space and in between fibroblast cells with co-localization of platelet derived growth factors in the orbital tissue.\textsuperscript{36, 37}

9.1.2 Effector cell in TO
Current evidence suggests the orbital fibroblast is the key effector cell in TO.\textsuperscript{38} Not only do orbital fibroblasts proliferate and differentiate into myofibroblasts and adipocytes, they produce GAG in excess, undergo adipogenesis and actively interact with mononuclear cells, produce chemoattractants and cytokines, which ensure perpetuation of orbital inflammation.\textsuperscript{36, 39-41} Most of our understanding of orbital fibroblasts in the pathophysiology of TO is derived from \textit{in vitro} culture studies. Orbital fibroblasts and preadipocyte cultures when subjected to differentiation medium underwent adipogenesis with increased peroxisome
proliferator-activated receptor-gamma (PPAR-γ) transcripts and lipoprotein lipase (LPL) expression, accompanied by increased HA production and hyaluronic acid synthase 2 (HAS2) mRNA transcripts. Interleukin (IL)-1β and leukoregulin stimulate a marked increase in HA secretion in TO orbital fibroblasts. Activated orbital fibroblasts from TO showed a robust response to pro-inflammatory cytokines compared with normal controls and secrete higher levels of pro-inflammatory cytokines including IL-1α, IL-1β, IL-6, IL-8, macrophage chemoattractant protein-1 (MCP-1), transforming growth factor (TGF)-β when stimulated by cytokines and growth factors. Heterogenous presentations of TO could be due to cellular divergence of orbital fibroblasts within the orbit. The fibroblast populations in the orbit are phenotypically heterogeneous, and differ with regards to surface glycoprotein, production of pro-inflammatory cytokines and cell surface receptors. The perimysial orbital fibroblasts uniformly express Thy-1, whereas adipose tissue orbital fibroblasts show bimodal distribution of both Thy-1-positive and Thy-1-negative cells. Both Thy-1-positive and Thy-1-negative fibroblasts express high levels of PPAR-γ but only the Thy-1-negative adipose orbital fibroblasts differentiate and accumulate lipid droplets. On the other hand, only Thy-1-positive orbital fibroblasts can differentiate into myofibroblasts on stimulation with TGF-β.

The innate depot differences in fibroblasts may also explain the predilection for orbital and pretibial extra-thyroidal involvement in GD. Adipogenesis and HA synthesis in orbital preadipocytes and fibroblasts is site specific, occurring in both TO and normal controls. Regional differences exist in basal PPAR-γ expression and responses of human pre-adipocytes to PPAR-γ and retinoid X receptor α agonists. Orbital fibroblasts also express considerably higher IL-6 and IL-6 receptor, and prostaglandin E2 (PGE2) than dermal fibroblasts when induced by IL-1β and leukoregulin respectively.

9.1.3 Molecular mechanisms underlying TO
The molecular mechanisms whereby recruitment of immune cells into the orbit, the molecular bridge between immune cells and orbital fibroblasts, molecular pathways leading to proliferation and differentiation of orbital fibroblast,
secretion of HA, adipogenesis and perpetuation of orbital inflammation are now better understood. (Figure 1)

9.1.3.1 Cellular Immunity

T cell infiltrates in TO orbital tissues are predominantly CD4+, with some studies suggesting presence of both CD8+ and CD4+ T cells.\textsuperscript{55-58} Th1 like cytokine profile predominates in TO retrobulbar tissue.\textsuperscript{55, 58} Th1-like cytokine expression profile consisting of interferon (IFN)\textsubscript{γ}, tumour necrosis factor (TNF)-\textalpha, IL-1β and IL-6 has been detected mainly in TO extraocular muscles, whereas IL-4 and IL-10, Th2-type cytokines were detected predominantly in orbital fat.\textsuperscript{48} Predominance of T cell subsets is also disease duration dependent, with Th1 cells dominating in the active phase of TO, shifting towards Th2 cells in the late phase.\textsuperscript{59}

Proliferation of orbital fibroblasts is activated by interaction of autoantigens on the fibroblasts with T cells that involve contact of T cell receptor with Major histocompatibility complex class II molecule (MHC II) and CD40: CD154 signalling.\textsuperscript{40} Co-culture of orbital fibroblasts with autologous T cells stimulates production of MHC II molecule and proliferation of orbital fibroblasts in a dose dependent manner; blocking antibodies to MHC II, CD40 and CD40 ligand (CD154) completely inhibit proliferation of orbital fibroblasts.\textsuperscript{40} CD40 expression is up-regulated in orbital fibroblasts by interferon-γ (IFN-γ) mediated through Jak2.\textsuperscript{46} Ligation of CD40 with CD154 increased secretion of intercellular adhesion molecule-1 (ICAM-1),\textsuperscript{60} nuclear translocation of nuclear factor-κβ (NF-κβ),\textsuperscript{61} IL-6, IL-8 and macrophage chemoattractant protein-1 (MCP-1) in TO orbital fibroblasts compared with normal controls.\textsuperscript{46} In addition, CD40 upregulates IL-1α secretion, HA and PGE2 synthesis.\textsuperscript{62} The molecular signaling triggered by CD40:CD154 ligation involve all three mitogen activated protein kinase (MAPK) pathways, p38, ERK1/2 and JNK, which mediate cellular activities such as gene expression, cellular proliferation, differentiation and apoptosis. ICAM expression is predominantly P38 MAPK and NF-κβ dependent, whereas ERK1/2 and JNK also activate the NF-κβ pathway, a transcription factor pathway that regulates genes involved in immune and inflammatory responses.\textsuperscript{60}
9.1.3.2 Role of cytokines

Study of the cytokine profile in orbital adipose tissue in TO and normal individuals shows over-expression of IL-1β, TNF-α, IFN-γ, IL-6 and IL-10 which are macrophage-derived and IL-8. IL-1β is expressed the most differentially.47 Similarly, patients with active TO have higher IL-1β, IL-6, IL-8 and IL-10 compared with inactive TO.63 Orbital fibroblasts from TO when stimulated by IL-1β up-regulate secretion of pro-inflammatory cytokines IL-6 and IL-8, PGE2, IL-6R and T cell chemoattractants, IL-16 and Regulated on Activation, Normal T Cell Expression and Secreted (RANTES), which recruit T cells into the orbit.49, 53, 54, 64 IFN-γ upregulates CD40 expression on orbital fibroblasts and fibrocytes.65 IL-6 increases the expression of thyrotropin receptor (TSHR) in orbital fibroblast pre-adipocytes, and promotes B cell differentiation and immunoglobulin production.66, 67 IL-1β uses p38 and ERK1/2 MAPK pathways to induce IL-6 gene expression.53 Immunoglobulin G from patients with GD substantially upregulates RANTES and IL-16, Akt/FRAP/mammalian target of rapamycin (mTOR)/p70 pathway is implicated in the induction of IL-16.64

9.1.3.3 Hyaluronan Synthesis

IL-1β, leukoregulin, CD154, TGF-β1 and platelet-derived growth factor (PDGF) are all involved in stimulating HA synthesis, likely via receptor and ligand binding on the orbital fibroblast.36, 44, 45, 68 Orbital fibroblast surface receptors for TSHR and insulin-like growth factor-1 receptor (IGF-1R) both appear to stimulate HA synthesis. TSHR activation alone is sufficient to upregulate expression of HAS1 and HAS2 and HA production via cyclin adenosine monophosphate (cAMP) and Akt/phosophoinositide 3-kinase (PI3K) signaling with upregulation of HA production.31, 69, 70. On the other hand both immunoglobulin G (IgG) from GD and IGF-1 stimulate an equivalent and substantial increase in HA synthesis in orbital fibroblasts, suggesting alternative IGF-1R pathways are also involved in HA synthesis.71, 72 However, the effect of IGF-1 on HA synthesis appears indirect as IGF-1 alone does not increase HAS2 transcription. The stimulatory effect of IGF-1 on HAS transcription is unmasked
by MAPK kinase inhibitor but not mTOR or PI3K inhibitors in orbital fibroblasts.\textsuperscript{42}

IL-1\(\beta\) is a potent stimulator for GAG synthesis. Increased secretion of HA in TO orbital fibroblasts by IL-1\(\beta\) is due to predominant induction of HAS2, and to a lesser extent HAS3. The effects of HAS mRNA induction by IL-1\(\beta\) can be inhibited by glucocorticoids.\textsuperscript{43} PDGF-\(\beta\) and TGF-\(\beta\) are growth factors that are significantly increased in TO orbital tissues. They induce orbital fibroblast proliferation and stimulate HAS1 and HAS2 expression in TO orbital fibroblasts.\textsuperscript{45,68} TGF-\(\beta\) acts via the Smad pathway.\textsuperscript{45} TGF-\(\beta\) treated orbital fibroblasts also bind activated human T cells through HA-CD44 interaction, thus promoting lymphocyte chemotaxis and adhesion to pro-inflammatory sites.\textsuperscript{68} Addition of PPAR-\(\gamma\) ligands on the other hand inhibits TGF-\(\beta\) induced HAS1 and HAS2 expression and attenuate HA synthesis independent of the PPAR-\(\gamma\) pathway.\textsuperscript{68}

\textbf{9.1.3.4 Adipogenesis}

\textit{De novo} adipogenesis is enhanced in TO as evidenced by increased expression of adipocyte specific genes leptin, adiponectin, fatty acid synthase, adipocyte fatty acid binding protein (AP2) and PPAR-\(\gamma\) mRNA in TO affected adipose tissue compared with normal orbital tissue.\textsuperscript{73, 74} Microarray studies provide further evidence that adipocyte related intermediate early genes, including CYR61, are over-expressed in active TO.\textsuperscript{75}

PPAR-\(\gamma\) is a potent stimulator for adipogenesis in TO, evident by increased expression of PPAR-\(\gamma\) in active TO adipose tissue compared with normal controls.\textsuperscript{76} PPAR-\(\gamma\) agonist, rosiglitazone increases TSHR expression, PPAR-\(\gamma\) mRNA and cAMP levels by 2.6-4.7 fold, resulting in adipogenesis in TO orbital fibroblasts both by proliferation and differentiation of adipocytes.\textsuperscript{77}

Signaling for adipogenesis has been shown to involve both TSHR and IGF-1R. It appears both TSHR and IGF-1R share the same intracellular AkT/PI3K signaling to affect adipogenesis. The close relationship of TSHR and IGF-1R in triggering adipogenesis in TO perhaps could be explained by co-localization of these two receptors on orbital fibroblasts.\textsuperscript{78} Stimulatory TSHR antibody increases phosphorylated AKT protein, cAMP levels and enhanced adipogenesis via the
phosphoinositide 3 kinase (PI3K) signaling cascade. On the other hand IGF-1 mediates proliferation and differentiation of human and murine 3T3-L1 preadipocytes into adipocytes. IGF-1 mediates its effect by binding to IGF-1R, and induces phosphorylation of Src homology 2 domain-containing protein (Shc) and insulin receptor substrate (IRS) and downstream AKT/PI3K pathway. IGF-1 uses Shc/IRS-1 to activate MAPK/ERK signaling in proliferating 3T3-L1 preadipocytes. Inhibiting MAPK by Shc proximal signaling switches off proliferation of preadipocytes and in turn permits differentiation into adipocytes with increased expression of PPAR-γ, LPL and AP2.

9.1.4 Autoantigens in TO

9.1.4.1 TSH receptor

Breaking of self tolerance to TSHR on thyroid epithelial cells, resulting in TSHR stimulating antibodies inducing thyrotoxicosis is well established in Graves' disease (GD). TSHR signals mainly by two G-protein mediated pathways: the adenylyl cyclase/cAMP pathway and the phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathway. Evidence from the temporal correlation of TO and GD, emerging TO animal models and correlation of disease activity and TSHR antibody increasingly point towards TSHR as the primary autoantigen in TO.

The observation that onset of TO is frequently within 18 months of diagnosis of GD, raised early on the concept that the two clinical entities are triggered by a common autoantigen. The first evidence of TSHR as an autoantigen came from identifying TSHR expression in retro-orbital tissue in cultured orbital fibroblast from patients with TO by polymerase chain reaction and liquid hybridization. Of note, the level of TSHR expression on orbital fibroblast is only of low abundance compared with thyrocytes but increases during adipogenesis and in active TO.

With improvement of TSHR assays, both thyroid binding inhibiting Ig (TBI) and thyroid stimulating Ig (TSI) TSHR titres are shown to be highly and significantly correlated with activity and severity of TO, thus inferring TSHR antigen is pathogenic in TO. The newer chimeric TSHR and cAMP response element
dependent luciferase MC4/TSI assay has higher sensitivity (97%) and specificity (89%) than the current TBI assay (77% and 43% respectively) in TO. The new MC4/TSI assay correlates strongly with clinical activity and clinical severity scores in both adults and children. In the uncommon patients with euthyroid TO, TSHR antibody was highly detectable at 93.8% using third generation TSI assay and 81.3% in second generation TBI assay, in comparison to the low TSHR positivity (18.8%) in first generation assays. Therefore insensitivity of earlier TSHR assays seems likely to explain the seemingly poor correlation of TSHR antibody with severity of TO seen in the past.

9.1.4.2 IGF-1 Receptor

IGF-1R is a ubiquitous cellular surface heterotetrametric receptor involved in diverse cellular responses including modulation of apoptosis, enhancing cell survival, growth and cellular proliferation, cell motility and migration. Evidence suggests IGF1/IGF-1R is involved in the pathogenesis of TO, but the auto-antigenic role of IGF-1R remains controversial. IGF-1R regulates lymphocyte trafficking in the orbit, HA synthesis, adipogenesis and defines T lymphocytes and B lymphocyte phenotypes and function. IGF-1R levels are three fold higher on TO compared to control fibroblasts. IgG from patients with GD induces IL-16 and RANTES secretion mediating T cell migration. These effects are shown to be induced by IGF-1 and IGF-1R specific ligand, Des(1-3) IGF-1 analogue, but not TSH. Moreover upregulation of IL-16, RANTES secretion and HA synthesis was restricted to GD orbital and dermal fibroblast and was not observed in normal control fibroblasts. Interfering with IGF1-R function completely abolished signaling induced by Ig G from GD, hence implying IGF-1R is a self-antigen mediating T cell migration, lymphocytes infiltration and HA synthesis in TO. A recent case-control microarray study also showed differentially expressed genes are dominated by IGF-1 signaling genes, with significant upregulation of IGF-1, IGF-1 signaling genes SOCS3 and SGK-1 (PDK/Akt signaling) and downregulation of IRS2 and IGFBP6 in TO.

It is now clear that once an IGF-1R antibody assay became available, that IGF-1R antibody is present in both patients with GD and healthy controls. The prevalence of IGF-1R antibody in patients with TO and healthy controls is similar
(11% in normal and 14% in TO), and there is no correlation of clinical activity score or severity of TO with IGF-1R antibody level; elevated IGF-1R antibody levels in TO also remains stable over 2 years. Furthermore IGF-1R antibody binds IGF-1R and interferes with IGF1-dependent receptor activation and signalling, its effect is inhibitory on hepatocarcinoma and breast cancer cells proliferation. Hence these findings do not support IGF-1R as an auto-antigen in TO. On the other hand in an animal model, mice challenged with IGF-1α plasmid produced strong IGF-1R antibody response, but did not induce hyperthyroidism or orbital changes. Conversely injection of TSHR A sub-unit plasmid combined with electroporation induces hyperthyroidism, and both TSHR stimulating antibody and IGF-1R antibody.

It has been shown that TSHR and IGF-1R co-localize in fibroblasts on cell membrane. More recent study revealed TSH and IGF-1 synergistically stimulate HA secretion. Upon M22 stimulation, a bidirectional cross talk between TSHR and IGF-1R mediates HA secretion. The M22 monoclonal antibody primarily actives TSHR and does not directly activate IGF-1R. The data further supports TSHR and IGF-1R cross talk having a major role in the pathogenesis of TO, and argues for TSHR as the primary antigen.
Figure 1 Model of pathogenesis of thyroid-associated orbitopathy

Figure 1 legend: T cell interacts with orbital fibroblast via CD40:CD154 ligation, and interaction of MHC II, autoantigen and T cell receptor activate orbital fibroblast with increase secretion of intercellular adhesion molecule-1 (ICAM-1),
nuclear translocation of nuclear factor (NF)-κβ, interleukin (IL)-1, IL-6, IL-8, macrophage chemoattractant and prostaglandin E2 (PGE2) secretion. Cytokines showed Th1 dominance with increase IL-1β, interferon (IFN)-γ, tumour necrosis factor (TNF)-α and IL-6. IFN-γ increases CD40 expression, IL-6 modulates B cell immunoglobulin secretion. Orbital fibroblast upregulates pro-inflammatory cytokines IL-1β, transforming growth factor (TGF)-β, leukoregulin that perpetuate orbital inflammation and increase hyaluronan (HA) synthesis. TGF-β induces myofibroblast proliferation and differentiation and promotes lymphocyte adhesion and chemotaxis by CD44 and HA interaction. Immunoglobulin G (Ig G) from Graves’ disease (GD) and insulin-like growth factor-1 (IGF-1) upregulates secretion of Regulated on Activation, Normal T cell Expression and Secreted (RANTES) and IL-16, which increase T cell migration into the orbit; the Akt/mammalian target of rapamycin (mTOR)/P70 pathway seems involve in IL-16 upregulation. Activating thyrotropin receptor (TSHR) increases hyaluronan synthase(HAS) via adenyl cyclase/cyclic adenosine monophosphate (cAMP) and Akt/phosphoinositide 3-kinase (PI3K) pathway. IGF-1 can also induce HAS and HA synthesis, the effect is unmasked by mitogen activated protein kinase (MAPK) inhibitor. Both TSHR and IGF-1R activate PI3K/Akt pathway to upregulate peroxisome proliferator-activated receptor-γ (PPAR-γ) expression, differentiation and proliferation of adipocytes and enhance adipogenesis. IGF-1R uses Src homology 2 domain-containing (SHC)/insulin receptor substrate (IRS)/MAPK signaling to increase proliferation of preadipocytes. Switching off proximal SHC signaling on MAPK in turn permit differentiation of preadipocyte to adipocytes and enhance adipogenesis.

9.1.5 Emerging TO animal model
Almost all animal models of GD utilize in vivo expression of TSHR either by transfected cells, plasmid or adenovirus. TSH subunit A seems to initiate the autoimmune response to TSHR. Many animal models developed for GD develop hyperthyroidism but fail to show TO manifestations. One that did induce orbital pathology using a splenocyte adoptive transfer model with observed extraocular muscle oedema, accumulation of PAS positive material, expansion of
adipose tissue, dissociation of muscle fibres, lymphocyte and mast cell infiltration, was not reproducible.102-104

A breakthrough in establishing a TO animal model was reported by Banga using TSHR A-subunit plasmid-immunised by muscle electroporation in BALB/c mice.105 It showed orbital remodeling with bilateral interstitial inflammatory infiltrate in the extraocular muscle, infiltration of CD3+ T cells, F4/80+ macrophages and mast cell, orbital fibrosis, GAG deposition and corresponding MRI changes of orbital muscle hypertrophy. A few mice also showed predominantly expansion of retro-orbital fat, proptosis, chemosis and congested orbital vessels. This is by far the most representative animal model of TO. TSHR and a lower level of IGF-1R antibodies were both induced.105 The less expected findings were predominance of TSH blocking antibodies, hypothyroid status and large inflammatory infiltrates around the optic nerve, which are not typical of GD. Nevertheless these findings support the pathogenic role of TSHR in the development of TO and open the door for investigating pathogenesis and therapeutic drugs in an animal TO model. Interestingly using a similar protocol with a slight alteration of the electroporation regime in an earlier study, TSHR plasmid induced a high frequency of hyperthyroidism (75%), TSHR stimulating antibodies, and in some animals, orbital connective tissue fibrosis.100

9.1.6 Oxidative Stress and TO

A state of oxidative stress has been described in GD and TO.106, 107 An increase in reactive oxygen species or reduced elimination of radicals by anti-oxidative enzymes will result in oxidative damage to cell membrane with lipid peroxidation and oxidative DNA damage, resulting in inflammation and loss of function.108

Both 8-hydroxy 2’-deoxyguanosine (8-OHdG) and malondialdehyde, as well as intracellular superoxide anion and hydrogen peroxide were significantly elevated in TO orbital fibroblast compared with normal controls.109 The 8-OHdG urinary levels correlate well with clinical activity score.110 These findings suggest increased oxidative DNA damage and lipid peroxidation may have a role in the pathogenesis of TO. Increased oxidative stress is also noted in vivo, where lipid hydroperoxide, superoxide dismutase (SOD), glutathione reductase and
glutathione peroxidase are significantly elevated in orbital fibroadipose tissue, while glutathione (anti-oxidant) is reduced compared with controls. Gluthathione levels are strongly negatively correlated with the ophthalmopathy index.\textsuperscript{106}

In hyperthyroid patients, achieving euthyroidism with methimazole results in all markers of oxidative stress being normalized in GD without orbitopathy, but not entirely in the TO group where oxidative stress indices remain significantly different from normal controls.\textsuperscript{107} Similarly oxidative stress marked by tert-butyl hydroperoxide initiated chemiluminescence remains high after radioactive iodine treatment.\textsuperscript{111} Treatment with oxygen radical scavengers and anti-thyroid drugs reduce hydrogen peroxide-induced and, to a lesser degree, heat-induced 72 kilodalton heat shock protein (HSP72). HSP72 is a cytosolic protein inducible by heat shock and ischaemia, and its expression is increased in autoimmune thyroid disease.\textsuperscript{112, 113}

Reactive oxygen species (superoxide anions and hydrogen peroxide) induce pro-inflammatory cytokines production (IL-1β, TGF-β1) and stimulate orbital fibroblast proliferation in a dose-dependent manner; the proliferative effect can be inhibited by multiple anti-oxidants, methimazole but not propylthiouracil.\textsuperscript{114, 115} Free radicals are also involved in IL-1β induced GAG production in TO orbital fibroblasts. IL-1β increases free radical production in both normal and TO orbital fibroblast, and stimulates SOD activity in TO orbital fibroblasts. Furthermore reducing oxygen free radicals with SOD and catalase partially blocked IL-1β induced GAG production.\textsuperscript{116} Nicotinamide reverses cellular injury in the orbit by inhibiting cytokine-induced activation in TO orbital fibroblasts.\textsuperscript{117}

Despite the established association of smoking with TO, the mechanism of smoking leading to TO remains less well defined. Cigarette smoke contain oxidants and radicals that cause oxidative burden systemically,\textsuperscript{118} hence it has been proposed that increased production of reactive oxygen species by smoking overwhelms oxidation reduction. Smokers had significantly higher 8-OHdG levels than non-smokers in TO, suggesting smoking has a higher impact on oxidative stress in patients with TO.\textsuperscript{110} Cigarette smoke extract can also stimulate HA
production and adipogenesis in a dose-related manner, and the effect on adipogenesis is synergistic with IL-1.\textsuperscript{119}

\subsection*{9.1.7 Putative role of fibrocytes in TO}
Fibrocytes are bone marrow derived pluripotent cells. They originate from monocyte and B cell lineages and circulate as peripheral blood mononuclear cells.\textsuperscript{38} Fibrocytes can differentiate into adipocytes, myofibroblast, chondrocytes, osteoblast, they are found in increased numbers in rheumatoid arthritis and scleroderma with interstitial lung disease.\textsuperscript{120} They infiltrate connective tissues in response to injury, and has been implicated in wound healing and in tissue remodeling and fibrosis in idiopathic pulmonary fibrosis, sclerosing cholangitis and kidney fibrosis; the circulating fibrocytes could differentiate into adipocytes and myofibroblast given PPAR-\gamma and TGF-\beta.\textsuperscript{38,120-122}

Early evidences inferred that a subset of orbital fibroblasts in TO might be derived from the circulating fibrocytes in GD. In GD, CD34+ TSHR+ fibrocytes in the blood were markedly increased compared to normal controls; a subpopulation of CD34+ fibrocytes were detected in cultures from TO and immunofluorescein staining of TO orbital tissue \textit{in vivo} showed co-localization of CD34 and TSHR.\textsuperscript{123} 25-35\% of TO orbital fibroblasts were CD34 positive in culture.\textsuperscript{124} Patients with TO also have an increased fraction of TSHR+ CD34+ fibrocytes in peripheral blood, however the frequency of CD34+ TSHR+ fibrocytes does not correlate with clinical activity score or smoking status in TO.\textsuperscript{125} These fibrocytes expressed higher level of TSHR and CD40 than orbital fibroblasts, which seems to have functional significance. Peripheral fibrocytes in culture responded to TSH and CD40 with increase expression of IL-8, RANTES, MCP-1 and additionally CD40 increased expression of IL-6 and TNF-\alpha.\textsuperscript{65,125} Thus, the concept appears appealing that circulating fibrocytes in GD could infiltrate orbital tissues and participate in the pathogenesis of TO, thus forming the potential link between systemic autoimmune thyroid disease and extra-thyroidal manifestations.

Although there is phenotypic resemblance between orbital fibroblasts and fibrocytes, they have divergent cytokines expression and IL-1 receptor antagonists (IL-1RA) expression in TO patients.\textsuperscript{124} When stimulated by IL-1\beta,
orbital fibroblasts produced high levels of IL-1α, IL-1β and prostaglandin endoperoxide H synthase -2 (PGHS-2), but only had minimal effect on CD34+ fibrocytes. CD34+ fibrocytes expressed higher basal level of serum IL-1RA and intracellular IL-1RA whereas CD34 negative orbital fibroblasts culture has undetectable basal serum and intracellular IL-1RA; upon stimulation by IL-1β, orbital fibroblasts showed dramatic rise in intracellular IL1-RA but not serum IL1-RA in contrast to fibrocytes serum IL1-RA which was dominantly up-regulated.

The complexity in identifying fibrocytes thickens as fibrocytes were known to loss haematopoietic surface markers during differentiation, similarly thyroid CD34+ fibroblast loss its surface markers in culture making it difficult to track these cells in vivo. Interestingly human adipose tissue-derived multipotent stromal cells and mature adipocytes found in subcutaneous and omental adipose tissues both express CD34 surface protein marker, which decreased in transition between stromal and mature adipocytes. On the other hand, multipotent stem cells from orbital fat tissues could differentiate into osteoblast, chondrocytes, adipocytes and corneal epithelial cells, these stem cells were negative for haematopoetic stem cell markers CD34 but have growth kinetics comparable to bone marrow stem cells. The bone marrow derived fibrocytes and adipose tissue derived multi-potent cells share similarities, further studies are required to clarify their differences.

Currently an in vivo model showing the functional response of fibrocytes in orbital tissue does not exist. There remains many questions to be answered: how does the biological behaviour of fibrocytes changes as it transit into the orbit, what impact does the fibrocytes have on orbital inflammation in vivo, are the heterogenous orbital fibroblast populations derived from CD34+ fibrocytes or was CD34+ orbital fibroblast a separate subpopulation of fibroblast? Further laboratory studies will be needed to evaluate the role of fibrocytes in TO.

9.1.8 Advances in therapeutic agents for TO
The mainstay treatments for TO have been systemic corticosteroids and orbital radiation for active TO, and surgical rehabilitation for inactive TO until
immunomodulators were trialed in TO targeting TSHR and IGF-1R on fibroblast, inflammatory cytokines IL-6, TNF, and CD20+ B cell depletion.\textsuperscript{130} (Table 1)

The better studied immunosuppressive therapy for TO is rituximab, an anti-CD20 monoclonal antibody that targets CD20 on B cells and its precursors. A systematic review of 43 TO cases treated with rituximab showed improvement in disease activity and severity in 91% cases, no improvement in 3 cases and worsening in 1 case.\textsuperscript{131} A randomized controlled trial (RCT) in Europe comparing rituximab to intravenous methylprednisolone in active moderate severe TO supports effectiveness and disease modifying effects with 100% response rate, no reactivation of TO at 24 weeks, and less rehabilitative surgery required at 76 weeks.\textsuperscript{132} An RCT in North America comparing rituximab to placebo (i.e., comparing to natural history) did not show a significant difference in the improvement of disease activity at 24 and 52 weeks, and there were more moderate-to-severe adverse events in the rituximab group.\textsuperscript{133} The conflicting results from the rituximab RCTs could be related to small sample sizes, and require clarification with larger RCTs.

Drug-like small molecule TSHR antagonists are emerging as a promising new treatment for TO and GD. M22, a small molecule TSH agonist increased cAMP production in a TSHR transfected ovary cell line and TO orbital fibroblasts, and the cAMP response was effectively abolished by low molecular weight TSHR antagonist.\textsuperscript{134} The results were replicated separately where small molecule TSHR antagonists can inhibit both basal and stimulated cAMP, pAKT and HA production in orbital fibroblast in a dose dependent manner.\textsuperscript{70, 135}

Teprotumumab, a humanized anti-IGF-1R monoclonal antibody is in phase II clinical trial for moderate severe active TO. Preliminary study shows teprotumumab can inhibit expression of TSHR and IGF-1R on CD34+ fibrocytes and TSH induced IL6 and IL8 production, by partially inhibiting phosphorylation of Akt.\textsuperscript{136}

Tocilizumab, a recombinant, humanized monoclonal antibody to IL-6 receptor has been trialed in 18 patients with active TO refractory to intravenous steroids. Tocilizumab significantly improved clinical activity score in all patients and disease activity remained stable up to 27 months after infusion.\textsuperscript{67} The anti-TNF
monoclonal antibodies infliximab, adalimumab and soluble TNF receptor etanercept have been trialled in small numbers of patients with active TO.\textsuperscript{137-139} Etanercept seems to be effective in controlling activity of TO, leading to a marked improvement in mainly soft tissue changes reported at 60%, but up to 30% had recurrence of TO activity after treatment cessation.\textsuperscript{137} Adalimumab reduced inflammatory score in 6 of 10 patients, the greatest benefit being seen in active TO with severe inflammatory signs.\textsuperscript{139} Apart from IL-6 and TNF antagonists, in-vitro use of anti-IL-1 antibody has been shown to reduce adipogenesis by 82% in orbital fibroblasts exposed to cigarette smoke extract.\textsuperscript{119} Novel therapeutic options for TO show some exciting developments, but large RCTs for these agents are needed to determine both efficacy and safety profile.

Anti-oxidants have a promising role in the treatment of mild to moderately active TO. In the first pilot study of antioxidant supplementation, allopurinol and nicotinamide therapy reduce soft tissue swelling and total eye score in 82% of patients accompanied by high patients’ satisfaction in mild to moderately severe TO.\textsuperscript{140} Selenium, a trace mineral incorporated into several selenoproteins and functions as anti-oxidant, reduces thyroperoxidase antibodies in autoimmune thyroiditis.\textsuperscript{141} A subsequent double-blind, RCT of selenium supplemented for 6 months in TO, was associated with improved quality of life, reduced soft tissue inflammation, improved appearance and slowed progression of TO, as compared with placebo.\textsuperscript{142}

\textit{Table 1} Novel and potential immunotherapies in clinical and pre-clinical trials for TO

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<th>Class of drugs</th>
<th>Mechanism of action</th>
<th>Example</th>
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<tr>
<td>CD 20 monoclonal antibody</td>
<td>Deplete B cells and precursor by recognizing surface CD20 marker</td>
<td>Rituximab\textsuperscript{131-133}</td>
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<tr>
<td>IL-6 receptor monoclonal antibody</td>
<td>Binding to soluble and membrane bound IL-6 receptor and inhibit pro-inflammatory cytokine IL-6</td>
<td>Tocilizumab\textsuperscript{67}</td>
</tr>
<tr>
<td>TNF-(\alpha) monoclonal</td>
<td>Bind and block TNF-alpha from interacting with cell surface TNF</td>
<td>Adalimumab\textsuperscript{139}</td>
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**Table:**

<table>
<thead>
<tr>
<th>Antibody Type</th>
<th>Description</th>
<th>Inhibitor(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soluble TNF receptor</td>
<td>A soluble TNF-α receptor-Fc protein that prevent TNF-α and TNF-β from binding to membrane bound TNF receptors</td>
<td>Infliximab$^{138}$</td>
</tr>
<tr>
<td>Small molecule TSHR antagonist</td>
<td>Binding within transmembrane region of TSHR receptor, blocking signaling of TSH either as allostERIC inverse agonist or neutral antagonist</td>
<td>Etanercept$^{137}$, Org 274179-0$^{134}$, NCGC00229600$^{135}$, NCGC00242595$^{70}$</td>
</tr>
<tr>
<td>IGF-1R monoclonal antibody</td>
<td>IGF-1R blocking, reduces both IGF-1R and TSHR expression</td>
<td>Teprotumumab$^{136}$</td>
</tr>
<tr>
<td>Anti-oxidant</td>
<td>Increase reserve for selenoproteins involve in oxidation reduction activity, eg, glutathione peroxidase, thioredoxin reductase, iodothyronine deiodinases</td>
<td>Sodium selenite$^{142}$</td>
</tr>
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In conclusion, cellular immunity has an important role in orbital inflammation in TO which involves interaction of T cells with orbital fibroblasts through specific receptor ligand bridges, with propagation of multiple intracellular signaling cascades leading to secretion of HA, adipogenesis, and the release of chemotactic factors and cytokines that ensure perpetuation of orbital inflammation. TSH receptor appears the likely candidate as an auto-antigen. IGF-1 receptor on orbital fibroblasts mediates some aspects of orbital changes and importantly it has a role in adipogenesis, HA synthesis and lymphocyte trafficking. Oxidative stress is increased in TO, and the increased oxidative burden appears to potentiate orbital inflammation, fibroblast proliferation and GAG production. With the emergence of animal models in TO and newer TSHR antibody assays, future studies will allow detailed evaluation of the heterogenous TSHR antibodies and their effects on TO, testing of new treatments targeting receptor ligand binding, signaling pathways and T and B cells. Further studies in signaling
networks and molecular triggers that lead to burnout of TO will improve our understanding of TO and can in turn open future therapeutic directions.

9.2 Risk factors for TO
The risk factors for development and exacerbation of TO is broadly defined as endogenous and exogenous risk factors. These include gender, age and ethnicity, genetic factors which are endogenous and non-modifiable, and exogenous risk factors including smoking, hyperthyroidism treatment modalities, TSH receptor auto-antibodies level and thyroid function status. This review will explore each of these risk factors and provide an update on the genetic susceptibility of GD and TO in the context of breakthrough discoveries afforded by genome-wide association studies.

9.2.1 Gender, age and ethnicity
GD like many other autoimmune disorders has a predilection in females. The reason underlying female preponderance of these autoimmune conditions remain unclear. Although GD is more common in female, TO occurs more severely in male patients and appears to involve a relatively greater proportion of males than females compared with patients with GD without TO.19, 143 The estimated prevalence rate of GD in the USA was 1151.5 per 100 000 with a female preponderance of 88% (female/male prevalence rate ratio=7.3).144 In contrast, the prevalence rate of TO in female is significantly lower when compared with GD, with a female to male ratio of 2.1, 3.4 and 4 in independent small TO case control studies.3, 19, 143 Female to male ratio is slightly below parity in severe TO with sight-threatening dysthyroid optic neuropathy (female/male ratio=0.9)145 Large case control studies consistently find the female to male ratio of GD with and without TO at 4:1.146, 147 In a Danish study, the risk for developing TO is similar in men and women with GD.8 Similarly in another large prospective study based in the United Kingdom, moderate to severe TO were recorded in equal proportion in male and female patients with GD, but higher proportion of male patients had severe TO compared to female (30.4% vs 21.3%, p<0.001).146

The frequency and severity of TO also seems to increase with age.146 In a study of 101 TO cases by Perros, the mean ophthalmopathy index of severity is significantly higher in male than female, in addition to a positive correlation
between age and ophthalmopathy index independent of gender. The study of clinical characteristics of TO revealed patients older than 50 years have greater degree of visual symptoms and signs with impaired ocular motility, soft tissue involvement, more severe restriction of eye movement, impaired visual acuity and asymmetrical TO. The incidence of TO is also much higher in older age. In a population based study in Denmark, <2% developed moderate-to-severe TO in GD less than 40 years, conversely the incidence rate climbed to 8% in the 40-60 years age group.

Population studies on prevalence rates for TO across different ethnicities and nations was well summarized by Lazarus, where estimates of prevalence rates for TO were similar ranging from 0.1-0.3% in parts of America, Sweden, England, Scotland, China and India. This is in contrast to previous findings of prevalence of TO in GD from a community referral based endocrine clinic consisting of 15% migrant population, where prevalence of TO in GD in Indians was significantly lower than Europeans (7.7% versus 42%), the study found the overall risk for Caucasians developing TO was 6.4 times higher than Asians. The results were surprising given the prevalence rates of TO in a multi-racial country showed comparable prevalence rates for TO at 35.1% in Malay, 34% in Chinese and 40% in Indian in their GD patients. Confounding factors to consider when interpreting prevalence rate of TO in different ethnic groups would potentially be differences in other factors such as smoking rate, thyroid function control, age of presentation of GD and selection bias arising from ethnic minority.

9.2.2 Genetics
The onset of TO mirrors the onset of GD with a close temporal relationship. 70% of patients with TO presented within the same year of the onset of GD. 66% of patients with GD presented with TO within 18 months of diagnosis of GD. This relationship strongly suggests TO shares a common aetiology with GD.

9.2.2.1 Genetic susceptibility in Graves’ disease
Family and twin studies found the development of GD is partly explained by genetic susceptibility. In the largest family study involving 15,743 hospitalized GD patients, familial GD accounted for 3.6% of all Graves’ patients. The familial standardized incidence ratio (SIR) for individuals whose parent or
one sibling is affected by Graves’ disease was 4.49 and 5.04 respectively. The SIR increases to 16.45 for twins and 310 when two siblings are affected. Interestingly clustering of GD was also noted in spouses (SIR was 2.75), this suggests environmental factors also influences the onset of GD. The concordance rates for monozygotic twins for developing GD is 35% to 36% and 0 to 7% for dizygotic twins. The estimated liability to the development of GD by genetic factors is 79%. Several different genetic study designs have been used over time to discover the genetic susceptibility for GD and they were linkage studies and association studies for candidate genes and genome wide screening. Linkage study is better at identifying genes of major effect in monogenic disease, but has limited success in studying complex trait such as GD that involves multiple susceptible gene loci. Early candidate genes association studies targeting genes involved in immune regulation and thyroid specific genes identified several susceptibility loci for GD, they were MHC class II alleles, CTLA4, PTPN22 and CD40. The genetic contributions from each of these genetic variants were modest. MHC class II alleles DRB1*03(DR3), DQA1*0501, DQB1*02 were significantly associated with GD with odds ratio of 2 to 3 compared to normal controls, and up to relative risk of 17.5 for GD in DR3 homogygotes. CTLA4 A49G, CT60, 3’UTR polymorphisms were more frequent in GD with an odds ratio of 1.4 to 2, and association with PTPN22 polymorphism had an odds ratio of 1.5 to 1.9. Cytotoxic T-lymphocyte associated antigen 4 (CTLA4) is expressed on surface of activated T lymphocytes and is a key inhibitor of T cell activation and immune response. Genetic polymorphism in CTLA4 loci was identified as a shared susceptibility loci for autoimmune diseases such as Type I diabetes in addition to autoimmune thyroid disease. Protein tyrosine phosphatase non-receptor 22 (PTPN22) is another important negative regulator of T cell activation. Genetic polymorphisms were associated with increase risk for autoimmune diseases including rheumatoid arthritis. With complete mapping of all common genetic variants in human genome and haplotype mapping by the international HapMap Consortium, linkage disequilibrium between single nucleotide polymorphisms (SNPs) is known, thus
enabling identification of tag SNPs. Combined with improvement in microarray technology that enables screening of millions of SNPS simultaneously, and recruitment of large cohorts of cases and controls, typically more than 1000 individuals, genome-wide association studies (GWAS) has allowed detection of minor allele frequency >1%. Using tag SNPs in candidate gene study, IL2RA and TSHR were further identified as susceptibility loci for GD. Using 98 SNPs including 70 tag SNPS across TSHR, 28 SNPs were associated with GD, the strongest replicable association was rs179247 with an odds ratio of 1.5. TSHR polymorphism association with TO was separately validated in other Caucasian and Japanese study populations. In addition polymorphisms in FCRL3, FCRL3_5 and FCRL3_6 SNPs were associated with GD with modest effect (odds ratio at 1.2), where FCRL3 was found on B cell surface that modulate NF-κβ binding and immune response.

Further new susceptibility loci for GD identified through GWAS studies were well summarized by Simmonds. The earliest simultaneous GWAS study on autoimmune thyroid disease, multiple sclerosis, ankylosing spondylitis and breast cancer genotyped 14 500 non-synonymous SNPs in 1000 cases and 1500 normal controls. The study identified multiple common genetic variants within both MHC class I and II regions with GD at genome-wide significance and confirmed TSHR and FCRL3 association with GD in an extended cohort of 2500 GD cases and 2500 controls. Association of multiple MHC loci with GD were further replicated in another GWAS study in the Japanese in two phases initially screening 268 000 SNPs in 1119 GD and 2718 unrelated control. 32 of the 34 SNPs were located within MHC region on chromosome 6p21 and 2 SNPs were located in SENP1 (sentrin/SUMO-specific protease 1) on chromosome 12 and SLAMF6 on chromosome 1. In the replication phase, 22 SNPs all in the MHC region were replicated in an independent cohort of 432 Japanese with GD and 1157 normal controls. The China Consortium for the genetics of autoimmune thyroid disease used GWAS to screen 650 000 SNP in 1536 GD cases and 1516 normal controls, the study detected 126 non-human leucocyte antigen (HLA) SNPs that represent 38 independent regions associated with GD, and replicated these regions in a second set of 3994 cases and 3510 controls. The study discovered 2 further new risk loci for GD, the RNASET2-FGFR1OP-CCR6 region at
6q27 and an intergenic region at 4p14, and additionally confirmed association of GD with rs2281388 located near HLA-DPB1 at MHC, rs12101261 at TSHR, rs1024161 at CTLA4 and rs3761969 at FCRL3, accounting for an overall estimated liability of GD heritability at 9.29%. Using custom array, an immuno-chip that contained SNPS across 186 susceptibility loci associated with autoimmune diseases, Cooper et al screened 100,000 common SNPs in 2285 GD, 462 Hashimoto’s thyroiditis cases and 9364 controls, this study confirmed association of autoimmune thyroid disease with PTPN22, CTLA4, IL-2RA and TSHR, and replicated the association of autoimmune thyroid disease with RNASET2-GFGR1OP-CCR6 locus and in addition found seven new susceptibility loci MMEL1 at 1p36.32, LPP at 3q28, BACH2 at 6q15, PRICKLE1 at 12q12, ITGAM at 16p11.2, rs1534422 at 2p25.1 and rs4409785 at 11q21. TSHR locus was only associated with GD but not Hashimoto thyroiditis.

9.2.2.2 Genetic susceptibility of thyroid-associated orbitopathy

The heritability of TO was largely uncertain. The progress in unraveling the genetic susceptibility of TO is hampered by under-power sample size, and limited replicability of the findings. The genetics of TO were mostly examined in candidate gene studies based upon known genetic variants in GD and selected genes related to pro-inflammatory cytokines and immune regulation in TO pathogenesis.

Genotyping the HLA 1 region reveals association with TO in a gender-dependent manner, the results are conflicting where the genetic variant in PRR3 gene showed protective effect in male but is a risk allele for TO in female, whereas polymorphism in ABCF-1 gene was associated with TO only in female. Genotyping HLA 2 region including HLA-DRB1, DQB1, DQA1 found no association with TO in caucasian. Meta analysis of association of HLA class II alleles with TO showed significant heterogeneous results among the studies, the combined odds ratio for association with DR3, DR4 and DR7 were 1.5, 0.9 and 0.8 but the results were not statistically significant.

In the candidate gene studies investigating immune response genes, Vaidya et al found significantly higher frequency of CTLA4 A49G and C1822T polymorphisms in TO compared to GD in their initial cohort and later in the extended cohort of
GD patients, even after correction for male gender, smoking and previous radio-active iodine treatment.\textsuperscript{190, 191} A third study in an Iranian population also show a significant association between \textit{CTLA4} A49G allele and TO.\textsuperscript{170} The odds ratio of \textit{CTLA4} association with TO compared to GD without TO was 1.7 to 2.\textsuperscript{170, 191} Functional analysis seems to correlate well with the genetic finding where increased serum \textit{CTLA4} was found in severe TO compared with non-severe TO and normal controls.\textsuperscript{192} However, the positive association of \textit{CTLA4} variants with TO has not been widely replicated in other Caucasian populations or other ethnicities.\textsuperscript{171, 187, 193} Bednarczuk et al found no association of \textit{CTLA4} A49G allele in both Polish Caucasian and Japanese patients when the sample sizes of GD were 264 and 319 respectively.\textsuperscript{172} Han et al found no association of \textit{CTLA4} A49G and CT60 with TO in Chinese patients; meta analysis of \textit{CTLA4} genetic variants in a total of 910 TO and 1245 GD without TO from Europe and Asia also did not show increase susceptibility of TO in GD.\textsuperscript{194, 195}

In a large candidate gene study of \textit{PTPN12}, a protein tyrosine phosphatase related functionally to \textit{PTPN22}, none of the 7 tagged SNPs were found associated with GD in 1058 British Caucasian with GD and 864 controls in despite adequate power, 3 of the \textit{PTPN12} tagged SNPs was associated with the presence of mild to moderate TO.\textsuperscript{196} Despite the close relation of \textit{PTPN12} with \textit{PTPN22} in terms of function, \textit{PTPN22} polymorphism has not yet been associated with TO.\textsuperscript{163, 197} Replications of these preliminary results are needed in other study populations. \textit{IL23R} were genotyped in 216 North American Caucasian with GD including 104 TO compared to 368 normal controls, 3 of the 4 SNPs was associated with GD and 2 of the 4 SNPs were associated with TO compared to GD and normal controls.\textsuperscript{198} However the association of \textit{IL23R} with TO was not replicated in another North American Caucasian population.\textsuperscript{187} Study on \textit{NFκβ1} showed NFκβ1 -94 ins/del ATTG was associated with GD in two Polish Caucasian cohorts but not in the Japanese; however in the Japanese study population NFκβ1 genotype was correlated with TO (OR 1.5, p=0.009).\textsuperscript{199} Similarly in a Turkish population with GD, \textit{NFκβ1} polymorphism did not affect risk for GD, this is in the context of having low power (23-28% power to detect positive association at p=0.05) attributable to small sample sizes, nevertheless the study found
polymorphism of del/ins of NFκB1 gene increased the risk of TO by 39%. B7 molecules (CD80 and CD86) are co-stimulatory molecules expressed on the surface of antigen-presenting cells. The polymorphism of CD86 gene was negatively correlated with TO (OR 0.73, p=0.0017), multifactor dimensionality reduction modeling suggested CD86 (rs9872483) and CD80 (rs9289131) genes interact to predict TO risk (p=0.001); the haplotype frequency G-A of the CD80 and CD86 SNPs was shown to have a protective effect against development of TO in GD patients, again the findings from this study will need replication.

Studies also explore genetic susceptibility in TO confer by adipogenesis related genes. In a Polish Caucasian study population with 276 GD cases including 213 TO and 466 healthy control, Pro12Ala PPARγ genotype decreased the risk of TO; the Ala12 variant (Ala12Ala and Pro12Ala genotype) possibly reduce the severity and activity of TO. On the other hand, no difference was noted for adipogenesis-related transcription factor, PPARγ Pro12Ala polymorphism when comparing allele frequency in 172 Dutch TO cases with 93 Greek GD controls without TO. However Pro12Ala PPARγ gene variant was associated with less active TO based on clinical activity score. Using tag SNPs, 4 SNPs in adipocyte related immediate early genes including CYR61, ZFP36 and SCD increase the odds of TO by 1.29 to 1.56 in a Swedish population when comparing 594 GD, inclusive of 327 TO, with 1147 normal controls.

Pro-inflammatory cytokines involved in the pathogenesis of TO were studied for genetic associations with TO. The reports of genetic association of IL-1α and IL-1β with TO are mixed for different ethnic groups. In a Chinese study using tag SNPs for IL-1β pre-conditioned to minor allele frequency of 10%, all eight SNPs in IL-1β did not differ significantly between TO and GD without TO, even though IL-1β plasma concentration was significantly higher in TO than GD without TO. In a different Chinese population with 760 GD including 190 TO and 735 normal controls, SNP rs1800587 tagging IL-1α T-889C and rs16944 tagging IL-1β A-511G showed significant association with GD and more so with TO, meta-analysis showed increased risk for TC and TT genotype in rs1800587 in GD and TO and protective effect of AA genotype in rs16944 in GD and TO. However no association of TO was found for both IL-1β -511 and +393 polymorphisms in
Polish.\textsuperscript{207} In an Iranian study \textit{IL-12}, \textit{IFN-\(\gamma\)}, \textit{TNF-\(\alpha\)}, \textit{IL-1\(\alpha\)}, \textit{IL-1R\(\alpha\)} all showed significant association with TO; the study sample was small (TO=50, GD=57), false positive results cannot be ruled out.\textsuperscript{208, 209} On the other hand \textit{TNF-\(\alpha\)} was found not to be associated with GD in Japanese, but \textit{TNF-\(\alpha\)} alleles T-1031C and G-863A were significantly associated with TO, however the results were not replicated in Polish.\textsuperscript{189, 210} \textit{TSHR}, \textit{IL-13}, \textit{IL-12B}, \textit{IL-18}, \textit{CD40}, glucocorticoid receptor, interferon induced helicase genes were found not to affect TO risk.\textsuperscript{164, 181, 187, 193, 211-214, 215}

9.2.3 Smoking
The evidences are strong for the association of smoking with TO. In case-control studies, smoking significantly increased the risk for TO. Prevalence of smokers and heavy smoking was higher in patients with more severe TO, the correlation is stronger for current smoker than ex-smoker.\textsuperscript{216-220} The quality of the studies researching relationship of smoking with TO were variable, affected by non-uniform criteria for defining smoking status, various control populations were used including GD without eye disease, other thyroid diseases, normal healthy controls, retrospective nature of most studies and in some studies insufficient control for confounding factors.\textsuperscript{19, 216-218, 221} Despite the heterogeneity of studies, smoking emerges strongly as a risk factor for TO with increase odds for developing TO in smokers compared to non-smokers in GD. In a systemic review of 15 case-control and cohort studies, smoking increased odds for TO (OR 1.94-10.1), when TO cases were compared with GD without TO; the odds ratio increased up to 20.2 when compared to normal controls.\textsuperscript{221} Current smokers had a relative increase risk of proptosis by 2.6-fold and diplopia by 3.1-fold when compared to patients with GD who never smoked; lifetime cumulative dose of tobacco use seemed less important in predicting risk of TO compared to current tobacco consumption.\textsuperscript{222} The severity of TO and smoking appears to be dose dependent. The relative risk of TO symptoms and signs increased proportionately with the current number of cigarettes smoked per day.\textsuperscript{148, 222} Current literatures await prospective studies that adequately measure the effects of stopping smoking to the progression and severity of TO.
In addition, smoking was associated with poorer response to medical treatment in TO, and in the context of radioiodine treatment showed more frequent exacerbation of TO and poorer response to oral prednisolone treatment.\textsuperscript{223} In a single-blind randomized study of mild TO receiving either radio-active iodine treatment alone or radio-active iodine treatment with 3 months of oral prednisolone, TO progressed more so in smokers compared to non-smokers (23.2% versus 5.9%, \textit{p}=0.007), and for the treatment arm with oral prednisolone cover during radio-active iodine treatment, TO was alleviated in 63.8% of non-smokers compared to 14.9% of smokers (\textit{p}<0.001).\textsuperscript{223} Reported in the same paper of a separate retrospective study, non-smokers responded better to medical treatment; 93.8% of non-smokers versus 68.2% of smokers responded to 6 months of high dose oral prednisolone and orbital irradiation (\textit{p}<0.001) in patients with severe TO.\textsuperscript{223} In a prospective cohort study, the clinical activity score became minimal (0-1) in 74% of non-smokers compared with 53% of smokers (\textit{p}<0.05) in patients with moderately severe TO treated with oral prednisolone for 6 weeks followed by orbital radiation; ocular motility also improved in 60% of non-smokers compared with 24% of smokers over 12 months (\textit{p}<0.017).\textsuperscript{224}

The biological mechanism of worsening TO by smoking remains unclear. Several laboratory studies suggest smoking enhances orbital inflammation and adipogenesis, reduces the body capacity to neutralize toxic metabolism and exerts synergistic effects to pro-inflammatory cytokines. In an innovative in-vitro model of TO, cigarette smoke extract stimulated adipogenesis and hyaluronic acid production in a dose dependent manner in orbital fibroblasts cultures derived from patients with TO, the effect of cigarette smoke extract and IL 1 on adipogenesis was synergistic, and hypoxic condition increased adipogenesis, production of leptin and MCP1.\textsuperscript{119} \textsuperscript{225} More recently microarray study showed smokers have more than 1.5-fold increase in immediate early genes expression, genes which were expressed in differentiating pre-adipocytes during adipogenesis; IL-1\textbeta{} and IL-6 were over-expressed in smokers compared to non-smokers’ intra-orbital fat in severe active TO cases, the results suggest smoking increases pro-inflammatory cytokines and enhances adipogenesis directly.\textsuperscript{226}
Inter-cellular adhesion molecules (ICAM) have an important role in autoimmunity, the level of ICAM1 increases upon stimulation by CD40-CD40 ligand interaction in orbital fibroblast from TO. Smoking and severity of TO were both found to be independent determinants of serum ICAM1 levels in TO. Smoking was associated with higher level of serum ICAM1 and lower level of vascular cell adhesion molecules 1 (VCAM1) in both TO and GD without TO. Separate study showed ICAM1 expression remained unchanged in TO orbital fibroblast cultures when exposed to cigarette smoke extract, suggesting an indirect mechanism of increased serum ICAM levels in smokers.

9.2.4 Radio-active iodine treatment
Achieving euthyroid status in GD could be achieved either by anti-thyroid medication, thyroidectomy or radioactive iodine. Overall, evidence suggest anti-thyroid medication and thyroidectomy do not influence the course of TO. The systemic review of effects of radioactive iodine on TO revealed an increased relative risk at 4.23 for developing TO when comparing radioactive iodine treatment to anti-thyroid drugs, but not compared to thyroidectomy. More recently large longitudinal cohort study of newly diagnosed GD found surgical thyroidectomy and statin use was significantly associated with 74% and 40% reduced hazard for TO respectively, neither antithyroid medications or radioactive iodine appeared to alter the risk for developing TO; that perhaps thyroidectomy might have a protective effect for TO.

There were three randomized control trials looking at the occurrence of TO after treatment for thyrotoxicosis. TO developed in 15% of patients treated with radioiodine two to six months after treatment, in contrast only 3% patients treated with methimazole developed or had worsening TO; whereas for patients treated with radioiodine and prophylactic oral prednisolone, no patients had progression of eye disease, 67% of patients with pre-existing TO showed improvement of their eye disease. Tallstedt found development or worsening of TO was similar in randomized groups for anti-thyroid medication treatment and thyroidectomy (15% versus 11%), TO occurs significantly more frequently in radioactive iodine group at 33% compared to medical treatment (10%) and thyroid surgery (16%). The only weakness of this study was hypothyroidism
was allowed to develop before thyroxine replacement, hence potentially a confounding factor for exacerbation of TO. In the third randomized study by Traisk, worsening or new onset of TO was still significantly greater in radioactive iodine group (38.7%) compared to anti-thyroid medication group (21.3%) even after early correction of hypothyroidism in both groups. In addition, de novo development of TO was significantly higher in radioactive iodine group (38%) than in the methimazole group (18%).

Prophylactic steroid treatment has been shown to prevent the progression of TO for a short term concomitantly with radioiodine therapy for patients with pre-existing TO, even at doses as low as 0.2mg/kg body weight. Other groups were less convinced about the need for prophylactic steroid treatment for patients with pre-existing minimal eye disease. Prospective cohort study found no deterioration of TO in patients with minimally active TO when post radioiodine hypothyroidism was prevented, transient eye changes post radioactive iodine treatment became insignificant at 1 year follow up. Consideration of concomitant steroid cover post radioiodine treatment for subset of patients at greater risk of worsening of TO remains less well studied, including smoker and fluctuating thyroid function.

9.2.5 TSH receptors auto-antibodies

With improvement of sensitivity and specificity in TSHR assays, both thyroid binding inhibiting Ig (TBI) and thyroid stimulating Ig (TSI) TSHR titres especially in their third generations are highly and significantly correlated with activity and severity of TO. Third generation TBI and TSI assays accurately diagnosed GD in untreated hyperthyroidism with close to 100% sensitivity and specificity, and hence the choice of which assay to use seems to make little difference. TSHR assays can accurately predict short term relapses of GD while on anti-thyroid drugs treatment, but are less effective in predicting long term remission.

The second generations TBI and TSI assays both correlate moderately well with clinical activity score (r=0.54 and r=0.50 respectively), and to a lesser degree with soft tissue inflammation and proptosis in TO. The novel chimeric TSHR and cAMP response element dependent luciferase MC4/TSI assay seems to
perform better than second generation TBI with higher clinic assay in terms of sensitivity and specificity and correlates strongly with clinical activity \( r=0.87 \) and clinical severity score \( r=0.87 \) in TO.\(^91\), \(^92\), TSHR antibody was detected in 98% with TO in the new TSI assay.\(^237\) In the largest multicentre TSHR antibody study in children and adolescents with GD, the new MC4/TSI titre was markedly higher in children with GD and TO than children with GD but no TO and correlated well with the severity of TO.\(^93\) The TSI assay seems more efficient as a functional biomarkers for TO than TBI assay both in terms of correlation with activity, severity and sensitivity.\(^237\)

Currently there is little data on the predictive value of TSHR assays in TO, or in monitoring treatment response for TO with TSHR assay.\(^85\) With the improvement of TSHR assay, future studies to develop predictive risk calculation for TO is keenly awaited.

9.2.6 Thyroid function status

Both poorly controlled hyperthyroidism and hypothyroidism may be associated with onset or worsening of TO. Prummel et al retrospectively correlated severity of TO with thyroid dysfunction whilst on anti-thyroid drug treatment in 90 patients. They observed significantly greater proportion of hyper- and hypothyroidism in groups with more severe eye disease compared to mild TO.\(^238\) Tallstedt et al showed pre-treatment serum T3 level was predictive for new onset or exacerbation of TO; the level of T3 at 5nmol/liter or greater increased the relative risk for TO to 5.8-fold for the iodine-131 treatment group and 12.7-fold for the anti-thyroid and thyroidectomy group.\(^231\) T3 level positively correlate with TSHR antibody level but the TSHR antibody itself does not independently predict risk of TO. Elevated T4 was also associated with increased risk of TO.\(^231\), \(^232\) A multi-centre cohort study of 2405 patients separately showed free T4 at diagnosis of GD was an independent predictor of the presence of TO.\(^146\) Conversely Kung et al did not find T3, or T4 levels associated with TO, but post radioiodine hypothyroidism with elevated TSH and low T4 was associated with increased risk of developing TO.\(^239\)

9.3 Summary and aims

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Over 30 years we begin to understand the molecular mechanisms involved in the pathogenesis of TO, yet a lot more is to be learnt about the factors that trigger the onset of TO which occur only in a proportion of GD patients, and to link these factors to the mechanisms underlying immune related orbital inflammation. More importantly, finding the mechanism that switches off the immune pathways for orbital inflammation will lead to development of new targeted treatment for TO. One of the most cost effective, rapid approaches for improving our understanding of TO is to study the genetics and gene expressions that may shed new lights on the pathogenesis of TO. Currently there is paucity of good evidence to support genetic risk factors as a significant component for the onset of TO, partly due to difficulty in amassing a large number of TO cases due to the uncommon occurrence of TO and partly due to the complex nature of genetic inheritance for GD. If there truly are genetic associations for TO the individual genetic variants effects are likely to be small and harder to detect.

High throughput transcriptomic and genomic study offers a promising approach to rapidly and effectively identify differential genetic expression and common genetic variants in TO with high sensitivity and without biases from prior knowledge. Orbital fat tissue could be harvested with ease and is a major tissue in the orbit affected by TO, gene expression profile could thus be readily investigated in orbital fat tissue to provide insights into the pathogenesis of TO. The potential discovery of genes that may affect phenotypic expression of TO in GD patients and correlating these genetic variants with the gene expression findings will further enhance our understanding of genetic susceptibility in TO.

The work undertaken in this thesis will test the null hypothesis that there is no genetic differences in GD patients with and without TO. Hence the alternative hypothesis is genetic susceptibility is present in TO, compared to GD without TO. This initially required exogenous risk factors associated with TO be examined, such that these factors could be adjusted for when analyzing genetic findings. In addition, the study aims to identify genes over- or under-expressed in TO compared to controls using microarray gene expression profiling to understand molecular mechanisms involved in the affected tissues in vivo. The research study will also examine the effectiveness of genome wide association study using
DNA pooling as the methodology for discovering common genetic variants using GD as the subject as a proof of principle. The final part of the thesis involved discovering genetic polymorphisms that may increase the genetic susceptibility for TO, and to correlate the genetic findings with the gene expression profile in TO.
10 Risk factors in thyroid-associated orbitopathy in Australian Thyroid-associated Orbitopathy Research (ATOR) Study

Publication arising from this chapter:


10.1 Background and Aims

Among the risk factors predisposing to thyroid-associated orbitopathy (TO), smoking, older age, radioactive iodine and thyroid dysfunction appear to correlate with onset of TO; the reasons underlying these predisposing factors remain unclear.240 As the incidence of Graves’ disease (GD) is low, the age-adjusted incidence rate for TO is even lower at 16 cases per 100,000 population per year for females and 2.9 cases per 100,000 population per year for males.2 As a result of the uncommon occurrence of TO, many earlier published studies examining risk factors for TO included only small numbers of cases and controls, except for smoking.19,216, 217

We wish to investigate the hypothesis that exogeneous risk factors exist for TO. To test this hypothesis, a case control study comparing known and exploratory variables in GD cohorts with and without TO was conducted.

The specific aims for this chapter were:

a) determination of risk factors for TO, using multiple logistic regression to estimate odds ratio for developing TO in patients with GD, including adjustment for explanatory variables based on know risk factors and exploratory variables.

b) The secondary outcome of the study was the determination of clinical characteristics of TO, including the predictors for dysthyroid optic neuropathy, an uncommon sight-threatening complication of TO.
10.2 Peer reviewed publication author version

Title: Risk factors for Graves’ Orbitopathy; the Australian Thyroid-associated Orbitopathy Research (ATOR) Study

Authors:
Jwu Jin Khong\textsuperscript{1,2,3}
Sue Finch\textsuperscript{4}
Chamika De Silva \textsuperscript{1}
Stacey Rylander \textsuperscript{5}
Jamie E Craig \textsuperscript{5}
Dinesh Selva \textsuperscript{6}
Peter R Ebeling \textsuperscript{7}

Affiliations:
1. North West Academic Centre, The University of Melbourne, St Albans, Melbourne, Victoria, Australia
2. Department of Ophthalmology, Department of Surgery, The University of Melbourne, Heidelberg, Victoria, Australia
3. Orbital, Plastics and Lacrimal Unit. The Royal Victorian Eye and Ear Hospital, East Melbourne, Victoria, Australia
4. School of Mathematics and Statistics, University of Melbourne, Victoria, Australia
5. Department of Ophthalmology, Flinders University of South Australia, Flinders Medical Centre, Bedford Park, South Australia, Australia
6. South Australian Institute of Ophthalmology, University of Adelaide, South Australia, Australia
7. Department of Medicine, School of Clinical Sciences, Monash University, Clayton, Victoria, Australia

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Corresponding author and address for reprint requests:
Dr Jwu Jin Khong
North West Academic Centre
University of Melbourne
176 Furlong Road, St Albans, VIC 3021, Australia
email: jwujinkhong@gmail.com

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Abstract

**Context:** Previous association studies suggest the development of Graves’ orbitopathy (GO) is variably influenced by environmental risk factors.

**Objective:** To determine the risk factors and predict odds for developing GO in Graves’ hyperthyroidism (GH).

**Design:** Case-control study

**Setting:** Multi-centre Australian Thyroid-associated Orbitopathy Research (ATOR) group consisting of tertiary endocrinology and ophthalmology outpatients and related private practices.

**Patients or Other Participants:** 1042 participants with GH were designated as cases if they had GO (n=604) and controls if they did not have GO (n=438).

**Main outcome measures:** Primary outcome was GO risk factors and secondary outcome was dysthyroid optic neuropathy (DON) with the effects of risk factors measured by odds ratio (OR) using multiple logistic regression, adjusted for known risk factors and exploratory variables.

**Results:** The odds of GO increased by 17% for each decade increase in the age of onset of GH (OR 1.17, CI: 1.06-1.29, p = 0.002) and by 7% for each year increase in the duration of GH (OR 1.07, CI:1.05-1.10, p<0.001)). Smoking increased the odds for GO by 2.22 for current smoker and 2.07 for ex-smoker (p<0.001), compared with never smoking. The odds of GO are 86% less in Graves’ patients using anti-thyroid medication than those not (OR 0.14, CI 0.06-0.34, p<0.001). Predictors for DON were older age, oculomotility restriction, strabismus, reduced palpebral aperture and active GO.
**Conclusions:** This study identified increase age of onset, duration of GH and smoking as risk factors for GO. Usage of anti-thyroid medication was negatively related to GO. Older patients with restricted ocular motility, strabismus and active GO are at higher risk of DON and may benefit from early medical intervention.
Introduction

Graves’ orbitopathy (GO) manifests clinically in 25% of patients with Graves’ hyperthyroidism (GH). Among the risk factors predisposing to GO, smoking, older age, radioactive iodine and thyroid dysfunction appear to correlate with onset of GO and males appeared to develop more severe GO; the reasons underlying these predisposing factors remain unclear. As the incidence of GH is low, reported at 21.4 per 100,000 population per year in Sweden, an iodine rich region, the age-adjusted incidence rate for GO is even lower reported at 16.1 cases per million per year for moderate to severe GO, with no changes in incidence rate after iodine fortification of salt in a Danish study. As a result of the uncommon occurrence of GO, many earlier published studies examining risk factors for GO included small number of cases and controls, and in some instances, no control group, with the exception for the inclusion of smokers and non-smokers.

This study aimed to determine the exogenous risk factors predisposing patients to GO in a large multi-centre Australian case-control study.
Materials and Methods:

Australian thyroid-associated orbitopathy research (ATOR) participants

1062 non-consecutive GH patients with and without GO were prospectively recruited from endocrinology and ophthalmology outpatient in tertiary hospitals and private practices across Victoria and South Australia from 2009-2013. The study was approved by multi-centre human research ethics committees, and conducted with adherence to the Declaration of Helsinki.

GH was defined by the presence of hyperthyroidism based on reduced TSH, elevated T3 or T4, and diffusely enlarged thyroid gland; and either presence of GO, positive thyrotropin receptor (TSHR) antibodies, or diffuse uptake on technetium-99m pertechnetate thyroid scan. The anti-TSHR used was an electrochemiluminescence immunoassay, a TSH binding inhibition (TBI) assay on Modular Analytics E170 (Roche Diagnostics, Risch-Rotkreuz, Switzerland) where a human thyroid stimulating monoclonal autoantibody (M22) labeled with ruthenium complex was used, the detection range was 0.3-40 IU/L, the lower limit was reported at <1 IU/L to reduce false positives. Cases were defined by the presence of GO and controls by the absence of GO. One individual who did not have GO status assessment was excluded. GO individuals who were euthyroid or hypothyroid at presentation and did not have positive TSHR antibody were also excluded from the study (n=19). A chart of cases and controls with their sources is illustrated in Figure 2.
Figure 2 Australian-associated Thyroid-associated orbitopathy Research (ATOR) Participants

N=1062

1 GO status unconfirmed
9 GO hypothyroid and 10 euthyroid cases without TSH receptor recorded

N=1042
Sources:
- Hospital ophthalmology outpatient 108 (10.37%)
- Hospital endocrinology outpatient 530 (50.86%)
- Ophthalmologists private rooms 181 (17.39%)
- Endocrinologists private rooms 151 (14.51%)
- Mail out recruitment 72 (6.92%)

N=604 GH with GO
N=438 GH without GO

N=283
No previous GO treatment

N=319
Previously had GO treatment

* 2 missing treatment data
**Graves’ orbitopathy evaluation:**

GO was defined by the presence of symptoms of GO and at least one sign of TO e.g. lid retraction. Early symptoms of GO were detected using the Vancouver Orbitopathy Rule symptom questionnaire, including red eyes, lids swelling, eye protrusion and stare and blurred vision. GO status was examined and classified using vision, inflammation, strabismus, appearance (VISA) classification. Ophthalmological measurements included the following: visual acuity, pupil response and colour vision, inflammatory index score, extra-ocular movement and strabismus, lid measurements including palpebral aperture, marginal reflex distance, lid retraction and Hertel exophthalmometry. Active GO was defined by an inflammatory index score ≥3 out of 8, where chemosis, lid oedema and retro-bulbar pain scored up to 2 points and conjunctival injection and lid erythema scored 1 point each. Based on Hirschberg test assessing the position of corneal light reflex, Grade 1duction restriction was defined as when the light reflex was between 30-45 degree, grade 2 restriction was when the light reflex was between 30-15 degree and grade 3 restriction was when the light reflex was within 15 degree of pupil centre on eye duction. Grade 1 strabismus was defined as when the eye deviation was between 15-30 degree (i.e. 2-4mm from pupil centre), grade 2 strabismus the light reflex was between 30-45 degree (i.e. 4-6mm from pupil centre), and grade 3 strabismus was when the light reflex was beyond 45 degree from the pupil centre (i.e. >6mm from pupil centre) on primary gaze fixation. Participants’ GO status was determined by an ophthalmologist (JJK).

Dysthyroid optic neuropathy (DON) was defined by visual dysfunction secondary to GO when other causes for visual impairment had been excluded. The diagnosis of DON was based on reduced visual acuity, relative afferent pupillary defect, reduced colour vision and perception of brightness in the affected eye and CT orbit showing apical crowding, supplemented when the diagnosis is uncertain by visual field test. For patients reporting previous treatment for DON, medical notes were reviewed to confirm DON as defined by the parameters above.

**Measured variables:**
Patient’s age at recruitment, the duration of having been diagnosed with GH and GO were documented, which allowed age at diagnosis of GH and onset of GO to be calculated. Family history was documented for GH, and other autoimmune diseases. Ethnicity of participants was determined by their parent’s country of origin and ancestry. Smoking status was classified as never smoked, current smoker and ex-smoker who had ceased smoking for 6 months or more. Lifetime cigarette consumption was calculated using cigarette pack years (daily cigarette consumption x years of smoking/20). Thyroid status of patients at initial diagnosis of GH was recorded as hyper-, hypo- or euthyroid. Euthyroid states were achieved with anti-thyroid medication as the first line treatment (carbimazole or propylthiouracil). For GH not in remission and in recurrent GH, radio-active iodine and total thyroidectomy were advocated in our study population. Treatments for thyrotoxicosis were recorded as binary data for anti-thyroid medication, radioactive iodine and thyroidectomy.

A secondary outcome of this study was to investigate the risk factors for dysthyroid optic neuropathy (DON) in the subset of GO cases (N=604). Variables explored for DON included age of onset of GO, GH duration, smoking status, lifetime cumulative cigarette consumption, hyperthyroidism treatment modalities, gender, clinical findings of visual acuity, extraocular muscle restriction, strabismus grades, palpebral aperture, marginal reflex distance, exophthalmometry, inflammatory index score, activity status of GO and corneal exposure.

Analysis:

Descriptive statistics and analysis comparing groups were performed using Minitab statistical software version 16. (Minitab Inc., Pennsylvania) For continuous variables, 2-sample t-tests were used to compare mean differences between cases and controls. For categorical variables, chi-square tests of association were carried out to investigate the association with GO status.
To investigate risk factors for GO comprehensively, we grouped measured variables into: a) known risk factors for GO including age of onset of GH, gender, smoking status and radioactive iodine treatment, and; b) exploratory variables which included ethnicity, family history, anti-thyroid medication, duration of GH since diagnosis and thyroid function status at initial diagnosis. We used GenStat® (VSN International Ltd, 15th Edition) to examine all the competing logistic regression models for each subset of variables and then combined all candidate variables from both subsets. We chose the best-fit logistic regression model from all possible models based on the 9 candidate explanatory variables. This was based on 1004 individuals, for whom all the explanatory variables were available. The ‘best’ logistic regression model was chosen based on Akaike’s information criteria (AIC). In general, models with smaller AIC values are preferred. It may be that there are one or more models whose AIC values are quite close to the smallest AIC, so we considered a principle of parsimony. The “best” model was the simplest model with an AIC value that is no more than two larger than the smallest AIC.\textsuperscript{243} We report the odds ratios, 95% confidence intervals and $P$-values for this best logistic regression model for predicting GO.

Similarly, to characterize predictors for DON, comparative variables were grouped into three subsets: a) clinical characteristics thought to predict DON including age of onset of GO, oculomotility restriction and GO activity; b) exploratory clinical characteristics which include strabismus grading, mean exophthalmometry, best corrected visual acuities, palpebral apertures and corneal exposure, and; c) exploratory demographic factors which include gender, smoking status, thyroid function test. Again, the best model was chosen based on the principles of using AIC described above. This was based on 471 individuals for whom all explanatory variables were available. We report this final logistic regression model for predicting DON.

**Results:**

GO was observed in 604 (58%) of the 1042 participants with GH. The female to male ratio in GO cases and non-GO controls did not differ (4:1). The mean age of onset of GH was about 2.5 years later in GO cases than in non-GO controls (mean
43.0 years versus 40.6 years, p = 0.008). The mean duration of GH since diagnosis was also longer in cases compared to controls, by just under 4 years (mean 8.8 years versus 5.0 years, p <0.001).

The largest proportion by ethnicity in the ATOR GH cohort was Caucasian (73.4%) with majority of Northern and Western European ancestry. The proportion of Caucasian in GH with GO (79.8%) was greater than non-GO controls (64.6%) (p<0.001).

The proportion of smokers (current and ex-smokers) was greater in GO compared to non-GO (58.8% versus 36.6%, p<0.001). The odds for having GO was 2.47 times higher for smokers than for non-smokers (95% CI 1.92-3.18, p<0.001). Breaking down the smoking status to never smoked, current smoker and ex-smoker, there were greater proportions of current smoker and ex-smoker in the GO than non-GO group. The mean cumulative lifetime cigarette consumption was also higher in cases (mean 12.9, SD 20.1 pack-years) than the controls (mean 6.3, SD 14.7 pack-years) (p<0.001).

There were greater proportions of hypothyroid and euthyroid status in GO cases than non-GO controls at initial diagnosis of GH. Of the GO cases, 95.9% were hyperthyroid, 1.8% were hypothyroid and 2.3% were euthyroid, compared with 99.5% non-GO GD controls who were hyperthyroid, 0.2% hypothyroid and 0.2% euthyroid. (p<0.001). Overall, 256 Graves' patients (24.6%) were treated with radio-active iodine. A greater proportion of GO than non-GO patients had radioactive iodine therapy (31.1% versus 15.8%) and total thyroidectomy (22.9% versus 4.6%) for the treatment of GH. A lesser proportion of GO cases used anti-thyroid medication (86.7% versus 98.6%) than non-GO controls (p <0.001). Patient's self-reported family history for GH and other autoimmune diseases did not differ substantially between GO and non-GO patients; nor did positive TSHR antibody status. (Table 2)
Table 2 Clinical characteristics of Graves' hyperthyroidism patients with and without Graves' orbitopathy

<table>
<thead>
<tr>
<th></th>
<th>TO (n=604)</th>
<th>No TO (n=438)</th>
<th>P-value from t-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age at Graves' diagnosis in years</td>
<td>43.0</td>
<td>40.6</td>
<td>0.008</td>
</tr>
<tr>
<td>Mean duration of Graves' hyperthyroidism in years</td>
<td>8.76</td>
<td>4.97</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean cigarette pack years</td>
<td>12.9</td>
<td>6.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Number</td>
<td>Percentage</td>
<td>Number</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>123</td>
<td>79.6%</td>
<td>347</td>
</tr>
<tr>
<td>Female</td>
<td>481</td>
<td>20.4%</td>
<td>91</td>
</tr>
<tr>
<td>Ethnic grouping</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>482</td>
<td>79.8%</td>
<td>283</td>
</tr>
<tr>
<td>Non-caucasian</td>
<td>122</td>
<td>20.2%</td>
<td>155</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never smoke</td>
<td>248</td>
<td>41.2%</td>
<td>277</td>
</tr>
<tr>
<td>Current smoker</td>
<td>150</td>
<td>24.9%</td>
<td>72</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>204</td>
<td>33.9%</td>
<td>88</td>
</tr>
<tr>
<td>Thyroid function at baseline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hyperthyroid</td>
<td>577</td>
<td>95.9%</td>
<td>436</td>
</tr>
<tr>
<td>Hypothyroid</td>
<td>11</td>
<td>1.8%</td>
<td>1</td>
</tr>
<tr>
<td>Euthyroid</td>
<td>14</td>
<td>2.3%</td>
<td>1</td>
</tr>
<tr>
<td>TSHR antibody</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>20</td>
<td>12.6%</td>
<td>19</td>
</tr>
<tr>
<td>Negative</td>
<td>139</td>
<td>87.4%</td>
<td>156</td>
</tr>
<tr>
<td>Family Hx</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nil</td>
<td>455</td>
<td>78.3%</td>
<td>351</td>
</tr>
<tr>
<td>Graves' Disease</td>
<td>89</td>
<td>15.3%</td>
<td>53</td>
</tr>
<tr>
<td>Other autoimmune Diseases</td>
<td>37</td>
<td>6.4%</td>
<td>21</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-thyroid drug</td>
<td>522</td>
<td>86.7%</td>
<td>432</td>
</tr>
<tr>
<td>Radio-active iodine</td>
<td>187</td>
<td>31.1%</td>
<td>69</td>
</tr>
<tr>
<td>thyroidectomy</td>
<td>138</td>
<td>22.9%</td>
<td>20</td>
</tr>
</tbody>
</table>

The best multiple logistic regression for GO after considering all 9 explanatory variables included age of onset of GH, duration of GH, smoking status, and usage of anti-thyroid medication (Table 3). Based on 1004 subjects used in the final multivariable binary logistic regression model, for each decade increase in age of diagnosis of GH, there is a 17% increase in the odds of GO. For each year increase
in the duration of GH at recruitment, there is a 7% increase in odds of GO. The odds ratio for GO increases for current smoker (2.22) and ex-smoker (2.07), relative to non-smokers. The odds of GO are 86% less in GH patients using anti-thyroid medication treatment alone, than those not on anti-thyroid medication.

Although the best logistic regression model in terms of parsimony and AIC included four explanatory variables, a five variable model can be considered equivalent in terms of the AIC. Importantly this included radio-active iodine treatment. Hence we have included the results for RAI in this model in Table 3. The positive association of radio-active iodine treatment with GO had an OR of 1.37, and a p-value of 0.085 in the five variable multivariate model, and is consistent with a weaker effect.
Table 3 Odds ratio for risk factors in predicting development of Graves’ orbitopathy in simple and multiple binary regression analysis (n=1004)

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Baseline</th>
<th>Level</th>
<th>Single explanatory variable models</th>
<th>Multiple explanatory variable model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Odds ratio (95% confidence interval)</td>
<td>Odds ratio (95% confidence interval)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>P-value</td>
<td>P-value</td>
</tr>
<tr>
<td>Age onset GH in decades</td>
<td>x</td>
<td>x + 1</td>
<td>1.12 (1.02-1.23) 0.013</td>
<td>1.17 (1.06-1.29) 0.002</td>
</tr>
<tr>
<td>GH duration</td>
<td>x</td>
<td>x + 1</td>
<td>1.08 (1.06-1.10) &lt;0.001</td>
<td>1.07 (1.05-1.10) &lt;0.001</td>
</tr>
<tr>
<td>Smoking status</td>
<td>Non-smoker</td>
<td>Current smoker</td>
<td>2.20 (1.57-3.07) &lt;0.001</td>
<td>2.22 (1.57-3.14) &lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ex-smoker</td>
<td>2.49 (1.83-3.39) &lt;0.001</td>
<td>2.07 (1.49-2.85) &lt;0.001</td>
</tr>
<tr>
<td>Anti-thyroid Med</td>
<td>None</td>
<td>Anti-thyroid Med</td>
<td>0.09 (0.04-0.22) &lt;0.001</td>
<td>0.14 (0.06-0.34) &lt;0.001</td>
</tr>
<tr>
<td>Radio-active Iodine</td>
<td>None</td>
<td>Radio-active iodine</td>
<td>2.38 (1.74-3.26) &lt;0.001</td>
<td>1.37 (0.96-1.95) 0.085</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>Non-Caucasian</td>
<td>Caucasian</td>
<td>2.08 (1.56-2.76) &lt;0.001</td>
<td>-</td>
</tr>
<tr>
<td>Family History</td>
<td>None</td>
<td>Graves’ disease</td>
<td>1.29 (0.90-1.87) 0.169</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Autoimmune disease</td>
<td>1.36 (0.78-2.36) 0.278</td>
<td>-</td>
</tr>
<tr>
<td>Thyroid function at initial diagnosis</td>
<td>Hyperthyroid</td>
<td>Hypothyroid</td>
<td>8.36 (1.08-65.04) 0.042</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Euthyroid</td>
<td>10.65 (1.39-81.26) 0.023</td>
<td>-</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>Female</td>
<td>1.00 (0.73-1.36) 0.999</td>
<td>-</td>
</tr>
</tbody>
</table>

- blank in multivariable model means these variables were considered during all possible subset selections but these variables were not included in the final model that accounted for interdependencies of the explanatory variables.
GO cases characteristics

The distribution of age of onset of GO was consistent with a normal distribution, the mean age was 44.8 ± SD 13.8 years (Figure 3). The onset of GO most frequently happens within the same year as the diagnosis of GD. 28 cases (4.8%) had onset of GO before the onset of GH. The mean duration of diagnosis of GO from GH was 1.5 ± SD 5 years (range -39 to 33 years). The clinical characteristics of GO were summarized in Table 4.

Figure 3 Histogram of age of onset of Graves’ orbitopathy
Table 4 Clinical characteristics of 604 participants with Graves’ orbitopathy in ATOR cohort

<table>
<thead>
<tr>
<th>Treatment and clinical characteristics of Graves’ orbitopathy cases</th>
<th>Mean (SD)</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>History</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oral prednisolone</td>
<td>149/602</td>
<td>24.8</td>
<td></td>
</tr>
<tr>
<td>Intravenous methylprednisolone</td>
<td>49/602</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>Orbital radiation</td>
<td>69/602</td>
<td>11.5</td>
<td></td>
</tr>
<tr>
<td>Immune-modulator</td>
<td>10/602</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>Orbital decompression</td>
<td>156/602</td>
<td>25.9</td>
<td></td>
</tr>
<tr>
<td>Strabismus surgery</td>
<td>55/602</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>Lid retraction surgery</td>
<td>111/602</td>
<td>18.4</td>
<td></td>
</tr>
<tr>
<td>Blepharoplasty</td>
<td>66/602</td>
<td>11.0</td>
<td></td>
</tr>
<tr>
<td>Diplopia</td>
<td>209/604</td>
<td>34.6</td>
<td></td>
</tr>
<tr>
<td><strong>Examination findings</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Right eye visual acuity (in decimal)</td>
<td>0.98 (0.24)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left eye visual acuity (in decimal)</td>
<td>0.96 (0.26)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative afferent pupillary defect</td>
<td>34/567</td>
<td>6.0</td>
<td></td>
</tr>
<tr>
<td>Altered red colour saturation</td>
<td>41/567</td>
<td>7.2</td>
<td></td>
</tr>
<tr>
<td>Optic disc pallor</td>
<td>6/567</td>
<td>1.1</td>
<td></td>
</tr>
<tr>
<td>Optic disc oedema</td>
<td>3/567</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Peri-ocular lid oedema</td>
<td>145/567</td>
<td>25.6</td>
<td></td>
</tr>
<tr>
<td>Inflammatory index score ≥3 out of 8</td>
<td>95/567</td>
<td>16.8</td>
<td></td>
</tr>
<tr>
<td>Oculomotility restriction</td>
<td>233/594</td>
<td>39.2</td>
<td></td>
</tr>
<tr>
<td>Strabismus/presence of tropia</td>
<td>82/594</td>
<td>13.8</td>
<td></td>
</tr>
<tr>
<td>Right palpebral aperture (mm)</td>
<td>9.56 (3.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Left palpebral aperture (mm)</td>
<td>9.58 (2.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palpebral aperture in the worse eye (mm)</td>
<td>10.7 (2.3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Upper lid retraction ≥1mm from limbus</td>
<td>176/587</td>
<td>30.0</td>
<td></td>
</tr>
<tr>
<td>Lower lid retraction ≥1mm from limbus</td>
<td>255/589</td>
<td>43.3</td>
<td></td>
</tr>
<tr>
<td>Lagophthalmos</td>
<td>100/585</td>
<td>17.1</td>
<td></td>
</tr>
<tr>
<td>Hertel exophthalmometry in the worse eye (mm)</td>
<td>21.6 (3.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proptosis ≥21mm</td>
<td>323/546</td>
<td>59.2</td>
<td></td>
</tr>
<tr>
<td>Proptosis ≥23mm</td>
<td>210/546</td>
<td>38.5</td>
<td></td>
</tr>
<tr>
<td>Exposure keratopathy</td>
<td>24/604</td>
<td>4.0</td>
<td></td>
</tr>
</tbody>
</table>

Active GO with inflammatory index score ≥3 was present in 95 of 567 recorded cases (16.8%). Dysthyroid optic neuropathy (DON) occurred in 51 of 604 GO cases (8.4%). DON occurred in GO cases with a more advanced age. The mean age of diagnosis of GO in DON cases (mean 54.9, SD 10.6 years) was higher by 11 years than in those without DON (mean 43.8, SD 13.7 years), p<0.001. The mean inflammatory index score was higher in DON cases (mean 2.2, SD 2.04) by 1.2 points than those without DON (mean 0.97, SD 1.34), p<0.001. In concordance with the higher inflammatory index score finding, active GO is more prevalent in GO with DON (48.0%) than those without (13.7%), p<0.001. Restriction of extraocular movement was present in a greater proportion of GO patients with DON and the proportion of DON increased with incremental worsening grades of
extra-ocular muscle restriction. Grade 1 oculomotility restriction was 18.9% in non-DON versus 20.0% in DON; grade 2 oculomotility restriction was 8.6% in non-DON and 20.0% in DON and grade 3 oculomotility restriction was 7.9% in non-DON versus 40.0% in DON GO cases (p<0.001). A greater proportion of GO patients with DON also had strabismus (42.0%) compared with GO patients without DON (11.2%) (p<0.001). Mean exophthalmometry reading was modestly higher in DON than non-DON GO patients by 2.7mm (95% CI 1.17-4.2mm, p=0.001). Mean best-corrected visual acuity annotated as fractions was marginally reduced in the right (mean difference 0.10, 95% CI: 0.02-0.19, p=0.02) and left eye (mean difference 0.17, CI: 0.07-0.28, p=0.002) in DON compared to non-DON GO cases.

When considered as single predictors of DON in simple logistic regression, duration of GH, duration of GO onset in relation to timing of diagnosis of GH, modality of treatments for hyperthyroidism, smoking status, lifetime cigarette consumption, gender, family history, thyroid function status, positive TSHR antibody, signs of corneal exposure, palpebral apertures and marginal reflex distances were not strongly associated with DON.

The multivariable model selected from the all subsets procedure, based on 471 patients, found age of onset of GO, palpebral aperture of the right eye, moderate to severe restriction of oculomotility and strabismus, and GO activity status as predictors for DON. For every decade increase in age of onset of GO, the odds of DON increased by 58%. For every millimeter increase in palpebral aperture, the odds of DON decreased by 19%. The odds of DON is higher by 6-9 times in those with moderate to severe oculomotility restriction compared to patients without oculomotility restriction. Similarly moderately severe strabismus had a 12 times increased odds of DON compared with no strabismus. Active GO is associated with 2.74 times increased odds for DON, compared with inactive GO (Table 5).
<table>
<thead>
<tr>
<th>Predictor</th>
<th>Baseline</th>
<th>Level</th>
<th>Single explanatory variable models</th>
<th>Multiple explanatory variable model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age onset GO in decades</td>
<td>x</td>
<td>x + 1</td>
<td>1.89 (1.42-2.52) &lt;0.001</td>
<td>1.58 (1.13-2.19) 0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.78 (0.27-2.25) 0.65</td>
</tr>
<tr>
<td>Strabismus</td>
<td>None</td>
<td>Grade 1</td>
<td>3.47 (1.52-7.96) 0.003</td>
<td>7.15 (1.40-39.28) 0.016</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grade 2</td>
<td>40.52 (7.47-21.79) &lt;0.001</td>
<td>2.35 (0.76-7.22) 0.14</td>
</tr>
<tr>
<td>Oculomotility restriction</td>
<td>None</td>
<td>Grade 1</td>
<td>3.22 (1.10-9.44) 0.033</td>
<td>9.07 (2.78-29.62) &lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grade 2</td>
<td>10.18 (3.57-29.03) &lt;0.001</td>
<td>5.96 (1.76-20.11) 0.004</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Grade 3</td>
<td>16.96 (6.47-44.45) &lt;0.001</td>
<td>2.35 (0.76-7.22) 0.14</td>
</tr>
<tr>
<td>TO activity</td>
<td>Inactive TO</td>
<td>Active TO</td>
<td>5.71 (2.85-11.41) &lt;0.001</td>
<td>2.74 (1.19-6.28) 0.017</td>
</tr>
<tr>
<td>Palpebral aperture right eye</td>
<td>x</td>
<td>x+1</td>
<td>0.83 (0.72-0.96) 0.011</td>
<td>0.81 (0.69-0.95) 0.012</td>
</tr>
<tr>
<td>Palpebral aperture left eye</td>
<td>x</td>
<td>x+1</td>
<td>0.87 (0.75-1.00) 0.056</td>
<td>-</td>
</tr>
<tr>
<td>Mean exophthalmometry</td>
<td>x</td>
<td>x+1</td>
<td>1.10 (1.00-1.21) 0.041</td>
<td>-</td>
</tr>
<tr>
<td>Best corrected vision right eye</td>
<td>Visual acuity 6/6 or better</td>
<td>Visual acuity worse than 6/6</td>
<td>2.19 (1.08-4.47) 0.038</td>
<td>-</td>
</tr>
<tr>
<td>Best corrected vision left eye</td>
<td>Visual acuity 6/6 or better</td>
<td>Visual acuity worse than 6/6</td>
<td>2.38 (1.20-4.75) 0.017</td>
<td>-</td>
</tr>
<tr>
<td>Corneal exposure</td>
<td>None</td>
<td>Corneal exposure</td>
<td>1.79 (0.39-8.27) 0.453</td>
<td>-</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>Female</td>
<td>0.47 (0.23-0.97) 0.041</td>
<td>-</td>
</tr>
<tr>
<td>Smoking status</td>
<td>None</td>
<td>Current smoker</td>
<td>1.50 (0.66-3.43) 0.338</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ex-smoker</td>
<td>1.14 (0.52-2.49) 0.747</td>
<td>-</td>
</tr>
<tr>
<td>Thyroid function at initial diagnosis</td>
<td>Hyperthyroid</td>
<td>Hypothyroid</td>
<td>3.14 (0.64-15.41) 0.158</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Euthyroid</td>
<td>3.77 (0.99-14.38) 0.052</td>
<td>-</td>
</tr>
</tbody>
</table>
Discussion:

In this study, we have described the specific demographics and clinical characteristics of the Australian thyroid-associated orbitopathy research (ATOR) cohort. The ATOR participants are derived from multiple tertiary hospital endocrinology and ophthalmology outpatient clinics and related private specialist practices. The results from this study will likely apply generally to the more severe spectrum of GO and GH requiring specialist management. This is demonstrated by a higher rate of reported dysthyroid optic neuropathy (DON) at 8.4% in this study population, compared with less than 2% in a population-based study of GO and 0.3% for a non-tertiary referral centre, 2 5 and a significant proportion of patients needing definitive treatment with radioactive iodine (24% overall) and total thyroidectomy (15% overall) for patients not in remission from GH. The patient profiles observed in our study were comparable to the study cohorts from combined thyroid eye clinic, eye and endocrinology referral centre setting in terms of the average age of GH (40.4-41.9 years), average age of GO cases (41.6-49 years), female to male gender ratio (4:1) and optic neuropathy complication rates, ranging from 3.9-9.9%.6,19,143,146,244,245

The ATOR cohort represents a large cohort of patients with a relatively uncommon disease, therefore this study population is ideal for studying both the genetic and environmental risk factors for both GO and GH. This study found an older age of diagnosis of GH, a longer duration of GH and smoking are risk factors associated with GO in GH. GO is positively associated with a lesser usage of antithyroid medication for GH, possibly reflecting greater proportion of euthyroid and hypothyroid status at diagnosis.

GH, like other autoimmune diseases, occurs more frequently in females. The female to male ratio for GH was estimated at 7.3:1 based on mean weighted prevalence data compared with United State census data.144 In contrast, large case-control studies consistently find the female to male ratio of GH with and without GO at 4:1,146,246 this is also true for our study. We did not find a relative male predilection for GO as noted in other case control studies.3 19,146 In fact, a large longitudinal cohort and a population study have both shown the risk for developing GO is similar in males and females.8 246
Our study confirmed the mean age of diagnosis of GH was higher in those with GO, consistent with findings of previous studies.\textsuperscript{143, 146} In a population based study in Denmark, less than 2\% developed GO in GH less than 40 years as compared with an incidence rate of 8\% for GO at 40-60 years.\textsuperscript{8} In addition, the GO cases in this study were diagnosed with GH for longer than those without GO.

It has been suggested that Europeans may have an increased risk of developing GO than Asians.\textsuperscript{148} The ethnicity mix of the ATOR cohort was heterogeneous but the majority were Caucasians. Initially on univariate analysis Caucasian patients had a higher odds of GO, compared with non-Caucasian patients; however when all explanatory variables were considered, ethnicity was no longer a predictor for the development of GO.

Smokers had increased odds for GO in this study, and a current smoker was at increased odds of GO compared with an ex-smoker. Smoking status remained in our model after adjusting for age of onset of GO, duration of GH, and usage of anti-thyroid medication. Smoking as a risk for GO has been well documented in case-control and cohort studies, where smoking also seems to correlate with severity of GO.\textsuperscript{216-218} Depending on which control groups were used and the size of the subjects studied, the odd ratios for smoking in GO varies between 1.94 and 10.1 for GH control without GO, and between 1.22 and 20.2 for normal healthy control.\textsuperscript{221} Smoking status and lifetime cigarette consumption are partly confounded; while there is a simple association between lifetime cigarette consumption and GO (OR 1.2, CI: 1.01, 1.03, p < 0.001), we chose to use smoking status rather than lifetime cigarette consumption in the multivariable model, as it performed better as a predictor in that model. Current number of cigarettes smoked a day dictate the incidence risk of GO, but not lifetime cigarette consumption.\textsuperscript{222} In Korean patients, being a current smoker is the strongest risk factor for the development of severe GO and DON.\textsuperscript{247} On the contrary, we did not find a positive correlation of severe GO, indicated by DON, with smoking status or lifetime cigarette consumption (OR 1.01, CI: 0.99, 1.02, p = 0.23); nor did we find a correlation of smoking status with severity of oculomotility restriction in GO (data not shown).
Treatment modalities for hyperthyroidism may affect the development of GO. It has been shown that radioactive iodine treatment is associated with development of GO in 15% of patients, in contrast with 3% GH treated with methimazole. The risk for developing or worsening of GO was significantly greater for radioactive iodine (38.7%) compared to anti-thyroid treatment group (2.1%) in a randomized trial when early correction of hypothyroidism was achieved. Conversely other groups did not find an association of radio-active iodine treatment with development or worsening of GO. Overall, evidence suggests anti-thyroid medications and thyroidectomy do not influence the course of GO. The largest longitudinal cohort study on the contrary found thyroidectomy reduces the hazard for GO. In our study, radioactive iodine treatment was found associated with GO in univariate analysis (odds ratio 2.37). However the effect of RAI is reduced when other explanatory variables were considered in multivariate analysis; the odds ratio became 1.37. Our findings indicate that proportionately more GO patients had radioactive-iodine and thyroidectomy treatments and proportionately fewer had anti-thyroid medication compared with GH patients without GO. The causal relationship of treatment modalities for hyperthyroidism with the onset of GO cannot be argued for due to the case-control design. In addition, we have not considered thyroidectomy in multivariable analysis because we are aware of the inherent selection bias with preference for thyroidectomy over radio-active iodine as the definitive treatment for refractory GH in GO patients in our study population.

A secondary outcome of this study is to study the predictors of DON in GO patients. This study found older age of onset of GO, oculomotility restriction, strabismus, reduced palpebral aperture and active GO were predictors for the development of DON in GO. The proportion of DON increased with increasing severity of oculomotility restriction, this clinical finding remains useful in predicting DON even in GO patients who have been treated previously. We found strabismus was predictive of the odds of DON; this is a novel finding and will prove to be useful in clinical assessment for DON. By clinical findings, only limitation of oculomotility is consistently correlated with the development of DON. Extraocular muscle volume and apical crowding on computed tomography were also found to be predictive of DON. The predictability of
DON is best when combining extraocular muscle duction restriction, reduced marginal reflex distance and apical crowding on radiology, than either clinical-only or radiological-only model alone.  

The correlation of older age of GO onset with DON is also consistent with observations that older patients develop more severe GO, and older patients often have more symptoms of impaired ocular motility and soft tissue involvement.  

While the mean difference in palpebral aperture in DON versus non-DON is marginal (0.06mm, CI: -0.88-0.99) therefore limiting the clinical applicability of this variable, the multivariable model for DON showed palpebral aperture is explaining some variability that is not otherwise accounted for. Interestingly one previous study that used the same GO classification found a positive correlation of marginal reflex distance with DON.  

Whilst palpebral aperture measurement is related to marginal reflex distance, we did not find a positive association of marginal reflex distance with DON in our cohort. This might relate to the inclusion criteria for DON, we included GO patients that presented with DON or had developed DON, whereas Weis et al. evaluated the GO patients initially presented with DON, hence marginal reflex distance as a clinical predictor may only be relevant to the prediction for patients that initially presented with DON. In addition we found a correlation of disease activity with DON, suggesting DON occurs predominantly during the active phase of GO. This is a novel finding for predicting DON.  

In conclusion, the predictors for GO include older age of onset of GH, a longer duration of GH, and smoking. Usage of anti-thyroid medication was negatively correlated with GO. DON could be predicted clinically by oculomotility restriction, strabismus and GO disease activity. Based on the study findings arguably older patients with severe restrictive oculomotility and active orbital inflammation would benefit from early medical treatment for GO to prevent the development of DON.
Acknowledgement: Drs Alan McNab, Garry Davies, Thomas Hardy, Richard Stawell, Simon Forehan, Mark Stein, Shane Hamblin, Rosemary Wong, Duncan Topliss, Peter Colman, Spiros Fourlanos, Cherie Chiang, Robert Thompson, Matthew Doogue for private patient sources. Ms Emmanuelle Souzeau for assistance in mail-out blood and data collection. Dr Matthew Lee for collating Graves’ disease patient database from Dr Rosemary Wong’s room.
11 Serum selenium status in Graves’ disease with and without thyroid-associated orbitopathy

Publication arising from this chapter:


11.1 Background and aim

Selenium is a trace element highly concentrated in the thyroid gland and functions as important anti-oxidants, predominantly as glutathione peroxidases. Increased oxidative stress in the retro-orbital tissues in TO coupled with the observation that selenium supplementation in TO appeared to improve orbital inflammation, seems to suggest that selenium deficiency could be a risk factor for exacerbation of thyroid-associated orbitopathy (TO) in Graves’ disease (GD).

This chapter will explored the null hypothesis that there is no difference in the serum selenium levels in GD with and without TO. The alternative hypothesis explored is that there is a significant difference in selenium status in TO compared to GD without TO. To test the hypothesis, we measured and compared serum selenium levels between patients with GD with and without TO in a case control study, taking into accounts other significant exogeneous risk factors associated with TO.

The specific aim for this chapter is:

a) to determine if serum selenium levels are reduced in GD patients with TO compared with GD patients without TO.
11.2 Peer reviewed publication author version

Title: Serum selenium status in Graves’ disease with and without orbitopathy: a case-control study

Short Title: Serum selenium status in Graves’ orbitopathy

Authors:

Jwu Jin Khong\textsuperscript{1,2} RANZCO, MMed, MBBS(Hons)
Rebecca F Goldstein\textsuperscript{1} FRACP, MBBS(Hons)
Kerrie M Sanders\textsuperscript{1} PhD
Hans Schneider\textsuperscript{3,4} FRACP, FRCPA, MD
Jeffrey Pope\textsuperscript{3} MAACB, B Apl Sc(Hons)
Kathryn P Burdon\textsuperscript{5} PhD, Bsc(Hons)
Jamie E Craig\textsuperscript{5} FRANZCO, DPhil, MBBS(Hons)
Peter R Ebeling\textsuperscript{1} FRACP, MD, MBBS

Author Affiliations:

1. NorthWest Academic Centre, The University of Melbourne, Western Health, St. Albans, Victoria, Australia
2. Orbital Plastics and Lacrimal Unit, The Royal Victorian Eye and Ear Hospital, East Melbourne, Victoria, Australia.
3. Clinical Biochemistry Unit, Alfred Pathology Service, Prahran, Victoria, Australia
4. Central Clinical School, Monash University, Prahran, Victoria, Australia
5. Department of Ophthalmology, Flinders University, Flinders Medical Centre, South Australia, Australia

Corresponding author:

Prof. Peter R Ebeling MBBS MD FRACP
NorthWest Academic Centre,
The University of Melbourne
Western Health, 176 Furlong Road, St Albans, VIC 3021
Telephone: +61 3 8395 8065
Fax: +61 3 8395 8258
Email: peterre@unimelb.edu.au

Key words: Selenium, Graves’ disease, Graves’ orbitopathy, thyroid ophthalmopathy
Disclosure summary: All authors have nothing to declare
Word count: 2439
Abstract

Objective: Selenium is effective in improving quality of life and reducing the progression of active Graves’ orbitopathy. The effect of correcting relative selenium deficiency on improving Graves’ orbitopathy is unknown, as baseline selenium levels have not previously been measured. The study aims to determine if serum selenium levels are reduced in patients with Graves’ orbitopathy (GO) compared with Graves’ without orbitopathy (GD).

Design: A prospective, case-control study performed between 2009 and 2012 at endocrine and ophthalmology clinics in Australia.

Patients: A total of 198 patients with Graves’ disease participated in the study: 101 with Graves’ orbitopathy and 97 without Graves’ orbitopathy.

Measurements: Serum selenium levels in both groups.

Results: Mean serum selenium levels were significantly lower in GO (1.10+/-0.18µmol/L) than GD (1.19+/-0.20µmol/L) (P=0.001). Mean selenium levels appeared to decrease in parallel with increasing severity of GO; selenium level was 1.19 +/-0.20 umol/L in GD, 1.10 +/-0.19 umol/L in moderate to severe GO and 1.09 +/-0.17 umol/L in sight-threatening GO (P 0.003). Serum selenium levels remained significantly lower in GO after adjusting for age, smoking status, thyroidectomy, radio-active iodine treatment and residential location.

Conclusion: Serum selenium levels are lower in patients with GO compared with GD in an Australian study population with marginal selenium status. Relative selenium deficiency may be an independent risk factor for orbitopathy in patients with Graves’ disease.
Introduction

Selenium, an essential trace element, has a number of actions, including the production of active thyroid hormone and as an anti-oxidant and anti-inflammatory agent. Selenium concentrations are highest in the thyroid gland. Reduced selenium levels have been reported in patients newly diagnosed with autoimmune thyroid disease. Multiple prospective randomized control trials showed successful serial reduction of thyroperoxidase (TPO) autoantibodies with selenium supplementation in Hashimoto’s thyroiditis after 3-12 months, whilst other studies did not show significant changes in TPO autoantibodies levels or thyroid function with selenium supplementation.

A randomized trial by the European Group on Graves Orbitopathy showed selenium is effective in improving the quality of life and reducing the progression of mild active Graves’ orbitopathy. The mechanism of improving Graves’ orbitopathy remains unclear, but one possibility is an effect mediated by reduction in oxidative stress, as the selenoproteins protect against damage caused by reactive oxygen species. Correcting selenium deficiency could also explain its effect on improving Graves’ orbitopathy. This hypothesis is currently untested because baseline selenium levels have not previously been measured in Graves’ orbitopathy (GO) compared with Graves’ without orbitopathy (GD). We aimed to determine whether serum selenium concentrations are reduced in patients with GO compared with GD in a prospective, case-control study.

Patients and Methods

Patients diagnosed with Graves’ disease were prospectively recruited from multiple endocrine and ophthalmology clinics in Victoria, Australia from 2009 to 2012. All participants were examined for the presence and severity of thyroid-associated orbitopathy by an ophthalmologist using VISA classification. Patients were interviewed to determine their ethnicity, age, address by postcodes, treatment for Graves’ disease and thyroid-associated orbitopathy, smoking status, family history and thyroid-specific ophthalmic symptoms. Thyroid function tests including thyroid stimulating hormone (TSH), free tri-
iodothyronine (fT3), and free thyroxine (T4), and TSH receptor antibody levels were obtained from medical records.

This prospective, case-control study was approved by each site’s human research ethics committees. Moderate and severe GO were included as cases. Cases either had sight-threatening complications (optic neuropathy, exposure keratopathy), thyroid-associated orbitopathy requiring surgical rehabilitative surgery (orbital decompression, lid recession, blepharoplasty, strabismus surgery), or untreated thyroid-associated orbitopathy with moderate to severe appearance on examination. Controls (GD) had Graves’ disease for more than 2 years, without clinical signs or symptoms of thyroid-associated orbitopathy. Participants’ locations of residence were defined into metropolitan and non-metropolitan categories according to Australian Standard Geographical Classification 2011, according to statistical division code 05.

Blood samples were collected and centrifuged and serum was stored at -80°C. Serum selenium was measured using graphite furnace atomic absorption spectrophotometry and compared between cases and controls. The method uses an AAAnalyst 800 Atomic Absorption Spectrophotometer (Perkin Elmer, Glen Waverley, Victoria, Australia) with a graphite furnace and a specific selenium electrode discharge lamp. Blood samples from 217 Victorian blood donors were used to establish a reference interval for adults (0.8-1.4µmol/L). There were no observed differences in selenium levels due to age or sex.

**Statistical analysis**

Statistical analysis was performed using Minitab v16. Serum selenium levels were normally distributed in cases and controls, hence a two-sample T test was used to compare the differences in selenium levels between GO and GD; \( P < 0.05 \) was considered significant. Mean serum selenium levels in GD, moderate to severe GO and sight threatening GO were analysed using one-way ANOVA. Thyroid function tests and TSH receptor antibody levels were not normally distributed, hence medians were compared using non-parametric Kruskal Wallis tests between cases and controls. We used the chi-square test to determine the statistical significance for differences noted in categorical variables. We used binary logistic regression with GO as the outcome for univariate analysis.
including selenium level, thyroid function tests, TSH receptor antibody, age, gender, smoking status, ethnicity, residential location, duration of Graves’ disease, anti-thyroid medication use, radioactive iodine treatment and thyroidectomy. Only variables associated with GO in univariate analyses were entered into a multivariate model, variables with p>0.05 were eliminated in stepwise analyses.

Results

198 patients were included in the study. 155 (78.3%) participants were female and 43 (21.7%) were male. Those with GO were older than GD (54.1 +/- 12.0SD years and 47.4 +/- 14.0SD years, respectively; p<0.001). The median duration of Graves’ disease diagnosis was 8 years in GO and 3 years in GD. There were more smokers and ex-cigarette smokers, non-metropolitan residents, radioactive iodine- and thyroidectomy-treatment in GO than GD (Table 6). GO had features of moderate to severe disease, 20 (19.8%) with optic neuropathy, 9 (8.9%) with exposure keratopathy, 84 (83.1%) required rehabilitative surgery for the complications of GO, and 76 (75.2%) had either a moderate or severe appearance. In total 26 cases had sight-threatening GO with either optic neuropathy, or exposure keratopathy, or both. There were 197 recordings of TSH, 190 recordings of fT4, 169 recordings of fT3 and 93 of TSH receptor antibodies. Median TSH level was significantly higher in GO (1.31uM/L{0.05, 3.21}) than GD (0.09uM/L{0.01,1.81}) (p<0.001). Median fT3 level was significantly lower in GO (4.5pmol/L{3.9, 5.3} than GD (5.35pmol/L{4.3,9.85}) (p<0.001). The median fT4 level was lower in GO (16.3pmol/L) than GD (17.2pmol/L) but was not statistically significant. (p 0.12) There was no significant difference in the median TSH receptor antibody levels in GO (1.58IU/L) and GD (1.10IU/L).
Table 6 Baseline demographics of Graves' orbitopathy versus Graves' control, variables having significant association with Graves' orbitopathy with p<0.05

<table>
<thead>
<tr>
<th>Variables++</th>
<th>Graves' Orbitopathy (GO)</th>
<th>Graves' no orbitopathy (GD)</th>
<th>All</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>n=101 (51%)</td>
<td>n=97 (49%)</td>
<td>198</td>
<td></td>
</tr>
<tr>
<td>Mean age</td>
<td>54.1 +/-12</td>
<td>47.4 +/-14</td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean serum selenium</td>
<td>1.10 +/-0.18 umol/L</td>
<td>1.19 +/-0.20 umol/L</td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>75 (74%)</td>
<td>80 (82%)</td>
<td>155</td>
<td>0.161</td>
</tr>
<tr>
<td>Male</td>
<td>26 (26%)</td>
<td>17 (18%)</td>
<td>43</td>
<td></td>
</tr>
<tr>
<td>Smoking status*</td>
<td></td>
<td></td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Non smoker</td>
<td>26 (26%)</td>
<td>54 (56%)</td>
<td>80</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cigarette Smoker</td>
<td></td>
<td>14 (14%)</td>
<td>41</td>
<td></td>
</tr>
<tr>
<td>Ex-cigarette smoker</td>
<td></td>
<td>29 (30%)</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>93 (92%)</td>
<td>79 (81%)</td>
<td>172</td>
<td>0.027</td>
</tr>
<tr>
<td>Metropolitan</td>
<td>78 (77%)</td>
<td>96 (99%)</td>
<td>174</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Non metropolitan</td>
<td></td>
<td>1 (1%)</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>Anti-thyroid medications</td>
<td>87 (86%)</td>
<td>95 (98%)</td>
<td>182</td>
<td>0.001</td>
</tr>
<tr>
<td>Radio-active iodine</td>
<td>36 (36%)</td>
<td>15 (15%)</td>
<td>51</td>
<td>0.001</td>
</tr>
<tr>
<td>Thyroidectomy</td>
<td>36 (36%)</td>
<td>6 (6%)</td>
<td>42</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TSH</td>
<td>1.31 uM/L (0.05, 3.21)</td>
<td>0.09 uM/L (0.01, 1.81)</td>
<td>197</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>T3</td>
<td>4.5 pmol/L (3.9, 5.3)</td>
<td>5.35 pmol/L (4.3, 985)</td>
<td>169</td>
<td>0.001</td>
</tr>
</tbody>
</table>

++ parametric data refers to mean +/-SD

*Missing value smoking status in 3 Graves' orbitopathy cases
Mean serum selenium levels were significantly lower in GO (1.10+/-0.18 µmol/L) compared with GD 1.19+/-0.20 µmol/L (Figure 4). The difference in mean levels between cases and controls was 0.09 µmol/L (95% CI{0.04, 0.14}, p 0.001). Mean selenium levels also appeared to decrease in parallel with increasing severity of GO; selenium level was 1.19 +/-0.20 umol/L in GD, 1.10 +/-0.19 umol/L in moderate to severe GO, and 1.09 +/-0.17 umol/L in sight-threatening GO (p 0.003) (Figure 5).

Figure 4 Serum selenium level in Graves' orbitopathy and Graves' disease

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Se</th>
<th>SD</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.189umol/L</td>
<td>0.197</td>
<td>97</td>
</tr>
<tr>
<td>1</td>
<td>1.097umol/L</td>
<td>0.180</td>
<td>101</td>
</tr>
</tbody>
</table>

Figure 4 legend: Individual value dot plot showing lower mean serum selenium level in patients with moderate to severe Graves' orbitopathy (Group1) compared to patients without Graves' orbitopathy (Group 0) The normal Victorian adult selenium level reference range is 0.8-1.4umol/L
In univariate analyses, the significant associations for GO were serum selenium (p<0.001), age (p<0.001), smoking status (p<0.001), TSH (p=0.007), fT4 (p 0.005), fT3 (0.001), ethnicity (p 0.025), residential location (p<0.001), duration of Graves’ disease (p< 0.001), use of anti-thyroid medication (p=0.001), radioactive iodine treatment (p=0.001) and thyroidectomy (p<0.001). The association of lower serum selenium with GO did not differ when variables that were significant in univariate analysis were included in a multivariate model. Serum selenium levels remained significantly lower in GO after adjusting for age, smoking status, thyroidectomy, radioactive iodine and residential location (Table 7). The Graves’ orbitopathy cases were 91% more likely to have lower selenium level than Graves’ without orbitopathy. (Odds ratio{95% CI};0.09{0.01,0.67}, p=0.019)
Table 7 Final multiple logistic regression model including variables having significant associations with Graves’ orbitopathy versus controls

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Coefficient</th>
<th>SE Coef</th>
<th>z</th>
<th>P value</th>
<th>Odd Ratio</th>
<th>95% CI for OR</th>
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<tr>
<td>Selenium level</td>
<td>-2.45</td>
<td>1.05</td>
<td>-2.34</td>
<td>0.019</td>
<td>0.09</td>
<td>0.01,0.67</td>
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<tr>
<td>Age</td>
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<td>0.015</td>
<td>3.00</td>
<td>0.003</td>
<td>1.05</td>
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<tr>
<td>Current Smoker</td>
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<td>3.21</td>
<td>0.001</td>
<td>5.85</td>
<td>1.99,17.22</td>
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<tr>
<td>Ex-smoker</td>
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<td>0.44</td>
<td>3.27</td>
<td>0.001</td>
<td>4.24</td>
<td>1.78,10.09</td>
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<tr>
<td>Radioactive iodine</td>
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<td>2.61</td>
<td>0.009</td>
<td>3.20</td>
<td>1.34,7.65</td>
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<tr>
<td>Thyroidectomy</td>
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<td>0.54</td>
<td>4.64</td>
<td>&lt;0.001</td>
<td>12.41</td>
<td>4.29,35.92</td>
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<td>Non metropolitan</td>
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<td>1.08</td>
<td>2.72</td>
<td>0.006</td>
<td>18.98</td>
<td>2.28,158.00</td>
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Discussion

We found a small, but significantly lower serum selenium level in patients with moderate to severe Graves’ orbitopathy (GO) compared with Graves’ without orbitopathy (GD). There appeared to be a graded response with reducing selenium level in increasing severity of GO, the mean selenium difference between moderate severe GO subgroup and sight threatening GO subgroup was marginal. The lower serum selenium level in GO remained significant after adjusting for age, smoking status, radioactive iodine, thyroidectomy, and non-metropolitan location of residence. As expected, we found patients with GO were significantly older, a higher proportion was current or ex-smokers compared with controls. More GO patients required radioactive iodine and thyroidectomy treatment compared with controls. The differences in baseline features between our cases and controls were consistent with previous studies. Serum selenium was not associated with thyroid function in our study population.

In a previous study, selenium significantly improved quality of life, reduced progression of mild active thyroid-associated orbitopathy and improved soft tissue changes, eyelid aperture and appearance in mild thyroid-associated orbitopathy. This was demonstrated in a double-blind, randomised controlled trial of 150 patients with GO, treated with 100µg selenium twice a day for 6
months compared with either pentoxyfylline 600mg twice a day, or placebo. This was the first randomized control study showing selenium supplementation is beneficial in GO. Trials on selenium supplementation in Graves’ disease are scarce. A randomized controlled trial (GRASS trial) to determine the effect of selenium supplementation in Graves’ disease was recently registered, and is currently recruiting. The supplementation of selenium for treating GO warrants further randomized controlled studies to validate the benefits in populations with differing baseline selenium status, and to determine the optimum dosage and selenium formulation. Selenium supplementation of 200 ug/day (as sodium selenite) over 6 months in GO was not associated with adverse events in a European population where marginal selenium deficiency was reported in the population.

Our study is the first to show lower selenium level in patients with GO compared with GD in an Australian population with adequate selenium intake. Selenium intake is sourced from proteinaceous food such as fish, shellfish, meat, offal, cereals and grains, notably Australian wheat is rich in selenium. Levels that we found in Victorian blood donors are consistent with the marginally lower levels found in British people (1.1 µmol/L) than the higher levels found in North Americans (1.75 µmol/L). Using a different technology selenium levels similar to our ranges were described in a South Australian study (1-1.6 µmol/L). This is different from another Australian study using 140 healthy subjects who found levels 0.8-2.2 mmol/L. Mean selenium levels found in two different healthy Australian populations was 1.27umol/L and 1.30umol/L. There is no consensus for the use of serum selenium as the marker of selenium status, or a universal normal reference range values. At a serum selenium level of 100ug/L (1.27umol/L), which correlates with optimum glutathione peroxidase activity, 76% of our study population fall below this level, that is 83 GO cases (83%) and 72 GD controls (72%). Serum selenium at 0.75umol/L or below is considered deficient, 1% of GO and GD respectively of our study population were in this category. Two GD controls (2%) and 9 GO cases (9%) are below the 95% confidence interval lower limits of 0.83umol/L. Thus, marginal selenium status is prevalent in our study population. This may imply that even as the
selenium level is only marginally lower in GO compared with GD, the capacity for handling increased oxidative stress in the orbit was compromised due to the presence of suboptimal selenium status.

In a Danish population, serum selenium levels in newly diagnosed Graves’ disease were lower than in randomly selected normal controls (mean 1.14 µmol/L versus 1.25 µmol/L, p<0.01) and were also marginally lower in patients with autoimmune hypothyroidism than in normal controls, selenium level was not associated with the thyroid function status of the patients.\textsuperscript{254} The study suggested a link between selenium deficiency and autoimmune thyroid disease.\textsuperscript{254} In another study that compared serum selenium levels in 83 patients in remission or relapse of Graves’ disease, there were no significant differences in the selenium levels between the two groups (mean 0.92 µmol/L, SD0.28 versus 0.91 µmol/L, SD0.21), however, the authors noted the highest level of selenium (>1.52 µmol/L) in patients with Graves’ disease in remission, and postulated a positive effect of selenium in Graves’ disease outcomes.\textsuperscript{266} Improving selenium status may improve GO in a number of ways including reducing oxidative stress in GO, modulating immune response and T cell functions. GD and GO inflammatory processes are dominated by increased oxidative stress with increased production of free radical oxygen species and cytokines.\textsuperscript{267, 268} Correcting selenium deficiency or supplementing selenium in individuals with adequate selenium may reinforce the activity of glutathione peroxidases and thioredoxin reductases in cells, or enhance proliferation of activated T cells.

Evidence of increased oxidative stress in GO comes from both laboratory and clinical studies. Normal retro-ocular fibroblasts demonstrated no measurable oxygen free radicals whereas retro-ocular fibroblasts from GO showed spontaneous oxygen free radicals production and has significantly higher intracellular superoxide dismutase(SOD) activity in resting state, indicating the orbital fibroblasts is subjected to increased oxidative stress. Retro-orbital fibroblasts were less responsive to interleukin 1B stimulation in free radical generation and SOD induction.\textsuperscript{116} Superoxide radicals induce a dose dependent orbital fibroblast proliferation in severe GO, not observed in fibroblast cultures
of normal controls.\textsuperscript{268} In patients with GO, reactive oxygen species decreased and antioxidants increased significantly in the blood with normalization of thyroid function. The antioxidants superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase levels in GO remained significantly reduced even after normalization of thyroid function compared with normal subjects.\textsuperscript{269}

Evidence of immune enhancement from selenium comes mainly from animal and \textit{in vitro} studies. Selenium supplementation of 400 µg/day in healthy elderly significantly increased total T cell count by 27\% more than placebo from an increase in CD4$^+$ T cells and enhanced natural killer cell activity.\textsuperscript{270} UK adults with low selenium status receiving 50 µg and 100 µg per day of sodium selenite was also noted to clear challenge dose of live attenuated poliovirus more readily than those given placebo.\textsuperscript{271} Selenium supplements augmented cellular immunity by increased production of interferon gamma and other cytokines, an earlier peak of T cell proliferation and an increase in T helper cells.\textsuperscript{271} Selenium supplementation also correlates with lymphocyte proliferation and increased expression of interleukin-2 receptor in humans.\textsuperscript{253} Selenium promotes T helper type 1 (Th1) differentiation i.e. cellular immunity and diverts away from T helper type 2 (Th2) differentiation i.e. humoral immunity.\textsuperscript{272}

The role of selenium in changing the Th1/Th2 balance and its role in T cell activation in GO will require further studies. Perpetuation of GO orbital inflammation results from infiltration of activated T cells into the orbit. Th1 cells and cytokines predominate early in thyroid-associated orbitopathy whereas Th2 cells and cytokines predominate later in its course.\textsuperscript{39} One study on the peripheral blood T cell profile in GO and GD showed a shift towards Th1 dominance in GO compared with GD and healthy controls, with a higher ratio of CD8$^-$/IFN\gamma$^+$ to CD8$^-$/IL4$^+$ T cells (Th1/Th2) and a predominance of CD4$^+$ T cells.\textsuperscript{273}

Our study has some limitations, Selenium levels in serum were only marginally lower in GO versus GD, with both groups well within the 95\% reference intervals. The physiological significance of the small mean selenium difference between GO and GD remains uncertain, as plasma selenium does not represent tissue selenium levels and the absolute measurement might underestimate local selenium requirements. The association of non-metropolitan location with
Graves’ orbitopathy and lower selenium levels in our view should not be over-interpreted as there was only one (1%) GD control from non-metropolitan area and the odds ratio for Graves’ orbitopathy from non-metropolitan area has a wide 95% confidence interval in multivariate analyses. There are also a number of missing values in fT3 and TSH receptor antibody levels as they were not routinely measured in our study, hence analyses pertaining to thyroid hormone and TSH receptor antibody measurements were limited.

In conclusion, we found a small, but significant, difference in selenium levels, being lower in GO than GD in a study population with marginal selenium status, not accounted for by differences in age, smoking status, residential location, antithyroid drug treatment or thyroidectomy. We conclude relative selenium deficiency may be an independent risk factor for orbitopathy in patients with Graves’ disease.

Acknowledgements

We acknowledge Dr. John Wentworth for his advice regarding the study,
12 Differential gene expression profiling of orbital adipose tissue in thyroid-associated orbitopathy

Publication arising from this chapter:


12.1 Background and aims

The complex molecular processes involved in the pathogenesis of thyroid-associated orbitopathy (TO), and the cross reactivity between the thyroid in Graves’ disease (GD) and TO remains incompletely understood. The study of genetics of TO were often based on candidate genes selection from known genetic risk factors for GD or based on current knowledge of pathogenesis in TO, which limits the discovery potential for new genetic susceptibility loci for TO. In addition there is paucity of literature on gene expression profiling in TO which could guide the selection of genes for investigation of their associations with TO. This chapter will focus on gene expression profiling in active TO with the intention of discovering differentially expressed genes *a priori* using a highly sensitive microarray platform, the results will then be correlated with findings of discovery genome-wide association scan for TO in the concluding chapter.

The null hypothesis is there is no difference in genes expressions between active and inactive phase of TO, or between active TO and normal subjects. The alternative hypothesis tested is that genes are differently expressed in active TO compared to inactive TO or normal subjects.

The specific aims investigated in this chapter were:

a) To determine differentially expressed genes relevant to orbital inflammation and orbital fat expansion in TO

b) To explore expressed genes interaction by gene enrichment analysis and molecular pathways analysis
12.2 Peer reviewed publication author version

Title: Differential gene expression profiling of orbital adipose tissue in thyroid-associated orbitopathy

Authors:

Jwu Jin Khong¹,⁴ FRANZCO, MMed, MBBS(Hons)
Lynn Yuning Wang² BCom
Gordon K Smyth²,³ PhD, BSc(Hons)
Alan A McNab⁴ FRANZCO, DMedSc
Thomas G Hardy⁴ FRANZCO, MBBS
Dinesh Selva⁵ FRANZCO, MBBS(Hons)
Bastien Llamas⁶,⁷ PhD
Chol-Hee Jung⁸ PhD, MSc, BSc
Shiwani Sharma⁷ PhD, MSc
Kathryn P Burdon⁷,⁹ PhD, BSc(Hons)
Peter R Ebeling¹,¹⁰ AO FRACP, MD, MBBS
Jamie E Craig⁷ FRANZCO, DPhil, MBBS(Hons)

Addresses:

1. Department of Medicine, North West Academic Centre, University of Melbourne, St Albans, Victoria, Australia
2. Bioinformatics Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, Victoria, Australia
3. Department of Mathematics and Statistics, University of Melbourne, Parkville, Victoria, Australia
4. Orbital, Plastics and Lacrimal Unit, The Royal Victorian Eye and Ear Hospital, East Melbourne, Victoria, Australia
5. Department of Ophthalmic and Visual Science, University of Adelaide, South Australia, Australia

6. School of Earth and Environmental Sciences, University of Adelaide, South Australia, Australia

7. Department of Ophthalmology, Flinders University, Bedford Park, South Australia, Australia

8. VLSCI, Life Sciences Computation Centre, The University of Melbourne, Carlton, Victoria, Australia

9. Menzies Institute for Medical Research, University of Tasmania, Hobart, Tasmania, Australia

10. Department of Medicine, School of Clinical Sciences, Monash University, Clayton, Victoria, Australia

Corresponding author:

Dr Jwu Jin Khong
Department of Medicine
NorthWest Academic Centre
University of Melbourne
Sunshine Hospital, PO Box 294
176 Furlong Road, St Albans, VIC 3021, Australia.

Tel: 613 8395 8098
Fax: 613 8395 8258
Email: jwujinkhong@gmail.com

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Keywords: gene expression profiling, cDNA microarray, thyroid ophthalmopathy, Graves' orbitopathy

Word count: 4482

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Abstract

Purpose: We aimed to determine differentially expressed genes relevant to orbital inflammation and orbital fat expansion in thyroid-associated orbitopathy (TO) using microarray gene profiling in a case-control study.

Methods: Human orbital adipose samples were obtained from individuals with active TO (n=12), inactive TO (n=21) and normal controls (n=21). Gene expression profiles were examined using microarray analysis and were compared between active and inactive TO, and between active TO and normal controls. Top ranked differentially expressed genes were validated by real-time RT-PCR in an additional eight active TO, 13 inactive TO and 11 normal controls and correlated with gene set enrichment analysis (GSEA) and molecular pathways analysis.

Results: Seven hundred twenty-one probes (683 genes) and 806 probes (735 genes) were significantly differentially expressed in comparing active to inactive TO and in comparing active TO to healthy controls respectively. All selected genes were confirmed to be differentially expressed by real time RT-PCR. Multiple top ranked genes in active versus inactive TO comparison are over-represented by immune and inflammatory response genes. They include defensins (DEFA1, DEFA1B, DEFA3) which were overexpressed by 3.05- to 4.14-fold and TIMD4 by 4.20-fold. Markers for adipogenesis were overexpressed including SCD, FADS1 and SCDP1. Gene set enrichment analysis revealed dysregulation of epigenetic signatures, T cell activation, Th1 differentiation, defensin pathway, cell adhesion, cytoskeleton organization, apoptosis, cell cycling, lipid metabolism in active TO.

Conclusions: Active TO is characterized by up-regulation of genes involved in cell-mediated immune, innate immune and inflammatory response and enhanced orbital adipogenesis. TIMD4, DEFA1, DEFA1B and DEFA3 genes may be involved in the innate immune mediated orbital inflammation in TO. Epigenetic mechanisms may play a role in the pathogenesis of TO.
Introduction

Thyroid-associated orbitopathy (TO) is an autoimmune disorder of the orbit related to Graves’ disease. TO occurs in 25% to 50% of patients with Graves’ disease, and 3% to 5% present with potentially blinding complications such as optic nerve compression or exposure keratopathy.4 Extra-ocular muscle enlargement from glycosaminoglycan deposition and interstitial swelling, connective tissue inflammation and orbital fat expansion are part of the observed pathological process, which leads to periocular oedema, proptosis, epiphora, retro-orbital pain and diplopia.29, 32, 34, 36 The molecular mechanisms involved in orbital fat tissue expansion remains incompletely understood. Previous microarray studies suggested peroxisome proliferator activated receptor gamma, secreted frizzled-related proteins, adipocyte related immediate early genes and lysosome related genes may play a role in orbital adipogenesis.74, 75, 274, 275

Expression microarray profiling uses high throughput technology to generate extensive gene expression data from small amounts of tissue. It is a rapid and highly effective way to discover differentially expressed genes with high sensitivity, thus advancing the understanding of pathogenesis without biases from prior knowledge of the disease. We aimed to determine differentially expressed genes that may be involved in stimulating orbital inflammation and orbital adipose tissue expansion in TO using microarray gene expression profiling in a case-control study to improve understanding of the molecular processes involved in this disease.

Methods

Tissue Samples

Human orbital fat samples were obtained during orbital decompression (extraconal orbital fat) and upper lid surgeries (pre-aponeurotic orbital fat) in patients with TO. Patients with TO were sub-classified as active (n=12) or inactive (n=21). Normal controls (n=21) were patients without autoimmune thyroid disease with adipose tissue harvested from corresponding anatomical locations at the time of unrelated orbital and lid surgeries. TO status was
determined by an ophthalmologist according to Vision, Inflammation, Strabismus and Appearance (VISA) classification.\textsuperscript{15} Active TO is defined by the presence of signs of orbital inflammation; inactive TO was defined by the absence of these inflammatory signs. Activity of thyroid eye disease was defined by inflammatory index score; the maximum inflammatory index score is 8, where chemosis could score up to 2, conjunctival redness up to 1, lid erythema up to 1, lid oedema up to 2 and retrobulbar pain up to 2 points. Seventy-five percent of active TO and 76% of inactive TO are euthyroid, 25% of TO patients had subclinical hyper- or hypothyroidism while on medical treatment but there was no statistically significant difference in the means of TSH and T4 levels. The research was conducted in compliance with the Declaration of Helsinki and was approved by human research ethics committee of the Royal Victorian Eye and Ear Hospital, Melbourne, Australia.

Following excision, orbital adipose tissue was immediately immersed in RNALater® solution (Ambion, Austin, TX, USA). The specimens were stored at 4°C for at least 24 hours before long-term storage at -80°C.

**RNA extraction and hybridization to microarrays**

Total RNA was extracted using RNeasy microarray tissue Mini Kit 50 (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The quality and quantity of RNA was ascertained on a Bioanalyser 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA) using NanoChip according to manufacturer's protocol. The mean RNA Integrity Number (RIN) for the RNA samples was $6.92 \pm 0.7$ SD. RNA samples were processed and hybridized to microarrays in two batches one year apart at the Australian Genome Research Facility using Illumina TotalPrep™ RNA Amplification Kit (Life Technologies, Carlsbad, CA, USA) protocol. A total of 750ng of amplified RNA was prepared for hybridisation to HumanHT-12 v4 Expression Beadchip (Illumina, San Diego, CA, USA) by preparing a probe cocktail (biotin labeled cRNA at 0.05ug/ul) that includes GEX-HYB Hybridisation Buffer supplied with the beadchip. A total hybridisation volume of 15µl for each sample was loaded into a single array on the beadchip, then hybridised at 58°C for 16 hours and coupled with Cy3 for scanning in the
Illumina iScan Reader. Raw intensity values for both regular and control probes were exported to text files using Illumina GenomeStudio software.

**Microarray data analysis**

Bioinformatics analysis was conducted using the limma software package. The raw intensity values were background corrected and normalized using the neqc function, which performs “normexp” background correction and quantile normalization using parameters estimated from the control probes. One probe (targeting long intergenic non-protein coding RNA 1239) was removed because it was available only in the batch 2 files. The proportion of probes expressed in each sample was estimated using the propexpr function, which compares regular probes to negative controls. The average proportion of expressed probes was found to be 53.6%, so the top 53.6% probes with highest average log normalized expression values were retained for subsequent analysis. This left 25,796 probes. The Illumina manifest file (HumanHT-12_V4_0_R2_15002873_B) was used to associate an Entrez Gene identifier with each probe. Other gene annotation was obtained from the National Centre for Biotechnology Information (NCBI) Homo sapiens gene information file downloaded on 21 November 2013.

Linear models were used to test for expression differences between disease categories while adjusting for gender differences and for differences between the hybridization batches. The correlation between repeated measurements from the same patient was estimated as 0.4 and incorporated into the linear models. Empirical array quality weights were estimated to allow for differences in quality between the RNA samples. Differential expression between active TO, inactive TO and normal tissue was assessed using empirical Bayes moderated t-statistics, allowing for an intensity-dependent trend in the standard errors. The false discovery rate (FDR) was controlled at less than 0.05 using the Benjamini and Hochberg method. Genes associated with the inflammatory index score were identified the same way, except that the quantitative index score was used in the linear model instead of the disease categories.

Molecular signature enrichment analyses were conducted using limma’s mroast function, which uses residual rotation to conduct gene set tests. Roast was
run with 99,999 rotations, using the same linear model settings as for the
differential expression analysis including batch correction, patient correlations
and quality weights. Gene sets representing expression signatures were
downloaded from the c2-curated collection of Version 4.0 of the Molecular
Signatures Database.\textsuperscript{283}

The lists of differentially expressed genes were uploaded to DAVID 6.7
webtool,\textsuperscript{284, 285} to test for over-representation of pathways from the Biological
Biochemical Image Database (BBID), BioCarta and KEGG databases.

**Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)
validation**

A total of eight active, 13 inactive TO and 11 normal control orbital adipose
samples with RNA extracted as above, were used to confirm findings. This
included three active TO, five inactive TO and three normal controls also used in
the microarray study. All RNA samples underwent in-solution DNase digestion
using RNAse-Free DNase Set (Qiagen) followed by on-column RNA cleanup using
RNeasy Mini Kit (Qiagen). The mean RIN for these RNA samples was $6.72 \pm 0.35$
SD.

Quantitative RT-PCR was performed in a 384-well plate with 19 gene targets
chosen from the top ranked and some lower ranked genes of interest. Expression
levels of \textit{SREBF1}, \textit{TIMD4}, \textit{DEFA3}, \textit{DEFA1B}, \textit{DEFA1}, \textit{FADS1}, \textit{SCD}, \textit{ELOVL6}, \textit{DYNCII1},
\textit{PLD2}, \textit{CHDH}, \textit{LTB}, \textit{CD3D}, \textit{CAMP}, \textit{EOMES}, \textit{CD8A}, \textit{CCL5}, \textit{GZMA} and \textit{SLAMF6} were
compared in duplicate. cDNA synthesis was performed using RT\textsuperscript{2} First strand Kit,
and cDNA amplification performed using RT\textsuperscript{2} SYBR® Green qPCR Mastermix in
an Applied Biosystems viiA 7 (Life Technologies, Carlsbad, CA, USA) thermal
cycler according to RT\textsuperscript{2} Profiler PCR Array protocol (Qiagen) according to MIQE
guidelines.\textsuperscript{286} Fold change and \textit{P} values were analyzed using the web-based
resource GeneGlobe Data Analysis developed by SABioscience

(\url{http://www.sabiosciences.com/pcrarraydataanalysis.php}; provided in the
public domain by SABioscience, a Qiagen company).
Results

The study was designed to determine the differences in genes expression profiles in TO by comparing diseased orbital adipose tissue when TO is in the active and inactive phases, and by comparing active TO with normal healthy tissues in a case-control study design. Orbital adipose tissue samples were harvested during orbital decompression and pre-aponeurotic orbital fat during eyelid surgeries in TO cases. Orbital fat from corresponding anatomical positions was harvested from unaffected healthy controls. TO status was determined by an ophthalmologist based on grading of vision, signs of orbital inflammation, strabismus and ocular motility, severity of appearance according to VISA classification. Active TO is defined by presence of signs of orbital inflammation including periocular oedema, lid erythema, conjunctival chemosis and injection; inactive TO defined by the absence of these inflammatory signs.

Differentially expressed genes between active and inactive TO identified by microarray analysis

The Illumina HumanHT-12 v4 contains 47,323 probes. Overall, the average proportion of expressed probes was 53.6%, hence the top 53.6% probes equivalent to 25,796 probes were retained for analysis. Seven hundred twenty-one probes (355 up and 366 downregulated) representing 683 annotated genes were significantly differentially expressed in the active TO versus inactive TO samples (FDR < 0.05). The top differentially expressed genes were dominated by overexpressed cell-mediated and innate immune response genes and adipogenesis markers (Table 8). Twenty of the 40 top differentially expressed probes represented immune and inflammatory response genes. Of these, 19 were upregulated and one was downregulated. TIMD4 was overexpressed by 4.20-fold and DEFA3 by 4.14-fold. DEFA1B was represented by three different probes and was upregulated by 3.05- to 4.04-fold and DEFA 1 by 3.95-fold. Genes overexpressed by 1.5- to 2-fold were CST7, CD247, NKG7, PTPRCAP, EOMES, GZMA, CYTL1, SLAMF6, KLRG1, CCL5 and CAMP. MASP1, a C4/C2 activating component of Ra-reactive factor, was significantly downregulated. Markers of adipogenesis were overexpressed by 2.36- to 6.12-fold including FADS1, SCD, and SCDP1. Lipid synthesis regulatory genes SREBF1 and DBI were upregulated
by 1.40- and 1.52-fold, respectively. *FZD9* was downregulated by 1.50-fold, whereas *GPX3* was upregulated by 1.83-fold.
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<th>AveExpr†</th>
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<td>7560100</td>
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<td>1.03x10^{-6}</td>
<td>Inositol phosphate metabolic process</td>
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</tr>
</tbody>
</table>

*LogFC is log 2 fold change, hence 1=2 fold, 2=4 folds, 3=8 folds
†AveExpr is average log2 expression
‡FDR is false discovery rate, <0.05 is significant
Differentially expressed genes between active TO and normal controls identified by microarray analysis

Of the 25,796 expressed probes, 806 probes (429 up- and 377 downregulated) representing 735 genes were differentially expressed in active TO compared to normal controls (FDR < 0.05). Ten of the 40 top ranked probes were from genes involved in adipogenesis, specifically unsaturated fatty acid and cholesterol synthesis including SREBF1, SCD, SCDP1, FADS1, PTPLB, ACSS2; these genes were upregulated by 1.57- to 5.37-fold (Table 9). Differential expression of other genes involved in metabolism included upregulation of PDXK and downregulation of CHDH. Eight of the top 40 ranked differentially expressed probes were from genes involved in cytoskeleton organization, collagen binding, cellular adhesion and migration. Upregulated genes were PLOD2, HOOK2 and SYNC with a fold difference of 1.45 to 1.91. Downregulated genes were FNDC5, DYNC111, TMSB15B, COLGALT2 and RADIL with fold change ranging from 1.51 to 2.10. Five immune and inflammatory response genes, TIMD4, KLRG1, DEFA3, PTGER3, CST7, were up regulated by 1.51- to 3.70-fold. Two genes coding protein with zinc binding domain (PDZRN4) and ubiquitin-protein ligase activity (TRIM9) were downregulated, and the specific functions of these genes have not been determined.
<table>
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<tr>
<th>Illumina probe identifier</th>
<th>Gene symbol</th>
<th>logFC*</th>
<th>AveExpr†</th>
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<th>Gene functions</th>
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<td>9.30</td>
<td>2.23x10⁻⁶</td>
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<td>FNDC5</td>
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<td>Positive regulation of brown fat cell differentiation</td>
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<td>-0.74</td>
<td>6.04</td>
<td>3.46x10⁻⁶</td>
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<td>0.001 Cysteine endopeptidase inhibitor, immune response</td>
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</table>
*LogFC is log 2 fold change, hence 1=2 fold, 2=4 folds, 3=8 folds
†AveExpr is average log2 expression
‡FDR is false discovery rate, <0.05 is significant
Correlation of differential expressed genes with inflammatory index score

To explore the effects of more graduated changes in orbital inflammation, we directly correlated the expression of each gene with the individual inflammation index scores instead of categorizing patients into normal, active TO and inactive TO. This analysis detected 188 probes positively correlated with inflammation and 162 negatively correlated (FDR < 0.05, Table 10). Genes previously found to be upregulated in active TO were generally found to be positively correlated with inflammation, while those previously found to be downregulated in active TO were negatively correlated. Table 11 shows correlation results for selected top ranking genes from the disease comparisons including all 19 genes selected for PCR validation. These results confirm negative correlations for CHDH and DYNC1I1 and positive correlations for the other genes.

Table 10 Top 40 genes correlated with inflammatory index score

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*LogFC gives the regression coefficient of the inflammatory score for predicting the log2-expression of each gene. Positive and negative values correspond to positive and negative correlation respectively.
†AveExpr is average log2 expression
‡FDR is false discovery rate, <0.05 is significant

Table 11 Correlation with inflammatory index score for selected genes

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<th>AveExpr†</th>
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*LogFC gives the regression coefficient of the inflammatory score for predicting the log2-expression of each gene. Positive and negative values correspond to positive and negative correlation respectively.
†AveExpr is average log2 expression
‡FDR is false discovery rate, <0.05 is significant

Validation of differential expression of genes in orbital adipose tissue specimens

Seventeen and eight gene targets were chosen for validation from the active versus inactive and active versus normal comparisons, respectively. Quantitative RT-PCR was used to analyse the expression of these genes in eight active TO, 13 inactive TO and 11 normal control orbital adipose samples. The qRT-PCR results were closely correlated with the microarray results, with all log 2-fold changes of comparable size and in the same direction as the microarray results for the same genes. This was true both for the active versus inactive TO comparison (Table 12) and for the active TO versus normal comparison (Table 13). Most chosen genes were confirmed to be significantly differentially expressed in the validation samples; even those genes that failed to achieve statistical significance still showed expression changes of similar size and direction to the microarray results.

<table>
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<tr>
<th>Gene targets</th>
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<th>Log FC Microarray</th>
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Table 12 Validation of differential express of genes in active vs inactive TO by quantitative RT-PCR
Columns 2-4 give PCR results while column 5 shows corresponding microarray results.

*Fold change values greater than one indicate an up-regulation, and values less than one indicate down-regulation.
†Log FC PCR and ‡Log FC microarray are log2 fold change from qRT-PCR and microarray analysis, respectively.
Range in LogFC microarray relates to LogFC of more than 1 probes for the gene.
§Differentially expressed genes of lower ranks from microarray study (below top 40) still validated by qRT-PCR.

Table 13 Validation of differentially expressed genes in active T0 vs normal healthy control by quantitative RT-PCR

<table>
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<th>*P value</th>
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<th>Log FC Microarray‡</th>
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<td>0.25</td>
<td>0.53-0.87</td>
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</tbody>
</table>

Columns 2-4 give PCR results while column 5 shows corresponding microarray results.

*Fold change values greater than one indicate an up-regulation, and values less than one indicate down-regulation.
†Log FC PCR and ‡Log FC microarray are log2 fold change from qRT-PCR and microarray analysis, respectively.
Range in LogFC microarray relates to LogFC of more than 1 probes for the gene.
Gene set enrichment analysis of molecular signatures

To understand the active TO expression profile more deeply, we conducted a gene set enrichment analysis using curated signatures from the Molecular Signatures Database. Each signature was tested using rotation gene set tests (ROAST), which are able to adjust for gender differences, patient effects and batch effects.

A number of epigenetic signatures were found significantly dysregulated in active TO, both in comparing to inactive TO and normal control. Upregulated hypomethylation of gene clusters in immunodeficiency signaling, cytotoxic T cell mediated apoptosis and T cell receptor, and hypomethylation of gene cluster representing epigenetic signature of PML-RARA leukaemia (Figueroa AML methylation cluster 5 and cluster 6) were noted in active compared to inactive TO. Conversely, active TO compared to normal showed negative correlation with unmethylated histone H3 in genes with high-CpG-density promoters (HCP) in embryonic fibroblast, downregulation of histone H3 dimethylation at K4 and trimethylation at K27 in brain and downregulation of genes methylated aberrantly in colon cancer cells. (Table 14 and Table 15)

Active TO versus inactive TO was characterized by upregulation of cell adhesion and cytoskeleton organization pathways including stathmin, cell-to-cell, E-cadherin nascent adhesion junction and e-cadherin stabilization pathway. Molecular processes for activation of T cell (CSK, TOB1, T cell receptor activation pathways), cytotoxic T cell (downstream CD8 T cell, cytotoxic T cell pathway), T helper cell function, IL-12 dependent Th1 development, Th1 specific genes and natural killer cell response, immunoregulation in lymphoid cells were upregulated in active versus inactive TO. Interestingly, CD40 signaling was negatively correlated with active TO. Gene sets controlling cell proliferation and cell cycling were downregulated as exemplified by downregulation of ERBB network, nuclear ERBB4 signaling, but genes involved in MAPK signaling, neuronal proliferation and differentiation were upregulated. Apoptosis was regulated by upregulation of caspase cascade and upregulation of genes negatively correlated with telomere shortening and telomerase reverse transcriptase, respectively. Lymphogenesis associated genes were upregulated. Multiple oncogenic genes, tumour suppressor
genes, homeobox genes were dysregulated in active TO in the context of comparing with cancer gene expression profiles. (Table 14)

Analysis of the active TO versus normal control expression changes revealed correlation with a number of signatures regulating lipid metabolism. These include genes involved in lipid digestion, mobilization and transport, integration of energy and metabolism, SREBF and PPAR targets, the WNT non-canonical pathway and biosynthesis of unsaturated fatty acids. Cytoskeleton control stathmin pathway was upregulated. Apoptosis pathways were upregulated with increased proportion of genes in caspase cascade, upregulation of proapoptotic genes regulated by telomerase reverse transcriptase, camptothecin and methotrexate. Cell cycling was downregulated with downregulation of genes involved in activation of pre-replicative complex and Rad3-related kinase (ATR), G2 checkpoint in mitosis and mini-chromosome maintenance pathway, whereas cell differentiation was upregulated (osteoblast differentiation in response to phenylamil). Genes related to hypoxia was upregulated. Generally cancer-related gene profiles are downregulated in active TO compared to normal. (Table 15)

Table 14 Gene enrichment analysis of active vs inactive TO using MSigDB curated C2 signatures

<table>
<thead>
<tr>
<th>Signature</th>
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<th>Direction</th>
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<th>FDR †</th>
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*number of genes in gene pathway
†false discovery rate from ROAST, FDR<0.05 significant, top signatures FDR<0.01 displayed

Table 15 Gene set enrichment analysis of active TO vs normal controls using MSigDB curated C2 signatures
<table>
<thead>
<tr>
<th>Pathway</th>
<th>No of Genes</th>
<th>Direction</th>
<th>FDR</th>
<th>P Value</th>
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</tbody>
</table>

*number of genes in gene pathway
†false discovery rate from ROAST, FDR<0.05 significant, top signatures FDR<0.01 displayed

Pathway analysis using DAVID functional annotation analysis

Functional annotation was examined more systematically for the entire set of differentially expressed genes by using the DAVID tool.\textsuperscript{284, 285} Analysis of
differentially expressed genes for the active versus inactive TO comparison revealed overrepresentation of the following pathways: KEGG’s regulation of actin cytoskeleton (17 genes, \( P = 2.9 \times 10^{-3} \)), adherens junction (eight genes, \( P = 0.017 \)), glycolysis (seven genes, \( P = 0.017 \)), MAPK signaling (17 genes, \( P = 0.022 \)), glutathione metabolism (six genes, \( P = 0.028 \)), biosynthesis of unsaturated fatty acids (four genes, \( P = 0.039 \)), focal adhesion (13 genes, \( P = 0.045 \)); Biocarta’s T cytotoxic cell surface molecules (five genes, \( P = 1.7 \times 10^{-3} \)), Lck and Fyn tyrosine kinases in initiation of TCR activation (four genes, \( P = 0.013 \)), T helper cell surface molecules (four genes, \( P = 0.016 \)), CTL-mediated immune response against target cells (four genes, \( P = 0.031 \)) and IL-17 signaling pathway (four genes, \( P = 0.031 \)).

Functional annotation of differentially expressed genes in active TO versus normal controls highlighted over-represented molecular pathways involved in KEGG’s PPAR signaling (13 genes, \( P = 5.6 \times 10^{-6} \)), citrate cycle (seven genes, \( P = 7.6 \times 10^{-4} \)), biosynthesis of unsaturated fatty acids (six genes, \( P = 9.8 \times 10^{-4} \)), insulin signaling (13 genes, \( P = 3.6 \times 10^{-3} \)), focal adhesion (16 genes, \( P = 6.4 \times 10^{-3} \)), butanoate metabolism (six genes, \( P = 7.3 \times 10^{-3} \)), pentose phosphate (five genes, \( P = 0.012 \)), glycerolipid metabolism (six genes, \( P = 0.023 \)) and glutathione metabolism (six genes, \( P = 0.035 \)).

Pathways for regulation of actin cytoskeleton, adherens junction, MAPK signaling, Lck and Fyn tyrosine kinases in initiation of TCR activation; PPAR signaling and insulin signaling pathways are illustrated in Figure 6, Figure 7, Figure 8, Figure 9, Figure 10 and Figure 11 with arrows pointing up for upregulated and down for downregulated gene probes. The corresponding Table 16, Table 17, Table 18, Table 19, Table 20 and Table 21 correlates dysregulated gene probes with the coded proteins shown on the illustrated pathways, and showed extent of gene probe dysregulation.
Figure 6 Kegg pathway database-regulation of actin cytoskeleton is over-represented in active compared to inactive TO
Figure 6 legend: Kegg pathway database-regulation of actin cytoskeleton is over-represented in active compared to inactive TO. 17 genes (and 17 gene probes) were differentially expressed, 12 genes were up regulated and 5 were down regulated. The stabilization of actin cystoskeleton involve focal adhesion assembly, adherens junction formation and actin polymerization. Note: 4 arrows on GF represent 4 different growth factors, 3 arrows on ITG represent 3 different integrins. Arp2/3 appears twice. Reprinted with permission from Kyoto Encyclopedia of Genes and Genomes.
<table>
<thead>
<tr>
<th>Illumina probe identifier</th>
<th>Gene Symbol</th>
<th>Protein identifier</th>
<th>logFC*</th>
<th>AveExpr†</th>
<th>P. Value</th>
<th>FDR‡</th>
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<td>1050671</td>
<td>EGFR</td>
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*LogFC is log 2 fold change, hence 1=2 fold, 2=4 folds, 3=8 folds
†AveExpr is average log2 expression
‡FDR is false discovery rate, <0.05 is significant
Figure 7 Kegg pathway database-adherens junction was upregulated in active compared to inactive TO
Figure 7 legend: Kegg pathway database-adherens junction was upregulated in active compared to inactive TO. 8 genes (and 8 gene probes) consisted of 6 up regulated and 2 down regulated genes were differentially expressed. Upregulation of β catenin and α actinin at multiple points in the pathway concurs with regulation of cell to cell adhesion and cell migration. Signal transduction from cadherin via cadherin-catenin complex and α actinin interaction affects actin cytoskeletal function. TGFβ signaling is upregulated with increased TGFβR. Note: 12 arrows represent 8 genes as nectin, β-catenin, α-actinin appear multiple times. Reprinted with permission from Kyoto Encyclopedia of Genes and Genomes.

Table 17 Genes up and downregulated in adherens junction pathway (active vs inactive TO)

<table>
<thead>
<tr>
<th>Illumina probe identifier</th>
<th>Gene Symbol</th>
<th>Protein identifier</th>
<th>logFC*</th>
<th>AveExpr†</th>
<th>P.Value</th>
<th>FDR‡</th>
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<td>ACTN1</td>
<td>α-actinin</td>
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<td>9.833</td>
<td>7.66x10^{-4}</td>
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<td>4260379</td>
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<td>ErbB1/2</td>
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<td>7.957</td>
<td>7.59x10^{-5}</td>
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*LogFC is log 2 fold change, hence 1=2 fold, 2=4 folds, 3=8 folds
†AveExpr is average log2 expression
‡FDR is false discovery rate, <0.05 is significant
Figure 8 Kegg pathway database-mitogen-activated protein kinase (MAPK) signaling
Figure 8 legend: Kegg pathway database-mitogen-activated protein kinase (MAPK) signaling have 17 differentially expressed genes (and 17 gene probes) which include 8 up regulated and 9 down regulated genes in active compared to inactive TO. MAPK control a diverse range of cellular activity including proliferation, differentiation of cells and inflammation. The dysregulated genes spread across both classic, JNK and p38 MAPK pathways modulating cellular proliferation. Note: cPLA2 has two arrows denoting two genes PLA2G2A and PLA2G4A. Reprinted with permission from Kyoto Encyclopedia of Genes and Genomes.

Table 18 Genes up and downregulated in MAPK pathway (active vs inactive TO)

<table>
<thead>
<tr>
<th>Illumina probe identifier</th>
<th>Gene Symbol</th>
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<td>CACN</td>
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*LogFC is log 2 fold change, hence 1=2 fold, 2=4 folds, 3=8 folds
†AveExpr is average log2 expression
‡FDR is false discovery rate, <0.05 is significant
Figure 9 Biocarta database-Lyn and Fyn tyrosine kinases in initiation of T cell receptor activation
Figure 9 legend: Biocarta database- Lyn and Fyn tyrosine kinases in initiation of T cell receptor activation. In this pathway all 4 genes represented 6 gene probes were upregulated in active compared to inactive T0. T cell receptor is a complex made up of CD247 and CD3A. CD45 protein tyrosine phosphatase activates Fyn which in turn phosphorylates T cell receptor complex and recruit ZAP70 to stimulate downstream pathway of T cell activation. Note: multiple arrows for CD247, CD3d TCR complex subunits and 2 arrows each for ZAP70 and FYN as they appear in the pathway. Reprinted with permission from BioCarta.

<table>
<thead>
<tr>
<th>Illumina probe identifier</th>
<th>Gene Symbol</th>
<th>Protein identifier</th>
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*LogFC is log 2 fold change, hence 1=2 fold, 2=4 folds, 3=8 folds
†AveExpr is average log2 expression
‡FDR is false discovery rate, <0.05 is significant
Figure 10 Kegg pathway database-peroxisome proliferator-activated receptor (PPAR) pathway is upregulated in active TO compared to normal control
Figure 10 legend: Kegg pathway database-peroxisome proliferator-activated receptor (PPAR) pathway is upregulated in active TO compared to normal control. 13 genes representing 15 gene probes, 11 genes were upregulated and 2 were downregulated which display over-expression of genes involved in lipogenesis, cholesterol synthesis, fatty acid transportation and oxidation. Activation of PPAR pathway leads to adipocyte differentiation and proliferation. Note: 14 arrows for 13 genes as FATCD36 appeared twice in the signaling pathway. Reprinted with permission from Kyoto Encyclopedia of Genes and Genomes.

Table 20 Genes up and down regulated in PPAR signaling pathway (active TO vs normal control)

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<th>Gene Symbol</th>
<th>Protein identifier</th>
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<th>AveExpr†</th>
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*LogFC is log 2 fold change, hence 1=2 fold, 2=4 folds, 3=8 folds  
†AveExpr is average log2 expression  
‡FDR is false discovery rate, <0.05 is significant
Figure 11 Kegg pathway - insulin pathway is upregulated in active TO compared to normal control.
Figure 11 legend: Kegg pathway database-insulin signaling pathway is upregulated in active TO compared to normal control. 13 genes representing 18 gene probes, 16 were upregulated, and 2 downregulated. Insulin signaling pathway governs adipocytes proliferation and differentiation. SOCS, PDK1/2, GSK-3B and PDE3 were upregulated, hence the phosphatidyl inositol (PI3K) pathway is active, effectively increases fatty acid biosynthesis, glycolysis, and glycogenesis. Conversely related MAPK pathway ERK1/2 was downregulated. Reprinted with permission from Kyoto Encyclopedia of Genes and Genomes.
Table 21 Genes up and down regulated in insulin signaling pathway (active TO vs normal control)

<table>
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<th>Gene Symbol</th>
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*LogFC is log 2 fold change, hence 1=2 fold, 2=4 folds, 3=8 folds  
†AveExpr is average log2 expression  
‡FDR is false discovery rate, <0.05 is significant
Discussion

This differential gene expression profiling study utilizes the largest number to date of orbital adipose tissues in TO patients and normal subjects. This is the first gene profiling study to compare TO in early and late phases and uses high density Illumina microarray platform to derive meaningful findings. The high throughput technology combined with selection of phenotypically well-characterized TO patients generated extensive gene expression data, which allowed examination of differentially expressed genes with high sensitivity. The results were validated by quantitative RT-PCR. Functional annotation of genes showed good correlation between different bioinformatics analysis.

The principal finding of this study is that cell-mediated and innate immune response genes and adipogenesis markers are concurrently overexpressed in active TO, and epigenetic pathways may be involved in the pathogenesis of TO. Among these, *TIMD4, DEFA3, DEFA 1* and *DEFA1B* were highly overexpressed. T cell immunoglobulin and mucin domain containing 4 (*TIMD4*, also known as TIM4) is a cell membrane receptor exclusively on antigen presenting cells including dendritic cells and macrophages that recognizes phosphatidyl serine, a specific marker of apoptosis, and via its immunoglobulin domain has a role in phagocytosis of apoptotic cells and in immune tolerance. Blockade of TIMD4 results in increased numbers of antigen specific T cells following the peak of immune response. Conversely, overexpression of TIMD4 on antigen presenting cells resulted in decreased antigen specific T cells and decreased secondary T cell response in TIMD4 transgenic mice. Overexpression of TIMD4 in peripheral blood mononuclear cells was noted in patients with systemic lupus erythematosus (SLE), especially during the active phase of the disease compared with healthy controls. Furthermore TIMD4 mRNA expression positively correlated with TIM1 and TNF-α in SLE but not in healthy controls.

TIM1 expression in polarized human Th2 cells is known to be upregulated in allergic rhinitis patients compared with healthy controls. TIM4 on dendritic cells played a critical role in maintaining skewed Th2 response by interacting with TIM1 on Th2 cells. We therefore hypothesize that TIMD4 is an important immune regulator in TO. Overexpression of TIMD4 in TO may induce innate
immune cells such as macrophages and dendritic cells to phagocytose apoptotic T cells to maintain immune balance in the context of increased apoptotic load during the active phase of the disease. Increased TIMD4 expression may also explain the Th2 dominance observed in the late phase of TO. The hypothesis that TIMD4 overexpression is a precursor for switching off active orbital inflammation in TO is now worth exploring.

We report here significantly higher levels of α-defensins (DEFA1 and DEFA3) in active TO. TO may share these biomarkers with other autoimmune disorders. There is considerable evidence that α-defensins are part of the innate immunity derived from neutrophils and monocytes. α-defensins are involved in the inflammatory response as a chemotactic factor for neutrophils, monocytes and T cells and promote adaptive immune responses. Conversely, α-defensins also reduces inflammatory injury through inhibiting lipopolysaccharide mediated responses, and inhibiting pro-inflammatory cytokine secretion by macrophages. The role of DEFA1-3 in the pathogenesis of autoimmune diseases such as rheumatoid arthritis (RA), SLE, Behcet’s disease and lung diseases has been increasingly recognized.

The α-defensin-1 promotes inflammation through induction of pro-inflammatory cytokines IL-6, IL-8 and matrix metalloproteinases, possibly through regulation of JNK and/or ERK MAP kinases and NF-κβ pathways. Furthermore, α-defensins induce synthesis of mucin and chemokines including IL-8, TNF-β and VEGF in lung epithelial cells and lung fibroblasts cultures. Even though α-defensins are mainly expressed by neutrophils, other studies suggest that T lymphocytes, NK cells and monocytes also produce DEFA1-3. DEFA1-3 are known to be significantly higher in T cells from peripheral blood and blister fluid in Stevens-Johnson syndrome/toxic epidermal necrolysis patients, where cytotoxic T cells are the main effector cells.

Our study confirms findings of previous microarray and cell culture studies that increased adipogenesis occur in active TO. A group of genes involved in fatty acid and cholesterol synthesis were upregulated in our study including SREBF1, SCD, FADS1 and SCDP1. Gene sets enriched in lipid metabolism including biosynthesis of unsaturated fatty acids, fatty acyl-CoA synthesis, triacylglycerol
and ketone body metabolism, glycolysis pathway and integration of energy metabolism, were upregulated. In addition, pathway analysis showed over-representation of pathways linked to adipogenesis including PPAR activation, insulin signaling pathway, unsaturated fatty acid synthesis and glycerolipid metabolism.

The overexpression of SCD in TO orbital fat tissue compared with normal controls was independently validated by Lantz et al., who further linked overexpression of immediate early genes in smokers with severe active TO. Upregulation of several adipocyte regulatory genes including PPARγ, adiponectin and leptin support increased de novo adipogenesis and/or fat cell expansion in TO. In addition adipogenesis may be regulated through Wnt-signaling with dysregulation of secreted frizzled-related protein-1, Wnt5a, sFRPs and DKK. Our study identified frizzled family receptor 9 (FZD9), a receptor for Wnt signaling protein, which was modestly down regulated in active compared to inactive TO. FZD9 was activated by Wnt-2 and functions in Wnt/β-Catenin signaling in mice. However, a pathogenic role for FZD9 in TO remains unclear as yet.

Multiple cell-mediated immune genes were overexpressed in TO confirming the importance of cellular immunity in the pathogenesis of TO. CD247, CD3D, CD8A, CST7, GZMA, CAMP, SLAMF6, EOMES, LTB and CCL5 were upregulated in active TO compared with inactive TO. CD3d and CD247 are components of the T cell receptor complex while Cd8a is a surface molecule on cytotoxic T cells. CST7 is a member of cysteine protease inhibitors expressed by T cells, natural killer cells, monocytes and mast cells. CST7 could be important in regulating serine protease activity in IL-12 activated natural killer (NK) cells by inhibiting cathepsin C. Granzyme A (GZMA) is a serine protease found in cytotoxic T cells and NK cells. There is mounting evidence that granzyme A induces caspase-independent apoptosis in targeted cells, as well as inducing IL-8, IL-6 and IL-1β production and facilitates lymphocyte migration through the extracellular matrix. SLAMF6 encodes a transmembrane protein of CD2 subfamily on NK cells, T and B lymphocytes, and functions as a co-stimulatory molecule, which primes T cells to produce Th1 cytokines. CAMP relates in function to defensin
in antimicrobial activity, cell chemotaxis, immune mediation and inflammation response regulation.\textsuperscript{293} PTPRCAP is a positive regulator of tyrosine phosphatase PTPRC (CD45), a key regulator of T and B lymphocytes activation.\textsuperscript{303} EOMES is a transcription factor crucial for mesoderm and neural development and a key regulator for differentiation of CD8+ T cells.\textsuperscript{304} LTB, also known as tumor necrosis factor (TNF) superfamily, member 3 is pro-inflammatory and is involved in normal development of lymphoid tissue.\textsuperscript{305} The upregulation of LTB supports the notion of a role of TNF-antagonist in the treatment of active TO.\textsuperscript{137} GPX3 functions in detoxification of reactive oxygen species and hydrogen peroxide that involves selenium binding at its active site (http://www.ncbi.nlm.nih.gov/gene/2878; provided in the public domain by the National Centre for Biotechnology Information, Bethesda, MD, USA). The upregulation of GPX3 in active TO may explain the observations that selenium supplementation helps with soft tissue signs in active TO, and the relative selenium deficiency in TO cases.\textsuperscript{142} 306 Of note, targets for novel and experimental therapies for TO including CD-20, IL-1, IL-6, IL-8 and IGF-1R were not differentially expressed in the orbital adipose tissue that we studied and TSHR was low ranking and marginally downregulated in active TO compared to inactive TO (data not shown).

CCL5, also known as RANTES, is an important chemo-attractant for blood monocytes, T helper cells and eosinophils. Immunoglobulins from Graves’ disease patients induced increased expression of RANTES in TO orbital fibroblast.\textsuperscript{64} The GSEA and pathway analysis are in keeping with translational studies highlighting the Th1 cytokine expression profile, T cell recruitment and activation by orbital fibroblasts, dysregulation of MAPK signaling, infiltration of lymphocytes, macrophages and monocytes in TO affected orbital tissues on histology.\textsuperscript{35, 36, 48, 53, 60, 64}

Our study revealed epigenetic factors may play a role in pathogenesis of TO. Active TO displayed gene set enriched with epigenetic signatures of acute myeloid leukaemia (AML). Hypermethylation of DNA leads to silencing of genes, conversely aberrant hypomethylation of cluster 5 epigenetic markers in AML relates to dysregulation of immunity-related pathways involving immune
deficiency signaling, cytotoxic T cell mediated apoptosis and T cell receptor signaling.\textsuperscript{307} Active TO also showed downregulation of genes with unmethylated histone 3 in high CpG density promoters (HCP). Methylation at K4 (H3K4me3) and K27 (H3K27me33) on histone 3, known as bivalent status on HCP is associated with cell lineage commitment, genes related to adipogenesis (PPAR\textgreek{y}) often remains bivalent in differentiating embryonic fibroblast.\textsuperscript{308}

Gene sets enriched with T cell-mediated immune response include T cell receptor signaling via COOH-terminal Srk kinase (CSK), Lck and Fyn tyrosine kinases, TOB1 nuclear transducer together modulate T cell activation, and proliferation. Gene sets involved in IL-12 and N02 dependent IL-12 signaling pathways were upregulated which induce Th1 cell differentiation and activation of both T and NK cells via JAK/STAT transcription factor signaling. Activation of T helper cell, cytotoxic T cell, caspase cascade and telomere shortening, mediated immune related apoptosis in target cells. Further gene sets enriched in cell-to-cell adhesion signaling, stathmin and E-cadherin signaling mediating intercellular communication may result in changes in cytoskeleton and facilitate cell mobility, migration and proliferation.

One potential limitation of the interpretation of the microarray data is that prior immunosuppressive treatment was not factored into the analysis. Despite more active TO cases had intravenous methylprednisolone within 2 months prior to surgery compared to inactive TO and normal controls, active TO cases still had signs of orbital inflammation at the time of surgery. The microarray results still detected significantly upregulation of genes involved in immune and inflammatory responses and showed positive correlation of inflammatory index score in vast majority of the top ranked genes expressions tested. In addition intravenous dexamethasone was routinely used at induction of general anaesthesia for orbital surgery in both active TO and inactive TO and normal controls and the prevalence of intravenous dexamethasone use between the groups of patients was statistically insignificant, hence the differential RNA expression observed is unlikely due to confounding steroid effects.

In conclusion, TIMD4, DEFA1, DEFA1B, and DEFA 3 were overexpressed in active TO compared with inactive TO suggesting a pathogenic role of the innate
immune response in TO. Active TO was marked by up-regulation of genes involved in cell-mediated, innate and inflammatory responses with concurrent enhancement of orbital adipogenesis. Epigenetic mechanisms may play a role in TO. The study gives new insights into the underlying complex molecular mechanisms in TO, and provides novel insights into candidate molecules and pathways which can be explored to develop alternative treatment strategies for the treatment of TO which carries substantial visual morbidity.

Acknowledgement

Supported by Ophthalmic Research Institute of Australia (ORIA), new investigator grant
13 Genome-wide Association study using DNA pooling strategy as a discovery tool for genetic variants- proof of principle in Graves’ disease

Publication arising from this chapter:


13.1 Background and aims
Graves’ disease (GD) is an autoimmune thyroid disease which typically presents as thyrotoxicosis and goiter; other clinical findings include Graves’ orbitopathy, dermopathy and acropachy. The incidence rate of GD is 15 per 100,000 persons per year with an overall mean prevalence of 1152 per 100,000 population in the United States. Women are disproportionately more affected with the female to male ratio reported at 7.3:1. The disease also occurs in children at one tenth the prevalence rate of all ages. The disease is a complex disease with a strong familial predisposition; the triggers are poorly understood but various environmental factors were linked to onset of GD such as stress, smoking and _Yersinia_ infection in genetically susceptible individuals. GD shows a strong familial predisposition. The concordance rate for GD in monozygotic twins is significantly higher than dizygotic twins and model-fitting analysis predicted that genetic factors account for 79% of disease heritability in twins. With a concordance rate of 22-35% for monozygotic twins, environmental and modifier factors are likely to play a key role. Clustering of GD with other autoimmune conditions including Addison’s disease, type I diabetes mellitus, myasthenia gravis, polymyositis and lupus erythematosus suggest genes regulating the immune system shared by these diseases might also contribute towards the genetic susceptibility for GD. The genetic susceptibility of GD can be broadly categorized into genes involved in immune regulation, and thyroid specific genes. Genes confirmed to be
associated with GD include the following: multiple loci within the HLA I and II region, *CTLA4, PTPN22, CD40, IL2A, FCRL3 and TSHR*. The contributions from each of these genetic variants are modest. From candidate gene studies, HLA class II alleles *DRB1*03(DR3), *DQA1*0501, *DQB1*02 were significantly associated with GD with odd ratios of 2 to 3 for *DR3*.*150, 161, 167, 168* *CTLA4* A49G, and CT60 3′UTR polymorphisms were more frequent in GD with an odds ratio of 1.4 to 2,*158, 159, 170-172* and association with *PTPN22* polymorphism has an odds ratio of 1.5 to 1.9.*160, 162, 163*

Genome wide association studies (GWAS) have replicated earlier candidate gene study findings and identified additional new genetic loci. The earliest GWAS studies on autoimmune thyroid disease showed association of GD with multiple loci within the HLA class I and II regions, *TSHR and FCRL3*. More recently two new risk loci for GD, the *RNASET2-FGFR1OP-CCR6* region at 6q27 and an intergenic region at 4p14 were reported, along with confirmation of positive association of GD with SNPs in HLA-*DPB1, TSHR, CLTA4 and FCRL3*. Using a custom array, association of SNPs near *PTPN22, CTLA4, TSHR, RNASET2-FGFR1OP-CCR6* loci with GD were replicated, and seven novel susceptibility loci, *MMEL1, LPP, BACH2, PRICKLE1, ITGAM*, rs1534422 at 2p25.1 and rs4409785 at 11q21 were identified but the results require confirmation in replication studies.*185*

A pooled GWAS strategy can effectively identify gene variants in multiple diseases. Using relatively small numbers of cases and controls, pooled GWAS has previously been used to identify the known susceptibility loci *CFH* and *ARMS2/HTRA1* in age related macular degeneration and *LOXL1* in pseudoexfoliation syndrome.*313* A pooled DNA study has also identified a new susceptibility locus, *HGF*, in keratoconus (a progressive corneal degeneration) and additional novel candidate genes related to platelet reactivity in diabetic patients.*313-315* The study design must be carefully considered to include multiple pools to minimize pooling errors and an appropriate array platform to extract maximal available information from the pooled DNA.*316, 317*

This aim of this study was to determine if GWAS using DNA pooling methodology could replicate genomic loci associated with GD and discover new genetic loci.
13.2 Methodology
The study was approved by human research ethics committee at multiple recruitment sites; the Royal Victorian Eye and Ear Hospital, Sunshine and Western Hospital, the Royal Melbourne Hospital and the Alfred Hospital in Melbourne, Victoria, Australia; Flinders Medical Centre and the Royal Adelaide Hospital in Adelaide, South Australia. The genetic research was conducted in accordance with the Declaration of Helsinki.

13.2.1 Definition of Graves’ disease
GD cases were defined by the presence of biochemically confirmed hyperthyroidism based on thyroid function tests including T3, T4 and TSH, and presence of thyroid-associated orbitopathy, elevated thyrotropin receptor (TSHR) antibodies or diffuse uptake on technetium-99m pertechnetate thyroid scan.

13.2.2 Discovery cohort
The Australian Thyroid-associated Orbitopathy Research (ATOR) discovery cohort consisting of 412 GD cases with and without thyroid-associated orbitopathy of European descent was recruited from endocrine and eye outpatient clinics in the hospitals and private practices from Victoria and South Australia.

A total of 498 controls were recruited from two Australian populations. These comprised 198 adult patients with keratoconus, an non-autoimmune eye disease unrelated to GD, who were recruited from eye clinics in Melbourne, and 300 healthy controls aged over 50 years recruited from retirement villages and the general community in South Australia. Neither control cohort was actively screened for GD, but no participant reported having this condition.

13.2.3 Replication cohort
An independent group of 539 GD cases and 1230 controls were recruited to form a replication cohort. The GD cases were new cases recruited into the ATOR cohort (n=373) from Melbourne and endocrine and ophthalmology clinics from Western Australia (n=166). The controls were research participants of European descent with eye diseases without recognizable association with GD or other autoimmune diseases (angle closure glaucoma or suspect, pigment dispersion
syndrome, steroid responsive ocular hypertension, myopia and keratoconus) recruited in a collaborative effort from South Australia and Victoria to study the genetics of these eye diseases. (age ranging from 9 to 92 years)

13.2.4 DNA pooling for GWAS study
Venous blood aliquots from all participants were thawed for 3 hours before construction of 3 pool comparisons (pool number 1: 154 GD cases versus 198 controls, pool number 2: 218 GD cases versus 213 controls, pool number 3: 40 GD cases versus 87 controls), totaling 412 cases and 498 controls from the discovery cohort. For pool 1, protease and lysis buffer were added to 100µl of thawed whole blood from each sample, vortexed and incubated, then samples were combined in one tube for each whole blood pool, 1 tube each for cases and controls. DNA was extracted from the pooled blood using QIA-amp DNA blood maxi kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. For the remaining discovery samples, equimolar DNA pools were generated in duplicate after step-wise dilutions of genomic DNA extracted using QIA-amp DNA blood maxi kit, to 75ng/µl. The DNA was quantitated using Fluoroskan Ascent microplate fluorometer (ThermoFisher Scientific, Waltham, MA, USA) together with double-stranded DNA quantitation assay using Picogreen reagents (Invitrogen, Carlsbad, CA) and comparison to a standard curve generated from serial dilutions of Lambda DNA (Invitrogen, Carlsbad, CA). A total of 451ng of DNA from each sample was added to the equimolar DNA pool.

13.2.5 Pooling error
Previous studies showed that the majority of pooling errors arise from errors on the arrays rather than pool construction errors.\(^{318}\) hence in this pooling design, multiple arrays were used for each pool to reduce array errors. The pooling standard deviation is very small for the Illumina bead arrays, pooling standard deviation for HumanHap300 was estimated between 0.007 to 0.011,\(^{317}\) and variance for the 1M array was 6.8x10^{-5}.\(^{313}\) this encompasses both assays and pool construction errors. The Illumina HumanOmni5-Quad beadchip array supersedes its predecessor arrays; the HumanHap300 and 1M array, by virtue of its comprehensive coverage of the human genome but are similar in the way of the bead array design.
13.2.6 Pooled genotyping and analysis

Genome-wide genotyping was conducted by hybridization to HumanOmni5-Quad beadchip microarray (Illumina, San Diego, CA, USA) containing approximately 4.3 million markers at QIMR Berghofer Medical Research Institute according to standard protocol for both case and control pools on two independent arrays for each pool. Three case pools and three control pools were generated and assigned into three case-control pool comparisons. Each pool was replicated 2-4 times to reduce measurement error. For the purpose of this analysis, we retained SNPs with minor allele frequency (MAF) over 1% from the reference panel of EUR samples in the 1000 Genome Project (excluded 1.8 million SNPs). Thus, the number of SNPs was reduced to 2.5 million. The output of the raw red and green bead scores from Illumina Beadstation were used for the pooled data analysis. The data cleaning and quality control procedures in array-based pooling have been described previously.\textsuperscript{317, 319, 320} Briefly, SNPs with more than 10% negative scores on each array were excluded, as well as the SNPs with the sum of mean red and green scores lower than 1200 across each array to ensure calibration was performed on a pre-cleaned dataset. A normalization/correction factor (corr) was calculated to make the mean value of the pooling allele frequency (PAF) 0.5 across all SNPs on each strands of the array. The PAF was then estimated based on the raw red intensities and the corrected green intensities for all the SNPs \[\text{PAF} = \frac{\text{red}}{\text{red} + \frac{\text{green}}{\text{corr}}}].\textsuperscript{319}

Further quality control included filtering non-autosomal SNPs and any SNPs with a significant variance difference between cases and controls.

After data cleaning, approximately 2.2M SNPs were left for association testing in the GD discovery cohort between cases and controls. A linear model was used to test for allelic association in the three case-control pool comparisons. A test statistic which corrects for pooling error was used to rank SNPs, with p values based on a chi-squared distribution with 1 degree of freedom. Pooling error is minimized by estimating the difference in pooling allele frequency between cases and controls, such that any unequal amplification of alleles effects cancel out.\textsuperscript{317} Specifically, a final set of autosomal SNPs meeting the criteria (1) have sufficient number of probes (i.e., over ½ of expected number of probes for MAF1-5%, or over 1/3 of expected number of probes for SNPs >5% ; (2) have no
large differences (>0.3) between PAF and reference allele frequency from EUR samples in 1000 Genome Project. We then performed a meta-analysis weighted by inverse variance, combining results from three case-control pool comparisons. The top-ranked SNPs in the meta-analysis were then counter checked for proxies (linkage disequilibrium r2>0.5 with the index SNPs in the EUR populations). To be considered a valid result, if proxy SNPs were available, they should also show evidence of association. A genome-wide significance threshold of 5x10^{-8} was set to account for multiple testing.

13.2.7 Individual genotyping and analysis
Top-ranked non-MHC SNPs with p-value ≤1x10^{-6} were selected for validation by genotyping in individual DNA samples as well as for genotyping in the replication cohorts. The top ranking SNPS were individually genotyped in the discovery cohort in 412 GD cases and 480 normal controls. A subset of 18 controls had insufficient residual samples for individual genotyping, hence the discrepancy in the final pooling and validation sample sizes.

In total 13 SNPS were genotyped on Sequenom iPLEX Gold assay using Sequenom MassARRAY® Analyzer in 2 batches, the ATOR discovery cohort and Western Australia replication cohort were genotyped at the Australian Genome Research Facility (Brisbane, Australia) and the ATOR replication cohort at GeneWorks Pty Ltd (Adelaide, Australia). The allelic association analysis was performed in PLINK,321 using a chi-square test (--assoc) and performed separately for ATOR pooled discovery cohort for validation, the replication cohorts and for combined discovery and replication cohorts. The odds ratio and 95% confidence internal for allelic association was further adjusted for covariates age and sex using logistic regression analysis (--logistic --covar). SNPs with p ≤ 1x10^{-4} were considered validated in validation genotyping. In the replication cohort p <0.05 was considered significant.

13.3 Results
The GD cases were predominantly female and younger when compared with controls (Table 22), hence subsequent individual genotyping analysis was adjusted for age and sex.
In the discovery GWAS, 19 of the top 30 ranking SNPs clustered within the MHC region on chromosome 6p21 including rs9272937 in HLA-DQA1 (p $10^{-7}$), two SNPs within C6orf10 (p $10^{-7}$), and rs1613056 reaching genome wide significance (p=$5\times10^{-8}$) (Table 23). One SNP, rs9676286 on chromosome 19, reached genome-wide significance in the pooled GWAS (p=$1.08\times10^{-8}$) for association with GD. However rs9676286 was not as strongly supported when we conducted technical validation genotyping of the individual DNA samples (p=$5.54\times10^{-3}$).
Table 22 Demographics of Graves' disease cases and controls in the discovery and replication cohorts

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean Age (SD)</th>
<th>Sex</th>
<th></th>
<th></th>
<th></th>
<th>p value</th>
</tr>
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<tr>
<td></td>
<td>Case</td>
<td>Control</td>
<td>Case</td>
<td>Control</td>
<td>p value</td>
<td>Graves' Case</td>
<td>Controls</td>
</tr>
<tr>
<td>Discovery cohort</td>
<td>412</td>
<td>498</td>
<td>45.3 (14.5)</td>
<td>56.3 (24.4)</td>
<td>&lt;0.001</td>
<td>Male 71 (17.23%)</td>
<td>Male 192 (41.03%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Female 341 (82.77%)</td>
<td>Female 276 (58.97%)</td>
</tr>
<tr>
<td>Replication cohort</td>
<td>539</td>
<td>1230</td>
<td>44.5 (14.8)</td>
<td>48.1 (17.4)</td>
<td>&lt;0.001</td>
<td>Male 109 (20.30%)</td>
<td>Male 596 (49.30%)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Female 428 (79.70%)</td>
<td>Female 613 (50.70%)</td>
</tr>
</tbody>
</table>
Table 23 Top 30 single nucleotide polymorphism associated with Graves’ disease compared with controls in genome wide association study

<table>
<thead>
<tr>
<th>SNP name</th>
<th>Chr</th>
<th>BP position</th>
<th>Effect allele</th>
<th>Effect allele frequency</th>
<th>OR</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs9676286*</td>
<td>19</td>
<td>58126481</td>
<td>A</td>
<td>0.1441</td>
<td>2.08</td>
<td>1.08x10^-8</td>
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<tr>
<td>rs1613056†</td>
<td>6</td>
<td>32668946</td>
<td>T</td>
<td>0.2313</td>
<td>1.87</td>
<td>5.04x10^-8</td>
</tr>
<tr>
<td>rs9644119*</td>
<td>8</td>
<td>26517817</td>
<td>T</td>
<td>0.297</td>
<td>1.78</td>
<td>9.10x10^-8</td>
</tr>
<tr>
<td>rs11722643*</td>
<td>4</td>
<td>10127484</td>
<td>T</td>
<td>0.1938</td>
<td>1.88</td>
<td>1.03x10^-7</td>
</tr>
<tr>
<td>Rs2395149†</td>
<td>6</td>
<td>32325562</td>
<td>A</td>
<td>0.2517</td>
<td>1.76</td>
<td>1.14x10^-7</td>
</tr>
<tr>
<td>rs2098230</td>
<td>4</td>
<td>10242924</td>
<td>A</td>
<td>0.9001</td>
<td>0.47</td>
<td>1.21x10^-7</td>
</tr>
<tr>
<td>Rs2647044†</td>
<td>6</td>
<td>32667910</td>
<td>A</td>
<td>0.2737</td>
<td>1.74</td>
<td>1.41x10^-7</td>
</tr>
<tr>
<td>rs674313†</td>
<td>6</td>
<td>32578082</td>
<td>T</td>
<td>0.3748</td>
<td>1.67</td>
<td>1.49x10^-7</td>
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<tr>
<td>Rs3132090†</td>
<td>6</td>
<td>31430752</td>
<td>A</td>
<td>0.2627</td>
<td>1.78</td>
<td>1.85x10^-7</td>
</tr>
<tr>
<td>rs1313179†</td>
<td>6</td>
<td>31721033</td>
<td>A</td>
<td>0.2381</td>
<td>1.76</td>
<td>2.25x10^-7</td>
</tr>
<tr>
<td>rs686806*</td>
<td>11</td>
<td>107228533</td>
<td>T</td>
<td>0.3005</td>
<td>1.70</td>
<td>2.36x10^-7</td>
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<td>rs62469516*</td>
<td>7</td>
<td>88958624</td>
<td>A</td>
<td>0.2349</td>
<td>0.51</td>
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</tr>
<tr>
<td>Rs9272190†</td>
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<td>32601760</td>
<td>A</td>
<td>0.7595</td>
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<td>Rs2395228†</td>
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<td>32623223</td>
<td>A</td>
<td>0.8464</td>
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<td>2.92x10^-7</td>
</tr>
<tr>
<td>rs73452600†</td>
<td>7</td>
<td>142828104</td>
<td>A</td>
<td>0.1889</td>
<td>1.90</td>
<td>3.49x10^-7</td>
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<tr>
<td>rs3132445†</td>
<td>6</td>
<td>31712196</td>
<td>A</td>
<td>0.2398</td>
<td>1.73</td>
<td>3.58x10^-7</td>
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<tr>
<td>rs9272937†</td>
<td>6</td>
<td>32611040</td>
<td>A</td>
<td>0.3969</td>
<td>1.64</td>
<td>4.43x10^-7</td>
</tr>
<tr>
<td>Rs9266001†</td>
<td>6</td>
<td>31316695</td>
<td>A</td>
<td>0.2301</td>
<td>1.74</td>
<td>5.20x10^-7</td>
</tr>
<tr>
<td>Rs3117109†</td>
<td>6</td>
<td>32340871</td>
<td>T</td>
<td>0.253</td>
<td>1.72</td>
<td>5.49x10^-7</td>
</tr>
<tr>
<td>rs1662312*</td>
<td>18</td>
<td>3220450</td>
<td>A</td>
<td>0.2089</td>
<td>0.51</td>
<td>5.72x10^-7</td>
</tr>
<tr>
<td>Rs1265757†</td>
<td>6</td>
<td>32302382</td>
<td>T</td>
<td>0.231</td>
<td>1.72</td>
<td>6.37x10^-7</td>
</tr>
<tr>
<td>Rs2517597†</td>
<td>6</td>
<td>30081189</td>
<td>A</td>
<td>0.2414</td>
<td>1.72</td>
<td>6.51x10^-7</td>
</tr>
<tr>
<td>rs1469893*</td>
<td>15</td>
<td>34988664</td>
<td>A</td>
<td>0.3369</td>
<td>0.58</td>
<td>6.58x10^-7</td>
</tr>
<tr>
<td>Rs3099844†</td>
<td>6</td>
<td>31448976</td>
<td>A</td>
<td>0.2668</td>
<td>1.68</td>
<td>6.96x10^-7</td>
</tr>
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<td>Rs313595†</td>
<td>6</td>
<td>32405192</td>
<td>T</td>
<td>0.4687</td>
<td>0.61</td>
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<tr>
<td>Rs2844559†</td>
<td>6</td>
<td>31340075</td>
<td>T</td>
<td>0.2834</td>
<td>1.68</td>
<td>7.79x10^-7</td>
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<td>rs17676303*</td>
<td>1</td>
<td>15767969</td>
<td>T</td>
<td>0.29</td>
<td>1.68</td>
<td>8.05x10^-7</td>
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<td>rs28578508*</td>
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<td>32316007</td>
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<td>8.16x10^-7</td>
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<td>Rs642093†</td>
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<td>32582075</td>
<td>A</td>
<td>0.3439</td>
<td>1.64</td>
<td>9.66x10^-7</td>
</tr>
<tr>
<td>Rs9272275†</td>
<td>6</td>
<td>32603603</td>
<td>T</td>
<td>0.3366</td>
<td>1.64</td>
<td>9.74x10^-7</td>
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<td>rs78542322*</td>
<td>6</td>
<td>77291670</td>
<td>T</td>
<td>0.2644</td>
<td>0.51</td>
<td>9.82x10^-7</td>
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<td>rs3818779*</td>
<td>10</td>
<td>121140671</td>
<td>A</td>
<td>0.1235</td>
<td>1.94</td>
<td>1.51x10^-6</td>
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<tr>
<td>rs2141440*</td>
<td>11</td>
<td>90898463</td>
<td>A</td>
<td>0.2653</td>
<td>0.58</td>
<td>4.48x10^-6</td>
</tr>
</tbody>
</table>

- † denotes top ranking non-HLA SNPs that were chosen for validation, rs2098230 was not typed due to failure to design the probe on Sequenom multiplex.
- † denotes SNPs in major histocompatibility complex region.
An additional 12 top ranked non-MHC SNPs suggestive of association (p~10^{-6}) were also selected for validation. Of these, four SNPs showed evidence of association with p<1x10^{-4} when we conducted technical validation genotyping. They are rs1469893 on chr 15, rs17676303 on chr 1, rs78542322 on chr 6 and rs62469516 on chr 7 (Table 24). Amongst these four, rs1767303 was most strongly associated with GD with a P value at 1.14x10^{-8} (OR=2.05, 95% CI 1.57-2.67). When correlating odds ratio of effect allele frequencies in pooled GWAS with individual validation genotyping, the results showed strong correlation (r=0.89, p <0.001). (Figure 12)

Figure 12 Scatterplot for odds ratio of 13 effect allele frequencies comparing pooled GWAS Graves’ disease with individual genotyping in the discovery cohort
Table 24 Top ranking SNPs identified from pooled genome wide association study and validation genotyping in the discovery cohort

<table>
<thead>
<tr>
<th>SNP name</th>
<th>Location bp</th>
<th>Gene</th>
<th>GWAS pooled genotyping (Cases=412, Controls=498)</th>
<th>Validation individual genotyping (Cases=412, Controls=480)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A1</td>
<td>P value</td>
</tr>
<tr>
<td>Rs9676286</td>
<td>58126481</td>
<td>ZNF134 intron</td>
<td>A</td>
<td>1.08x10^-8</td>
</tr>
<tr>
<td>rs9644119</td>
<td>26517817</td>
<td>DPYSL2</td>
<td>T</td>
<td>9.10x10^-8</td>
</tr>
<tr>
<td>rs11722643</td>
<td>10127484</td>
<td>Intergenic WDR1 upstream, ZNF518B downstream</td>
<td>T</td>
<td>1.03x10^-7</td>
</tr>
<tr>
<td>Rs686806</td>
<td>107228533</td>
<td>CWF19L2 intron</td>
<td>T</td>
<td>2.36x10^-7</td>
</tr>
<tr>
<td>rs62469516</td>
<td>88958624</td>
<td>ZNF804B intron</td>
<td>A</td>
<td>2.68x10^-7</td>
</tr>
<tr>
<td>rs73452600</td>
<td>142828104</td>
<td>PIP 1070bp upstream</td>
<td>A</td>
<td>3.49x10^-7</td>
</tr>
<tr>
<td>Rs1662312</td>
<td>3220450</td>
<td>MYOM1 upstream</td>
<td>A</td>
<td>5.72x10^-7</td>
</tr>
<tr>
<td>rs1469893</td>
<td>34988664</td>
<td>GJD2</td>
<td>A</td>
<td>6.58x10^-7</td>
</tr>
<tr>
<td>Rs17676303</td>
<td>157679691</td>
<td>FCRL3</td>
<td>T</td>
<td>8.05x10^-7</td>
</tr>
<tr>
<td>rs28578508</td>
<td>32316007</td>
<td>DTNA intron</td>
<td>A</td>
<td>8.16x10^-7</td>
</tr>
<tr>
<td>rs78542322</td>
<td>77291670</td>
<td>Intergenic IMPG1 upstream HTR1B downstream</td>
<td>T</td>
<td>9.82x10^{-7}</td>
</tr>
<tr>
<td>Rs3818779</td>
<td>121140671</td>
<td>GRK5 intron</td>
<td>A</td>
<td>1.51x10^{-6}</td>
</tr>
<tr>
<td>rs2141440</td>
<td>90898463</td>
<td>Intergenic MIR4490 and FAT3</td>
<td>A</td>
<td>4.48x10^{-6}</td>
</tr>
</tbody>
</table>

P values are uncorrected for multiple testing

F_U is individual genotyping minor allele frequency A1 in normal control, F_A is the allele frequency in Graves’ disease.

OR is odd ratio for having minor allele in Graves’ cases compared to normal controls.

*P values and odds ratios in validation genotyping were adjusted for age and sex*
Genotyping in the replication cohort showed positive association of GD with rs17676303, located upstream of FCRL3 on chromosome 1q23.1 (OR=1.22, 95% CI 1.01-1.46, p=0.04). The minor T allele was more frequent in GD cases than in normal controls (OR=1.42, 95% CI 1.22-1.64, p=3.41x10⁻⁶) in the combined discovery and replication cohorts, adjusted for sex and age. Rs62469516, rs1469893 and rs78542322 minor alleles were not associated with GD in the replication cohort, or the combined cohorts (Table 25).
### Table 25: Genotyping results for top ranking single nucleotide polymorphisms in replication cohorts, and combined analysis of discovery and replication cohorts using individual genotyping.

<table>
<thead>
<tr>
<th>SNP name</th>
<th>Location chr and bp</th>
<th>Gene</th>
<th>Replication cohorts (Cases=539, Controls=1230)</th>
<th>Combined discovery and replication cohorts (Cases=951, Controls=1710)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>F_A</td>
<td>F_U</td>
</tr>
<tr>
<td>Rs9676286</td>
<td>Chr19q13.43 58126481</td>
<td>ZNF134 intron</td>
<td>A</td>
<td>0.045</td>
</tr>
<tr>
<td>rs9644119</td>
<td>Chr8p21.2 26517817</td>
<td>DPYSL2 2124 bp downstream</td>
<td>T</td>
<td>0.219</td>
</tr>
<tr>
<td>rs11722643</td>
<td>Chr4p16.1 10127484</td>
<td>Intergenic WDR1 upstream, ZNF518B downstream</td>
<td>T</td>
<td>0.096</td>
</tr>
<tr>
<td>Rs686806</td>
<td>Chr11q22.3 107228533</td>
<td>CWF19L2 intron</td>
<td>T</td>
<td>0.198</td>
</tr>
<tr>
<td>rs62469516</td>
<td>Chr7q21.13 88958624</td>
<td>ZNF804B intron</td>
<td>A</td>
<td>0.053</td>
</tr>
<tr>
<td>rs73452600</td>
<td>Chr7q34 142828104</td>
<td>PIP1 1070bp upstream</td>
<td>A</td>
<td>0.090</td>
</tr>
<tr>
<td>Rs1662312</td>
<td>Chr18p11.31</td>
<td>MYOM1 upstream</td>
<td>A</td>
<td>0.070</td>
</tr>
<tr>
<td>rs1469893</td>
<td>Chr 15q14</td>
<td>GJD2</td>
<td>A</td>
<td>0.074</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>---------------</td>
<td>---</td>
<td>-------</td>
</tr>
<tr>
<td>Rs17676303</td>
<td>Chr 1q23.1</td>
<td>FCRL3</td>
<td>T</td>
<td>0.233</td>
</tr>
<tr>
<td>rs28578508</td>
<td>Chr 18q12.1</td>
<td>DTNA intron</td>
<td>A</td>
<td>0.069</td>
</tr>
<tr>
<td>rs78542322</td>
<td>Chr 6q14.1</td>
<td>Intergenic IMPG1 upstream HTR1B downstream</td>
<td>T</td>
<td>0.094</td>
</tr>
<tr>
<td>Rs38187799</td>
<td>Chr 10q26.11</td>
<td>GRK5 intron</td>
<td>A</td>
<td>0.055</td>
</tr>
<tr>
<td>rs2141440</td>
<td>Chr 11a14.3</td>
<td>Intergenic MIR4490 and FAT3</td>
<td>A</td>
<td>0.094</td>
</tr>
</tbody>
</table>

P values and odds ratios are adjusted for age and sex.
We further examined the relationship between SNP rs17676303 and other annotated SNPs in *FCRL3* gene or its promoter region, namely rs7528684, rs3761969 and rs11264798, which have previously been identified as positively associated with GD, rheumatoid arthritis and systemic lupus erythematosus. All three SNPs previously associated with GD showed high linkage disequilibrium with rs17676303 in the Caucasian HapMap population. (Table 26) The result suggests that these SNPs reported with various auto-immune diseases may be tagging the same functional genetic variants around the *FCRL3* gene locus.

<table>
<thead>
<tr>
<th>Locus 1</th>
<th>Locus 2</th>
<th>D’</th>
<th>Log of the Odds (LOD)</th>
<th>R²</th>
<th>CI low</th>
<th>CI High</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs11264798</td>
<td>rs17676303</td>
<td>1.0</td>
<td>4.44</td>
<td>0.142</td>
<td>0.64</td>
<td>1.0</td>
</tr>
<tr>
<td>rs3761959</td>
<td>rs17676303</td>
<td>1.0</td>
<td>5.83</td>
<td>0.221</td>
<td>0.71</td>
<td>1.0</td>
</tr>
<tr>
<td>rs7528684</td>
<td>rs17676303</td>
<td>1.0</td>
<td>5.73</td>
<td>0.224</td>
<td>0.7</td>
<td>1.0</td>
</tr>
</tbody>
</table>

A second SNP, rs9644119, located downstream of *DPYSL2* on chromosome 8p21 is highly ranked for its association with GD in pooled GWAS with a *P* value approaching genome wide significance (*p*=9.10×10⁻⁸). The association was not validated in the discovery cohort and for the combined cohort the association with GD is less strong than the discovery pooled GWAS would suggest (*p*=4.02×10⁻³). In the replication cohort, the minor T allele conferred increased odds for GD compared to controls (OR=1.33, 95% CI 1.11-1.59, *p*=1.95×10⁻³); after adjusting for sex and age, the result remained significant. (OR=1.28, 95% CI 1.06-1.54, *p*=9.34×10⁻³). The results suggested this allele might be a genetic locus of interest for GD, but will need further confirmation in future studies.

**13.4 Discussion**
This study investigated the genetic associations of GD using a pooled genome wide association study (GWAS) in 910 samples. Despite the relatively small number of samples for discovery GWAS, the pooled strategy in combination with
increased sample size in a second stage of individual genotyping in both the validation and replication cohorts, readily detected evidence of association of a genetic locus upstream of *FCRL3* on chromosome 1q23. The association of *FCRL3* with GD has previously been identified in large scale GWAS studies.\textsuperscript{183, 184, 312} The pooled GWAS also detected association of GD with multiple SNPs within the MHC I and MHC II regions on chromosome 6p21. Susceptibility loci within both these regions which contain human leucocyte antigen (HLA) genes involved in antigen presentation, are well established for GD by association studies, candidate gene analysis and GWAS.\textsuperscript{150, 167, 183, 312, 323} Rs9272937 in the untranslated region of *HLA-DQA1* within the MHC class II region showed evidence of association with Graves’ disease (p=4.43x10^{-7}) in our study. The positive association of *HLA-DQA1* with Graves’ disease has been replicated in multiple studies.\textsuperscript{161, 323, 324} Two SNPs within the intron of the *C6orf10* gene suggesting association with GD (p 10^{-7}), were also independently validated as a locus of susceptibility in GD in a previous GWAS study.\textsuperscript{183}

These replicative findings in GD prove the robustness of the two-stage study design for pooled GWAS. This pooled GWAS study also found evidence of association for a gene locus marked by rs9644119, downstream of *DPYSL2* on chromosome 8, but the significance is less certain. The risk attributions from individual genetic variants for GD are known to be small with odds ratios <1.5,\textsuperscript{177} therefore large sample sizes in the thousands will be needed to increase power for detecting significant association, and may in the future prove or disprove the significance for the rs9644119 locus.

For the two compelling signals we have detected in the vicinity of *FCRL3* and *DPYSL2*, genetic variants in *FCRL3* were associated with a modest increase in odds for GD at 1.2 to 1.35.\textsuperscript{182, 184, 312} Genetic variation in the promoter region of *FCRL3* i.e. *FCRL3* -169T>C allele is reportedly associated with other autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus,\textsuperscript{322, 325, 326} suggesting that the *FCRL3* locus may influence the susceptibility to multiple autoimmune diseases in a similar manner to the immune regulating *CTLA4*, *PTPN22* and MHC genes.\textsuperscript{174, 185, 312} To pinpoint the exact locus associated with *FCRL3*, the entire FCRL region on chromosome 1q21 was previously investigated.
using imputation, and the strongest signals associated with GD were located in a cluster of SNPs including rs7528684 and rs3761959. SNP rs7528684 in the promoter region of FCRL3 was also previously reported as positively associated with rheumatoid arthritis, systemic lupus erythematosus and GD. Rs3761959 tagging rs7522061 and rs7528684 in the FCRL3 gene showed association with GD in the Wellcome Trust Case Control Consortium GWAS study (p=9.4x10⁻³), and the association was further confirmed by The China Consortium for the Genetics of Autoimmune Thyroid disease, reaching genome wide significance (p=2.22x10⁻⁸). In addition, rs11264798 in FCRL3 was also associated with GD (p=1.6x10⁻⁵). We found high linkage disequilibrium of rs17676303, the SNP associated with GD in our study, with rs7528684, rs3761959 and rs11264798, that strongly suggest these SNPs were tagging the same functional variants in the vicinity of the FCRL3 gene.

Functional analysis suggests FCRL3 has immune regulating roles. FCRL3 is an Fc receptor like molecule that shares a genomic location (chr 1q21-23) and gene structure as a receptor for the Fc portion of immunoglobulin with other members of the immunoglobulin superfamily. Unlike the Fc receptor, the FCRL molecule has not been shown biochemically to bind immunoglobulin. FCRL3 molecules are preferentially expressed by B cells. FCRL3 possesses both tyrosine based activating and inhibitory motifs in its cytoplasmic tail, indicating these may be used to transmit immune-modulatory signals via tyrosine phosphorylation in regulating B cell differentiation and response. The strongest evidence linking FCRL3 polymorphisms with autoimmunity is derived from linkage disequilibrium mapping of the chr1q21-23 region in rheumatoid arthritis, which identified 4 SNPs (rs7528684, rs11264799, rs945635 and rs3761959 in a Japanese population. Of the 4 SNPs positively associated with rheumatoid arthritis, FCRL3 -169T>C (marked by rs7528684 in the promoter region of FCRL3) substitution substantially increased promoter activity in FCRL3 and enhanced binding affinity to the consensus NF-κβ binding motif, where NF-κβ widely regulates genes involved in immune response. In addition, FRCL3 transcripts in peripheral B cells and genomic DNA are higher in heterozygous individuals with genotype -169C/T +358C/G, and increased FCRL3 expression in synovial tissue and peripheral CD19+ B cell population suggesting that higher
expression of FCRL3 is potentially pathogenic. Further correlation of B cell abnormalities with FCRL3 expression is afforded by significant positive correlation of rheumatoid factor autoantibody titre in individuals with rheumatoid arthritis with the number of susceptible -169 C alleles. However not all studies showed consistent association with polymorphisms in FCRL3, specifically no discernible association with FCRL3 were noted in some other rheumatoid arthritis populations including Caucasians in North American and European countries. When the susceptibility effect of FCRL3 was stratified by NF-κβ1 genotypes, association of FCRL3 polymorphisms with rheumatoid arthritis was observed in patients heterozygous for the NF-κβ1 promoter in a Spanish population.

The second gene of interest, DPYSL2, produces collapsin response mediator protein 2 (CRMP2). It was first described in the central nervous system as an effector cytosolic protein for semaphorin mediated axonal growth cones navigation during neural development and plasticity. Dysregulation of CRMP2 phosphorylation and expression, have been associated with neuropsychiatric diseases including Alzheimer’s disease and schizophrenia. CRMP2 is also highly expressed in activated T lymphocytes from peripheral blood bearing CD69 and HLA-DR markers. CRMP2 is distributed in the trailing uropod in polarized T cells binding to cytoskeletal structures particularly to vimentin. The observations of enhanced spontaneous and chemokine-directed T cell transmigration by induced expression of CRMP2 in Jurkat T cells, and high expression of CRMP2 in migrating T cells suggest CRMP2 may regulate T cell motility and migration. The mechanism underlying CRMP2 induced T cell migration seems to involve phosphorylation of CRMP2 Tyr-479 residue, acting as transducer for chemokine stromal cell-derived factor-1α (CXCL12) signaling. The discovery of CRMP2 cellular function in T cells defines its potential role in the pathogenesis of immune mediated inflammation, specifically neuroinflammation.

In conclusion, the pooled GWAS is an effective methodology for detecting common genetic variants in GD. The study confirmed FCRL3 region as a susceptibility locus for GD in addition to MHC. A second locus downstream of
DPYSL2 is potentially a novel genetic variant in GD that requires further confirmation.
14 Genome-wide association study to find genetic variants in thyroid-associated orbitopathy

Publication arising from this chapter:


14.1 Background and aims

The introduction of high throughput microarray technology, in combination with large cohorts of cases and controls, has seen genome-wide association studies (GWAS) revolutionise the study of the genetics of complex traits and has allowed detection of association of common genetic variants with many diseases. Although genetic risk factors for Grave’s disease (GD) are becoming well defined, limited progress has been made with TO, due largely to small sample sizes limiting power to detect modest genetic effects and failure to replicate genetic findings. Candidate gene studies, predominantly of genes and polymorphisms associated with Graves’ disease or genes involved in immune response and inflammation have been investigated for a contribution to TO.

The pooled GWAS strategy principally showed that discovery GWAS is a cost effective screening strategy for identifying gene variants in Graves’ disease, and the pooled DNA strategy has successfully identified known susceptibility loci in multiple eye diseases e.g. CFH, ARMS2/HTRA1 in age related macular degeneration, LOXL1 in pseudoexfoliation syndrome, and new susceptibility locus HGF in keratoconus.

The specific aim investigated in this chapter was:

1) to determine the genomic susceptibility loci associated with TO.

The null hypothesis states that there is no difference in allele frequencies comparing GD with and without TO. The alternative hypothesis tested is genetic variants exist for TO.
14.2 Peer reviewed publication author version

Title: Association of polymorphisms in MACRO domain containing 2 with thyroid-associated orbitopathy

Authors:
Jwu Jin Khong\textsuperscript{1,2,3} FRANZCO, MMed, MBBS(Hons)
Kathryn P Burdon\textsuperscript{4} PhD, BSc(Hons)
Yi Lu\textsuperscript{5} PhD
Lefta Leonardos\textsuperscript{6} BSc(Hons)
Kate J Laurie\textsuperscript{6} BSc
John P Walsh\textsuperscript{7,8} FRACP, PhD
Adam D Gajdatsy\textsuperscript{9} FRANZCO, MRCP(UK), MB ChB, BSc(Hons)
Peter R Ebeling\textsuperscript{10} FRACP, MD, MBBS
Alan A McNab\textsuperscript{2,11} FRANZCO, DMedSc
Thomas G Hardy\textsuperscript{2,3} FRANZCO, MBBS
Richard J Stawell\textsuperscript{11} FRANZCO, FRACS
Garry J Davis\textsuperscript{12} FRANZCO, FRACS, MBBS
Dinesh Selva\textsuperscript{12} FRANZCO, FRACS, MBBS(Hons)
Angelo Tsirbas\textsuperscript{13} AAFPS, FRANZCO, MBBS(Hons)
Grant W Montgomery\textsuperscript{14} PhD
Stuart Macgregor\textsuperscript{5} BSc, MSc, PhD
Jamie E Craig\textsuperscript{6} FRANZCO, DPhil, MBBS(Hons)

Affiliations:
1. North West Academic Centre, Department of Medicine, University of Melbourne, Sunshine Hospital, St Albans, Victoria, Australia
2. Orbital, Plastics and Lacrimal Unit, The Royal Victorian Eye and Ear Hospital, Victoria, Australia
3. Department of Surgery, University of Melbourne, Victoria, Australia
4. Menzies Institute for Medical Research, University of Tasmania, Hobart, Tasmania, Australia
5. Statistical Genetics, QIMR Berghofer Medical Research Institute, Herston, Queensland, Australia
6. Department of Ophthalmology, Flinders University of South Australia, Bedford Park, South Australia, Australia
7. Department of Endocrinology and Diabetes, Sir Charles Gairdner Hospital, Nedlands, Western Australia, Australia
8. School of Medicine and Pharmacology, The University of Western Australia, Crawley, Western Australia, Australia
9. Centre for Ophthalmology and Visual Sciences, University of Western Australia, Western Australia, Australia
10. Department of Medicine, School of Clinical Sciences, Monash University, Clayton, Victoria, Australia
11. Centre for Eye Research Australia, University of Melbourne, East Melbourne, Victoria, Australia
12. South Australian Institute of Ophthalmology, University of Adelaide, South Australia, Australia
13. Australian School of Advanced Medicine, Macquarie University, Sydney, Australia
14. Molecular Epidemiology, QIMR Berghofer Medical Research Institute, Herston, Queensland, Australia

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Corresponding author:

Jwu Jin Khong

162
Melbourne Medical School-Western Campus

Department of Medicine

University of Melbourne

176 Furlong Road, St Albans, VIC, 3021, Australia

Email: jwujinkhong@gmail.com
Abstract

Purpose: Thyroid-associated orbitopathy (TO) is an autoimmune mediated orbital inflammation that can lead to disfigurement and blindness. Multiple genetic loci have been associated with Graves’ disease, but the genetic basis for TO is largely unknown. This study aimed to identify loci associated with TO in individuals with Graves’ disease, using a genome-wide association scan (GWAS) for the first time in TO.

Methods: GWAS was performed on pooled DNA from an Australian Caucasian discovery cohort of 265 participants with Graves’ disease and TO (cases) and 147 patients with Graves’ disease without TO (controls). Top-ranked single nucleotide polymorphisms (SNPs) were then genotyped in individual DNA samples from the discovery cohort, and two replication cohorts totaling 584 cases and 367 controls.

Results: In the GWAS of pooled DNA samples, several SNPs showed suggestive association with TO at genome-wide \( p \leq 10^{-6} \); rs953128 located on chr10q21.1, rs2867161 on chr7q11.22, rs13360861 on chr5q12.3, rs7636326 on chr3q26.2, rs10266576 on chr7q11.22, rs60457622 on chr3q23, and rs6110809 on chr20p12.1. However, the only SNP consistently associated with TO on individual genotyping in the discovery and replication cohorts was rs6110809, located within MACROD2 on chromosome 20p12.1. On combined analysis of discovery and replication cohorts, the minor A allele of rs6110809 was more frequent in TO than in Graves’ disease controls without TO (\( p=4.35 \times 10^{-5}, \ OR=1.77, \ 95\% \ CI \ 1.35-2.32 \)) after adjusting for age, sex, duration of Graves’ disease and smoking.

Conclusions: In patients with Graves’ disease, a common genetic variant in MACROD2 may increase susceptibility for thyroid-associated orbitopathy. This association now requires confirmation in additional independent cohorts.
Introduction

Thyroid-associated orbitopathy (TO) is an autoimmune-mediated orbital inflammation affecting 25-50% of patients with Graves’ disease (GD). The overall age-adjusted incidence rate of TO is 16 cases per 100 000 per year for females and 2.9 cases per 100 000 population per year for males. The average age at diagnosis is 44.7 years. The onset of TO mostly occurs within 18 months of diagnosis of Graves’ disease, and the close temporal relationship suggests the two diseases share a common autoantigen.

Genetic susceptibility for Graves’ disease is complex and involves multiple genetic loci regulating both immune system and thyroid specific genes. The HLA class II alleles, CTLA4, PTPN22, CD40 polymorphisms were identified early on by candidate gene association studies as genetic risk factors for Graves’ disease, even though the overall contributions from each of these genetic variants are modest. The introduction of high throughput microarray technology, in combination with large cohorts of cases and controls, has seen genome-wide association studies (GWAS) revolutionise the study of the genetics of complex traits and has allowed detection of association of common genetic variants with many diseases. The earliest GWAS studies of autoimmune thyroid disease reported associations with HLA class I and II regions, TSHR and FCRL3 genes. Subsequent GWAS studies replicated association of FCRL3, HLA loci, TSHR, CTLA4, PTPN22, IL2RA and discovered new susceptibility loci at SENP1, SLAMF6, RNASET2-FGFR10P-CCR6, an intergenic region at 4p14, MMEL1, LPP, BACH2, PRICKLE1 and ITGAM.

Although genetic risk factors for Grave’s disease are becoming well defined, limited progress has been made with TO, due largely to small sample sizes limiting power to detect modest genetic effects and failure to replicate genetic findings. Candidate gene studies, predominantly of genes and polymorphisms associated with Graves’ disease or genes involved in immune response and inflammation have been investigated for a contribution to TO. While some studies showed CTLA4, HLA I and II alleles, and IL-23R rs10889677 and rs2201841 SNPs were associated with TO compared to Graves’ disease controls, other studies have not supported these associations. TNF-α alleles were significantly associated with
TO in a Japanese but not in a Polish study.\textsuperscript{189, 210} The adipogenesis-related gene \textit{PPAR\textsubscript{\gamma}} Pro12Ala polymorphism was found to decrease the risk of TO in one study but not in another, but both studies found the Pro12Ala variant possibly reduces the severity and activity of TO.\textsuperscript{202, 203} Using tag single nucleotide polymorphisms (SNP), adipocyte-related immediate early genes showed increased risk for TO with genetic variants in \textit{CYR61} (rs1378227), \textit{ZFP36} (rs1057745, rs11083522) and \textit{SCD} (rs1393491) with odds between 1.3 to 1.4 compared to GD, but the findings need to be replicated.\textsuperscript{204} Similarly using tag SNPs, SNPs in \textit{IL-1\beta} did not differ between TO and non-TO in GD in one study but C allele of rs1143634 was found to increase the risk both in TO and GD compared to normal controls, \textsuperscript{205} whereas another identified rs1800587 polymorphism at \textit{IL-1\alpha} and rs16944 polymorphism at \textit{IL-1\beta} significantly associated with GD and TO.\textsuperscript{206} \textit{PTPN12} (rs1468682, rs4729535 and rs17467232), functionally related to \textit{PTPN22}, were associated with TO compared to non-TO in GD with odds at 1.4 and 2 of the SNPs (rs1468682 and rs4729535) showed interactions with \textit{TSHR} rs2268458 SNP, the findings again require replication.\textsuperscript{196} However, TSHR rs2268458 SNP previously found to be associated with GD, were not associated with TO.\textsuperscript{187}

Pooled GWAS study is a cost effective screening strategy for identifying gene variants in multiple diseases. Pooled DNA strategy has successfully identified known susceptibility loci \textit{CFH}, \textit{ARMS2/HTRA1} in age related macular degeneration, \textit{LOXL1} in pseudoexfoliation syndrome, and discovered new susceptibility locus \textit{HGF} in keratoconus.\textsuperscript{313, 314} This study aims to use a genome-wide association study to discover genomic loci associated with TO. We report the first attempts at using a genome-wide association scan for discovering genetic variants in TO, and an association of variants in the \textit{MACROD2} gene suggesting it as a new TO susceptibility locus.
Methods
The genetic study of thyroid-associated orbitopathy (TO) was approved by human research ethics committee at multiple recruitment sites; the Royal Victorian Eye and Ear Hospital, Sunshine and Footscray Hospitals, the Royal Melbourne Hospital and the Alfred Hospital in Melbourne, Victoria, Australia and Flinders Medical Centre and the Royal Adelaide Hospital in Adelaide, South Australia. The genetic research was conducted in accordance with the Declaration of Helsinki.

Australian thyroid-associated orbitopathy research (ATOR) cohorts

Definition of Graves’s disease and thyroid-associated orbitopathy

Graves’ disease was defined by the presence of hyperthyroidism based on thyroid function test including T3, T4 and TSH, and either elevated thyrotropin receptor (TSHR) antibodies or diffuse uptake on technetium-99m pertechnetate thyroid scan. Cases are defined by the presence of TO clinically and controls by the absence of TO.

TO was defined by the presence of symptoms of TO and at least one sign of TO e.g. lid retraction. Early symptoms of TO were detected using the Vancouver Orbitopathy Rule symptom questionnaire, including red eyes, lids swelling, eye protrusion and stare and blurred vision. TO status was examined and classified using the Vision, Inflammation, Strabismus and Appearance (VISA) classification. Ophthalmological measurements included the following: visual acuity, pupil response and colour vision, inflammatory index score, extra-ocular movement and strabismus, lid measurements including palpebral aperture, marginal reflex distance, lid retraction and Hertel exophthalmometry. The status of TO was determined by ophthalmologist.

ATOR discovery cohort

TO cases (n=265) and Graves’ disease without TO controls (n=147) of European descent were recruited from endocrine and eye hospital outpatient clinics and private practices of endocrinologists and ophthalmologists from Victoria and South Australia. Whole blood samples in EDTA were stored at 4°C.

Replication cohorts
A further 319 TO cases and 220 Graves’ disease controls were recruited from Victoria and Western Australia as replication cohorts. These comprised 231 TO cases and 142 Graves’ disease controls recruited from Victoria using the same protocol as the ATOR discovery cohort and 88 TO cases and 78 Graves’ disease controls of European descent recruited from endocrine and ophthalmology clinics in Western Australia. TO was defined clinically by presence of eye symptoms and signs with severity classified according to European group on Graves’ orbitopathy (EUGOGO) recommendations (by co-author JW)\textsuperscript{338} or VISA classification (by co-author AG).\textsuperscript{15} DNA samples (50ng/µl) from venous blood were used for individual genotyping.

282 normal controls consisting of healthy hospital volunteers and retirement village residents over 50 years of European descent from South Australia were used for additional individual genotyping for comparison with TO cohort and Graves’ disease cohort (with and without TO) respectively. The normal control cohort was not actively screened for Graves’ disease, but no participant reported having this condition.

**DNA pooling for GWAS study in the discovery cohort**

Venous blood aliquots from the discovery cohort were stored at 4°C before construction of 2 comparative blood pools (pool number 1: 154 TO cases versus 102 controls, pool number 2: 116 TO cases versus 40 controls), totaling 270 cases and 142 controls. In between the construction of the blood pools and individual genotyping, the phenotypes of 5 individuals were updated (5 from cases to controls), these small numbers do not materially affect the overall blood pool results. Protease and lysis buffer were added to 100µl of thawed whole blood from each sample, vortexed and incubated, then samples were combined in one tube for each pool. DNA was extracted from the pooled blood using QIA-amp DNA blood maxi kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. For the remaining discovery samples, equimolar DNA pools were generated in duplicate after step-wise dilutions of genomic DNA extracted using QIA-amp DNA blood maxi kit to 75ng/µl as described previously.\textsuperscript{313,314} The DNA was quantitated using Fluroskan Ascent (ThermoFisher Scientific, Waltham, MA, USA) together with double-stranded DNA quantitation assay using Picogreen reagents.
(Invitrogen, Carlsbad, CA, USA). A total of 451ng of DNA from each sample was added to the DNA pool.

**Pooled genotyping and analysis**

Genome-wide genotyping was conducted by hybridization to HumanOmni5-Quad (Illumina, San Diego, CA, USA) beadchip microarray containing approximately 4.3 million markers, at QIMR Berghofer Medical Research Institute, according to standard protocol for both case and control pools on two independent arrays for each pool. Two case pools and two control pools were generated and assigned into two case-control pool comparisons. Each pool was replicated 2-4 times to reduce measurement error. For the purpose of this analysis, we retained SNPs with minor allele frequency (MAF) over 1% from the reference panel of EUR samples in the 1000 Genome Project (excluded 1.8M SNPs). Thus, the number of SNPs was reduced to 2.5 million. The output of the raw red and green bead scores from Illumina Beadstation were used for the pooled data analysis. The data cleaning and quality control procedures in array-based pooling have been described previously.\(^{317, 319, 320}\) Briefly, SNPs with more than 10% negative scores on each array were excluded, as well as the SNPs with the sum of mean red and green scores lower than 1200 across each array to ensure calibration was performed on a pre-cleaned dataset. A normalization/correction factor (corr) was calculated to make the mean value of the pooling allele frequency (PAF) 0.5 across all SNPs on each strands of the array. The PAF was then estimated based on the raw red intensities and the corrected green intensities for all the SNPs \[PAF=\frac{\text{red}}{\text{red} + \frac{\text{green}}{\text{corr}}}\].\(^{319}\) Further quality control included filtering non-autosomal SNPs and any SNPs with a significant variance difference between cases and controls.

After data cleaning, 2.2M SNPs were left for association testing in the ATOR discovery cohort. A linear model was used to test for allelic association in the two case-control pool comparisons. A test statistic which corrects for pooling error was used to rank SNPs, with \(P\) values based on a chi-squared distribution with 1 degree of freedom. The application of the test statistic is based upon contrasting case and control pools, the effect of unequal amplification of alleles is minimal as such effects cancel out.\(^{317}\) Specifically, a final set of autosomal SNPs meeting the criteria (1) have sufficient number of probes (i.e., over \(\frac{1}{2}\) of expected number of probes
for MAF1-5%, or over 1/3 of expected number of probes for SNPs >5%; (2) have no large differences (>0.3) between PAF and reference allele frequency from EUR samples in 1000 Genome Project. We then performed a meta-analysis weighted by inverse variance, combining results from two case-control pool comparisons. The top-ranked SNPs in the meta-analysis were then counter checked for proxies (linkage disequilibrium r2>0.5 with the index SNPs in the EUR populations). To be considered a valid result, if proxy SNPs were available, they should also show evidence of association. A genome-wide significance threshold of 5x10^{-8} was set to account for multiple testing.

**Validation genotyping and analysis**

Top-ranked SNPs with p-value threshold ≤1x10^{-6} indicative of association, or corresponding proxy if failed genotyping, were selected for individual genotyping in the discovery and replication cohorts, in order to validate pooled genotyping results as well as to replicate the associations in independent samples. In total 7 SNPs were genotyped on Sequenom iPLEX Gold assay using Sequenom MassARRAY® Analyzer in 2 batches, the ATOR discovery cohort and Western Australia replication cohort were genotyped at the Australian Genome Research Facility (Brisbane, Australia) and the ATOR replication cohort at GeneWorks Pty Ltd (Adelaide, Australia). The allelic association analysis was performed in PLINK,\textsuperscript{321} using a chi-square test and performed separately for ATOR pooled discovery cohort for validation, the replication cohorts and for combined discovery and replication cohorts, the odds ratio and p values were adjusted for age of disease onset, duration of Graves’ disease, sex using logistic analysis. SNPs with p ≤ 1x10^{-4} were considered validated in validation genotyping. In the replication cohort p <0.05 was considered associated. In the secondary analysis, we further adjusted the individual genotyping results for smoking status (smoker, ex-smoker and never smoked) in addition to sex, age and duration of disease for the discovery, replication and combined cohorts, bearing in mind there are missing data points for smoking in 10.9% in the replication cohorts. Further individual genotyping for the top ranking SNPS were compared between TO and normal controls; and between Graves’ disease and normal control in order to differentiate whether the validated SNPs were associated with TO and Graves’ disease as a
whole or the risk variant is specific for TO in Graves’ disease. As smoking status data were not available from the healthy controls, the genotyping results for these genotyping analyses were adjusted for sex and age only.

Following individual genotyping in discovery and replication cohorts revealing rs6110809 in MACROD2 as locus of interest, further SNPs residing in MACROD2 with nominal \( p \leq 5 \times 10^{-4} \) were extracted from pooled GWAS top ranking data. They were assessed for linkage disequilibrium with rs6110809, to determine if multiple independent risk alleles were potentially present within MACROD2. Genotype data limited to a 100kb region of MACROD2 capturing all 4 additional SNPs were downloaded from International Hapmap Project (http://hapmap.ncbi.nlm.nih.gov/cgi-perl/gbrowse/hapmap24_B36/#search). Genotype data were analysed in HaploView (Version 1.0, Broad Institute) for pairwise comparisons of marker SNPs with minor allele frequency >0.01.

**Results**

The age of disease onset, duration of Grave's disease, sex and smoking status distributions of the three cohorts of participants are described in Table 27. TO cases were slightly older compared with Graves’ without TO at diagnosis and the duration of Grave’s disease in TO cases were longer. The proportions of male to female were similar between the groups and the proportion of smokers was greater in cases compared to controls for ATOR cohorts. The age and sex distribution of the normal controls in comparison with TO as a whole and Graves’ disease as a whole were shown in Table 28.
### Table 27 Clinical characteristics of GWAS discovery and replication cohorts

<table>
<thead>
<tr>
<th></th>
<th>ATOR discovery cohort N=412</th>
<th>ATOR replication cohort N=373</th>
<th>WA replication cohort N=166</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Case</td>
<td>Control</td>
<td>P value</td>
</tr>
<tr>
<td>N</td>
<td>265</td>
<td>147</td>
<td></td>
</tr>
<tr>
<td>Mean Age in years (SD)</td>
<td>46.7 (13.8)</td>
<td>42.7 (15.2)</td>
<td>0.01</td>
</tr>
<tr>
<td>Graves' disease Duration in years (SD)</td>
<td>10.91 (9.66)</td>
<td>5.42 (6.83)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>47 (20.4%)</td>
<td>24 (16.3%)</td>
<td>0.717</td>
</tr>
<tr>
<td>Female</td>
<td>218 (79.7%)</td>
<td>123 (83.7%)</td>
<td></td>
</tr>
<tr>
<td>Smoking Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoker</td>
<td>66 (25.2%)</td>
<td>25 (17.1%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ex-smoker</td>
<td>110 (42%)</td>
<td>42 (28.8%)</td>
<td></td>
</tr>
<tr>
<td>Never smoked</td>
<td>86 (32.8%)</td>
<td>79 (54.1%)</td>
<td></td>
</tr>
<tr>
<td>* 3 missing</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*40 missing</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 28 Demographics of overall cohort with Graves' disease, thyroid-associated orbitopathy compared with normal healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Thyroid-associated orbitopathy compared with normal controls</th>
<th>Graves’ disease compared with normal controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TO cases</td>
<td>Normal controls</td>
</tr>
<tr>
<td>N</td>
<td>584</td>
<td>282</td>
</tr>
<tr>
<td>Mean Age in years</td>
<td>46.5 (13.7)</td>
<td>75.9 (8.18)</td>
</tr>
<tr>
<td>(SD)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>107 (18.38%)</td>
<td>116 (42.96%)</td>
</tr>
<tr>
<td>Female</td>
<td>475 (81.62%)</td>
<td>154 (57.04%)</td>
</tr>
<tr>
<td></td>
<td>*2 missing</td>
<td>*12 missing</td>
</tr>
</tbody>
</table>
Pooled DNA was used to identify genetic variants associated with TO. None of the top ranked SNPs reached the genome-wide significance threshold of $5 \times 10^{-8}$ (Table 29), nevertheless multiple top ranked SNPs approached genome-wide significance with $p \leq 10^{-6}$ and in particular with rs2867161 on chr7q11.22 ($p=5.94 \times 10^{-8}$) and rs60457622 on chr3q23 ($p=9.28 \times 10^{-8}$) almost reaching significance (Table 30).

The seven top ranked SNPs from pooled GWAS were individually genotyped in the ATOR discovery cohort. rs6110809 was validated with $p<1 \times 10^{-4}$, the minor allele is associated with TO risk, showing higher frequency in cases compared with controls (Table 30). After further adjustment for smoking, rs6110809 was associated with TO with odds ratio (OR) at 2.66 (95% CI 1.69-4.18, $p=2.45 \times 10^{-5}$) in the discovery cohort.
Table 29 Top 20 single nucleotide polymorphisms associated with TO compared to Graves’ without TO

<table>
<thead>
<tr>
<th>SNP name</th>
<th>Chr</th>
<th>BP position</th>
<th>Illumina ID</th>
<th>Effect allele</th>
<th>Effect allele Frequency</th>
<th>Log OR</th>
<th>Std Err</th>
<th>OR</th>
<th>P-value</th>
<th>Genes at locus</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs2867161*</td>
<td>7</td>
<td>67637342</td>
<td>rs2867161</td>
<td>A</td>
<td>0.221</td>
<td>0.917</td>
<td>0.169</td>
<td>2.50</td>
<td>5.94x10^-8</td>
<td></td>
</tr>
<tr>
<td>rs60457622*</td>
<td>3</td>
<td>141549404</td>
<td>kgp2485806</td>
<td>A</td>
<td>0.105</td>
<td>1.047</td>
<td>0.196</td>
<td>2.85</td>
<td>9.28x10^-8</td>
<td></td>
</tr>
<tr>
<td>rs13360861*</td>
<td>5</td>
<td>65724966</td>
<td>rs13360861</td>
<td>T</td>
<td>0.147</td>
<td>0.949</td>
<td>0.178</td>
<td>2.58</td>
<td>1.02x10^-7</td>
<td></td>
</tr>
<tr>
<td>rs10266576*</td>
<td>7</td>
<td>67862566</td>
<td>rs10266576</td>
<td>A</td>
<td>0.061</td>
<td>1.199</td>
<td>0.238</td>
<td>3.32</td>
<td>4.67x10^-7</td>
<td></td>
</tr>
<tr>
<td>rs175805</td>
<td>20</td>
<td>15832282</td>
<td>rs175805</td>
<td>T</td>
<td>0.220</td>
<td>0.815</td>
<td>0.164</td>
<td>2.26</td>
<td>7.00x10^-7</td>
<td>MACROD2</td>
</tr>
<tr>
<td>rs953128*</td>
<td>10</td>
<td>56502386</td>
<td>rs953128</td>
<td>A</td>
<td>0.160</td>
<td>0.874</td>
<td>0.176</td>
<td>2.40</td>
<td>7.13x10^-7</td>
<td></td>
</tr>
<tr>
<td>Rs28986350</td>
<td>6</td>
<td>32749175</td>
<td>kgp7043619</td>
<td>T</td>
<td>0.333</td>
<td>-0.879</td>
<td>0.183</td>
<td>0.42</td>
<td>1.58x10^-6</td>
<td></td>
</tr>
<tr>
<td>rs10479597</td>
<td>5</td>
<td>180452273</td>
<td>kgp1394432</td>
<td>A</td>
<td>0.066</td>
<td>1.817</td>
<td>0.380</td>
<td>6.15</td>
<td>1.69x10^-6</td>
<td></td>
</tr>
<tr>
<td>rs2318784</td>
<td>17</td>
<td>49239431</td>
<td>kgp5552211</td>
<td>A</td>
<td>0.170</td>
<td>0.852</td>
<td>0.179</td>
<td>2.34</td>
<td>1.87x10^-6</td>
<td>NME1-NME2</td>
</tr>
<tr>
<td>rs6110809*</td>
<td>20</td>
<td>15851077</td>
<td>rs6110809</td>
<td>A</td>
<td>0.242</td>
<td>0.763</td>
<td>0.161</td>
<td>2.14</td>
<td>2.13x10^-6</td>
<td>MACROD2</td>
</tr>
<tr>
<td>rs4282275</td>
<td>5</td>
<td>53747307</td>
<td>kgp7813857</td>
<td>A</td>
<td>0.158</td>
<td>0.856</td>
<td>0.181</td>
<td>2.35</td>
<td>2.28x10^-6</td>
<td></td>
</tr>
<tr>
<td>rs76429681</td>
<td>2</td>
<td>83507626</td>
<td>kgp6559527</td>
<td>A</td>
<td>0.895</td>
<td>-0.957</td>
<td>0.204</td>
<td>0.38</td>
<td>2.75x10^-6</td>
<td></td>
</tr>
<tr>
<td>rs9560142</td>
<td>13</td>
<td>112272866</td>
<td>kgp662896</td>
<td>T</td>
<td>0.322</td>
<td>-0.867</td>
<td>0.185</td>
<td>0.42</td>
<td>2.93x10^-6</td>
<td></td>
</tr>
<tr>
<td>rs117168488</td>
<td>17</td>
<td>49246495</td>
<td>kgp10832184</td>
<td>A</td>
<td>0.102</td>
<td>0.973</td>
<td>0.210</td>
<td>2.64</td>
<td>3.58x10^-6</td>
<td>NME1-NME2</td>
</tr>
<tr>
<td>rs219341</td>
<td>14</td>
<td>60451770</td>
<td>rs219341</td>
<td>A</td>
<td>0.974</td>
<td>-2.121</td>
<td>0.463</td>
<td>0.12</td>
<td>4.74x10^-6</td>
<td></td>
</tr>
<tr>
<td>rs7636326*</td>
<td>3</td>
<td>170236594</td>
<td>kgp12260280</td>
<td>T</td>
<td>0.100</td>
<td>1.023</td>
<td>0.225</td>
<td>2.78</td>
<td>5.32x10^-6</td>
<td>SLC7A14</td>
</tr>
<tr>
<td>rs10889117</td>
<td>1</td>
<td>40471236</td>
<td>rs10889117</td>
<td>T</td>
<td>0.144</td>
<td>0.822</td>
<td>0.184</td>
<td>2.28</td>
<td>8.02x10^-6</td>
<td></td>
</tr>
<tr>
<td>rs74678447</td>
<td>17</td>
<td>49339268</td>
<td>kgp6867329</td>
<td>A</td>
<td>0.926</td>
<td>-1.006</td>
<td>0.226</td>
<td>0.37</td>
<td>8.33x10^-6</td>
<td>UTP18</td>
</tr>
<tr>
<td>rs13270118</td>
<td>8</td>
<td>110698456</td>
<td>kgp9627683</td>
<td>T</td>
<td>0.186</td>
<td>0.764</td>
<td>0.173</td>
<td>2.15</td>
<td>1.01x10^-5</td>
<td>SYBU</td>
</tr>
<tr>
<td>rs12256054</td>
<td>10</td>
<td>130097698</td>
<td>kgp1496492</td>
<td>T</td>
<td>0.196</td>
<td>0.707</td>
<td>0.168</td>
<td>2.03</td>
<td>2.65x10^-5</td>
<td></td>
</tr>
</tbody>
</table>

*denotes SNPs that was chosen for validation. Highly ranked rs175805 was not typed at the validation stage due to failure in designing the probe on Sequenom multiplex, hence proxy of rs175805 i.e. rs6110809 was used for validation genotyping.
Table 30 Top ranking pooled genome-wide association study and validation genotyping in Australian Thyroid-associated Orbitopathy Research discovery cohort

<table>
<thead>
<tr>
<th>SNP name</th>
<th>Location Chr and bp</th>
<th>Genes</th>
<th>GWAS pooled genotyping association (cases=270, controls=142)</th>
<th>Validation individual genotyping association (cases=265, controls=147)</th>
<th>P value adjusting for sex, age, duration of Graves' disease</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs953128</td>
<td>Chr10q21.1 56502386</td>
<td>Intronic PCDH15</td>
<td>A 7.13x10^{-7} 2.40 0.133 0.048 3.35 (1.75, 6.51)</td>
<td>F_A F_U OR_A1 (95% CI) adjusting for sex, age, duration of Graves' disease</td>
<td></td>
</tr>
<tr>
<td>rs2867161</td>
<td>Chr7q11.2 67637342</td>
<td>Intergenic STAG3L4, AUTS2</td>
<td>A 5.94x10^{-8} 2.50 0.135 0.052 2.59 (1.42, 4.72)</td>
<td>1.97x10^{-3}</td>
<td></td>
</tr>
<tr>
<td>rs7636326</td>
<td>Chr3q26.2 170236594</td>
<td>Intronic SLC7A14</td>
<td>T 5.32x10^{-6} 2.78 0.095 0.024 4.34 (1.84, 10.26)</td>
<td>8.15x10^{-4}</td>
<td></td>
</tr>
<tr>
<td>rs13360861</td>
<td>Chr5q12.3 65724966</td>
<td>Intergenic SREK1, MAST4</td>
<td>T 1.02x10^{-7} 2.58 0.087 0.024 3.28 (1.44, 7.47)</td>
<td>4.62x10^{-3}</td>
<td></td>
</tr>
<tr>
<td>rs60457622</td>
<td>Chr3q23 141549404</td>
<td>Intergenic GRK7, ATP183</td>
<td>A 9.28x10^{-8} 2.85 0.085 0.028 3.18 (1.43, 7.09)</td>
<td>4.68x10^{-3}</td>
<td></td>
</tr>
<tr>
<td>rs10266576</td>
<td>Chr7q11.2 67862566</td>
<td>Intergenic STAG3L4, AUTS2</td>
<td>T 4.67x10^{-7} 3.32 0.061 0.014 5.23 (1.75, 15.65)</td>
<td>3.12x10^{-3}</td>
<td></td>
</tr>
<tr>
<td>rs6110809</td>
<td>Chr20p12.1 15851077</td>
<td>Intronic MACROD2</td>
<td>A 2.13x10^{-6} 2.14 0.247 0.120 2.77 (1.77, 4.33)</td>
<td>8.17x10^{-6}</td>
<td></td>
</tr>
</tbody>
</table>

F_A is the minor allele frequency in TO
F_U is frequency of minor allele A1 in Graves' disease without TO control

OR_A1 is the odd ratio of minor allele in cases versus controls.

P values are uncorrected
All 7 SNPs were also assessed in the replication cohorts. The only SNP consistently associated with TO in replication cohorts was rs6110809, located within an intron of *MACROD2* on chromosome 20p12.1 with an age, duration, sex-adjusted OR 1.42 (95% CI 1.04-1.95; p=0.03); after further adjustment for smoking, the OR was 1.35 (95% CI 0.96-1.91, p=0.08). Overall, for the combined discovery and replication cohorts, the *MACROD2* rs6110809 was associated with TO compared to Graves’ disease controls without TO (OR=1.81, 95% CI 1.40-2.34, p=6.78 x10^{-6}) (Table 31). After adjusting for age, duration of Graves’ disease, sex and smoking, the minor A allele was more frequent in TO than in Graves’ disease without TO with OR 1.77 (95% CI 1.34, 2.32), p=4.35 x10^{-5}.  
Table 31 Replication individual genotyping of top ranking single nucleotide polymorphisms from discovery GWAS in replication cohorts and in combined discovery and replication cohorts

<table>
<thead>
<tr>
<th>SNP name</th>
<th>Location Chr and bp</th>
<th>Genes</th>
<th>Replication cohorts association results (Cases=319, controls=220)</th>
<th>Combined GWAS and replication cohorts individual genotyping results (cases=584, controls=367)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>A_F_A, F_U, OR_A1 (95% CI) adjusted for age/sex/duration Graves’ disease, P value adjusted for age/sex/duration Graves’ disease</td>
<td>A_F_A, F_U, OR_A1 (95% CI) adjusted for age/sex/duration Graves’ disease, P value adjusted for age/sex/duration Graves’ disease</td>
</tr>
<tr>
<td>rs953128</td>
<td>Chr10q21.1 56502386</td>
<td>Intronic PCDH15</td>
<td>0.114 0.094 1.29 (0.84, 1.98) 0.24</td>
<td>0.122 0.076 1.78 (1.25, 2.51) 1.37x10^-3</td>
</tr>
<tr>
<td>rs2867161</td>
<td>Chr7q11.2 67637342</td>
<td>STAG3L4, AUTS2</td>
<td>0.093 0.093 0.90 (0.57, 1.42) 0.65</td>
<td>0.112 0.077 1.40 (0.99, 1.98) 0.06</td>
</tr>
<tr>
<td>rs7636326</td>
<td>Chr3q26.2 170236594</td>
<td>Intronic SLC7A14</td>
<td>0.069 0.070 0.99 (0.60, 1.63) 0.97</td>
<td>0.081 0.052 1.59 (1.06, 2.39) 0.03</td>
</tr>
<tr>
<td>rs13360861</td>
<td>Chr5q12.3 65724966</td>
<td>Intergenic SREK1, MAST4</td>
<td>0.104 0.081 1.38 (0.87, 2.19) 0.18</td>
<td>0.096 0.058 1.77 (1.20, 2.60) 3.86 x10^-3</td>
</tr>
<tr>
<td>rs60457622</td>
<td>Chr3q23 141549404</td>
<td>Intergenic GRK7, ATP183</td>
<td>0.057 0.075 0.74 (0.44, 1.25) 0.26</td>
<td>0.070 0.056 1.25 (0.83, 1.89) 0.28</td>
</tr>
<tr>
<td>rs10266576</td>
<td>Chr7q11.2 67862566</td>
<td>STAG3L4, AUTS2</td>
<td>0.041 0.048 0.90 (0.48, 1.70) 0.75</td>
<td>0.050 0.034 1.63 (0.97, 2.74) 0.06</td>
</tr>
<tr>
<td>rs6110809</td>
<td>Chr20p12.1</td>
<td>Intronic MACROD 2</td>
<td>A</td>
<td>F_A</td>
</tr>
<tr>
<td>-----------</td>
<td>------------</td>
<td>------------------</td>
<td>---</td>
<td>-----</td>
</tr>
</tbody>
</table>

F_A is the minor allele frequency in TO

F_U is frequency of minor allele A1 in Graves’ disease without TO control

OR_A1 is the odd ratio of minor allele in cases versus controls

P values are uncorrected
Additional individual genotyping analysis were undertaken to determine if the top ranking SNPs from pooled GWAS in TO versus non-TO in Graves’ disease were associated with TO or Graves’ disease as a whole or the risk variant was specific to TO in Graves’ disease. We found that none of the seven top ranking SNPS were associated with TO when compared to normal individuals or with Graves’ disease when compared to normal individuals. (Table 32) Hence SNP at MACROD2 locus was an association specific to risk of TO in Graves’ disease.
Table 32 Individual genotyping of top ranking single nucleotide polymorphisms from discovery GWAS in TO cohort compared to healthy normal controls adjusted for age and sex.

<table>
<thead>
<tr>
<th>SNP name</th>
<th>Location Chr and bp</th>
<th>A1</th>
<th>F_A</th>
<th>F_U</th>
<th>OR_A1 (95% CI) adj for age/sex</th>
<th>P value adj for age/sex</th>
<th>F_A</th>
<th>F_U</th>
<th>OR_A1 (95% CI) adj for age/sex</th>
<th>P value adj for age/sex</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs953128</td>
<td>Chr10q21.1 56502386</td>
<td>A</td>
<td>0.122</td>
<td>0.114</td>
<td>1.29 (0.73, 2.28)</td>
<td>0.38</td>
<td>0.104</td>
<td>0.114</td>
<td>1.03 (0.61, 1.73)</td>
<td>0.91</td>
</tr>
<tr>
<td>rs2867161</td>
<td>Chr7q11.2 67637342</td>
<td>A</td>
<td>0.112</td>
<td>0.091</td>
<td>0.80 (0.43, 1.48)</td>
<td>0.47</td>
<td>0.098</td>
<td>0.091</td>
<td>0.88 (0.50, 1.55)</td>
<td>0.67</td>
</tr>
<tr>
<td>rs7636326</td>
<td>Chr3q26.2 170236594</td>
<td>T</td>
<td>0.081</td>
<td>0.061</td>
<td>1.19 (0.59, 2.39)</td>
<td>0.63</td>
<td>0.070</td>
<td>0.061</td>
<td>1.25 (0.66, 2.35)</td>
<td>0.50</td>
</tr>
<tr>
<td>rs13360861</td>
<td>Chr5q12.3 65724966</td>
<td>T</td>
<td>0.096</td>
<td>0.065</td>
<td>1.56 (0.81, 3.01)</td>
<td>0.19</td>
<td>0.082</td>
<td>0.065</td>
<td>1.46 (0.80, 2.69)</td>
<td>0.22</td>
</tr>
<tr>
<td>rs60457622</td>
<td>Chr3q23 141549404</td>
<td>A</td>
<td>0.070</td>
<td>0.079</td>
<td>0.84 (0.43, 1.63)</td>
<td>0.61</td>
<td>0.064</td>
<td>0.079</td>
<td>0.75 (0.41, 1.36)</td>
<td>0.34</td>
</tr>
<tr>
<td>rs10266576</td>
<td>Chr7q11.22 67862566</td>
<td>T</td>
<td>0.050</td>
<td>0.052</td>
<td>0.79 (0.33, 1.91)</td>
<td>0.60</td>
<td>0.044</td>
<td>0.052</td>
<td>0.67 (0.30, 1.47)</td>
<td>0.32</td>
</tr>
<tr>
<td>rs6110809</td>
<td>Chr20p12.1 15851077</td>
<td>A</td>
<td>0.243</td>
<td>0.175</td>
<td>1.48 (0.92, 2.38)</td>
<td>0.10</td>
<td>0.211</td>
<td>0.175</td>
<td>1.36 (0.89, 2.08)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

F_A is the minor allele frequency in cases
F_U is frequency of minor allele A1 in controls
OR_A1 is the odd ratio of minor allele in cases versus controls adjusted for sex and age
$P$ values were uncorrected
Four additional highly ranked SNPs at \textit{MACROD2} gene locus with p<10^{-4} extracted from the discovery pooled GWAS data were also assessed for their linkage disequilibrium to rs6110809 in the Caucasian HapMap population. They are rs978767 (p=1.8x10^{-4}), rs175805 (p=3.05x10^{-7}), rs6135575 (p=4.32x10^{-4}) and rs761684 (p=3.2x10^{-4}). Two SNPs were highly correlated with rs6110809 (rs6135575 and rs761684) and two SNPs showed more moderate linkage disequilibrium with rs6110809 (rs978767 and rs175805) (Figure 13). This suggests the top ranking \textit{MACROD2} SNPs may represent two separate loci within \textit{MACROD2} associated with TO, although further genotyping and analysis are required to confirm this.

\textbf{Figure 13} Haploview linkage disequilibrium plot for 5 highly ranked single nucleotide polymorphisms within \textit{MACROD2} in HapMap CEU population data.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure13.png}
\caption{Haploview linkage disequilibrium plot for 5 highly ranked single nucleotide polymorphisms within \textit{MACROD2} in HapMap CEU population data.}
\end{figure}

\textbf{Discussion}

This genome-wide association study discovered a positive association of SNP on chromosome 20 within the \textit{MACROD2} gene with TO with replication in an independent cohort. The genetic variant appeared specific to the risk for TO in patients with Graves’ disease and not for the risk of Graves’ disease itself, potentially implicating \textit{MACROD2} as a new genetic susceptibility locus for TO.
Multiple genetic loci on chromosome 10, 7, 5 and 3 trended towards positive association with TO but the associations dropped out of significance in subsequent individual genotyping.

This study is the first attempt to characterize the genetic risk factors for TO using a GWAS approach, where associated loci were discovered *a priori* and confirmation of these findings in additional independent cohorts was performed. Whilst none of the SNPs reached genome-wide significance, there was evidence of association of *MACROD2* with TO first reported by this study. The evidence of association with *MACROD2* stays true overall with adjustment for potential confounders age, duration of Graves' disease, sex and smoking in the combined dataset, bearing in mind that the smoking data is absent in 10.9% of the replication cohorts, and 6.6% in the combined cohorts dataset which might affect the final estimation of odds ratio and its significance level when smoking was taken into consideration. Hence we have presented the data in this study for confounder adjustments with and without smoking and found p values slightly increased after smoking adjustment. A further limitation of this study is the use of pooled DNA, which although highly economical, reduces the ability to correct for confounders in the GWAS stage, or to assess population substructure, which may lead to false positive findings. While we and others find limited evidence for genes with major effects affecting risk for TO, the number of cases studied in this study is still relatively small, limiting the power and certainty of the association found. Much larger samples will be required to confirm our findings and to prove genetic associations in TO. Nevertheless the identification of genetic variants even of modest effects will improve our understanding of pathogenesis of TO and identify pathways for therapeutic considerations. In the context of these provisions, we will discuss the results of interest from this study that are potentially relevant to the genetic risk of TO.

The odds ratio for rs6110809 A allele in predicting risk for TO ranges between 2.77 for the pooled discovery cohort and 1.42 in the replication cohorts, with an odds ratio of 1.81 for the extended cohort when all cohorts were combined after adjusted for age of disease onset, duration of Graves' disease and sex. The odds ratio is notably smaller in the replication cohorts, which is consistent with “the winner's curse” where locus-specific size estimate bias upwards in GWAS.\(^{339}\)
Hence if the association of rs6110809 is true, the estimated conferred risk is more realistic from the replication samples, where the estimated effect size is also more in line with unbiased estimates of odd ratios discovered by GWAS studies around 1.1-1.3.\textsuperscript{340}

The MACROD2 gene encodes a protein with a highly conserved macrodomain binding module in the N-terminal domain that interacts with mono-ADP-ribosylated proteins.\textsuperscript{341, 342} There are no existing data as to whether MACROD2 is expressed in the orbital adipocytes or myofibroblasts, and further studies are required to examine this. However, MACROD2 is expressed strongly in the brain, and is moderately to strongly expressed in cuboidal epithelium of the lens and the inner nuclear layer of the retina.\textsuperscript{341} The association of MACROD2 with eye diseases has not been previously reported, but genetic variants in MACROD2 have been reported to be associated with neuropsychiatric disorders. De-novo deletion of exon 5 within MACROD2 was identified in Kabuki syndrome, where the index case suffered from mental retardation and had ophthalmic features including ptosis, hypermetropic astigmatism, alternating strabismus and blue sclera.\textsuperscript{341} MACROD2 allelic variants and common copy number gain has been linked to several diseases.\textsuperscript{340, 343-345} Recent GWAS identified SNPs within MACROD2 as a risk variant in autism spectrum disorder,\textsuperscript{340} and possibly a protective factor for MRI-defined brain infarcts.\textsuperscript{343} A candidate gene study investigating autistic-like traits also reported an association between rs4141463 SNP in intron 5 of MACROD2.\textsuperscript{344} Overexpression of MACROD2 mediates oestrogen-independent growth and tamoxifen resistance in breast cancer, suggesting the expressed gene confers advantage to cellular survival.\textsuperscript{345}

The MACROD2 protein binds to mono-ribosylated proteins and its catalytic domain functions as O-acetyl-ADP-ribose deacetylases and hydrolases which reverses mono-ADP ribosylation,\textsuperscript{342, 346} thus underlying the key role of MACROD2 as an eraser of mono-ADP-ribosylation.\textsuperscript{347} The function of macrodomain molecules in general are tightly linked to ADP-ribosylation that modulate functions of protein by post-transcriptional modification. The addition of ADP-ribose to proteins affects diverse cellular processes including DNA repair, chromatin remodeling, gene transcription, lipid metabolism and apoptotic processes.\textsuperscript{347,349} To understand the
The role of MACROD2 in disease, we need to understand the function of intracellular ADP-ribosyltransferase Cholera toxin-like (ARTD) which inserts ADP-ribose monomers to protein that MACROD2 erases. ARTD10 regulates cell signaling, represses nuclear factor-κβ by mono-riboylation of NF-κβ essential modulator (NEMO), where NF-κβ signaling pathway promotes inflammation, innate immune response, cellular proliferation and cell survival.347,349-352

Overexpression of ARTD10 promotes apoptosis in HeLa cells whereas ARTD10 knockdown increases cell survival upon DNA damage caused by DNA damaging agents.353 ARTD10 is inducible by inflammatory and immunogenic stimuli, hence suggesting its role in innate immunity.347 ARTD10 also inhibits glycogen synthase kinase 3β (GSK3β), a key enzyme which modulates WNT signaling via β-catenin phosphorylation that regulate apoptosis, immunity and development of neurodegenerative diseases.342,347,354,355 Hence inhibition of both GSK3β and NF-κβ signaling by ARTD10 may explain enhanced apoptosis and MACROD2 on the other hand counteracts the actions of ARTD10.

The role of MACROD2 in modulating DNA repair is yet unknown, but it is recruited to poly-ADP-ribose synthesized by ARTD1 in response to DNA damage.349 MACROD2 removes the most proximal ADP ribose linked to the target amino acid after partial breakdown of poly-ADP-ribosylation by poly-ADPr-glycohydrolase to fully reverse ARTD1 regulatory modification.356 This functional link of MACROD2 to ARTD1 becomes more noteworthy in light of the findings of carriers of G allele of ARTD1 G1672A polymorphism are at risk of Graves' disease and a positive association of ARTD1 C410T polymorphism with TO confers an increase risk of TO by 1.7 times.200

MACROD2 and ARTD10 regulation of apoptosis and NF-κβ signaling may be relevant to the mechanism of immune-mediated orbital inflammation in TO. Gene set enrichment analysis of molecular signatures of TO revealed dysregulation of epigenetic signatures, T cell activation, Th1 differentiation, defensin pathway, apoptosis amongst a few in active TO.357 Dysregulation of WNT signaling was also evident in TO from microarray studies.74,98 Orbital inflammation in TO is mediated by communication of orbital fibroblast and fibrocytes with immune cells via CD40-CD40 ligand. This is inducible by interferon-γ, evidenced by nuclear translocation.
of NF-κβ and increase secretion of pro-inflammatory cytokine interleukin (IL)-6 and IL-8 upon triggering of CD40 bearing orbital fibroblast by CD40 ligand.\(^{61, 65}\) CD40 ligand significantly increases intercellular adhesion molecule-1 (ICAM-1) protein in a dose- and time-dependent manner, where ICAM-1 plays a role in initiating and sustaining inflammatory immune response.\(^{60}\) The signaling pathways of increased ICAM-1 involve both mitogen activated protein kinases and NF-κβ; CD40 ligand at 100ng/ml induced a moderate activation of NF-κβ, which increases in a time-dependent manner.\(^{60}\) In addition polymorphisms of del/ins of \(NFκβI\) gene may be related to the development of TO, further supporting the role of NF-κβ in TO pathogenesis.\(^{200}\) Over-expression of MACROD2 is expected to enhance NF-κβ signaling and increase cell survival, cell proliferation, inflammation and immune response, mechanisms all relevant to the pathogenesis of TO. The functional study of MACROD2 is beyond the scope of this study; this study provided grounds for further candidate gene study and future functional studies looking at expression levels of MACROD2 in orbital fibroblasts culture, and it’s effects on IL-6, IL-8, ICAM-1, NF-κβ expression levels \textit{in vitro}.

In conclusion this exploratory GWAS study of TO suggests that in patients with Graves’ disease, a common variant at the \textit{MACROD2} locus increases susceptibility to thyroid-associated orbitopathy. This novel finding should prompt further replication studies to confirm its role in the pathogenesis of TO.
15 Concluding Discussion

15.1 Rational for studying genetics of thyroid-associated orbitopathy
Graves’ disease (GD) is an auto-immune thyroid disease that demonstrate strong familial tendency, with the attributable genetic risk estimated at 79%, meaning that environmental factors then account for the remaining 21% of risk for developing GD. Given that GD is partly a genetically determined condition, it is therefore possible that the subset of patients affected by GD may have distinct genetic predisposition for the eye complications. However the genetic contributions to thyroid-associated orbitopathy (TO) susceptibility is largely unknown, and in conjunction with incomplete understanding of the pathogenesis of TO hampers the progress of treatment advancement in TO.

Vast majority of genotyping in common immune regulatory gene variants in HLA I and II, PTPN22 and CTLA-4 thus far found little contribution to TO genetic susceptibility. Earlier genetic study in TO by candidate genes association study for HLA, CTLA-4, TSHR and TNF-β found little genetic contributions to TO when compared to controls except for a weak association of HLA-DR3 and CTLA-4 for TO, which were inseparable from the genetic risk for GD; the segregation ratio for TO in nuclear families was zero from the same study suggesting limited genetic risk in TO. The same research group repeated the genetic study using candidate genes association study 12 years later in a larger cohort of TO patients (n=256) and GD controls (n=90) and genotyped for HLA, CTLA-4, IL-23R and TSHR and again did not find genetic differences. On the other hand other candidate gene association studies have found variable genetic associations for TO mostly with risk ratio <2 in comparison with GD without TO for common immune regulatory and pro-inflammatory genes in CTLA-4, IL-23R, PTPN12, TNF-α, CD86, NF-κβ1, PARP-1 (also known as ARTD1) and IL-1. Meanwhile environmental risk factors also appear to contribute towards onset of TO, TO was more severe in older individuals and in smokers, and associated variably to radioactive iodine use and poorly controlled hypothyroidism and hyperthyroidism. With the completion of genotyping of the human genome down to minor allele frequency of at least 5% by the international HapMap consortium in 2005, and
increasingly high density single nucleotide polymorphisms (SNPs) typed to the millions in microarray chips, array-based genome wide association studies (GWAS) that relate thousands to millions of SNPs to disease or trait, have unprecedentedly increase the discovery of genetic loci, some still of unknown gene identities, with common diseases and traits, previously not suspected of having a role in the disease. The discovery nature of GWAS, represent an important step forward from candidate gene studies, which are often limited by the number of genetic variants genotyped to at most a few hundreds at a high cost per genetic variant assayed, and limited also by the selection biases of genetic variants for investigations from the incomplete understanding of the pathogenesis of the disease, of which TO is a good example. Identifying genetic variants in TO can therefore provide important clues to the genetic risk of TO and its pathophysiologic mechanisms, especially when coupled with gene expression profiling using high throughput array technology that can demonstrate differential gene expressions, functional annotation of genes, gene-gene interactions and molecular pathways involved.

This thesis is designed to investigate if genetic susceptibility is present in TO by using discovery GWAS in a case-control study design for GD patients with and without TO, followed by validation of the top ranking SNPs by individual genotyping and replication study. This thesis represents the first GWAS in TO, a new milestone for the genetic study of TO. Environmental risk factors known to be important determinants for TO were incorporated into the genetic analysis to increase the strength of the genetic findings. Finally, the thesis hypothesized that if there was an important genetic contribution to TO, the genetic findings will correlate with gene expression in TO in the active phase when compare to inactive TO or normal unaffected individuals, or link in some ways to the molecular signatures.

15.2 Overview of findings from the thesis

15.2.1 Exogenous risk factors
The strength of the exogenous risk factors association study in this thesis is based upon a large sample population of well-characterized GD patients with and without TO in a relatively uncommon disease, this allowed for a well powered
association study for confirmation of characteristics and environmental risk factors for TO. The sample population is from the tertiary hospitals and related private practices of ophthalmologists and endocrinologists, hence the true population that the samples inferred to likely represents the more severe spectrum of TO and GD that require specialists management. The sample population inferred that the onset of TO frequently occur within the same year as GD, TO was diagnosed at a mean duration of 1.5 years later than the diagnosis of GD. The mean age of onset of TO was 2.5 years older than GD without TO. The proportions of current and ex-smokers were greater in TO compared to non-TO and the mean cumulative lifetime cigarette consumption was also higher in TO compared to non-TO. The duration of having a diagnosis of GD was longer in TO cases than GD without TO controls by 3.8 years. Considering the best multiple logistic regression model, the study found older age, longer duration of having diagnosed with GD, being a current smoker or ex-smoker are the significant exogenous risk factors for TO. The gender ratio did not differ in TO cases and non-TO controls, and the association of TO with radio-active iodine treatment was weak after correcting for all exploratory factors. As we do not have sufficient data on T3, T4 and TSH levels recorded at recruitment, and there were missing data for TSH receptor antibody level, the study cannot comment on the relationship between TO status with thyroid dysfunction or TSH receptor levels with certainty.

Sampling of selenium levels in TO and non-TO GD patients further provided insights on the redox status in TO. The study found GD cohort as a whole has marginally normal level of serum selenium levels, the TO cases’ selenium level was significantly lower than the non-TO controls, and there appeared to be a graded reduction in serum selenium level with increasing severity of TO. Correspondingly in the gene expression profiling of orbital adipose, glutathione peroxidase 3 (GPX3) was amongst the top differentially expressed genes and was upregulated in active TO by 1.83-fold compared with inactive TO. GPX3 is a major oxidation-reduction enzyme that neutralises reactive oxygen species and hydrogen peroxide, and it is a selenoprotein. The combination findings from the selenium status study showing relative selenium deficiency in TO and the gene expression profiling study showing up-regulation of selenoprotein with crucial role in redox reaction support the use of selenium supplementation in active TO.
In the context of genetic study, at the very least the age of diagnosis of GD and TO, duration of GD and smoking status are environmental risk factors to be taken into consideration as covariates when analyzing genotyping frequency.

15.2.2 Genome-wide association study methodology and genetics findings
The pooling of blood and DNA methodology employed by the GWAS approach in this thesis is unique in a number of ways. Usually arrays were run and analyzed for every individuals, for thousands of samples the cost of genotyping is still relatively prohibitive for many research centers. Equimolar DNA pooling strategically reduce the arrays to two, whereas pooling of blood methodology further reduces the time consumed for DNA extraction and pooling constructions.\textsuperscript{313} The simplicity of the blood pooling method does not seem to detract from the effectiveness of detecting genes of smaller effect sizes in large pools, and has the real advantage of expediting genetic discovery in a cost effective way, as exemplified by validating genetic variants known to be associated with age-related macular degeneration, eye colour and pseudo-exfoliation.\textsuperscript{313}

As a proof of principle, the thesis examined the genetic variants using pooling GWAS in GD. The pooling GWAS readily identified multiple loci within the MHC locus on chromosome 6 in the top ranking SNPs, with the smallest $P$-value exceeding genome wide significance, and known variants HLA-\textit{DQA1}, \textit{C6orf10} approaching genome wide significance. With the relatively small sample pools for GWAS (N=412 for Graves' disease and N=498 for normal controls), the study found evidence of positive association with rs17676303, in the upstream region of \textit{FCRL3} on chromosome 1 across the discovery cohort, replication cohort and in the combined cohorts in the second stage individual genotyping. Both the MHC loci and \textit{FCRL3} locus have been previously identified as genetic variants in Graves’ disease in multiple large-scale GWAS studies.\textsuperscript{183, 184, 312} These findings support the hypothesis that pooling GWAS is a cost effective methodology for discovery common genetic variants in Graves’ disease.

When pooling GWAS was applied to the genetic study of TO, none of the top ranked SNPs reached the genome-wide significance threshold of $5 \times 10^{-8}$. Out of the top ranked SNPs close to genome-wide significance ($p \leq 10^{-6}$), rs6110809 within intron of \textit{MACROD2} on chromosome 20 showed evidence of association with TO in
validation genotyping. The positive association of TO with MACROD2 was replicated in an independent replication cohort. The minor A allele in MACROD2 was more frequent in TO than non-TO with GD with an odds ratio of 1.77 and p at 4.35 x10^-5 for the combined discovery and replication cohort (N=584 cases, N=367 controls) in individual genotyping after adjusting for age, duration of Grave's disease, sex and smoking. In the replication cohort the minor A allele predicted the odds for TO is 1.35 higher compared to non-TO after adjusting for age, duration of Graves’ disease, sex and smoking.

MACROD2 as a genetic variant in TO was first implicated by GWAS a priori. The genetic effect is small with an overall odds ratio of less than 2 in TO compared to non-TO with GD, the level of statistical significance is tantalizingly close. This finding from the Australian population is applicable to GD patients of western and northern European origin, as the patterns of linkage disequilibrium and performance of chosen tag SNPs in the Australian sampling from Tasmania and Victoria and the HapMap Utah residents of CEU samples are similar.\textsuperscript{359} Functionally MACROD2 is an eraser of mono-ADP ribosylation that modulates function of proteins by post-transcriptional modification.\textsuperscript{349} In theory MACROD2 could be involved in the pathogenesis of TO as MACROD2 affects the function of ARTD10, which affects nuclear factor-κβ(NF-κβ) signaling and apoptosis.\textsuperscript{347} NF-κβ signaling is known to be involved in regulating inflammation and cellular proliferation in TO.\textsuperscript{60, 65} MACROD2 is also functionally linked to ARTD1, a poly-ADP-ribose polymerase that modulates pro-inflammatory genes like interleukin, response to oxidative stress, DNA repair, NF-κβ signaling.\textsuperscript{200, 349, 356} Importantly positive association of ARTD1 polymorphism has also been identified in TO and GD by candidate gene study.\textsuperscript{200} Functional studies such as expression of MACROD2 in TO, MACROD2 effects on orbital fibroblast proliferation, inflammatory cytokine levels and NF-κβ signaling is now worth exploring in order to understand the role of MACROD2 in the pathophysiology of TO. This could be a focus of future studies.

For a disease with complex inheritance, multiple genes usually of small effect sizes contribute towards the genetic susceptibility. This thesis did not find evidence for genes with major effects affecting the genetic risk of TO. The thesis also did not find compelling evidence to disproof the null hypothesis that there is no difference
in genetic polymorphisms between TO and non-TO in GD. One of the limitations of the GWAS in TO is the relatively small discovery cohort at the time of DNA pooling, which limits the certainty of the association found. It is difficult to ascertain if the SNPs associations trending towards genome-wide significance are due to insufficient power to prove a weak genetic effect, and power calculation for high through-put genotyping is almost impossible to obtain, or the results obtained is truly negligible. The evidence of association with MACROD2 with TO cannot be disregarded without a real risk of committing a type II error in hypothesis testing, given that pooling GWAS in this study has shown evidence of association of FCRL3 with GD, a known association with GD, also showing trend towards genome-wide significance. To confirm genetic association between TO and MACROD2 in the future, a much larger sample size preferably in the thousands for GWAS is more likely achievable by multinational collaboration in a consortium set-up.

Due to the inherent nature of pool construction in pooling GWAS, allele genotyping only allow for comparison of mean allele frequency, and covariates cannot be adjusted for in pooled genotyping analysis. This limitation is counteracted in the second stage of study by making adjustment to the allele individual genotyping results by covariates analysis in age, duration of Graves’ disease, gender and smoking. We found that after adjustment for age, duration of Graves’ disease and gender the genotyping results are fairly similar. When smoking status was further adjusted for, odds ratio for MACROD2 were slightly reduced (still less than 2) for the replication cohort and the combined cohorts, with P values slightly increased.

15.2.3 Gene expression profile in TO and correlation with genetics and epigenetics

The discovery of over-expression of TIMD4, DEFA3, DEFA1 and DEFA1B, up-regulation of adipogenesis markers and adipogenesis regulatory genes, up-regulation of other cell-mediated and innate immune response genes and gene sets enriched with epigenetics signatures are the most important findings arising from the differential gene expression study in active TO. TIMD4 is exclusively present on antigen presenting cells that recognizes phosphatidyl serine, a cell surface marker of apoptosis, and plays a role in phagocytosis of apoptotic cells and in inducing immune tolerance. α-defensins form part of the innate immune system and are involved in pro-inflammatory response, chemotaxis in monocytes and T
cells and in promoting adaptive immune responses. In addition, multiple cell-mediated immune genes such as CD247, CD3D, CD8A, CST7, GZMA, CAMP, SLAMF6, EOMES, LTB and CCL5 were also up-regulated in active TO compared to inactive TO. Active TO was characterized by up-regulation of cell adhesion and cytoskeletal organization pathways, T cell signaling pathways, IL-12 dependent Th1 development, defensin and natural killer cell response, and apoptosis compared to inactive TO. Furthermore active TO in comparison to normal showed up-regulation of gene sets involved in lipid metabolism: WNT non-canonical pathway, SREBF, PPAR and insulin signaling pathways. Gene sets controlling cellular proliferation and cell cycling were down-regulated.

The gene expression profiling study did not find differential gene expression for MACROD2 in active TO either compared to inactive TO or normal control, nor did the study found dysregulation of CD-20, IL-1, IL-6, IL-8, IGF-1R which were previously implicated in the pathogenesis in TO by bench experiments or targeted for novel treatments. TSHR was lowly ranked at position 715th for differentially expressed genes in active TO compared to inactive TO and was down-regulated in TO by 1.75 fold with marginal significance (false discovery rate 0.05).

The strength of the gene expression profiling in TO lies in the use of large number of tissue samples and high-density array platform, which generated extensive gene expression data. The validity of the microarray differential gene expression findings was well founded. The microarray findings were confirmed by real-time reversed transcriptase polymerase chain reaction, the fold changes and direction of expression of chosen genes were very closely correlated; the functional gene annotations between separate bioinformatics analysis was also well correlated.

The major advantage of studying gene expression in the active phase of TO is the study design enables detection of genes involved in the molecular processes in real time when active orbital inflammation is present. This point is illustrated by the study when exploring correlation of each gene expression with the effects of graduated changes in orbital inflammation. In summary genes found to be up-regulated in active TO were positively correlation with severity of inflammation, where those down-regulated in active TO were negatively correlated.
In terms of gene set enrichment analysis that delve deeply into functional connectedness between expressed genes, the study found a number of epigenetic signatures dysregulated in active TO, either when comparing to inactive TO or normal control. These include up-regulation of gene clusters in immunodeficiency signaling, cytotoxic T cell mediated apoptosis, T cell receptor hypomethylation when active TO was compared to inactive TO, down-regulation of unmethylated histone H3 in genes with high-CpG-density promoters (HCP) in embryonic fibroblast, downregulation of histone H3 dimethylation at K4 and trimethylation at K27 in brain and downregualtion of genes methylated aberrantly in colon cancer cells when active TO was compared to normal control. For the very first time, epigenetic factors was implicated in the pathogenesis of TO. Future research to characterize epigenetic mechanism in TO is advocated.

15.3 Implications for novel therapy in TO
This thesis has enriched our understanding on the molecular mechanisms in TO on many levels. By the in-depth study of gene expression, this thesis has allowed us to identify possible molecular targets for the treatment of TO. Given that immune response genes were over-expressed, novel therapy could be designed as specific immune-suppression therapy, or immune-modulator to induce immune tolerance. TIMD4 over-expression on antigen presenting cells was associated with reduced T cell response in transgenic mice, and helped maintain a skewed Th2 response in allergy. With TIMD4 noted to be over-expressed in active TO, it’s role in inducing immune tolerance or as a switch to inactive phase of TO with Th2 dominance will need further investigations.

The findings of this thesis suggest activation of T cells and T cell signaling play a major role in cellular immune response in TO. Anti-CD3 monoclonal antibody including otelixizumab and teplizumab are potentially useful in TO. Anti-CD3 monoclonal antibody reached phase III trial in type 1 diabetes, and preliminary phase II trial showed promising results with induction of immune-tolerance at least for 24 months after a short course of treatment. Monoclonal antibody directed against IL-2R, the CD25 molecule, on the surface of activated T cells such as basiliximab and daclizumab potentially could be useful in reducing T cell
proliferation in the orbit, both drugs have shown equal efficacy and safety profile in kidney transplant patients.\textsuperscript{361}

\textit{LTB} was up-regulated in active TO. \textit{LTB} is also known as tumour necrosis factor superfamily member 3 and as such is pro-inflammatory. Hence there is a role for TNF-antagonist such as adalimumab, infliximab in the treatment of TO. Although \(\alpha\)-defensins and several other innate immune response genes were over-expressed in TO suggesting the innate immune system as a potential target of treatment, there is no specific therapeutic agent yet approved for clinical use, and the risk of disrupting first line immune defense needs to be carefully considered.

Genes involved in adipogenesis showed increased expression in active TO including the regulatory transcription factor \textit{SREBF1}. One potential target for treatment is to counteract up-regulated adipogenesis by developing intracellular inhibitor for adipogenic transcription factors. The other novel therapy to consider is topical prostaglandin analogues such as bimatoprost or travaprost which have been known to cause reversible prostaglandin-associated periorbitopathy, the mechanism may directly lead to inhibition of adipogenesis through prostanoid receptors.\textsuperscript{362} The use of topical prostaglandin analogues in TO has never been studied before and this would warrant further investigation.

\subsection*{15.4 Future Directions}

Gene expression studies in TO will benefit from more sophisticated technology such as RNA sequencing to investigate the molecular pathways and the relationship of pathways in the context of co-expression network. An integrated analysis with epigenomic and genomic data using next generation sequencing will be the future for investigating the genetics of TO and help us deeply understand its pathophysiology.

The completion of 1000 genomes project (1KGP) has laid a strong foundation for future GWAS studies.\textsuperscript{363} Using whole genome sequencing, the 1000 genome project data allow detection of almost all minor genetic variants at a frequency greater than 1\%, the estimated power to detect SNPs at 1\% frequency is 99\%.\textsuperscript{363} At the time of completion of the GWAS project of TO in this thesis, the 1KGP population genome data has just become available and it is on this population genomic database that the genotyping analysis was based upon. The location, allele
frequency and haplotype structure fine mapping of the human genome in 1KGP database is unprecedented, with validated genotyping of 38 million SNPs, 1.4 millions short insertions and deletions and 14,000 larger deletions. Using exome sequencing, for the first time low-frequency to rare variants with minor allele frequency at 0.1% to 1% can be compared with increased certainty, allowing studying of low frequency and rare variants; the power to detect minor allele at 0.1% frequency is more than 90%. The implication is that an alternative common disease/rare variants hypothesis can now be investigated, where rare variants are postulated to be functionally deleterious and displayed higher penetrance in causing disease, each variants exhibit high allelic heterogeneity and mutation rate, and in combination has high total frequency leading to pathology.

To accurately identify the causal relationship between low-frequency variants with disease, the pre-requisite for future GWAS is ever larger number of samples, which may mean the need to conduct meta-analysis to increase statistical power; and well-characterized samples to reduce phenotypic heterogeneity that will reduce power. Other complexities such as data quality, batch effects, relatedness of samples and genetic outliers also needs to be accounted for.

Next generation sequencing (NGS) is a high-throughout technology that is highly sensitive and cost-effectively in generating the entire genome sequence, and it has taken the study of genomics, transcriptomics and epigenetics into the next generation of genetic and molecular researches. The next generation sequencing platform focused on library template preparation, sequencing, signal detection, alignment of read and data analysis by specialized software packages. The NGS technology is advantageous compared to microarray platform given the higher level of resolution down to the single bases, low level of background noise, and in the context of transcriptomic studies the NGS technology is not reliant on known genomic sequence in detecting transcripts and has an impressive dynamic range in quantifying gene expression as compared to microarray. Hence the future of GWAS is likely NGS-based as the cost of sequencing becomes cheaper and the technology is made more widely available.

Depending on the target enrichment strategies, selective regions of DNA can be isolated for sequencing. Whole exome sequencing serves to discover coding region
of the genome with an obvious advantage of allowing high coverage of disease causing mutations and in-depth sequencing in the targeted region with reduced cost, which comes with the caveat that only 1% of the whole genome is sequenced; the untranslated region, promoters and other functioning regions could be missed. Targeted DNA sequence capture is useful for determining DNA sequence variation, and is most useful in the context of GWAS in deep re-sequencing of the most strongly associated variant at the locus associated with disease, in order to find the likely causal genetic variants for prioritization of subsequent functional studies.

Developed in parallel with whole genome sequencing, is RNA sequencing (RNA-Seq), the state-of-the-art for transcriptomic study. Genome-wide expression analysis is crucial to the understanding of key molecular mechanisms in diseases and in correlating genotype to phenotype expression. RNA-seq is gradually replacing microarray in high throughput gene expression studies because RNA-seq can quantify the abundance levels and relative changes of each transcript, can more fully quantify both coding and non-coding RNAs including small RNAs, can map out transcription initiation sites, is strand-specific, can characterize alternative splicing patterns and allows gene fusion detection. Direct RNA sequencing is an emerging RNA-seq technology that require only femtomole quantities of RNA, and by not requiring the intermediate steps of cDNA synthesis, biases introduced by cDNA synthesis, end repair, ligation and amplification procedures will be eliminated. However many challenges remain before direct RNA sequencing is used widely, limited by read quantities and error rates. The massive data generated by RNA-seq requires an equally sophisticated computational program for data quality assessment and pre-processing, reads mapping, and choosing an appropriate algorithm for analyzing differential expression, alternative splicing and variants detection, and this is an area of considerable growth. Previous pathway analysis tool and co-expression network analysis algorithm have been adapted from microarray data analysis or developed new for RNA-seq data, refinement of methodologies for integrated analysis of gene expression is required to extract biological meaning.
The study of epigenetics complements genomic study in understanding heritability of disease. Epigenetics is the study of the regulation of gene expression that is heritable that does not involve changes in DNA sequence, through DNA methylation and post-translational modification of chromatin. The study of epigenetics is still predominated by the use of methylation arrays, because the array technology is considered robust and cost-efficient in this area; a current standardized platform is the Infinium Human-Methylation450 BeadChip Kit (450K). Over the last 5 years, enrichment for methylated DNA by various approaches such as enzyme digestion, affinity enrichment and bisulfite sequencing leads to exponential growth of whole genome methylation studies. Bisulfite sequencing in particular have a high resolution and provide both qualitative and quantitative assessment of the methylome; reduced representation bisulfite sequencing (RRBS) and whole genome bisulfite sequencing (WGBS) are both superior in terms of its massive capacity for reads per sample, single nucleotide resolution and extensive coverage for CpG islands.

15.5 Conclusion
Overall the genetic study of TO found limited evidence for susceptible genes with major effects in TO. This discovery of MACROD2 as a potential TO susceptible gene, together with it’s functional link to NF-κβ signaling should prompt future research to replicate the MACROD2 association with TO in another study population, and to characterize it’s functional effects using experimental studies. The findings from this thesis also contribute significantly to our understanding of the gene expression in TO. Multiple genes TIMD4, DEFA1, DEFA1β and DEFA3 are highly expressed in active TO, concurrently up-regulation of multiple innate and cell mediated immune genes that were novel and for the first time were differentially profiled in TO. Not only do we begin to understand the importance of cell adhesion, cytoskeletal organization, T cell signaling, Th1 differentiation, defensin, natural killer cell response and apoptosis regulation in TO, the gene expression findings also directly inform the choices of immune modulating therapy worth investigating in TO. Epigenetic mechanisms might have a role in the pathophysiology of TO, this is first implicated in TO in the transcriptomic study, and is now worth exploring through epigenetic study. Lastly, multiple environmental factors were associated with TO in this study population including
smoking status, age, duration of GD and selenium status, findings suggesting that
gene-environment interactions for TO may have ubiquitous effects on the
phenotypic expression of TO, requiring careful consideration for future genetic
studies of TO.
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Appendix A: Publication “Pathogenesis of thyroid eye disease: review and update on molecular mechanisms”

Title: Pathogenesis of thyroid eye disease: review and update on molecular mechanisms

Authors:

Jwu Jin Khong\(^{1,2,6}\) FRANZCO, MMed, MBBS(Hons)

Alan A McNab\(^{2,7}\) FRANZCO, DMedSc

Peter R Ebeling\(^{1,5}\) FRACP, MD, MBBS

Jamie E Craig\(^{3}\) FRANZCO, DPhil, MBBS(Hons)

Dinesh Selva\(^{4}\) FRANZCO, FRACS, MBBS(Hons)

Author Affiliations:

1. North West Academic Centre, The University of Melbourne, Western Hospital, St Albans, Victoria, Australia
2. Orbital Plastics and Lacrimal Unit, Royal Victorian Eye and Ear Hospital, East Melbourne, Victoria, Australia
3. Department of Ophthalmology, Flinders University, Flinders Medical Centre, South Australia, Australia
4. South Australian Institute of Ophthalmology, University of Adelaide, South Australia, Australia
5. Department of Medicine, School of Clinical Sciences, Monash University, Clayton, Victoria, Australia
6. Austin Health, Department of Surgery, University of Melbourne, Heidelberg, Victoria, Australia
7. Centre of Eye Research Australia, University of Melbourne, East Melbourne, Victoria, Australia

Corresponding author:
Dr Jwu Jin Khong
Orbital Plastics and Lacrimal Unit
The Royal Victorian Eye and Ear Hospital
East Melbourne, VIC 3002, Australia
Telephone: +61 3 9929 8666
Fax: +61 3 9973 1421
Email: jwujinkhong@gmail.com

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Dr Jwu Jin Khong, Dr Alan McNab and Dr Dinesh Selva has made substantial contributions to the perspectives on the topic, structure of the review paper, intellectual content, writing and revision of the review paper.

Dr Jamie Craig and Dr Peter Ebeling has made substantial contribution to the intellectual content in the revision of the review paper.
Abstract

Orbital changes in thyroid orbitopathy (TO) result from de novo adipogenesis, hyaluronan synthesis, interstitial oedema and enlargement of extraocular muscles. Cellular immunity, with predominantly CD4+ T cells expressing Th1 cytokines, and over-expression of macrophage derived cytokines, perpetuate orbital inflammation. Orbital fibroblasts appear to be the major effector cells. Orbital fibroblasts express both thyrotropin receptor (TSHR) and insulin-like growth factor-1 receptor (IGF-1R) at higher levels than normal fibroblasts. TSHR expression increases in adipogenesis; TSHR agonism enhances hyaluronan production. IGF-1R stimulation leads to adipogenesis, hyaluronan synthesis, and production of the chemokines, IL-16 and RANTES (Regulated on Activation, Normal T Cell Expression and Secreted), which facilitate lymphocyte trafficking into the orbit. Immune activation uses a specific CD40:CD154 molecular bridge to activate orbital fibroblasts, which secrete pro-inflammatory cytokines including IL-1β, IL-1α, IL-6, IL-8, MCP-1 and TGF-β, to perpetuate orbital inflammation. Molecular pathways including adenylyl cyclase/cAMP, PI3K/AKT/mTOR, MAPK are involved in TO. The emergence of a TO animal model and a new generation of TSHR antibody assays increasingly point towards TSHR as the primary autoantigen for extrathyroidal orbital involvement. Oxidative stress in TO resulting from imbalances of the oxidation-reduction state, provides a framework of understanding for smoking prevention, achieving euthyroidism and the use of anti-oxidants such as selenium. Progress has been made in the understanding of the pathogenesis of TO, which should advance development of novel therapies targeting cellular immunity, specifically the CD40:C40 ligand interaction, antibody producing B cells, cytokines, TSHR and IGF-1R.
and its signaling pathways. Further studies in signaling networks and molecular triggers leading to burnout of TO will further our understanding of TO.

**Introduction**

Thyroid orbitopathy (TO) is an autoimmune inflammatory disorder involving the orbit. 90% of patients with TO have Graves’ disease (GD) and are hyperthyroid, 5% are hypothyroid and another 5% are euthyroid. Many patients with TO develop eye symptoms within the first 18 months of autoimmune thyroid disease, with 13% of patients presenting beyond 2 years, and 3% preceding the diagnosis of Graves’ disease by more than 12 months.

In the United States the age-adjusted incidence rate of TO was 16/100,000 population/year in females and 2.9 cases/100,000/year in males. When only moderate to severe TO is considered, the incidence rate reduces to 16.1 cases/million/year, regardless of salt iodination. Predicted prevalence rates of TO are stable across different countries, ranging from 0.1%-0.3%. In newly diagnosed Graves’ disease, 20% have mild and inactive TO, 5.8% present with moderate to severe, active TO and 0.3% develop compressive optic neuropathy in a non-tertiary setting. The evidence is strong for the association of smoking and TO; smokers have an increased risk for TO, and severity of TO correlates with smoking in a dose-dependent manner. In addition, uncontrolled hypo- and hyperthyroidism, and radioactive iodine therapy have been associated with development of TO in clinical studies. Cessation of smoking, achievement of euthyroidism and prophylactic oral prednisolone prior to radio-active iodine therapy in at-risk patients form important preventive steps to control these modifiable risk factors for TO.
Recent advances in transcriptomics and proteomics have brought new insights into the molecular basis of thyroid orbitopathy. These discoveries have lead to the emerging use of monoclonal antibodies and will undoubtedly eventually lead to more specific therapies for this challenging condition. This review explores the underlying molecular mechanisms of TO, highlighting the basis for emergent prevention and treatment options.

**Pathological changes in TO**

Pathological changes of TO in the orbit appear to involve both the extraocular muscles and the orbital fat compartments with computed tomography indicating most patients have a mixture of both extraocular muscle enlargement and orbital fat expansion.\(^{18}\) Proptosis is due to expansion of orbital tissue within the unyielding confines of the bony orbit. The consequent increase in orbital pressure can also lead to venous outflow congestion and chronic periorbital oedema.\(^{19}\)

Histological examination of affected extraocular muscles shows extraocular muscle enlargement is due to deposition of glycosaminoglycan (GAG), predominantly hyaluronan (HA) within the muscles’ endomysial space.\(^{20}\) Total orbital GAG in TO is markedly elevated with significant increase in chondroitin sulfate and HA; correspondingly 24 hour urinary total GAG, dermatan sulfate and HA are also elevated in TO compared to normal controls.\(^{21,22}\) HA from orbital cells is primarily >500 000 daltons high molecular weight polymers.\(^{23}\) As HA is highly anionic, intense water binding leads to pronounced orbital interstitial oedema and extraocular muscle expansion without disruption of muscle fibres.\(^{20,21,23}\)
Furthermore, histology of extraocular muscles shows diffuse and focal lymphocytic infiltrates and fibrosis, whereas orbital fat and connective tissue contains few infiltrating cells. The majority of mononuclear cells are T cells, along with a few B cells, macrophages and mast cells in the intercellular space.\textsuperscript{24,25} Macrophages, monocytes and mast cells are also located in the perivascular interstitial space and in between fibroblast cells with co-localization of platelet derived growth factors in the orbital tissue.\textsuperscript{26,27}

**Effector cell in TO**

Current evidence suggests the orbital fibroblast is the key effector cell in TO.\textsuperscript{28} Not only do orbital fibroblasts proliferate and differentiate into myofibroblasts and adipocytes, they produce GAG in excess, undergo adipogenesis and actively interact with mononuclear cells, produce chemoattractants and cytokines, which ensure perpetuation of orbital inflammation.\textsuperscript{26,29-31} Most of our understanding of orbital fibroblasts in the pathophysiology of TO is derived from *in vitro* culture studies. Orbital fibroblasts and preadipocyte cultures when subjected to differentiation medium underwent adipogenesis with increased peroxisome proliferator-activated receptor-gamma (PPAR-\(\gamma\)) transcripts and lipoprotein lipase (LPL) expression, accompanied by increased HA production and hyaluronic acid synthase 2 (HAS2) mRNA transcripts.\textsuperscript{32} IL-1\(\beta\) and leukoregulin stimulate a marked increase in HA secretion in TO orbital fibroblasts.\textsuperscript{23,33,34} Activated orbital fibroblasts from TO showed a robust response to pro-inflammatory cytokines compared with normal controls and secrete higher levels of pro-inflammatory cytokines including IL-1\(\alpha\), IL-1\(\beta\), IL-6, IL-8, MCP-1, TGF-\(\beta\) when stimulated by cytokines and growth factors.\textsuperscript{35-38}
Heterogenous presentations of TO could be due to cellular divergence of orbital fibroblasts within the orbit.\textsuperscript{31} The fibroblast populations in the orbit are phenotypically heterogeneous, and differ with regards to surface glycoprotein, production of pro-inflammatory cytokines and cell surface receptors.\textsuperscript{36,39,40} The perimysial orbital fibroblasts uniformly express Thy-1, whereas adipose tissue orbital fibroblasts show bimodal distribution of both Thy-1 positive and negative cells.\textsuperscript{39,40} Both Thy-1 positive and negative fibroblasts express high levels of PPAR-\(\gamma\) but only the Thy-1 negative adipose orbital fibroblasts differentiate and accumulate lipid droplets.\textsuperscript{40} On the other hand, only Thy-1+ orbital fibroblasts can differentiate into myofibroblasts on stimulation with TGF-\(\beta\).\textsuperscript{41}

The innate depot differences in fibroblasts may also explain the predilection for orbital and pretibial extra-thyroidal involvement in GD. Adipogenesis and HA synthesis in orbital preadipocytes and fibroblasts is site specific, occurring in both TO and normal controls.\textsuperscript{32} Regional differences exist in basal PPAR-\(\gamma\) expression and responses of human pre-adipocytes to PPAR-\(\gamma\) and retinoid X receptor \(\alpha\) agonists.\textsuperscript{42} Orbital fibroblasts also express considerably higher IL-6 and IL-6 receptor, and prostaglandin E2 (PGE2) than dermal fibroblasts when induced by IL-1\(\beta\) and leukoregulin respectively.\textsuperscript{43,44}

**Molecular mechanisms underlying TO**

The molecular mechanisms whereby recruitment of immune cells into the orbit, the molecular bridge between immune cells and orbital fibroblasts, molecular pathways leading to proliferation and differentiation of orbital fibroblast, secretion of HA,
adipogenesis and perpetuation of orbital inflammation are now better understood. (Figure 1)

**Cellular immunity**

T cell infiltrates in TO orbital tissues are predominantly CD4+, with some studies suggesting presence of both CD8+ and CD4+ T cells.\(^{45-48}\) Th1 like cytokine profile predominates in TO retrobulbar tissue.\(^{45,48}\) Th1 like cytokine expression profile consisting of IFN-\(\gamma\), TNF-\(\alpha\), IL-1\(\beta\) and IL-6 has been detected mainly in TO extraocular muscles, whereas IL-4 and IL-10, Th2 type cytokines were detected predominantly in orbital fat.\(^{38}\) Predominance of T cell subsets is also disease duration dependent, with Th1 cells dominating in the active phase of TO, shifting towards Th2 cells in the late phase.\(^{49}\)

Proliferation of orbital fibroblasts is activated by interaction of autoantigens on the fibroblasts with T cells which involve contact of T cell receptor with MHC II and CD40:CD154 signalling.\(^{30}\) Co-culture of orbital fibroblasts with autologous T cells stimulates production of MHC II molecule and proliferation of orbital fibroblasts in a dose dependent manner; blocking antibodies to MHCII, CD40 and CD40 ligand (CD154) completely inhibit proliferation of orbital fibroblasts.\(^{30}\) CD40 expression is up-regulated in orbital fibroblasts by interferon-\(\gamma\) (IFN-\(\gamma\)) mediated through Jak2.\(^{36}\) Ligation of CD40 with CD154 induces secretion of intercellular adhesion molecule-1 (ICAM-1),\(^{50}\) nuclear translocation of nuclear factor-\(\kappa\)\(\beta\) (NF-\(\kappa\)\(\beta\)),\(^{51}\) IL-6, IL-8 and macrophage chemoattractant protein-1 (MCP-1) in TO orbital fibroblasts compared to normal controls.\(^{36}\) In addition, CD40 upregulates IL-1\(\alpha\) secretion, HA and PGE2 synthesis.\(^{52}\) The molecular signaling triggered by CD40:CD154 ligation involve all three mitogen activated protein kinase (MAPK) pathways, p38, ERK1/2 and JNK, which mediate
cellular activities such as gene expression, cellular proliferation, differentiation and apoptosis. ICAM expression is predominantly P38 MAPK and NF-κβ dependent, whereas ERK1/2 and JNK also activate the NF-κβ pathway, a transcription factor pathway that regulates genes involved in immune and inflammatory responses.50

**Role of cytokines**

Study of the cytokine profile in orbital adipose tissue in TO and normal individuals shows over-expression of IL-1β, TNF-α, IFN-γ, IL-6 and IL-10 which are macrophage-derived and IL-8. IL-1β is expressed the most differentially.37 Similarly active TO patients have higher IL-1β, IL-6, IL-8, IL-10 compared to inactive TO.53 Orbital fibroblasts from TO when stimulated by IL-1β up-regulate secretion of pro-inflammatory cytokines IL-6 and IL-8, PGE2, IL-6R and T cell chemoattractants, IL-16 and RANTES (Regulated on Activation, Normal T Cell Expression and Secreted), which recruit T cells into the orbit.39,43,44,54 IFN-γ upregulates CD40 expression on orbital fibroblasts and fibrocytes.55 IL-6 increases the expression of TSHR in orbital fibroblast preadipocytes, and promotes B cell differentiation and immunoglobulin production.56,57 IL-1β uses p38 and ERK1/2 MAPK pathways to induce IL-6 gene expression.43 Immunoglobulin G from GD patients substantially upregulates RANTES and IL-16, Akt/FRAP/mTOR/p70 pathway is implicated in the induction of IL-16.54

**Hyaluronan synthesis**

IL-1β, leukoregulin, CD154, TGF-β1 and PDGF are all involved in stimulating HA synthesis, likely via receptor and ligand binding on the orbital fibroblast.26,34,35,58 Orbital fibroblast surface receptors for TSHR and IGF-1R both appear to stimulate HA synthesis. TSHR activation alone is sufficient to upregulate expression of HAS1 and HAS2 and HA production via cAMP and Akt/PI3K signaling with upregulation of HA production.23,59,60.
On the other hand both IgG from GD and IGF-1 stimulate an equivalent and substantial increase in HA synthesis in orbital fibroblasts, suggesting alternative IGF-1R pathways are also involved in HA synthesis. However, the effect of IGF-1 on HA synthesis appears indirect as IGF-1 alone does not increase HAS2 transcription. The stimulatory effect of IGF-1 on HAS transcription is unmasked by MAPK kinase inhibitor but not mTOR or PI3K inhibitors in orbital fibroblasts.

IL-1β is a potent stimulator for GAG synthesis. Increased secretion of HA in TO orbital fibroblasts by IL-1β is due to predominant induction of HAS2, and to a lesser extent HAS3. The effects of HAS mRNA induction by IL-1β can be inhibited by glucocorticoids. PDGF-β and TGF-β are growth factors that are significantly increased in TO orbital tissues. They induce orbital fibroblast proliferation and stimulate HAS1 and HAS2 expression in TO orbital fibroblasts. TGF-β acts via the Smad pathway. TGF-β treated orbital fibroblasts also bind activated human T cells through HA-CD44 interaction, thus promoting lymphocyte chemotaxis and adhesion to pro-inflammatory sites. Addition of PPAR-γ ligands on the other hand inhibits TGF-β induced HAS1 and HAS2 expression and attenuate HA synthesis independent of the PPAR-γ pathway.

**Adipogenesis**

*De novo* adipogenesis is enhanced in TO as evidenced by increased expression of adipocyte specific genes leptin, adiponectin, fatty acid synthase, adipocyte fatty acid binding protein (AP2) and PPAR-γ mRNA in TO affected adipose tissue compared to normal orbital tissue. Microarray studies provide further evidence that adipocyte related intermediate early genes, including CYR61, are over-expressed in active TO. PPAR-γ is a potent stimulator for adipogenesis in TO, evident by increased expression of PPAR-γ in active TO adipose tissue compared to normal controls. PPAR-γ agonist,
Rosiglitazone increases TSHR expression, PPAR-γ mRNA and cAMP levels by 2.6-4.7 fold, resulting in adipogenesis in TO orbital fibroblasts both by proliferation and differentiation of adipocytes.67

Signaling for adipogenesis has been shown to involve both TSHR and IGF-1R. It appears both TSHR and IGF-1R share the same intracellular AkT/PI3K signaling to effect adipogenesis. The close relationship of TSHR and IGF-1R in triggering adipogenesis in TO perhaps could be explained by co-localization of these two receptors on orbital fibroblasts.68 Stimulatory TSHR antibody increases phosphorylated AKT protein, cAMP levels and enhanced adipogenesis via the phosphoinositide 3 kinase (PI3K) signaling cascade.69 On the other hand IGF-1 mediates proliferation and differentiation of human and murine 3T3-L1 preadipocytes into adipocytes.70,71 IGF-1 mediates its effect by binding to IGF-1R, and induces phosphorylation of Src homology 2 domain-containing protein (Shc) and insulin receptor proteins (IRS) and downstream Akt/PI3K pathway.71,72 IGF-1 uses Shc/IRS-1 to activate MAPK/ERK signaling in proliferating 3T3-L1 preadipocytes. Inhibiting MAPK by Shc proximal signaling switches off proliferation of preadipocytes and in turn permits differentiation into adipocytes with increased expression of PPAR-γ, LPL and AP2.73

**Autoantigens in TO**

**TSH receptor**

Breaking of self tolerance to TSHR on thyroid epithelial cells, resulting in TSHR stimulating antibodies inducing thyrotoxicosis is well established in Graves’ disease (GD).74,75 TSHR signals mainly by 2 G-protein mediated pathways: the adenyl cyclase/cAMP pathway and the phosphoinositide 3-kinase(PI3K)/AKT/mammalian
target of rapamycin (mTOR) pathway. Evidence from the temporal correlation of TO and GD, emerging TO animal models and correlation of disease activity and TSHR antibody increasingly point towards TSHR as the primary autoantigen in TO.

The observation that onset of TO is frequently within 18 months of diagnosis of GD, raised early on the concept that the two clinical entities are triggered by a common autoantigen. The first evidence of TSHR as an autoantigen came from identifying TSHR expression in retro-orbital tissue in cultured orbital fibroblast from TO patients by polymerase chain reaction and liquid hybridization. Of note, the level of TSHR expression on orbital fibroblast is only of low abundance compared to thyrocytes but increases during adipogenesis and in active TO.

With improvement of TSHR assays, both thyroid binding inhibiting Ig (TBI) and thyroid stimulating Ig (TSI) TSHR titres are shown to be highly and significantly correlated with activity and severity of TO, thus inferring TSHR antigen is pathogenic in TO. The newer chimeric TSHR and cAMP response element dependent luciferase MC4/TSI assay has higher sensitivity (97%) and specificity (89%) than the current TBI assay (77% and 43% respectively) in TO. The new MC4/TSI assay correlates strongly with clinical activity and clinical severity scores in both adults and children. In the uncommon patients with euthyroid TO, TSHR antibody was highly detectable at 93.8% using third generation TSI assay and 81.3% in second generation TBI assay, in comparison to the low TSHR positivity (18.8%) in first generation assays. Therefore insensitivity of earlier TSHR assays seems likely to explain the seemingly poor correlation of TSHR antibody with severity of TO seen in the past.

**IGF-1 Receptor**
IGF-1R is a ubiquitous cellular surface heterotetrametric receptor involved in diverse cellular responses including modulation of apoptosis, enhancing cell survival, growth and cellular proliferation, cell motility and migration.\textsuperscript{68,85} Evidence suggests IGF1/IGF-1R is involved in the pathogenesis of TO, but the auto-antigenic role of IGF-1R remains controversial. IGF-1R regulates lymphocyte trafficking in the orbit, hyaluronan synthesis, adipogenesis and defines T and B lymphocyte phenotypes and function.\textsuperscript{86} IGF-1R levels are three fold higher on TO compared to control fibroblasts.\textsuperscript{68} Immunoglobulin G from patients with GD induces IL-16 and RANTES secretion mediating T cell migration.\textsuperscript{54} These effects are shown to be induced by IGF-1 and IGF-1R specific ligand, Des(1-3) IGF-1 analog, but not TSH.\textsuperscript{87} Moreover upregulation of IL-16, RANTES secretion and hyaluronan synthesis was restricted to GD orbital and dermal fibroblast and are not observed in normal control fibroblasts. Interfering with IGF1-R function completely abolished signaling induced by Ig G from GD, hence implying IGF-1R is a self-antigen mediating T cell migration, lymphocytes infiltration and hyaluronan synthesis in TO.\textsuperscript{61,87} A recent case-control microarray study also showed differentially expressed genes are dominated by IGF-1 signaling genes, with significant upregulation of \textit{IGF-1}, IGF-1 signaling genes \textit{SOCS3} and \textit{SGK-1} (PDK/Akt signaling) and downregulation of \textit{IRS2} and \textit{IGFBP6} in TO.\textsuperscript{88} It is now clear that once an IGF-1R antibody assay became available, that IGF-1R antibody is present in both GD patient and healthy controls. The prevalence of IGF-1R antibody in TO patients and healthy controls is similar (11\% in normal and 14\% in TO) and there is no correlation of clinical activity score or severity of TO with IGF-1R antibody level; elevated IGF-1R antibody levels in TO also remains stable over 2 years.\textsuperscript{89} Furthermore IGF-1R antibody binds IGF-1R and interferes with IGF1-dependent
receptor activation and signalling, its effect is inhibitory on hepatocarcinoma and breast
cancer cells proliferation.\textsuperscript{89} Hence these findings do not support IGF-1R as an auto-
antigen in TO. On the other hand in an animal model, mice challenged with IGF-1\textsubscript{α}
plasmid produced strong IGF-1R antibody response, but did not induce
hyperthyroidism or orbital changes.\textsuperscript{90} Conversely injection of TSHR A sub-unit plasmid
combined with electroporation induces hyperthyroidism, and both TSHR stimulating
antibody and IGF-1R antibody.\textsuperscript{90}

\textbf{Emerging TO animal model}

Almost all animal models of GD utilize in vivo expression of TSHR either by transfected
cells, plasmid or adenovirus. TSH subunit A seems to initiate the autoimmune response
to TSHR.\textsuperscript{91} Many animal models developed for GD develop hyperthyroidism but fail to
show TO manifestations. One that did induce orbital pathology using a splenocyte
adoptive transfer model with observed extraocular muscle oedema, accumulation of
PAS positive material, expansion of adipose tissue, dissociation of muscle fibres,
lymphocyte and mast cell infiltration, was not reproducible.\textsuperscript{91-93}

A breakthrough in establishing a TO animal model was reported by Banga using TSHR
A-subunit plasmid-immunised by muscle electroporation in BALB/c mice.\textsuperscript{94} It showed
orbital remodeling with bilateral interstitial inflammatory infiltrate in the extraocular
muscle, infiltration of CD3\textsuperscript{+} T cells, F4/80\textsuperscript{+} macrophages and mast cell, orbital fibrosis,
GAG deposition and corresponding MRI changes of orbital muscle hypertrophy. A few
mice also showed predominantly expansion of retro-orbital fat, proptosis, chemosis and
congested orbital vessels. This is by far the most representative animal model of TO.
TSHR and a lower level of IGF-1R antibodies were both induced.\textsuperscript{94} The less expected
findings were predominance of TSH blocking antibodies, hypothyroid status and large inflammatory infiltrates around the optic nerve, which are not typical of GD. Nevertheless these findings support the pathogenic role of TSHR in the development of TO and open the door for investigating pathogenesis and therapeutic drugs in an animal TO model. Interestingly using a similar protocol with a slight alteration of the electroporation regime in an earlier study, TSHR plasmid induced a high frequency of hyperthyroidism (75%), TSHR stimulating antibodies, and in some animals, orbital connective tissue fibrosis.90

**Oxidative Stress and TO**

A state of oxidative stress has been described in Graves' disease and TO.95,96 An increase in reactive oxygen species or reduced elimination of radicals by anti-oxidative enzymes will result in oxidative damage to cell membrane with lipid peroxidation and oxidative DNA damage, resulting in inflammation and loss of function.97 Both 8-hydroxy 2'-deoxyguanosine (8-OHdG) and malondialdehyde, as well as intracellular superoxide anion and hydrogen peroxide were significantly elevated in TO orbital fibroblast compared to normal controls.98 The 8-OHdG urinary levels correlate well with clinical activity score.99 These findings suggest increased oxidative DNA damage and lipid peroxidation may have a role in the pathogenesis of TO. Increased oxidative stress is also noted in vivo, where lipid hydperoxide, superoxide dismutase (SOD), glutathione reductase and glutathione peroxidase are significantly elevated in orbital fibroadipose tissue, while glutathione (anti-oxidant) is reduced compared to controls. Gluthathione levels are strongly negatively correlated with the ophthalmopathy index.95
In hyperthyroid patients, achieving euthyroidism with methimazole results in all markers of oxidative stress being normalized in Graves’ disease without orbitopathy, but not entirely in the TO group where oxidative stress indices remain significantly different from normal controls.\textsuperscript{96} Similarly oxidative stress marked by tert-butyl hydroperoxide initiated chemiluminiscence remains high after radioactive iodine treatment.\textsuperscript{100} Treatment with oxygen radical scavengers and anti-thyroid drugs reduce hydrogen peroxide-induced and, to a lesser degree, heat-induced 72 kilodalton heat shock protein (HSP72). HSP72 is a cytosolic protein inducible by heat shock, ischaemia, and its expression is increased in autoimmune thyroid disease.\textsuperscript{101,102}

Reactive oxygen species (superoxide anions and hydrogen peroxide) induce pro-inflammatory cytokines production (IL-1\(\beta\), TGF-\(\beta\)1) and stimulate orbital fibroblast proliferation in a dose-dependent manner; the proliferative effect can be inhibited by multiple anti-oxidants, methimazole but not propylthiouracil.\textsuperscript{103,104} Free radicals are also involved in IL-1\(\beta\) induced glycosaminoglycan production in TO orbital fibroblasts. IL-1\(\beta\) increases free radical production in both normal and TO orbital fibroblast, and stimulates SOD activity in TO orbital fibroblasts. Furthermore reducing oxygen free radicals with SOD and catalase partially blocked IL-1\(\beta\) induced glycosaminoglycan production.\textsuperscript{105} Nicotinamide reverses cellular injury in the orbit by inhibiting cytokine-induced activation in TO orbital fibroblasts.\textsuperscript{106}

Despite the established association of smoking with TO, the mechanism of smoking leading to TO remains less well defined. Cigarette smoke contain oxidants and radicals that cause oxidative burden systemically,\textsuperscript{107} hence it has been proposed that increased production of reactive oxygen species by smoking overwhelms oxidation reduction. Smokers had significantly higher 8-OHdG levels than non-smokers in TO, suggesting
smoking has a higher impact on oxidative stress in TO patients. Cigarette smoke extract can also stimulate hyaluronic acid production and adipogenesis in a dose-related manner, and the effect on adipogenesis is synergistic with IL-1.

**Advances in therapeutic agents for TO**

The mainstay treatments for TO have been systemic corticosteroids and orbital radiation for active TO, and surgical rehabilitation for inactive TO until immunomodulators were trialed in TO targeting TSHR and IGF-1R on fibroblast, inflammatory cytokines IL-6, TNF, and CD20+ B cell depletion. (Table 1)

The better studied immunosuppressive therapy for TO is rituximab, an anti-CD20 monoclonal antibody that targets CD20 on B cells and its precursors. A systematic review of 43 TO cases treated with rituximab showed improvement in disease activity and severity in 91% cases, no improvement in 3 cases and worsening in 1 case. A randomized controlled trial (RCT) in Europe comparing rituximab to intravenous methylprednisolone in active moderate severe TO supports effectiveness and disease modifying effects with 100% response rate, no reactivation of TO at 24 weeks, and less rehabilitative surgery required at 76 weeks. A RCT in North America comparing rituximab to placebo (i.e. comparing to natural history) did not show a significant difference in the improvement of disease activity at 24 and 52 weeks, and there were more moderate to severe adverse events in the rituximab group. The conflicting results from the rituximab RCTs could be related to small sample sizes, and require clarification with larger RCTs.

Drug-like small molecule TSHR antagonists are emerging as a promising new treatment for TO and GD. M22, a small molecule TSH agonist increased cAMP production in a
TSHR transfected ovary cell line and TO orbital fibroblasts, and the cAMP response was effectively abolished by low molecular weight TSHR antagonist. The results were replicated separately where small molecule TSHR antagonists can inhibit both basal and stimulated cAMP, pAKT and HA production in orbital fibroblast in a dose dependent manner. Teprotumumab, a humanized anti-IGF-1R monoclonal antibody is in phase II clinical trial for moderate severe active TO. Preliminary study shows teprotumumab can inhibit expression of TSHR and IGF-1R on CD34+ fibrocytes and TSH induced IL6 and IL8 production, by partially inhibiting phosphorylation of Akt.

Tocilizumab, a recombinant, humanized monoclonal antibody to IL-6 receptor has been trialed in 18 patients with active TO refractory to intravenous steroids. Tocilizumab significantly improved clinical activity score in all patients and disease activity remained stable up to 27 months after infusion. The anti-TNF monoclonal antibodies infliximab, adalimumab and soluble TNF receptor etanercept have been trialled in small numbers of active TO patients. Etanercept seems to be effective in controlling activity of TO, leading to a marked improvement in mainly soft tissue changes reported at 60%, but up to 30% had recurrence of TO activity after treatment cessation. Adalimumab reduced inflammatory score in 6 of 10 patients, the greatest benefit being seen in active TO with severe inflammatory signs. Apart from IL-6 and TNF antagonists, in-vitro use of anti-IL-1 antibody has been shown to reduce adipogenesis by 82% in orbital fibroblasts exposed to cigarette smoke extract. Novel therapeutic options for TO show some exciting developments, but large randomized controlled trials for these agents are needed to determine both efficacy and safety profile.

Anti-oxidants have a promising role in the treatment of mild to moderately active TO. In the first pilot study of antioxidant supplementation, allopurinol and nicotinamide
therapy reduce soft tissue swelling and total eye score in 82% of patients accompanied by high patients’ satisfaction in mild to moderately severe TO. Selenium, a trace mineral incorporated into several selenoproteins and functions as anti-oxidant, reduces thyroperoxidase antibodies in autoimmune thyroiditis. A subsequent double-blind, randomized control trial of selenium supplemented for 6 months in TO, was associated with improved quality of life, reduced soft tissue inflammation, improved appearance and slowed progression of TO, as compared to placebo.

**Conclusion**

Cellular immunity has an important role in orbital inflammation in TO which involves interaction of T cells with orbital fibroblasts through specific receptor ligand bridges, with propagation of multiple intracellular signaling cascades leading to secretion of hyaluronan, adipogenesis, and the release of chemotactic factors and cytokines that ensure perpetuation of orbital inflammation. TSH receptor appears the likely candidate as an auto-antigen. IGF-1 receptor on orbital fibroblasts mediates some aspects of orbital changes and importantly it has a role in adipogenesis, hyaluronan synthesis and lymphocyte trafficking. Oxidative stress is increased in TO, and the increased oxidative burden appears to potentiate orbital inflammation, fibroblast proliferation and GAG production. With the emergence of animal models in TO and newer TSHR antibody assays, future studies will allow detailed evaluation of the heterogenous TSHR antibodies and their effects on TO, testing of new treatments targeting receptor ligand binding, signaling pathways and T and B cells. Further studies in signaling networks and molecular triggers that lead to burnout of TO will improve our understanding of TO and can in turn open future therapeutic directions.
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Table 1. Novel and potential immunotherapies in clinical and pre-clinical trials for TO

<table>
<thead>
<tr>
<th>Class of drugs</th>
<th>Mechanism of action</th>
<th>Example</th>
</tr>
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<tbody>
<tr>
<td>CD 20 monoclonal antibody</td>
<td>Deplete B cells and precursor by recognizing surface CD20 marker</td>
<td>Rituximab\textsuperscript{130-132}</td>
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<tr>
<td>IL-6 receptor monoclonal antibody</td>
<td>Binding to soluble and membrane bound IL-6 receptor and inhibit pro-inflammatory cytokine IL-6</td>
<td>Tocilizumab\textsuperscript{67}</td>
</tr>
<tr>
<td>TNF-alpha monoclonal antibody</td>
<td>Bind and block TNF-alpha from interacting with cell surface TNF receptors.</td>
<td>Adalimumab\textsuperscript{138}</td>
</tr>
<tr>
<td>Soluble TNF receptor</td>
<td>A soluble TNF-alpha receptor-Fc protein that prevent TNF-alpha and TNF-beta from binding to membrane bound TNF receptors</td>
<td>Etanercept\textsuperscript{136}</td>
</tr>
<tr>
<td>Small molecule TSHR antagonist</td>
<td>Binding within transmembrane region of TSHR receptor, blocking signaling of TSH either as allosteric inverse agonist or neutral antagonist</td>
<td>Org\textsuperscript{274179-0}\textsuperscript{133}</td>
</tr>
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<td></td>
<td></td>
<td>NCGC00229600\textsuperscript{134}</td>
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<td></td>
<td></td>
<td>NCGC00242595\textsuperscript{70}</td>
</tr>
<tr>
<td>IGF-1R monoclonal antibody</td>
<td>IGF-1R blocking, reduces both IGF-1R and TSHR expression</td>
<td>Teprotumumab\textsuperscript{135}</td>
</tr>
<tr>
<td>Anti-oxidant</td>
<td>Increase reserve for selenoproteins involve in oxidation reduction</td>
<td>Sodium selenite\textsuperscript{141}</td>
</tr>
<tr>
<td>activity eg glutathione peroxidase, thioredoxin reductase, iodothyronine deiodinases</td>
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**Figure Legend**

Figure 1. Model of pathogenesis of TO. T cell interacts with orbital fibroblast via CD40:CD154 ligation, and interaction of MHC II, autoantigen and T cell receptor activate orbital fibroblast with increase secretion of ICAM-1, nuclear translocation of nuclear factor-κβ, IL-1, IL-6, IL-8, macrophage chemoattractant and prostaglandin E2 secretion. Cytokines showed Th1 dominance with increase IL-1β, IFN-γ, TNF-α and IL-6. IFN-γ increases CD40 expression, IL-6 modulates B cell immunoglobulin secretion. Orbital fibroblast upregulates pro-inflammatory cytokines IL-1β, TGF-β, leukoregulin that perpetuate orbital inflammation, increase HA synthesis. TGF-β induces myofibroblast proliferation and differentiation and promotes lymphocyte adhesion and chemotaxis by CD44 and HA interaction. Ig G from GD and IGF-1 upregulates secretion of RANTES and IL-16, which increase T cell migration into the orbit; the Akt/MTOR/P70 pathway seems involve in IL-16 upregulation. Activating TSHR increases hyaluronan synthase(HAS) via adenyl cyclase/cAMP and Akt/PI3K pathway. IGF-1 can also induce HAS and HA synthesis, the effect is unmasked by MAPK inhibitor. Both TSHR and IGF-1R activate PI3K/Akt pathway to upregulate PPAR-γ expression, differentiation and proliferation of adipocytes and enhance adipogenesis. IGF-1R utilizes SHC/IRS/MAPK signaling to increase proliferation of preadipocytes. Switching off proximal SHC signaling on MAPK in turn permit differentiation of preadipocyte to adipocytes and enhance adipogenesis.
Author/s: Khong, Jwu Jin

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