

Investigating the functionality of candidate susceptibility genes in ophthalmoplegic myasthenia gravis using patient-derived material



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Lihle Bayavuya Moyakhe, 10Th February 2019.

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List of Abbreviations

%	percentage
°C	degrees celsius
μl	microlitre
Ab	antibody
AChR	acetylcholine receptor
DNA	deoxyribonucleic acid
EOM	extraocular muscle
FBS	fetal bovine serum
MG	myasthenia gravis
MGS	myasthenia gravis sera
ml	millilitre
mM	millimolar
MT	mutant
ng	nanogram
OP-MG	ophthalmoplegic myasthenia gravis
PBS	phosphate-buffered saline
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
V	volt
WES	whole-exome sequencing
WT	wild type

FOREWORD

This thesis is presented in fulfilment of the Master of Science in Medicine at the Faculty of Health Sciences, University of Cape Town. The subject of this thesis is the molecular genetic investigation of a subphenotype of an autoimmune disease, myasthenia gravis (MG). This subphenotype is characterised by extraocular muscle (EOM) weakness which does not respond to treatment (treatment-resistant ophthalmoplegia or OP-MG).

This research builds on the previous work and findings of Dr M. Nel who performed a candidate gene study and identified a functional, African-specific regulatory region variant in the transforming growth factor-beta 1 (*TGFB1*) gene which is associated with juvenile-onset OP-MG (Nel *et al.*, 2016). However, this gene variant was not present in all OP-MG cases which led to the further investigation of other candidate risk genes and pathways that could contribute to the development of the OP-MG subphenotype in susceptible individuals. Whole exome sequencing (WES) was performed, including interrogation of the flanking untranslated regions (UTRs), in a discovery sample comprising highly selected individuals of African genetic ancestry with juvenile-onset MG (11 with OP-MG and 4 with control MG, i.e. no EOM weakness). Candidate OP-MG susceptibility variants were identified by filtering for variants which were more common in the OP-MG group and were found in genes expressed in extraocular muscles. A number of gene variants were identified including *IL6R* c.*3043 T>C and *ST8SIA1* c.-477_-475 delCCC. Although the IL-6 signalling pathway has previously been implicated in MG (Maurer *et al.*, 2015), the identification of *ST8SIA1* (an enzyme involved in the synthesis of gangliosides) was a novel and biologically relevant finding related to OP-MG pathogenesis. Therefore, the focus of this study was to investigate the functionality of 5' UTR *ST8SIA1* c.-477_-475 delCCC promoter variant using luciferase promoter-reporter assays in relevant cell lines and in patient-derived ocular fibroblasts. In addition, since the functionality of the OP-MG susceptibility variants in *TGFB1* and *IL6R* have only been investigated in transformed human and murine cell lines to date, the expression of these genes was also profiled in the patient-derived ocular fibroblasts. As this study has built on a lot of fundamental work previously performed by members of our group, where I have not done the work, I have made it a point to acknowledge.

ABSTRACT

Ophthalmoplegic myasthenia gravis (OP-MG) is a subphenotype of an autoimmune disease, myasthenia gravis (MG). This subphenotype described by our group is characterised by extraocular muscle (EOM) weakness which does not respond to standard immunosuppressive therapy whilst the non-ocular muscles do respond to treatment. OP-MG predominantly affects patients of African genetic ancestry and most commonly those with juvenile-onset. The cause of OP-MG is unknown.

Previously, in efforts to understand the pathophysiology of this subphenotype, whole-exome sequencing was undertaken which identified amongst others a putatively functional multi-allelic deletion in the 5' untranslated regulatory region of the *ST8SIA1* gene. The most frequent deletion was *c.-477_475 delCCC*. The first aim of this thesis was to assess the functionality of this variant. To this end, *in vitro* promoter-reporter assays were performed after the *delCCC* deletion (MT) was created by site-directed mutagenesis of the wild-type (WT) promoter. Transient transfection assays showed that basal promoter activity of the MT promoter was lower than the WT promoter in the human fibroblast cell line (HT1080, $p=0.031$) but not in a mouse myoblast cell line (C2C12, $p=0.33$). Next, gene expression analysis was performed in opportunistically sampled orbital fibroblasts ($n=5$ controls, $n=2$ OP-MG) and the endogenous expression levels of *ST8SIA1* was determined under basal conditions and following treatment with homologous MG sera to mimic MG conditions. Although there was no significant difference in the basal *ST8SIA1* expression levels in OP-MG ocular fibroblasts when compared to the control ocular fibroblasts ($p=0.091$), there were significantly lower *ST8SIA1* expression levels in OP-MG ocular fibroblasts in response to MG sera ($p=0.024$).

Previous work by others also identified two other African specific gene variants in *IL6R* and *TGFB1* which associated with OP-MG and in which luciferase reporter work showed functional implications. This thesis conducted the first examination of the endogenous expression levels of these genes in patient-derived ocular fibroblasts. There was no difference in *TGFB1* expression levels between OP-MG and control ocular fibroblasts under basal conditions or following treatment with MG sera ($p>0.3$). However, *IL6R* expression showed a similar trend to the luciferase reporter assays results in HT1080 cells. Both the OP-MG ocular fibroblasts tested harboured the 3'UTR *IL6R* *c.*3043T>C* variant, which was predicted to alter a putative miRNA binding site and showed a trend towards repression of *IL6R* expression in response to MG sera ($p=0.083$).

In conclusion, although faced with the scarcity of sample tissue, we were able to use patient-derived ocular fibroblasts to validate previous genetic association studies and *in vitro* assays. Although the ocular fibroblasts are not EOM, they are both specialised tissue existing in the same microenvironment. Since MG sera modulate the expression of *ST8SIA1* and *IL6R* differently between OP-MG and controls, our

findings suggest that these genes may play a role in the OP-MG pathogenesis. Larger sample sizes are required for a more accurate representation of endogenous gene expression within these cell lines as the sample size is a factor when determining the significance of data. This is, however, a challenge due to the scarcity and unavailability of EOM for cell culture.

1. INTRODUCTION

1.1 Myasthenia Gravis

Myasthenia gravis (MG) is an autoimmune disorder which results in muscle fatigue and weakness due to the impairment of neuromuscular transmission. It is a rare disease which can occur in both sexes and can affect all races across all ages(Engel, Sahashi and Fumagalli, 1981). The majority of affected individuals produce pathogenic acetylcholine receptor antibodies (AChR-Abs) which target the acetylcholine receptors (AChRs) on the postsynaptic muscle, the reason behind which is not yet fully understood (Engel, Sahashi and Fumagalli, 1981). While functional blockade of AChRs by auto-antibodies contributes to impaired transmission at the neuromuscular junction, the main AChR-Ab effector mechanism is complement-mediated endplate damage(Engel, Sahashi and Fumagalli, 1981;Sahashi *et al.*, 1980).

Although the main immunogenic target in MG appears to be the AChR, however not all individuals with MG will be AChR-Ab positive, this describes the detection of characteristic AChR-Abs in their sera(Hoch *et al.*, 2001) A group of “seronegative “ MG individuals exist whereby they have antibodies directed against two other neuromuscular junction proteins, muscle-specific kinases (MUSK)(Hoch *et al.*, 2001) or LDL receptor-related protein 4 (LRP4)(Higuchi *et al.*, 2011). In this study, however, the focus was only on those patients with AChR-Abs (seropositive individuals).

The incidence rate of MG in South Africa (8.5 per million) is similar to the worldwide incident rate of 7.3 per million (Carr *et al.*, 2010; Mombaur *et al.*, 2015). MG results in weakness of skeletal muscle which includes limb muscles, muscles involved in breathing, speaking and eating as well as eye muscles (extraocular muscles). This disease is treatable usually with a combination of acetylcholinesterase inhibitors and immunosuppressive therapy. The acetylcholinesterases work to boost the concentrations of acetylcholine (ACh) in the postsynaptic cleft by impairing its degradation whereas the immunosuppressive therapy aims to decrease the production of pathogenic AChR-Abs and modulate the inflammatory response(Mehndiratta, Pandey and Kuntzer, 2014). While most patients respond to symptomatic and immunosuppressive therapy. However, a distinct subgroup of patients with African genetic ancestry has been identified in whom the extraocular muscles do not respond to immunosuppressive therapy. These patients develop severe eye muscle weakness or ophthalmoplegia (Heckmann, Owen and Little, 2007). This treatment-resistant subphenotype, termed ophthalmoplegic myasthenia gravis (OP-MG), is most common in children and young adults. The research focused on understanding the pathogenesis of OP-MG is important since it results in significant visual impairment and its management is still unclear.

1.1.1 Pathophysiology of MG

Myasthenia gravis (MG) is an autoimmune antibody-mediated disease in which pathogenic antibodies (Ab) are produced which most frequently target the acetylcholine receptors (AChRs) located on the postsynaptic muscle membrane of the neuromuscular junction (NMJ) (Figure 1 A and B). These antibodies either block or destroy AChRs; resulting in impaired neuromuscular transmission by decreasing the number of available AChR binding sites for acetylcholine which is released pre-synaptically when the nerve is excited (Vaphiades, Bhatti and Lesser, 2012).

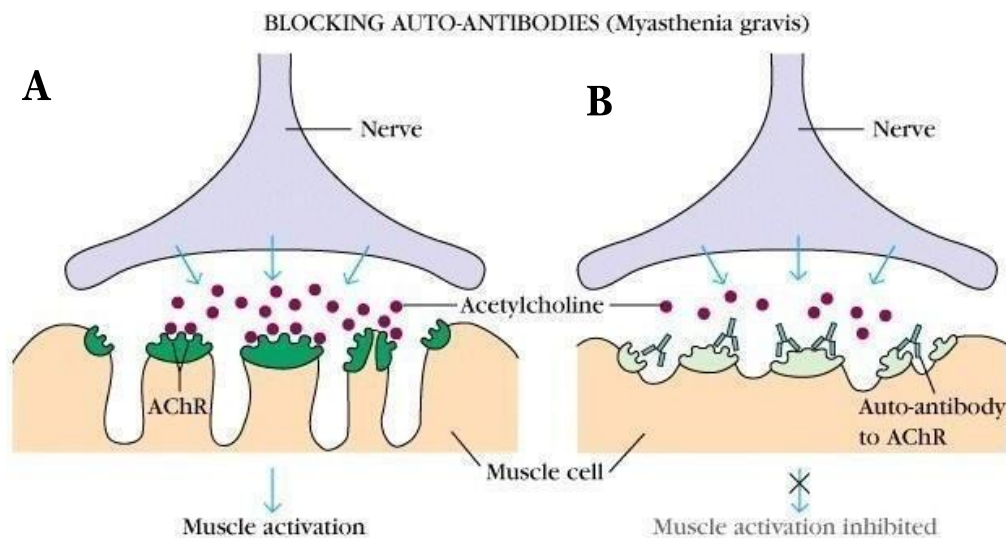


Figure 1: Schematic diagram of the neuromuscular junction. A: Normal neuromuscular junction, muscle activation is not interrupted as result acetylcholine is able to bind to the acetylcholine receptors thus allowing muscle activation. B: Myasthenia gravis, pathogenic antibodies bind to the acetylcholine receptors which disrupts the ability of acetylcholine to bind to the receptors thereby inhibiting muscle activation (Pathologicallyspeaking.blogspot.com, 2019).

The AChR-Abs can be either classified as being part of the immunoglobulin (Ig) G1 or the IgG3 subclass. These divalent antibodies have the capacity to trigger complement activation by binding to the AChRs causing the assembly of the membrane attack complex (MAC) (Phillips and Vincent, 2016). The MAC creates punctures within the membrane which causes calcium influx accompanied by the release of AChR-containing membrane debris into the synaptic cleft. The damaged postsynaptic membrane shows a diminished response to acetylcholine and this impairs neuromuscular transmission due to the loss of AChRs. Clinically this results in muscle fatigability and muscle weakness (Phillips and Vincent, 2016).

Complement activation is crucial in innate and adaptive immunity for protecting the host against invading pathogens. It does so through mechanisms of opsonisation with complement fragments, cell lysis, chemotaxis of inflammatory cells and formation of MAC (Kusner, Kaminski and Soltys, 2008).

The complement system is comprised of an assembly of proteins of various biological activities. These proteins include decay-accelerating factor (DAF) which acts to prevent the cascade from progressing towards the formation of MAC and tissue damage due to the inadvertent binding of activated complement components (Kusner, Kaminski and Soltys, 2008). The complement system is comprised of three main pathways: the classic, lectin as well as alternative pathways. The classic complement pathway is activated by antigen-antibody complexes, and, as such, it is implicated in the pathogenesis of a variety of immune-complex and antibody-associated diseases such as myasthenia gravis (Huda, Tüzün and Christadoss, 2014). The significance of the complement system in the pathogenesis of myasthenia gravis was highlighted by studies which found varying levels of complement factors such as C3, C5 convertases in the sera of MG patients which suggested the use of the complement system in the pathogenic process (Huda, Tüzün and Christadoss, 2014). In humans, DAF, CD59 and membrane cofactor protein (MCP or CD46) are the three cell-associated complement regulators. DAF and CD59 play a vital role in inhibiting complement activation by accelerating the decay of C3 and C5 convertases (Kusner, Kaminski and Soltys, 2008). Complement activation and MAC formation damage the postsynaptic membrane at the muscle endplate by altering the architecture of the endplate folds which in turn reduces the surface area, the number of AChRs and voltage-gated sodium ion channels. All these factors can lead to abnormal neuromuscular transmission and the characteristic muscle weakness associated with MG (Kusner, Kaminski and Soltys, 2008; Heckmann *et al.*, 2009).

The identification of lower levels of DAF in experimental autoimmune myasthenia gravis (EAMG) led Soltys *et al* (2008) to hypothesise that EOMs could be particularly susceptible to the complement-mediated injury in EAMG and by extension MG. EAMG mice model which were induced by immunisation with recombinant human AChR subunits and characterised as having severe ptosis accompanied with mild generalised muscle weakness were found to have abundant IgG deposits at the extraocular muscle NMJs. The EOM of these mice were found to have reduced amounts of complement regulators compared to limb muscles; thus, suggesting possible susceptibility of EOM to autoimmune attack when compared to limb muscles (Huda, Tüzün and Christadoss, 2014). Supporting this hypothesis is a study by Heckmann *et al* (2010) which found a SNP within the *DAF* regulatory region of MG patients who had ocular involvement. This SNP was shown to play a role in the prevention of the upregulation of lipopolysaccharide-induced *DAF*. This result thus suggested that reduction in expression levels of complement regulators renders individuals susceptible to ocular MG (Heckmann *et al.*, 2010). Human extraocular muscles (EOM) are among the most complex muscles in the body. They have unique features such as multiple-innervated fibres in addition to the normal single-innervated fibres normally seen in other muscles. Human EOMs also contain an atypical content of AChR isoforms. EOMs express gene transcripts specific for adult and foetal isoforms of AChR, unlike the other mature muscles which

contain only adult AChR isoforms. EOMs are particularly susceptible to weakness in MG since they have structural and functional properties which set them apart from skeletal and cardiac muscles (Yu Wai Man, Chinnery and Griffiths, 2005). These differences may be critical in explaining why the extraocular muscles are preferentially involved in some disease states and spared in others (Yu Wai Man, Chinnery and Griffiths, 2005).

It has been suggested that EOM and orbital tissue possess immunological features which make them vulnerable to immune attack such as in MG (Soltys *et al.*, 2008). A study by Porter *et al* (2001) which analysed gene expression profiles of limb, masticatory muscle and EOMs showed that many genes are differentially expressed in EOMs. The genes which were differentially expressed in EOMs reflect fundamental aspects of muscle biology which include: transcriptional regulation, sarcomeric organization, excitation-contraction coupling, and intermediary metabolism and immune response.

Other differences between EOM and skeletal muscles include differences in their classification profiles and disease susceptibility. For example, EOMs tend to be selectively spared in motor neuron diseases and Duchenne muscular dystrophy, however, is selectively targeted in MG, chronic progressive external ophthalmoplegia (CPEO) and Graves' ophthalmopathy. The differences which exist between EOM and limb muscle allotypes may account for the selective vulnerability of EOMs to these disorders (Yu Wai Man, Chinnery and Griffiths, 2005).

Extraocular muscle and skeletal muscle have different innervation patterns. In limb muscles, the endplate potential amplitude is larger than the minimum depolarisation needed to trigger a propagated action potential called the safety factor. EOM twitch fibres have a lower safety factor as they have less prominent synaptic folds and by extension/prediction fewer AChR on the postsynaptic membrane. This low safety factor may make EOM twitch fibres more vulnerable to the reduction in synaptic depolarization that occurs in myasthenia (Yu Wai Man, Chinnery and Griffiths, 2005).

Skeletal and EOM also differ in motor units and contractile properties. Ocular motor units are an order of magnitude smaller than limb muscle motor units which are consistent with the capacity of EOMs to vary their contractile forces by small increments. The maximum firing frequencies of ocular motor units in the phasic and sustained phases are four times greater than those of limb muscle motor units. The higher firing frequencies of EOM, therefore, make them more prone to neuromuscular transmission failure in MG (Yu Wai Man, Chinnery and Griffiths, 2005).

Extraocular muscles also have a smaller twitch: tetanic tension ratio than limb muscles which is consistent with their high capacity to frequency modulate their force output. The higher firing frequencies, faster contractile properties and higher percentage of recruitment of ocular motor units in

almost every eye movement make the properties of EOMs more energy demanding than those of limb muscles (Yu Wai Man, Chinnery and Griffiths, 2005).

Limb and EOM also differ in their structural and metabolic adaptations. Extraocular muscles need a higher fatigue resistance to fulfil their more energy-demanding properties. To enjoy such a high fatigue resistance, EOMs possess a highly developed microvascular bed, a higher blood flow, higher mitochondrial content and higher metabolic rate. These patterned differences in gene expression define EOM as a distinct muscle class and may explain the unique response of these muscles in neuromuscular diseases. Therefore, the selective vulnerability of EOMs to certain disorders such as MG may be explained by their fundamentally distinct structural, functional, biochemical and immunological properties compared to those of skeletal muscles (Yu Wai Man, Chinnery and Griffiths, 2005).

1.1.2 MG Subtypes

Myasthenia Gravis may be categorised into two main subtypes depending on the clinical features: ocular myasthenia gravis in which the patient only has ocular symptoms and generalised MG in which the patient has generalised proximal limb muscle weakness which may include weakness of the EOMs. Most patients with generalised myasthenia gravis often present with ocular manifestations which may include diplopia (double vision due to misalignment of the visual axis) and ptosis (drooping of the eyelids) (Elrod and Weinberg, 2004). Diplopia occurs due to weakness of the various extraocular muscles that move the globe (Figure 2A; Figure 2B) and ptosis results from weakness of the levator palpebrae superioris muscle that elevates the eyelid (Figure 2B). In addition, weakness of the orbicularis oculi (a facial muscle) can also contribute to weak eyelid closure (Figure 2A).

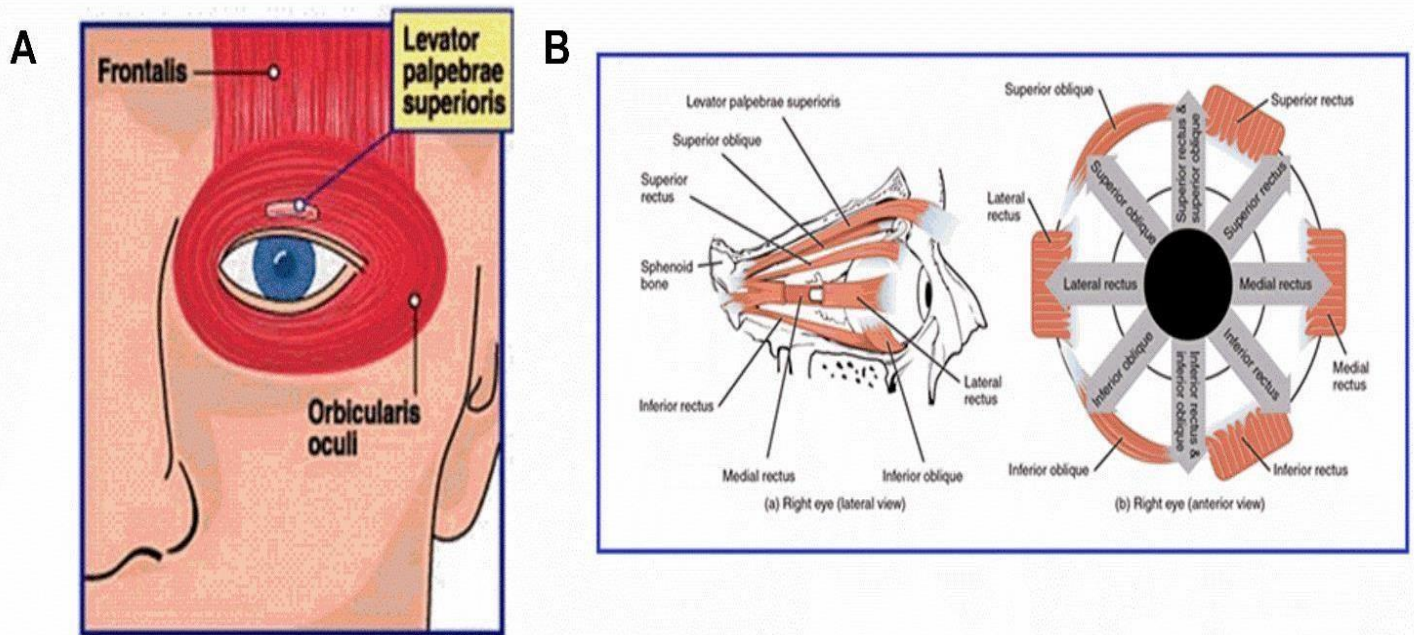


Figure 2: Images of ocular muscles affected in Myasthenia Gravis. A Levatorpalpebraesuperioris (opens the eyelid) and Orbicularis oculi (closes the eye). B, Extraocular muscles which move the eyeball (Sources: Figure A: Know your body.net. Image by Madhulima Acharya; Figure B: Humananatomy101.com).

EOMs may be affected in generalised MG, but they respond to standard immunosuppressive therapy much like other skeletal muscles. However, our research group has identified a subphenotype of MG which is characterised by eye muscles that do not respond to treatment (treatment-resistant ophthalmoplegia). Patients with the OP-MG subphenotype typically have early-onset MG and are of African genetic ancestry. These individuals develop severe eye muscle weakness (ophthalmoplegia) and eyelid weakness (ptosis) (Heckmann, Owen and Little, 2007). Most of these patients have AChR- Ab positive generalised MG, although some may have ocular MG (Heckmann *et al.*, 2012). An individual is classified as having OP-MG if there is a weakness in 6 of the 12 EOMs. Weakness may be mild (25%) but the clinicians only regard at least 50% weakness of an EOM as a relevant weakness for the definition of OP-MG. This weakness can range from complete OP-MG whereby all 12 EOMs are affected or partial OP-MG with only 6-11 EOMs being affected (Heckmann, Owen and Little, 2007).

Although the incidence of MG in Africa is similar to elsewhere, a high incidence of OP-MG has been reported in individuals with African genetic ancestry and juvenile-onset MG (Heckmann, Owen and Little, 2007). Juvenile MG is frequently defined as symptom onset before age 20 and is rare in comparison to adult MG (Heckmann *et al.*, 2012; Heckmann and Nel, 2018).

1.2 Previous work: OP-MG susceptibility pathways

1.2.1 Decay accelerating factor (DAF)

Acetylcholine receptor antibodies in MG activates complement at the muscle endplate which causes damage. Decay accelerating factor is one of the complement regulatory proteins which reduces complement-mediated damage and is particularly important in EOMs. Heckmann *et al.* (2010) reported a functional polymorphism (-198C>G) in the regulatory region of *DAF* in OP-MG subjects with African genetic ancestry. The authors found that the *DAF* promoter variant resulted in lower *DAF* expression levels during lipopolysaccharide (LPS)-induced immune activation (Heckmann *et al.*, 2010). This led the authors to hypothesize that in extraocular muscles, at critical periods, inadequate *DAF* upregulation and the resultant decrease in complement protection increases susceptibility to develop OP-MG (Heckmann *et al.*, 2010). Due to this variant being present in only a few of the OP-MG patients and not entirely explained by the *DAF* -198C>G hypothesis led the authors to question whether additional muscle endplate damage may result in altered muscle repair pathways.

1.2.2 Transforming growth factor-beta 1 (TGFB1)

The transforming growth factor-beta (TGFB) superfamily consists of a variety of cytokines expressed in various cell types which include skeletal muscle. It plays a crucial role in normal physiology and pathogenesis in several tissues. Members of this superfamily that are of particular importance in skeletal muscle are TGFB1, mitogen-activated protein kinases (MAPKs) and myostatin (Burks and Cohn, 2011). These signalling molecules play important roles in skeletal muscle homeostasis and in a variety of inherited and acquired neuromuscular disorders. The downstream effects of this signalling cascade are often tissue-specific; thereby dictating which target genes will be activated in response to the transduction signal. Given its multifaceted effects in different tissues, the deregulation of TGFB cascades can lead to a multitude of developmental defects and/or diseases (Burks and Cohn, 2011). Several members of the TGFB family have been shown to play important roles in regulating muscle growth and atrophy. Expression of these molecules is linked to normal processes in skeletal muscle such as growth, differentiation, regeneration and stress response (Burks and Cohn, 2011; Saika, 2006). However chronic elevation of TGFB1, MAPKs and myostatin are linked to various features of muscle pathology, including impaired regeneration and atrophy (Burks and Cohn, 2011). *TGFB1* is also implicated in muscle endplate damage and its expression has been shown to be upregulated by inflammatory cells in/around the muscle (Burks and Cohn, 2011). TGFB1 has also been shown to drive the trans differentiation of satellite cells into myoblasts or myofibroblasts (Burks and Cohn, 2011).

Transforming growth factor beta 1 is involved in muscle healing responses and was reported to upregulate

DAF expression. Previously a candidate gene approach identified functional promoter variants in DAF and transforming growth factor beta-1 (TGFB1) which were more common in OP-MG patients compared to control MG patients (patients with generalised MG but no ophthalmoplegia (Heckmann *et al.*, 2010; Nel *et al.*, 2016). The cytokine *TGFB1* plays a critical role in muscle response to injury and muscle endplate damage. Shah *et al.* (2006) found genetic diversity in the *TGFB1* promoter of African subjects and found a novel single nucleotide polymorphism (SNP) in a South African control. Some of these African *TGFB1* promoter polymorphisms were predicted to alter *TGFB1* expression (Shah *et al.*, 2006). Nel *et al.* (2016) showed the association of an African-specific SNP -387C>T found in the extended *TGFB1* regulatory region with OP-MG more so juvenile-onset OP-MG. The authors demonstrated the functionality of the -387C>T SNP. The *in silico* analysis predicted the SNP to influence *TGFB1* transcriptional activity along with the activity of two other closely linked SNPs in the distal regulatory region (rs11466313 and rs1800469). The SNPs in the distal regulatory region were found to be underrepresented in OP-MG compared with MG controls and were reported to be functional (Nel *et al.*, 2016). The MG subjects had one of four possible *TGFB1* promoter haplotypes (pHap-1 to pHap-4) (Figure 3). pHap3, containing the -387T allele, was found to be frequent in juvenile OP-MG cases compared with MG controls. The authors then investigated whether the -387T allele impacts *TGFB1* promoter activity by measuring luciferase activity of the luciferase promoter-reporter constructs of pHap-1 to pHap-4 which were transiently transfected into HT1080 human fibrosarcoma cells and C2C12 mouse myoblast cells (Figure 3). They found that pHap-3 which contains the African-specific -387T allele showed loss of basal promoter activity relative to the other 3 haplotypes (Figure3).

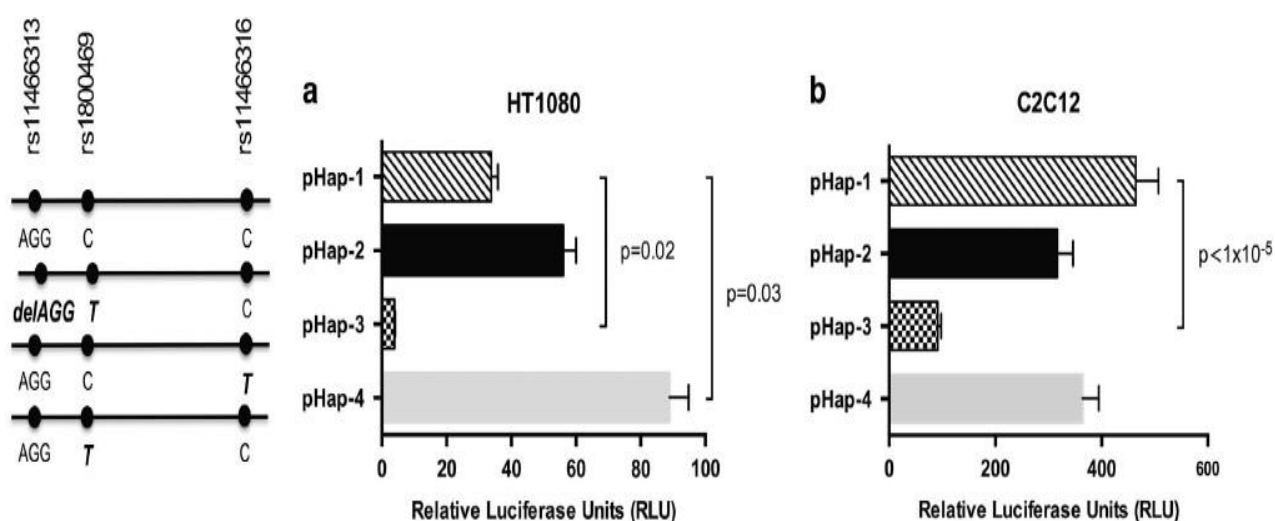


Figure 3: pHap-3 containing the African specific -387T allele reduces basal transforming growth factor beta 1 transcriptional activity as measured in HT1080 (A) and C2C12 (B) cell lines (Nel *et al.*, 2016). Cells were transiently transfected with pL3 luciferase reporter gene constructs containing *TGFB1* promoter haplotypes pHap-1 to pHap-4 together with TK renilla reporter gene constructs as an internal control for transfection efficiency. pHap-3 which contains the African specific -387T allele, showed a loss of basal promoter activity relative to the other 3 haplotypes.

The authors then went on to determine whether the observed differences in promoter activity of pHap-1 and pHap-3 translate into altered *TGFB1* expression levels in phenotype-specific dermal fibroblasts. They found that consistent with the promoter studies (figure 3), fibroblasts from OP-MG patients with the -387C/T or T/T genotype had >2-fold lower *TGFB1* mRNA transcript abundance than those of control MG patients with the -387C/C genotype (figure 4). The authors speculated that control fibroblasts will have higher levels in comparison to OP-MG fibroblasts. To test this, they performed western blot analysis of phosphorylated smad3 (p-Smad3) which is a downstream marker of the canonical *TGFB1* signalling pathway. Densitometric as well as quantitative PCR results corroborated their prediction, p-Smad3 was found to be lower in the OP-MG fibroblasts (-387C/T) when compared to control fibroblasts (-387C/C).

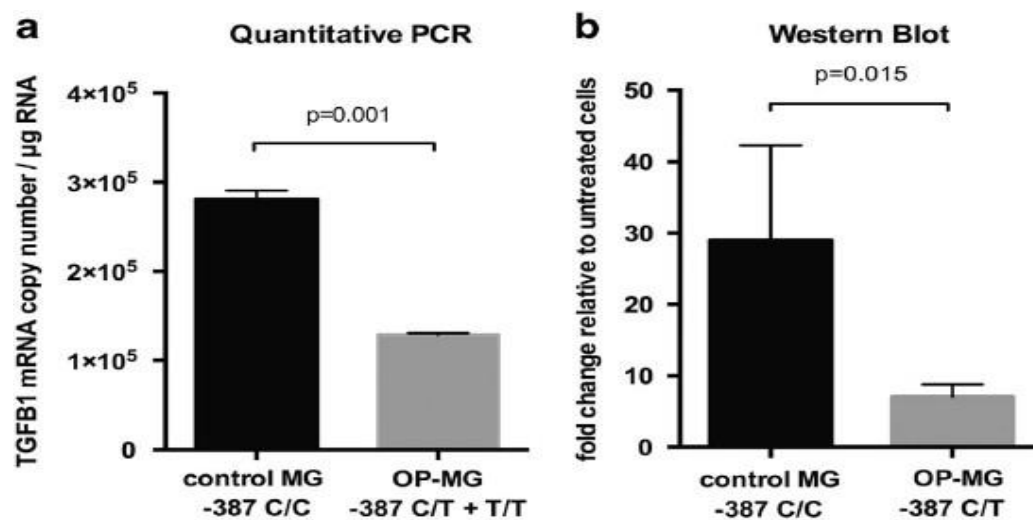


Figure 4: *TGFB1* -387 C>T is an expression quantitative trait in phenotype-specific dermal fibroblasts (Nel et al., 2016). The observed differences in promoter activity of pHap-1 and pHap3 were investigated to determine whether they translated into *TGFB1* expression levels in phenotype-specific dermal fibroblasts by quantitative PCR. The data suggest that at least in fibroblasts, the *TGFB1* -387 promoter genotype strongly predicts *TGFB1* mRNA levels.

Taken together, the African-specific *DAF* and *TGFB1* SNPs suggest that critically low extraocular muscle *DAF* expression levels as a result of either MG-induced *DAF* repression (susceptible subjects with *DAF* -198C>G) and/or low *TGFB1*/*Smad3* canonical signalling contribute to the manifestation of the ophthalmoplegic complication in juvenile MG. However, not all individuals with OP-MG harboured the variants in *DAF* and *TGFB1*. This, therefore, led the authors to hypothesize that there may be other gene variants and/or other pathways, in addition to excessive complement activation, which triggers the progression of MG to the OP-MG subphenotype.

In order to identify other OP-MG susceptibility genes, WES was performed on a small sample of well-characterised patients with OP-MG and control MG cases (i.e patients with MG but without ophthalmoplegia

as controls) in an unbiased manner. Only those genes expressed in extraocular muscles were considered. The authors identified four genes which harbour potentially functional variants in their untranslated regions (UTR) (Table 1 highlights two of the four genes which were the main focus of this study)(Nel *et al.*, 2017). These variants were found in genes involved in the complement, myogenesis and gangliosphingolipid pathways; which suggest that these pathways might be perturbed in the extraocular muscles of OP-MG individuals (Nel *et al.*, 2017).

Table 1: Gene variants identified by whole-exome sequencing in Nel et al. 2017.

Gene Variant	Location	Predicted transcription factor binding site
<i>ST8SIA1c</i> .-477_-475 <i>delCCC</i>	5'UTR	RXR α ; MAZ
<i>IL6Rc</i> . *3043T>C	3'UTR	

1.2.3 Interleukin 6 receptor (IL6R)

Interleukin 6 (IL-6) is a T cell-derived cytokine capable of inducing B lymphocyte differentiation into Ig-producing cells, and, as a result, was named B cell stimulatory factor 2 (Ghasemi and Ghasemi, 2017) (Chen, Tsui and Smith, 2005). It is a pleiotropic cytokine that mediates several biological functions which include; regulation of the immune system, regenerative processes, metabolism, bone homeostasis, cardiovascular protection and neural function (Chen, Tsui and Smith, 2005). IL-6 plays a vital role in the development and activation of the innate and adaptive immune system. In the innate immune system, IL-6 induces the differentiation of monocytes to macrophages instead of dendritic cells and is associated with immunosuppressive properties in dendritic cells. Many inflammatory responses in tissues are also initiated by IL-6 which promotes the infiltration and activation of mononuclear leukocytes (Chen, Tsui and Smith, 2005).

Interleukin 6 has also been implicated in several diseases which associate with extraocular tissues such as thyroid-associated ophthalmopathy where connective tissue and extraocular muscles become infiltrated with T and B lymphocytes. The IL-6R belongs to the class I receptor family, and functions as a complex which includes the receptor and a ubiquitous 130-KDa signalling membrane glycoprotein called gp130. This complex is essential in signal IL6 transduction. Interleukin 6 binds to IL-6R, and the IL- 6/IL-R complex then associates with gp130 allowing to homodimerize (Taga and Kishimoto, 1997). The IL-6R does not need to be membrane-anchored as soluble forms of this receptor can bind IL-6 and signal through membrane-bound gp130 (Taga and Kishimoto, 1997). Orbital fibroblasts express *IL-6R* and can respond to exogenous *IL-6*. A study by Maurer et al. (2015) found the expression of *IL6* and *IL6R* to be altered in muscles of EAMG rats and mice when compared to their control animal

counterparts. They showed that IL6 deficient mice are resistant to the development of autoimmune diseases such as EAMG. This, therefore, led Perek et al. (2001) and subsequently Aricha et al (2011) to conclude that the therapeutic downregulation of *IL6* could control the deleterious events occurring at the neuromuscular junction in EAMG and likely in the early stages of MG (Perek *et al.*, 2001)(Aricha *et al.*, 2011). In muscle biopsies from MG patients, IL-6 protein level was higher than in control muscles (Perek *et al.*, 2001). They concluded that perhaps the increased production of IL-6 by MG muscle may influence the muscle function and on systemic inflammation. Modulating their protein products could represent a new therapeutic strategy for MG (Perek *et al.*, 2001).

Previously, Nel et al. (2017) identified an African-specific variant *IL-6R c.*3043T>C* which associated with the OP-MG phenotype. This variant implicates the IL6/IL6R pathway which has been shown to be involved in MG-induced muscle responses. This variant was predicted to result in the loss of miR-338-3p and miR-364 binding sites. These two miRNAs were reported to be upregulated in the sera of MG patients when compared to controls (Nel *et al.*, 2017). N. van Geunen's unpublished work showed the functionality of the *IL6R c.*3043C>T* variant in the HT1080 and C2C12 cell lines shown in figure 5 (citation of honours work listed in the references section).

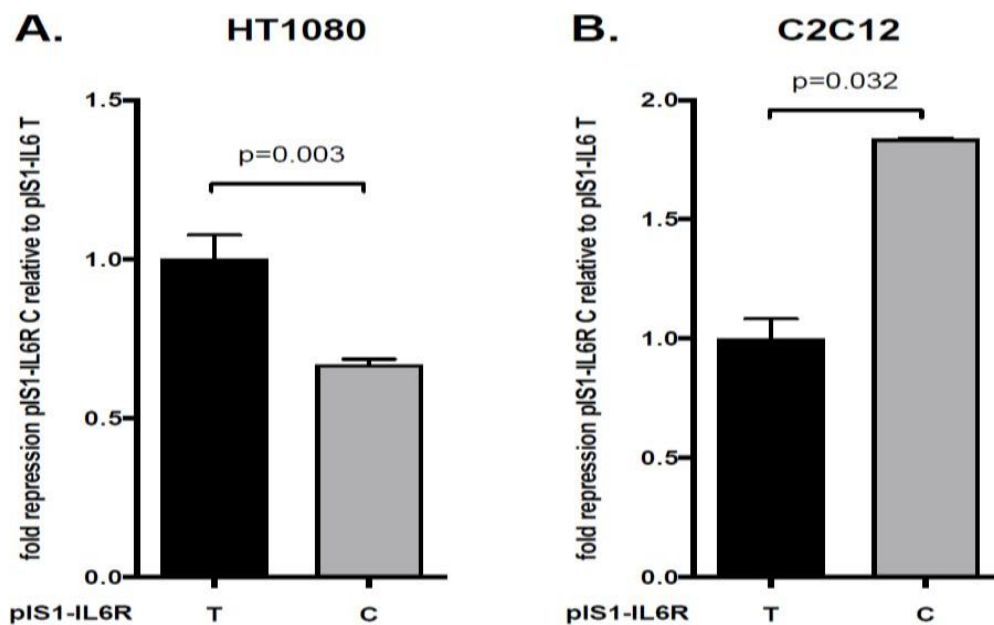


Figure 5: *IL6R c.*3043T>C* (rs11344481) regulates reporter gene expression in two cell lines. HT1080 cells (A) and C2C12 cells (B) were transiently transfected with pIS1-IL6R renilla luciferase 3'UTR constructs (200ng and 1000ng respectively) together with 40ng pGL3 firefly luciferase construct. Cells were transfected with IL-6R constructs containing either the wildtype (T allele) or the mutant (C allele) created by site-directed mutagenesis. Thirty hours after transfection cells were lysed and dual-luciferase reporter activity measured. Renilla luciferase activity was normalised to firefly luciferase activity to control for transfection efficiency. The average fold repression of pIS1-IL6R-C relative to pIS1-IL6R-T vector is shown for each cell line (representing pooled results from 3 independent experiments). Error bars show SEM. Student's unpaired t-test was used to calculate the statistical significance of the fold change differences.

1.2.4 ST8 alpha-N-acetyl-neuraminide alpha-2, 8-sialyltransferase 1 (ST8SIA1)

One interesting gene variant discovered through the WES study is a three-base pair deletion (c.-477_-475 delCCC) in the promoter region of the *ST8SIA1* gene. The *ST8SIA1* (ST8 alpha-Nacetyl-neuraminide alpha-2, 8-sialyltransferase 1) gene encodes an enzyme, GD3 synthase which is involved in the synthesis of b-series gangliosides such as GQ1b which are highly expressed at pre- and postsynaptic EOM endplates. This 5' UTR variant was predicted to be functional using the Algene Promo webserver bioinformatic tool which suggested that it may interfere with transcription factor binding (Melissa Nel, PhD Thesis, 2016). The GD3 synthase is a membrane protein that catalyses the transfer of sialic acid from CMP-sialic acid to GM3 to produce GD3 and GT3 (Figure 6). Consequently, it is the principal enzyme controlling the biosynthesis of b- and c-series gangliosides (Steenackers *et al.*, 2012).

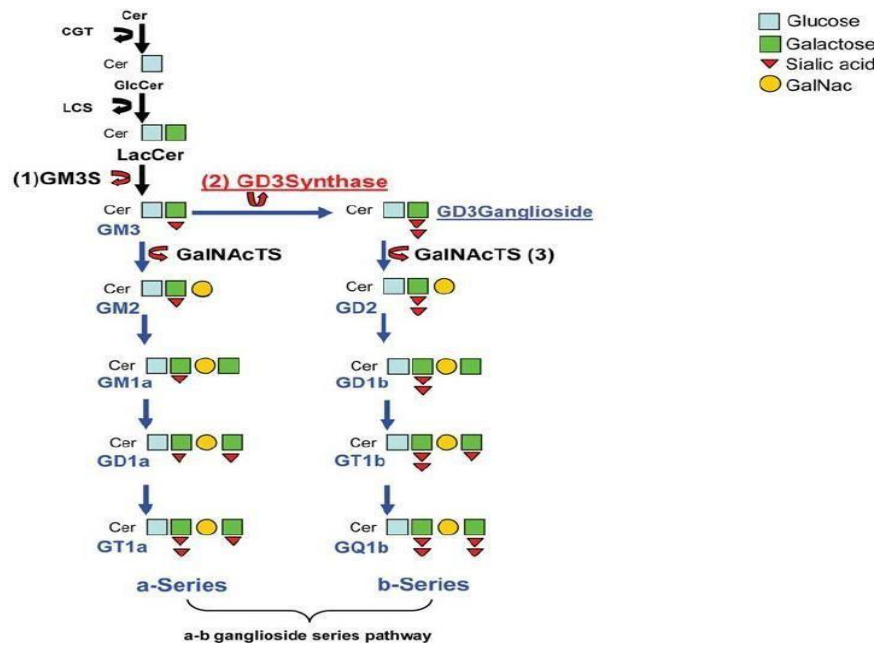


Figure 6: Partial ganglioside biosynthetic pathway. The 3 key regulatory enzymes in ganglioside biosynthesis, GM3 synthase (1), GD3 synthase ((2) encoded by ST8SIA1) and N-acetylgalactosaminyltransferase ((3) GalNAcT) are shown (Husain et al., 2008).

The potential role of altered ganglioside biosynthesis in OP-MG pathogenesis

Gangliosides are sialic acid-containing glycosphingolipids (Figure 3). They are ubiquitously found in tissues and body cells and are more abundantly expressed in the nervous system (Yu *et al.*, 2011). Gangliosides have also been considered to play a role in the development, differentiation and function of nervous systems. Gangliosides are typically anchored in the outer leaflet of the plasma membranes where long saturated hydrocarbon chains of ceramide drive gangliosides to partition laterally into lipid rafts which are membrane microdomains that contain other sphingolipids, cholesterol, and selected signalling molecules. Lipid rafts concentrate receptors and proteins that are involved in specific signalling pathways (Simons and Toomre, 2000). They form concentrating platforms for individual receptors. In the context of EOM, these receptors would include AChR, DAF and IL-6R. Lipid rafts are in extension stabilised by gangliosphingolipid pathways. If receptor activation takes place within a lipid raft, the signalling complex is protected from non-raft enzymes such as membrane phosphatases that otherwise could affect the signalling process (Simons and Toomre, 2000).

There is growing evidence which shows that gangliosides are present on nuclear membranes and they have been recently proposed to play a pivotal role in modulating intracellular and intranuclear calcium homeostasis and the ensuing cellular functions (Ledeen and Wu, 2002). The biological importance of gangliosides has been revealed mainly through the analyses of genetically engineered mice deficient in ganglioside synthase (Yu *et al.*, 2011).

ST8SIA1 Transcription Factor Binding sites

To assess the potential functionality of the *ST8SIA1* c.-477_-475 delCCC 5'UTR variant, a 45 bp sequence (with 21 bp upstream and 21 bp downstream of the variant) was screened using the Algene Promo webserver (Nel *et al.*, 2017). This tool enables identification of putative transcription factor binding sites in a given sequence. The results showed that the Retinoid X receptor alpha (RXR-alpha) and myc-associated zinc finger protein (MAZ) transcription factors potentially bind to the repetitive C sequence element which flanks the *ST8SIA1* c.-477_-475 delCCC variant (Figure 7).

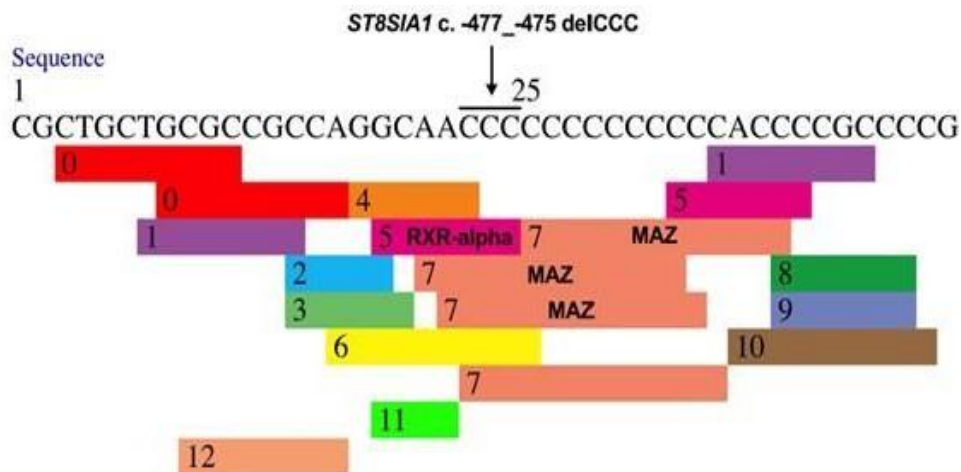


Figure 7: A schematic diagram depicting the predicted transcription factor binding sites in the 5'UTR of *ST8SIA1* gene promoter identified using the Algene Promo webserver. The result shows possible binding sites for myc-associated zinc finger protein to the repetitive C sequence element flanking the *ST8SIA1* variant. The arrow indicates the site of the CCC deletion which is observed as the *ST8SIA1* c.-477_-475 delCCC variant (Melissa Nel, PhD Thesis, 2016).

Myc-associated zinc finger binding protein (MAZ)

Myc-associated zinc finger binding protein is a transcription factor that consists of proline-rich repeats and six Cys₂ His₂ zinc type finger motifs along with five putative sites of phosphorylation by casein kinase II. It was identified as binding to a GA box (GGGAGGG) at the ME1a1 site, to the attenuator region of p2 within the first exon of the c-myc gene and to a related sequence that is involved in the termination of transcription of the gene for complement 2 (Komatsu *et al.*, 1997). MAZ has been implicated in the activation of several genes and has been shown to repress its own gene, as well as c-myc (Komatsu *et al.*, 1997). Despite its abundant expression in skeletal and cardiac muscle, its functional roles in these have not been explored. To date, no MAZ knockout mouse model has been described (Himeda, Ranish and Hauschka, 2008b).

Retinoid x receptor alpha (RXR-alpha)

Retinoid x receptor alpha (RXR-alpha) also known as NR2B1 (nuclear receptor subfamily 2, group B, member 1) is a nuclear receptor which in humans is encoded by the *RXRA* gene. The retinoid receptors fall into two classes: (a) that bind all-trans-retinoic acids (RA), called retinoic acid receptors (RARs) and (b) those for which the ligand is 9-cis-RA, called RXRs. The RXR and RARs are nuclear receptors that mediate the biological effects of retinoid through their involvement in retinoic acid-mediated gene activation (Mangelsdorf *et al.*, 1990). These retinoic acid receptors share homology with a superfamily of steroid hormone and thyroid hormone receptors and have been shown to regulate specific gene expression by a similar ligand-dependent mechanism (Mangelsdorf *et al.*, 1990). These receptors exert their action by binding as homodimers or heterodimers to specific sequences in the promoters of target genes and regulate their transcription (Mangelsdorf *et al.*, 1990). In the absence of ligand, the RXR-RAR heterodimers associate with a multiprotein complex containing transcription corepressors that induce histone deacetylation, chromatin condensation and transcriptional suppression. Upon ligand binding, the corepressors dissociate from the receptors and associate with coactivators, leading to transcriptional activation (Mangelsdorf *et al.*, 1990). Recent studies have shown that the RXRs can function alone as homodimers on certain response elements, but their major function is to serve as a heterodimeric partner with the RARs. The RXR β is found in low levels in nearly all tissues, whereas RXR α is abundantly expressed in the intestine, heart, kidney and spleen. Preliminary studies have suggested RXR in mediating immune response to infection (Núñez *et al.*, 2010). The RXR α has also been suggested to be involved in skeletal muscle differentiation (McBurney, Costa and Pratt, 1993).

2. RESEARCH AIMS AND OBJECTIVES

Although OP-MG is not life-threatening, its pathogenesis is not yet understood, and it results in significant functional impairment in affected individuals.

Previous WES studies from our group identified OP-MG associated gene variants namely *ST8SIA1* c-477_-475 delCCC, *IL6R* c.*3043T>C and *TGFB1* -387C>T. These genes are involved in the gangliosphingolipid, complement and the myogenesis pathways which may be relevant disease pathways in the EOMs of OP-MG individuals.

Aim 1

The first aim of this study is to investigate the putative functionality of the *ST8SIA1* 5'UTR variant using *in vitro* promoter-reporter assays in relevant cell lines.

Aim 1: Hypothesis

We hypothesise that OP-MG may be a result of a complex genetic network which is activated by autoimmune MG in a tissue-specific setting such as extraocular muscles. Since the *ST8SIA1* 5'UTR variant associates with OP-MG, we hypothesize that this variant may have functional consequences for the expression of *ST8SIA1* in the extraocular muscle microenvironment. We postulate that the CCC deletion may abrogate the binding of transcription factors such as MAZ and RXR α , to the *ST8SIA1* regulatory region and hence alter the expression levels of this gene.

Aim 1: Objectives

1. To investigate the functionality of the 5'UTR variant on *ST8SIA1* gene expression. Site-directed mutagenesis will be used to create a mutant plasmid (referred to henceforth as MT) by introducing a CCC deletion into a wild type (WT) promoter-reporter plasmid containing the human *ST8SIA1* regulatory region upstream of a luciferase gene. Both the WT and MT plasmids will be transfected into HT1080 cells (a human fibrosarcoma cell line) and C2C12 cells (a mouse myoblast cell line) and their respective basal promoter activities compared.
2. MG sera from patients with OP-MG may contain growth factors and cytokines which may impact on gene expression. To investigate whether MG sera influences both WT and MT *ST8SIA1* promoter activity in C2C12 myoblasts, luciferase assays will be performed in the presence of MG sera.

Aim 2

The second aim is to assess the endogenous levels of the genes harbouring the OP-MG-associated gene variants (*ST8SIA1*, *IL6R* and *TGFB1*) in patient-derived orbital fibroblasts using qPCR and, lastly, assess their expression in an MG specific context by treating ocular fibroblasts with homologous MG sera.

Aim 2: Hypothesis

Recently we (Dr Europa) sampled orbital fibroblasts from cases with OP-MG and controls without MG. These cells were grown in culture and afforded the opportunity to assess endogenous gene expression in the candidate genes *ST8SIA1*, *TGFB1* and *IL6R* at basal levels in OP-MG cells vs controls, and after treating MG-specific stimulus we postulate that the gene expression in OP-MG cells will differ for control cells if the gene variant is functional and relevant in orbitally-derived tendon fibroblast. Previously 5'UTR variant in the *ST8SIA1* gene was found to be functional by bioinformatics tools and associates with OP-MG phenotype (Nel *et al.*, 2017). We postulate lower *ST8SIA1* expression in OP-MG vs control patients in response to MG sera which may have negative implications for extraocular muscle endplate repair and vulnerability to complement damage.

Aim 2: Objectives

1. The endogenous basal *ST8SIA1*, *IL6R* and *TGFB1* expression levels in patient-derived ocular fibroblasts will be determined (n=5 controls and n=2 OP-MG) using qPCR.
2. Cells will be treated with MG sera from OP-MG patients to mimic the MG disease state (environment) within these cells, and the basal vs MG sera treated endogenous gene expression levels determined using qPCR and compared.

This study was approved by the University of Cape Town Health Sciences Faculty Research Ethics committee (ethics number: HREC 320/2009) and all individuals (or their parents if <18 years) signed informed consent to participate.

3. METHODS

3.1 *ST8SIA1* promoter-reporter plasmid construct

A pGL3-Enhancer luciferase vector (Promega) containing a 2307 bp region of the human *ST8SIA1* promoter region cloned into the vector using Xho1 and Nhe1 restriction enzyme sites as previously described (Bobowski *et al.*, 2013) was obtained as a gift from Dr Delannoy (University of Lille, France). This reporter construct served as the wild type (WT) plasmid-reporter construct for subsequent experiments. A schematic of this *ST8SIA1* promoter-reporter plasmid construct is shown in Figure 8 and will be referred to as pGL3 -*ST8SIA1* WT.

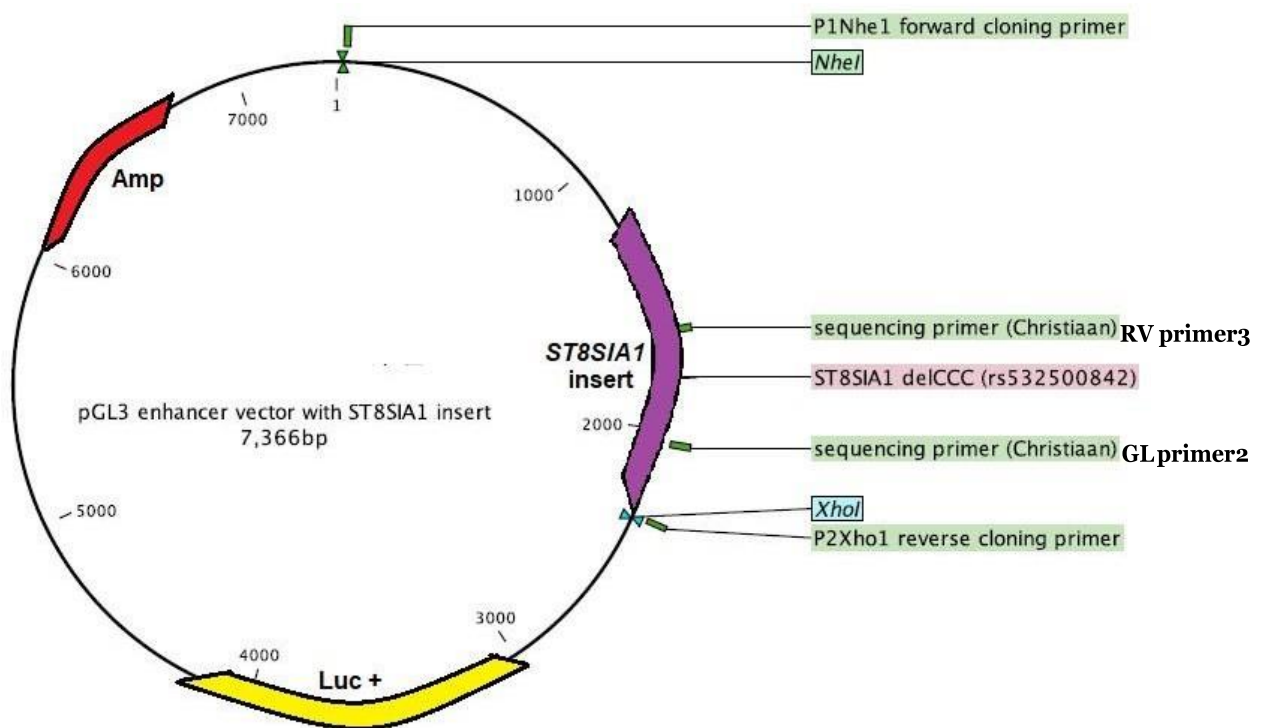


Figure 8: A schematic diagram of pGL3-ST8SIA1 wild type (WT) plasmid. This reporter vector has a human *ST8SIA1* promoter region (*ST8SIA1* insert indicated in purple) subcloned into a pGL3-Enhancer vector (Promega) upstream of the firefly luciferase gene (indicated in yellow) at the Nhe1/Xho1 sites. pGL3 (-2307/+1) and has an ampicillin resistance gene (indicated in red). The sequencing primers used (RV and GL) are indicated by green highlighted region on the pGL3 vector.

3.1.1 Plasmid extraction from filter paper

The pGL3-ST8SIA1 plasmid was received as a blot on filter paper where a circle had been drawn by the sender to indicate the location of the plasmid. This is a suitable method to transport DNA to ensure the stability of the DNA at room temperature for transportation. Having cut the circle out of the filter paper it was then divided in half into two separate pieces. One half was stored in an empty 1.5ml centrifuge tube. The second half was placed in a 1.5 ml microcentrifuge tube containing 50µl of 10 mM Tris (pH8) (Tris was used as a buffer to maintain a constant pH for the DNA throughout the extraction process). The tube with Tris buffer was pulse vortexed for 10 seconds and then placed in a heating block at 63°C for 30 seconds. This heating step is necessary to ensure degradation of nucleases and facilitates the extraction of DNA from the filter paper and increases DNA yield. The vortex and heating steps were repeated twice after which the tube was centrifuged for 30 seconds to pellet the filter paper fragments. The supernatant containing the plasmid was transferred to a clean tube.

3.1.2 Preparation of competent cells

Competent bacterial cells were generated by inoculation of the *DH5α Escherichia coli* (*E.coli*) bacterial strain in Luria Broth (LB) and incubated overnight in a shaker at 37°C. 1ml of this overnight culture was used to inoculate 100ml LB in a 500ml flask and allowed to grow to reach mid-log phase (which is an optical density (OD₅₉₅) of 0.5 - 0.8) at 37°C after 3 hours. The overnight culture was pelleted by centrifugation at 3000 rpm for 10 min at 4°C and subsequently resuspended in freshly prepared ice-cold 100mM calcium chloride (CaCl₂) (see appendix). The CaCl₂ provides calcium cations which neutralise the negatively charged phosphate backbone of the DNA molecule and the negatively charged surface of the bacteria. Following resuspension, the cells were kept on ice for 1 hour (for the stabilisation of the bacterial membrane whilst facilitating the interaction between the cations and the negatively charged components) and centrifuged at 3000 rpm for 10 minutes at 4°C. The pellet was resuspended in CaCl₂ and kept on ice in the refrigerator overnight at 4°C. 50% glycerol was added to the remaining bacterial culture and snap-frozen in liquid nitrogen for long term storage at -80°C for future use.

3.1.3 Transformation of competent cells

The transformation was carried out by adding 50µl of solubilised plasmid DNA (section 3.1.1) to a thawed 100µl competent *E. coli DH5α* glycerol stock and shaken gently on ice for 30 minutes at 12 000 rpm. The DNA-cells mixture was heat shocked in a water bath for 1 minute at 42°C to facilitate the uptake of the plasmid DNA by the bacterial cells. The heat-shock process creates pores in the bacterial plasma membrane which allows the plasmid DNA to enter the bacterial cells. This was followed by rapid cooling on ice for 5 minutes. This cooling allows the cell membrane to

normalise/stabilise following the heat shock process which briefly alters membrane fluidity. LB (see appendix) was subsequently added to the competent DNA-cells mixture and incubated at 37 °C with gentle shaking at 220 rpm for 2 hours. Following the incubation, the mixture was spread plated aseptically on 100mg/ml ampicillin Lauria Agar (LA) plates, incubated overnight at 37 °C and the growth of discrete bacterial colonies closely monitored. The bacterial colonies were grown in ampicillin plates which allow the growth of only ampicillin-resistant bacteria (the pGL3-*ST8SIA1* plasmid has an ampicillin resistance gene thus the assumption is that only competent cells which successfully incorporated this plasmid are selected for growth).

3.1.4 Preparation of Plasmid DNA

Ampicillin positive LA plates were checked for bacterial colony growth following the transformation of DH5 α bacterial cells with pGL3-*ST8SIA1* WT plasmid. Upon visible bacterial growth, plasmid DNA (pGL3-*ST8SIA1* WT) was isolated from the transformed DH5 α bacterial cells and prepared for subsequent use using the Pure Yield maxiprep system (Promega). A single bacterial colony was selected aseptically and inoculated into 100mg/ml ampicillin positive LB and incubated overnight at 37°C with gentle shaking at 220 rpm. Following observed bacterial growth (murky LB is an indicator of bacterial growth), 1 ml of this bacterial culture was used to inoculate 200ml LB containing 1% ampicillin. The culture was placed on a shaker and allowed to grow overnight at 220 rpm. The cultures were pelleted by centrifugation at 5000 xg for 10 minutes.

The maxipreps were carried out according to manufacturer's instructions (Promega) being summarised: The bacterial cells were lysed using lysis buffer which contains sodium hydroxide and detergent sodium dodecyl sulfate (SDS) which acts to solubilise the cell membrane whilst denaturing most proteins and chromosomal DNA in the cells. This assists in the effective separation of proteins from the plasmid further along within the maxiprep process. The cell lysis process was facilitated by gently inverting the 50ml screw-up tubes followed by a three min incubation for at room temperature. The lysed cells were neutralised with neutralisation buffer along with gentle inversion for 10 to 15 times (this allows the chromosomal DNA to re-aggregate with cellular proteins).

The lysate was centrifuged at 7000 xg for 30 minutes at 24°C which concentrates and precipitates cellular material (including DNA) into a pellet at the bottom of the vessel thus separating the chromosomal DNA, protein and cell debris from the supernatant containing the plasmid DNA. This supernatant lysate containing plasmid DNA was purified by passing through a maxi binding column (Promega) using a vacuum manifold. The lysate was subjected to several washes using endotoxin removal wash. Endotoxin (lipopolysaccharide) is a cell wall component of gram-negative bacteria such as *E. coli*. The endotoxin removal wash, therefore, reduces the number of endotoxins, proteins as well as other contaminants

which may get eluted with the plasmid DNA. Upon elution of plasmid DNA, nuclease-free water was added to the binding column and allowed to stand for 5 min after which it was centrifuged at 3000 rpm for 5 minutes. The elute was stored in a 1.5ml tube at -20°C. The integrity of the eluted plasmid DNA was determined using nanodrop (ND- 1000 v.3.5.2 software, Inqaba biotechnical industries) and analysed by gel electrophoresis by running plasmid DNA on 0.5% agarose gel at 80V for 1 hour (Results section 4.1). The plasmid DNA was also sent for Sanger sequence verification at Inqaba Biotechnical industries.

3.2 Verification of the pGL3-ST8SIA1 plasmid

The *pGL3-ST8SIA1* plasmid was confirmed by diagnostic digests as well as Sanger sequencing. Sanger sequencing allows for a thorough analysis of DNA by providing us with the sequence of nucleotides which allows us to locate regulatory regions, identify mutations as well as draw comparisons between homologous genes across species.

Sanger sequencing was performed on the *pGL3-ST8SIA1* plasmid to ensure that the correct plasmid was received (i.e. a pGL3 enhancer vector with the *ST8SIA1* insert incorporated in the correct location within the vector). The sequencing was also critical in determining the presence of any unintended mutations.

Sanger sequencing, in this case, was performed in order to confirm receipt of the correct plasmid in terms of its size and confirm correct incorporation of the *ST8IA1* insert (*pGL3-ST8SIA1*) as well as to ensure that the obtained plasmid which had no additional or unintended mutations.

3.2.1 Restriction enzyme digest

The diagnostic digest was performed sequentially using XhoI and NheI NEB high fidelity restriction enzymes respectively, according to NEB Timesaver protocol. High fidelity enzymes differ from the “normal” commercial enzymes in those reactions which usually take up to 3 hours to complete are cut down to 15 minutes of reaction time. In the case of a double digest, 0.5µl of each enzyme was added whilst all the other reagents and reaction protocol were maintained. 1 µg of plasmid DNA was combined with 1X NEBuffer with the total reaction volume of 50µl. The reaction was incubated for 10 minutes at 37°C. The reaction was stopped by adding 10µl of 6x gel loading dye. The digest products were run on a 0.5% agarose gel alongside a 1000 bp molecular weight marker (NEB) used as a sizing reference at 80 V for 1 hour (see Figure 10).

3.2.2 Sanger sequencing of pGL3-ST8SIA1

The *pGL3-ST8SIA1* WT plasmid was purified from bacteria using maxipreparation kit (Promega) after which it was sent to Inqaba Biotechnical Industries (Pty Ltd) using the GL primer2 forward primer (5'-CTAGCAAAATAGGCTGTCCC-3') and RV3 reverse primer(5'-TGGAAGACGCCAAAACATAAAG-

3'). The sequencing data was returned as a FASTA file. Sequence alignment to a reference sequence was created to enable insertion and viewing of the sequencing file. The reference sequences for both pGL3 enhancer vector as well as that off *ST8SIA1* were retrieved in FASTA format from Addgene (Addgene.org, 2017) and imported to CLC Bio Main Workbench (Qiagen). The pGL3 Enhancer vector, although it is a plasmid circular in conformation, its FASTA format is such that it is presented in a linear configuration. This, therefore, displaces the location of the multiple cloning site (region) at its starting point thus making the alignment of the sequencing primers difficult. While the location of the GL primer2 may not pose any issues, this linear configuration makes aligning of the RV3 primer location towards the 5000-base pair region which is located near the end of the sequence (Figure 9A). The location makes the reading the alignment close to the multiple cloning site difficult due to the primers being sparsely distributed and it makes aligning of the primers to the reference sequence difficult to analyse as the sequencing information from the forward primer will not align beyond the end of the reference sequence and as a result will not provide any information in the region of the multiple cloning site (as it is located very far from this region of interest). This impacts the ability to accurately detect the presence and/or absence of an insert. This was rectified through linearizing the sequence manually such that the sequencing primers were repositioned to flank the multiple cloning site to enable both the GL primer2 and RV3 primers to “read through” the multiple cloning site to verify the presence of the insert and its nucleotide sequence (Figure 9B).

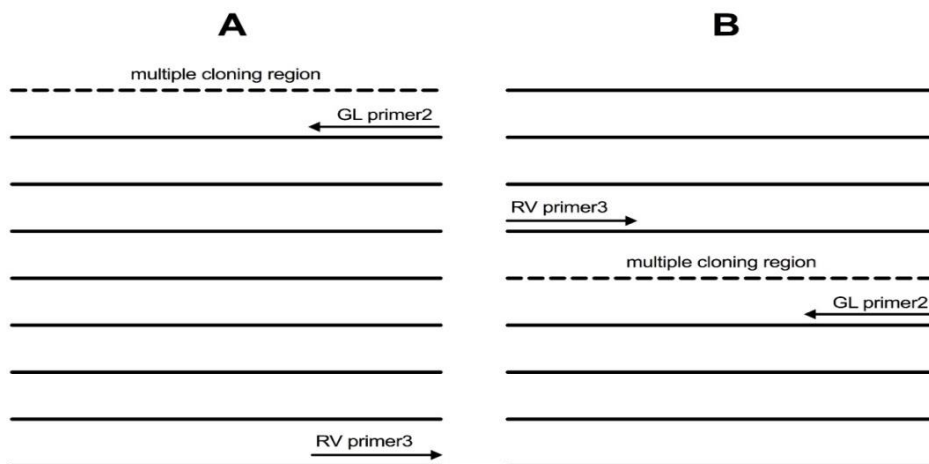


Figure 9: A schematic diagram showing the sequencing primer positions (GL primer2 and RV primer3) relative to the reference sequence in FASTA format. The multiple cloning regions indicated by a dashed line is located towards the start of the reference sequence. The reference sequence is indicated by the solid black lines. Arrows are the primers. In (A) the multiple cloning site is located towards the start of the sequence. Whilst it is possible to align primer GLprimer2 it is difficult to align RV primer3 as this primer will align towards the end of the actual sequence itself making it difficult to analyse as this primer will not have any reference sequence to align to. (B) This is the adjusted sequence whereby the multiple cloning site was repositioned to a more centred position so as to allow GL primer 2 and primer RV primer3 to flank the multiple cloning site for a better “read-through” of the multiple cloning site by both primers.

3.3 Site-Directed mutagenesis (SDM)

To create a mutant pGL3-ST8SIA1 (MT) a CCC deletion was generated in the 5'UTR of wild type pGL3-ST8SIA1 (WT) plasmid for subsequent experiments, by site-directed mutagenesis (Figure 10).

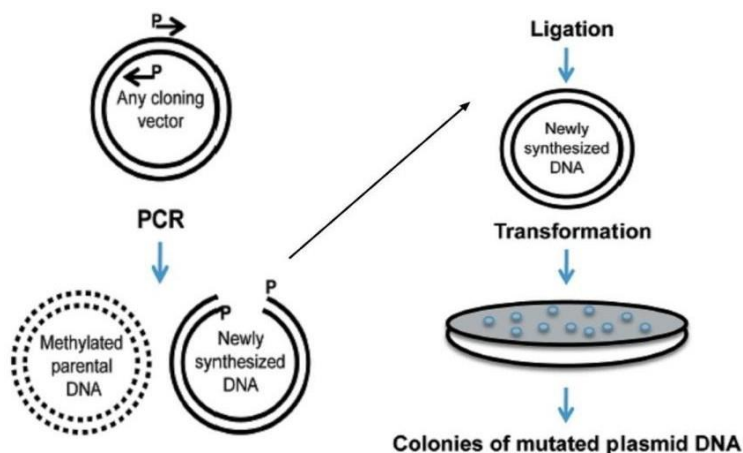


Figure 10: Site-directed mutagenesis workflow. The pGL3-ST8SIA1 construct was amplified with phosphorylated mutagenic primers. This plasmid DNA was amplified by PCR. The PCR product was DpnI treated and ligated. The newly synthesised DNA is re-ligated with KAPA rapid Ligase. The mutant transformants were then plated on ampicillin Lauria Agar plates.

3.3.1 Mutagenic Primer design & Mutagenesis Reactions

Table 2: Mutagenic Primers

Forward Primer	Reverse primer
5'P- CCCCCCCCCACC CGCCCCGAG	5'P- TTGCCTGGCGGCGCAGCAGCGCG

To generate the CCC deletion, primers were designed with a gap between them corresponding to the region to be deleted (Figure 11). Due to the G-C rich nature of the sequence flanking the deletion site, the primers were phosphorylated (Table 2). This was to aid in the PCR efficiency of the primers. 5'phosphorylation is important (and advantageous) if an oligo is used as a substrate for DNA ligation. Oligo's containing 5'-phosphate group are able to act as linkers and adapters within cloning and ligation processes. G-C nucleotides bind more strongly than A-T nucleotides. Having too many G and C nucleotides at the end of a primer can cause the melting temperature (T_m) to increase causing the G- C clamp of the primer to be too strong which then prevents it from denaturing at the correct temperature. G-C rich primers also have an increased likelihood of mispriming. G-C rich content at the 3'end decreases specificity (Itdna.com, 2017).

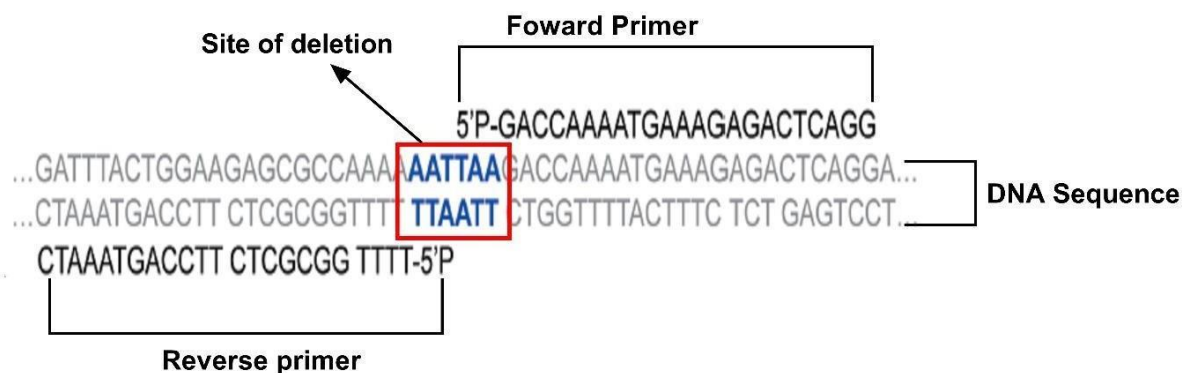


Figure 11: The approach for SDM PCR primer design. A deletion in the DNA sequence (blue) is introduced by designing primers with a gap between them corresponding to the region to be deleted (adapted from Kapa HIFI Site-Directed Mutagenesis, n.d). (The primer sequence in this Figure serves as an example and is not the actual primer/ DNA sequence used)

3.3.2 Mutagenesis PCR

The PCR amplification was carried out in a 50 µl reaction, using Kapa HotStart ready Mix with 1 ng template DNA and 1 U of Kappa Hot Start ready mix. Each primer was used at a concentration of 0.3µM. Due to the primers being GC rich 5% DMSO was added to enhance the deletion. This addition of DMSO to GC rich primers aids in the denaturing of templates with high GC contents. The cycling protocol is specified in Table 3. The cycling protocol was performed according to the manufacturer's specifications (Kapa Biosystems, Cape Town, SA).

Table 3: Site-directed mutagenesis cycling protocol.

Process	Temperature	Time	Number of cycles
Initial Denaturation	95 °C	2 minutes	1 cycle
Denaturation	98 °C	20 seconds	16 cycles
Anneal	75 °C	15 seconds	
Extension	72 °C	3.65 seconds	
Final extension	72 °C	2 min	1 cycle

3.3.3 DpnI digestion and ligation

Once successful amplification was confirmed by running the PCR product on a 0.5% agarose gel it was then treated with DpnI which digests methylated and hemimethylated DNA. DpnI serves to remove the parental DNA by digestion of the methylated plasmid template to ensure that that nearly all of the resultant DNA has the mutation incorporated. The reaction was set up as shown in Table 4. The reaction was incubated for 2 hours and heat-inactivated at 80°C for 20 minutes. The DpnI treated PCR product was purified using Promega Wizard SV gel and PCR clean up as per manufacturer's instructions. The concentration of the purified product was analysed by Nanodrop and gave a final concentration of 34.2ng/µl.

Table 4: DpnI reaction Mixture set up.

Reagent	Volume
DpnI Buffer (10 X Tango Buffer)	2µl
DpnI	2µl
Nuclease free water	16µl
PCR reaction (0.5 – 1 µg/ µl)	10µl

Following the DNA clean-up of the SDM product using the Promega Wizard SV gel cleanup system. Preparation for ligation was performed using reaction setup in Table 5.

Table 5: T4 ligase reaction setup.

Reagent	Concentration	Volume
Product [34.2 ng/µl]	50ng	1.462
T4DNA (Thermo scientific)	5 Weiss U	1
T4 DNA ligase buffer	10 X	5
PEG	5%	5
Water	-	37.5

The ligation reaction was incubated overnight at 22°C and the reaction inactivated at 65°C for 10 minutes. The entire ligation product was transformed into DH5α bacteria; bacterial colony growth was checked after 24 hours. The transformants were selected by their ability to propagate on ampicillin-containing agar plates. Since the pGL3-ST8SIA1 plasmid has an ampicillin resistance gene it is expected that only those bacteria which had successfully taken up pGL3-ST8SIA1 plasmid would therefore grow. This clone was maxi prepped and designated as ST8SIA1 MT for transfections. This clone was run on a gel after the maxiprep procedure to ensure that its integrity was preserved. The plasmid was sent for Sanger sequencing (Inqaba Biotech, Pretoria, South Africa) and the sequence alignment generated in CLC Main Workbench.

3.4 Cell culture

The human fibrosarcoma (HT1080) and mouse myoblast (C2C12) cell lines (T-box Lab stocks) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose GlutaMAX supplement pyruvate medium (Gibco, Life Technologies). The medium was supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin and 100µg/ml streptomycin. Cells were maintained at 37°C in a 95% air and 5% CO₂ humidified incubator. Cells were cultured in the fresh medium which was replaced every 3 days. The cells were routinely subjected to mycoplasma testing. Cells were subjected to mycoplasma testing as mycoplasmas can modify many aspects of a cell's physiology and metabolism. This, therefore, renders experiments conducted with mycoplasma positive cells worthless and questionable. Only mycoplasma negative cells were used in experiments.

3.4.1 Mycoplasma Testing

Approximately 1×10^5 cells were grown on a coverslip in a 35 mm dish for 24 hours in antibiotic-free medium. The cells were fixed in a 1:3 glacial acetic acid and methanol mixture for 5 seconds, washed briefly with water (to remove the fixing solution) and air-dried at room temperature for a couple of minutes. Once dried, 500µl of 0.5µg/ml Hoechst 33258 (Sigma-Aldrich) was applied for 7 seconds to stain the DNA, followed by a brief wash with water to remove excess stain. The coverslip was mounted on a slide with mounting fluid (see appendix) and viewed with a fluorescent microscope (Zeiss Axioskop 2 MOT Fluorescent Microscope, Germany) using the DAPI filter. Mycoplasma negative cells stained positive with Hoechst 33258 only in the nucleus, whilst mycoplasma infected cells show positive staining in both the nucleus and the cytoplasm. Staining in the latter is due to the presence of bacterial DNA, which is not confined to the nucleus of the host cell.

3.5 Luciferase assays

Luciferase can be used as a reporter enzyme to estimate gene expression in prokaryotic or eukaryotic cells. When the luciferase gene containing a target promoter region is transfected into target cells using a plasmid (i.e. a promoter-reporter gene construct), the activity of the promoter can be inferred based on the measured luciferase activity (estimated by bioluminescence) (Figure 12). Luciferases form part of a group of oxidative enzymes which allow certain organisms to bioluminesce. Firefly luciferases emit light via a chemical reaction in which luciferin is converted into oxyluciferin by the luciferase enzyme. The energy released in this reaction is in the form of light. Transient transfection assays such as the luciferase assay are used to quantify promoter strength. In these assays, the promoter of interest (in this case WT and MT pGL3-ST8SIA1) is placed upstream of a luciferase gene. The resultant reporter construct is then transferred into fibrosarcoma and mouse myoblast cells by chemical transfection. Expression of the luciferase gene is measured to quantify the activity of the promoter.

Based on this principle, luciferase assays can be used to study the functionality of promoter variants, by comparing the resultant activity of wild type versus mutated constructs which carry the variant of interest.

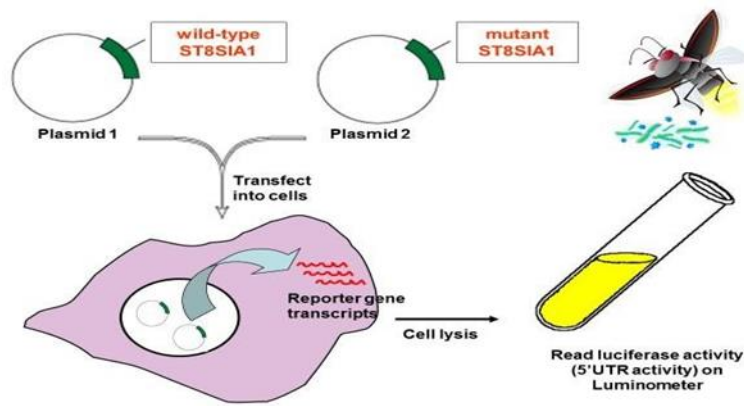


Figure 12: Luciferase assay principle. A DNA sequence of interest (ST8SIA1 promoter) is cloned into an expression vector (pGL3 Enhancer plasmid) upstream of the luciferase reporter gene. Wild type pGL3-ST8SIA1 and mutant pGL3-ST8SIA1 plasmids were then transfected into cells (HT1080 & C2C12 cell lines). Following the lysis of these cells and the addition of the luciferin substrate, a luciferase assay is performed to directly measure the enzymatic activity of the luciferase reporter protein using a luminometer. Adapted from: (Van Greunen, 2016).

3.5.1 Transfection

Luciferase assays were performed in transparent 12 well plates using the Dual-Luciferase Reporter assay system (Promega). The cells were seeded at a density of 5×10^4 cells per well (C2C12) and 7.5×10^4 per well (HT1080) and were grown to a confluency of 50 to 80% for optimum transfection efficiency. The type of transfection reagent used was chosen based on which reagent resulted in less cell cytotoxicity whilst producing luciferase activity of greater than 1.

HT1080 cell line

24 hours after seeding cells, X-tremeGene transfection reagent (Merck) was used to facilitate the transfection of pGL3-ST8SIA1 wild type (WT) and mutant (MT) plasmids into the HT1080 cell line according to manufacturer's instructions. X-tremeGene is a non-viral transfection reagent. This transfection reagent is comprised of lipids and acts by forming a complex with the transfected DNA, which is then transported into the cells. A dose curve of varying concentrations of pGL3-ST8SIA1 promoter constructs (WT and MT) was performed to determine the optimal DNA concentration for transfection in subsequent experiments. The total amount of DNA per well was 500ng, empty pGL3-

basic vector was used to adjust the amount of DNA to 500ng. Each well was also transfected with 40ng of renilla luciferase (T.K renilla).

C2C12 cell line

C2C12 cells were seeded in growth medium in the presence of 400 µl matrigel substrate (Corning). Matrigel is a basement membrane rich in extracellular matrix proteins as well as a number of growth factors. Matrigel assists (facilitates) cell growth and differentiation. These cells were transfected with a total of 1000 ng of plasmid DNA (i.e 1000 ng of either the pGL3-*ST8SIA1* WT or 1000ng of the pGL3-*ST8SIA1* MT plasmid DNA) per well which was adjusted with empty pGL3-basic vector and 40 ng of renilla luciferase (T.K Renilla) added along with the 1.5µl lipofectamine dose according to manufacturer's instructions (ThermoFisher Scientific). Lipofectamine, also a non-viral transfection reagent is based on the lipofection method whereby negatively charged vector DNA is trapped in a cationic lipid vacuole (called a liposome) which is formed by the reagent. The liposome fuses with the cell membrane thereby releasing the vector DNA into the cell. Following transfection, the cells were treated with 5% MG sera for 24 hours.

3.5.2 Harvesting Cells

24 hours (HT1080) and 48 hours (C2C12) post-transfection the cells were washed with cold phosphate buffer saline (PBS) and lysed with 1X passive lysis buffer (Promega). The cells were lysed for 30 minutes on a shaker after which the cells were scraped with 1ml syringe plunger and collected into sterile 1.5ml tubes and frozen at -80°C to enhance the lysis process. Ten microlitres of the lysate were used for luminescence measurement on a white bottom 96 well plate.

Reporter activity is measured by the reaction of firefly luciferase enzyme catalysing the conversion of luciferin to oxyluciferin, which emits light that is measured by the luminometer. Renilla luciferase is then measured, which converts the coelenterazine to coelenteramide in the presence of oxygen. The dual-luciferase reporter assay utilizes these reporter enzymes to simultaneously measure both the reporter gene activity of the transfected pGL3-*ST8SIA1* promoter construct as well as the renilla internal control.

3.5.3 Reading luciferase activity

Following lysis, cells were thawed on ice and spun down to pellet the cell debris. 10µl of cells were added to the wells of a white bottom 96 well plate. To induce the initial luminescent reaction, 50µl Luciferase Assay Reagent (LAR) II (Promega) containing the luciferin substrate of the firefly luciferase was added to the wells containing cell lysate. This sample was mixed, and luminescence quantified using luminometer (Thermo Lab Systems, Luminoskan Accent). LARII functions as a substrate in the

enzymatic reaction to activate the luciferase enzyme. After quantifying the firefly luminescence, this reaction was quenched and the Renilla luciferase initiated simultaneously by adding Stop &Glo Reagent to the same sample. The Stop &Glo reagent quenches firefly luminescence and provides the coelenterazine substrate for renilla luciferase enabling quantification of renilla luminescence without disturbance from firefly luminescence. 50µl of the Stop &Glo reagent was added to the wells, mixed and renilla luminescence quantified. Firefly luciferase and renilla luciferase make use of different substrates in their luminescent reactions. The luminescence of firefly can be quenched without impeding the activity of the renilla luminescence thus enabling for the quantification of each protein individually.

The addition of internal control such as renilla is important as it allows for the determination of transfection efficiency. TK renilla yields low-to-moderate levels of expression in most cell lines and its promoter activity is lower compared to CMV or SV40 promoter. It is also less likely to be affected by other experimental treatments. This, therefore, makes TK renilla an ideal initial vector to choose for normalisation (Worldwide.promega.com, 2017). The values generated by the luminometer from these reactions were quantified and recorded using the Ascent software 2.6 (ThermoFisher Scientific) as well as Microsoft Excel (Windows Vista). Relative luciferase activity values were normalised to the renilla activity to account for variation in transfection efficiency before making comparisons between the activity of the pGL3-*ST8SIA1* WT and MT promoters.

3.5.4 Luciferase assay optimization experiments

The transfection step is critical in luciferase assays. Given the diversity of cell types, there isn't a single transfection method nor transfection reagent which can be applied across all cultures (cell types) under all conditions. As a result, the optimization of experimental conditions becomes imperative. There are several factors which affect transfection efficiency such as quality and type of molecule to be transfected (DNA, RNA etc.), the passage number of the cells and the quality of the cells to be transfected all of which are key in the overall transfection result. Optimization, therefore, is critical to ensure viability and reproducibility.

3.5.5 Choosing the internal control vector

The renilla luciferase gene is commonly used as an internal control in luciferase-based reporter gene assays to normalise the values of the experimental reporter gene for variations that could be caused by transfection efficiency and sample handling. Plasmids encoding renilla luciferase under different promoter constructs are used (in this case TK renilla and pCMV). The validity of the use of renilla luciferase as an internal control is based on the assumption that it is constitutively expressed in transfected cells and that its constitutive expression is not modulated by experimental factors which could result in either the upregulation or downregulation of the amounts of the enzyme produced.

When choosing a co-reporter for normalisation of transfection variability, there are several factors to be considered such as the relative light units (RLU) from the expression of the co-reporter. The co-reporter signal should be high enough to obtain reliable readings and better signal-to-noise ratios. Promoter crosstalk or interference is another factor to be considered (Daniell, 2012). A strong promoter may cause a “squenching” effect soaking up general transcription factors or it may specifically interfere with the test promoter by competing for the same factor(s) that are required for transcription for both promoters (Thermofisher.com, 2018). There is a possibility that co-reporter expression may also be influenced by the same treatment making it unreliable as an internal control vector (Thermofisher.com, 2018).

In transient transfections, the primary objective is to quantify promoter strength. Due to the possibility of low transfection efficiency, promoters fused to reporter genes encoding enzymes that can be quantified are used. The reporter protein’s activity or fluorescence within a transfected cell population is approximately proportional to the steady-state mRNA level. Co-reporters (namely pCMV and TK renilla) were used to normalise the values of the experimental reporter gene for variations in transfection efficiency and sample handling.

3.5.6 Choosing a transfection reagent

Transfection is the process by which foreign material such as DNA, RNA etc. is introduced into cells. These nucleic acids can be transported by polymeric or lipidic transfection reagents either through mechanical means i.e. electroporation, microinjection etc. or chemically using compounds such as calcium phosphate and transfection reagents. No transfection reagent is ideal for use in all cell types and under all conditions therefore, optimization of experimental conditions is required with any reagent.

Optimization experiments compared transfection efficiency of XtremeGene vs Lipofectamine transfection reagents within the C2C12 cell line. The effects of adding substrate to supplement/assist in the improvement of transfection were also investigated by adding 400 µl of matrigel. The effects of substrate on cell growth or toxicity were assessed by observing cell growth under a light microscope at 40X objective magnification.

Transfection efficiency was assessed through the basal *ST8SIA1* expression of WT and MT using the different XtremeGene and Lipofectamine. Furthermore, cytotoxicity of Lipofectamine transfection reagent was assessed by looking at the growth of the cells at 3 µl as well as 1.5 µl lipofectamine dose. C2C12 cells were grown in the presence and absence of matrigel substrate at both lipofectamine doses and the growth of the cells observed (assessed) by viewing under a light microscope at 40x objective magnification. Matrigel has been shown to enhance the transfection efficiency of cultured cells

(Hughes, Postovit and Lajoie, 2010). Matrigel is the trade name for a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumour rich in extracellular matrix proteins. It resembles the complex extracellular environment found in many tissues (Hughes, Postovit and Lajoie, 2010). Its major components are laminin, collagen IV, heparan sulfate proteoglycans, entactin/nidogen, TGFB, epidermal growth factor, insulin-like growth factor and tissue plasminogen activator, and other growth factors which occur naturally in the EHS tumour (Hughes, Postovit and Lajoie, 2010).

3.6 Preparation and use of MG sera

The sera used in these experiments were obtained from different patients who were newly diagnosed as having AChR-antibody MG, had extraocular muscle involvement and were also treatment-naïve. The sera collection was carried out as described by Nel et al. (2016). All the sera used in this study are shown in Table 6. All the sera collected were used at 5% concentration at 24-hour incubation periods.

Table 6: MG sera used as a treatment in this study

MG sera used	The experiment in which sera were used
MG 201	Luciferase assays (C2C12 cell line treatment) & qPCR experiments
MG 206	qPCR experiments
MG 218	qPCR experiments

3.7 Patient-derived ocular fibroblasts

Perimysial ocular fibroblasts were opportunistically sampled ocular tendon from patients who required ocular alignment surgeries (5 control MG and 2 OP-MG cases). The ocular tendon was established after EOM tendons were harvested by (Professor Murray and Dr Lenake who are both surgeons and collaborators of our research group) with written informed consent from patients. Table 7 indicates the type of surgery from which the tendon was sampled along with the patient diagnosis. The cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C (95% air, 5%CO₂ and 65% humidity).

Table 7: Patient-specific ocular fibroblast information.

MG Sample Number	Diagnosis	Phenotype	Origin of EOM tendon fibroblasts
MG 64	MG	OP-MG	Medial rectus
MG 162	MG	OP-MG	Medial rectus
MG 189	MG	Control	Lateral rectus
MG 267	Congenital blindness	Control	Medial rectus
MG 268	Congenital strabismus	Control	Lateral rectus
MG 272	Traumatic extraocular muscle injury	Control	Lateral rectus
MG 273	Intermittent exotropia	Control	Medial rectus

3.8 Quantitative polymerase chain reaction (qPCR)

Quantitative PCR is a type of reverse transcription PCR and measures the number of transcripts present per gene. It is used when the starting material is RNA. In this method, RNA is first transcribed into complementary DNA (cDNA) by reverse transcriptase from total RNA or messenger RNA (mRNA). The cDNA is then used as the template for the qPCR reaction. Unlike other methods used to quantify mRNA (e.g Northern blotting and ribonuclease protection assays), qPCR requires small amounts of RNA, is less labour intensive and produces large amounts of data in a short period of time. Its relative ease-of-use and sensitivity have made it an invaluable tool. It differs from conventional PCR in that it incorporates the use of a fluorescent signal, which is monitored by a special, computerised thermocycler.

3.8.1 RNA extraction and quantification

Expression profiles for *ST8SIA1*, *TGFB1* and *IL6R* were measured in perimysial ocular fibroblasts (derived from extraocular tendon explants). The basal and expression levels, as well as expression levels following treatment with sera, were measured for the above-mentioned genes. The cells were grown to confluency in 6cm dishes (confluency was reached after approximately 4 days on average). RNA was extracted from both plates using the HighPure RNA extraction kit (Roche) according to the kit protocol. RNA was quantified using the Nanodrop 1000 v3.5.2 software (Inqaba Biotechnical Industries) and all A260/A280, as well as A260/A230 ratios, were ≥ 1.8 and ≥ 2.0 respectively were accepted as pure forms of RNA and only samples which matched these ratios were used.

The A260/280 ratio is used to assess the purity of DNA and RNA. A ratio of ≈ 1.8 is generally accepted as “pure” for DNA whereas a ratio of ≈ 2.0 is accepted as “pure” for RNA. If the ratio is low in each of the above-mentioned cases then this could be indicative of the presence of protein, phenol or other possible contaminants which absorb strongly at or near 280 nm. The 260/230 ratio is used as a secondary measure of nucleic acid purity. Expected 260/230 values are commonly in the range of 2.0-

2.2. If the ratio is lower than expected, it may indicate the presence of contaminants which absorb at 230 nm.

3.8.2 Reverse Transcription

326 ng of RNA extracted from the perimysial ocular fibroblasts was reverse transcribed using the ImPromII Reverse Transcription kit (Promega) according to manufacturer’s instruction. The reverse transcription reaction for each sample was performed on the same day in the same run to minimise experimental variability introduced in reverse transcription efficiency. A calibrator sample was loaded with each qPCR run. The calibrator sample serves as a control with a known concentration. It is used to verify the efficiency of each qPCR run and plays a vital role when analysing reproducibility between qPCR runs. It is important in qPCR to verify the reagents used and to ensure standards are working well for normalization when running unknown samples on different plates.

3.8.3 Quantitative PCR reactions

The qPCR reactions were performed on the Roche light cycler 2.0 instruments. All reactions were completed in duplicate using Kapa SYBR FAST qPCR Master Mix (Kapa Biosystems). Each 10 μ l PCR reaction contained, 2.6 μ l water, 1 μ l BSA (2.5 mg/ml), 0.4 μ l *ST8SIA1* primer(1X), 5 μ l Kapa and 1 μ l of cDNA (104.9 ng/ μ l). The reaction mixture was carried out in glass capillaries, with cDNA experimental samples as in Table 9. qPCR has four fundamental steps which contain their own separate cycling parameters, namely a hot start denaturing step; a PCR step; a melting step and lastly a cooling step. The qPCR cycling parameters were as shown in table 10.

Table 8: MG samples used in qPCR experiments.

Sample	Treatment type	Sample phenotype
MG 64	Untreated	OP-MG Sample
	MG sera treated	
MG 162	Untreated	
	MG sera treated	
MG 267	Untreated	Control Samples
	MG sera treated	
MG 268	Untreated	
	MG sera Treated	
MG 188	Untreated	
	MG sera treated	

Table 9: Thermal cycling conditions for qPCR.

Step/Phase		Temperature	Duration	Number of Cycles
HotStart Denaturation		95°C	3 Min	1
PCR	Denaturation	95°C	3 sec	40
	Annealing	55°C	20 sec	
	Elongation	72°C	01 sec	
Melting	Final extention	65°C	15 sec	1
Cooling		40°C	30 sec	1

Legend: min= minutes and sec= seconds

3.9 DNA extraction

DNA was extracted from a single control ocular fibroblast sample for storage and use in future experiments such as genetic analysis, PCR and Sanger Sequencing. Patient-derived ocular fibroblasts were grown as a monolayer to confluency in a 6 cm dish in supplemented medium (DMEM; Gibco, Life Technologies, New York) with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin and 100µg/ml streptomycin. The cells were trypsinized in 0.5% trypsin for 5 minutes to lift (detach) the cells off the dish and 5×10^6 cells were transferred to a 1.5ml tube. The tube was centrifuged for 5 minutes at 300×g; the supernatant was aspirated without disturbing the cell pellet. The remainder of the DNA extraction was performed using the QIAmp Mini spin column according to manufacturer's instructions (Qiagen) and the concentration along with the purity ratios quantified using the Nanodrop 1000 v3.5.2 software (Inqaba Biotechnical Industries).

3.10 Statistical analysis

All statistical analysis of the raw luciferase data was calculated using Microsoft excel. Luciferase values were calculated for each sample and the average determined to account for changes in the transfection efficiency for each technical repeat by calculating the firefly to renilla ratios. ST8SIA1 promoter activity was normalised to pGL3-basic empty vector. For transient and co-transfection experiments, the fold activations and standard deviations were pooled across biological repeats and summarised on a single graph. ST8SIA1 promoter activity was compared with unpaired student's t-test between the WT and MT, while the paired student's t-test was used for comparisons between the different treatments (untreated vs MG sera) for each promoter constructs (WT or MT). Variation within a sample set was tested by calculating the standard deviation. Statistically, the significant value was defined as $p < 0.05$.

All statistical analysis for qPCR experiments were carried out using Microsoft Excel using the student's t-test function for continuous normally distributed data. The t-test compares sample means from two independent groups for an interval-scale variable when the distribution is approximately normal. In this case, we performed an unpaired t-test to determine whether there was significance in the endogenous gene expression between control and OP-MG ocular fibroblasts in the presence and absence of MG sera. The paired t-test can also be used to compare two sample means where there is a one-to-one correspondence (or pairing) between the samples. This was performed by comparing relative fold expression in the presence and absence of sera where the basal and sera treated fold expression were compared between the control and OP-MG samples. Basal vs sera activated fold expression in each construct (i.e. in WT and MT plasmid constructs). Statistically, the significant value was defined as $p < 0.05$.

4. RESULTS

Previous work in a highly selected group of MG individuals identified several gene variants associated with the OP-MG phenotype (Nel *et al.*, 2017). The first objective of this study was to ascertain the functionality of a *ST8SIA1* promoter variant (c.-477_-475 del CCC) using site-directed mutagenesis of a *ST8SIA1* promoter-reporter construct (Bobowski *et al.*, 2013). The activity of *ST8SIA1* reporter construct containing the delCCC deletion was assessed in transfected HT1080 fibrosarcoma and C2C12 mouse myoblast cell lines using luciferase promoter-reporter assays.

4.1 Sequence verification of the *pGL3-ST8SIA1* wild type plasmid

The pGL3-*ST8SIA1* plasmid comprising a pGL3 enhancer vector (Promega, Madison, USA) and a 2307 bp fragment of the human *ST8SIA1* promoter (insert) cloned upstream of the luciferase gene was obtained as a gift from Dr Delannoy (Bobowski *et al.*, 2013). To verify that we had indeed received the correct plasmid containing the insert of interest, a diagnostic digest was performed. The diagnostic digest uses restriction enzymes to cut the plasmid to release the insert from the vector backbone and the resultant fragments (insert and backbone) can be visualised by gel electrophoresis. The pattern of the fragments on the gel can be analysed to infer that the digested plasmid contains the expected fragment sizes (in this case 2307 bp for the insert and 5064 bp for the backbone). Xho1 and NheI restriction enzymes were used to perform the diagnostic digest. This diagnostic double digest gave the expected band sizes of approximately 2500 bp corresponding to the insert and approximately 5000 bp for the pGL3 enhancer backbone (see lane 2, Figure 13). In contrast, when only one restriction enzyme is used to perform the digest, it simply linearizes the plasmid and the insert is therefore not visible on the gel (see lanes 3 and 4, Figure 13).

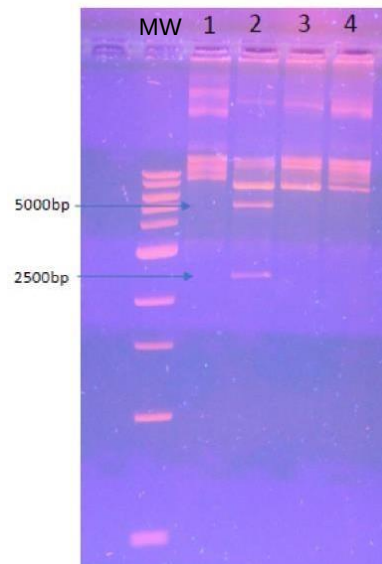


Figure 13: Agarose gel (0.5%) of the digested pGL3-ST8SIA1 plasmid using XhoI and NheI restriction enzymes. Lane MW: 100 bp molecular weight marker (NEB), Lane 1: undigested plasmid. Lane 2: Double digest (XhoI and NheI). Lane 3: Single digest (NheI), Lane 4: Single digest (XhoI). The pGL3-ST8SIA1 plasmid is expected to be a total size of 7371 bp. The double digested plasmid shows 2 bands: one of ~5 Kb representing the vector backbone and one of ~2.5 Kb which represents the insert.

Sanger sequencing was used to confirm the presence of the correct insert and the absence of mutations in the plasmid construct by determining the precise order of nucleotides within the plasmid construct. Figure 14 depicts a portion of the sequencing alignment which confirms the presence of the correct insert with no mutations in the sequence.

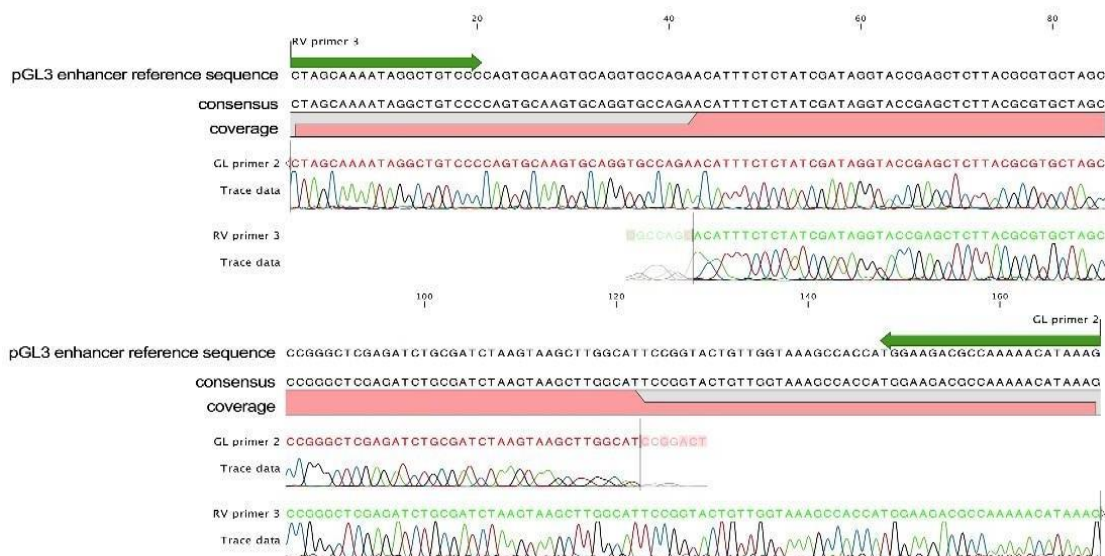


Figure 14: Alignment of Sanger sequencing chromatograms against the pGL3-ST8SIA1 plasmid sequence which shows consensus with the reference sequence. The well-resolved peaks in this chromatogram, when compared against the reference sequence, confirm the presence of the correct plasmid with the absence of mutations. The pGL3 enhancer was used as the reference sequence.

4.2 Generation of the pGL3-ST8SIA1 mutant plasmid

To study the functionality of the *ST8SIA1* c.-477_-475 *delCCC* OP-MG associated variant identified in the WES study (Nel *et al.*, 2017), a CCC deletion was introduced into the wild type pGL3-*ST8SIA1* plasmid. This was achieved using site-directed mutagenesis (see methods, section 3.3). Following the successful amplification of the mutated PCR product (Figure 15), the PCR product was treated with DpnI and re-ligated. An aliquot of the ligation mix was used to transform *E. coli* cells which were grown on ampicillin agar plates to select for cells containing the mutant plasmid (Figure 16). A single colony was picked, and DNA was extracted which was sent for sequence verification using RV primer3 and GL primer2 (methods, Table 2).

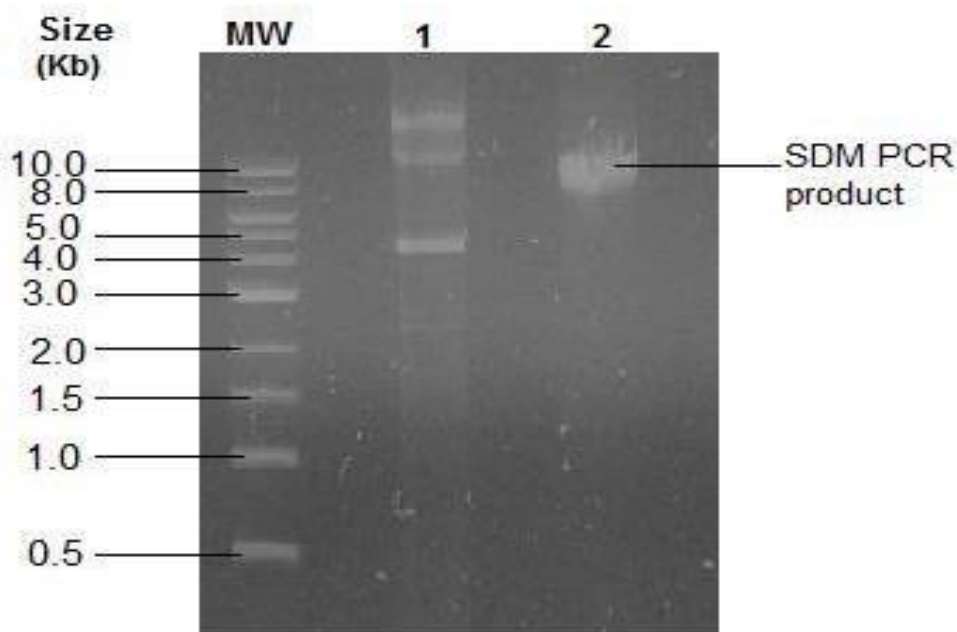


Figure 15: Agarose gel (0.5%) electrophoresis of SDM PCR product. The mutated PCR product was run on an agarose gel to confirm its successful amplification. The resultant gel image includes a 1Kb ladder (NEB) as the molecular weight marker (lane MW), the undigested pGL3-*ST8SIA1* plasmid DNA with 3 bands which represent the different plasmid conformations (lane 1) and the SDM PCR product (lane 2).

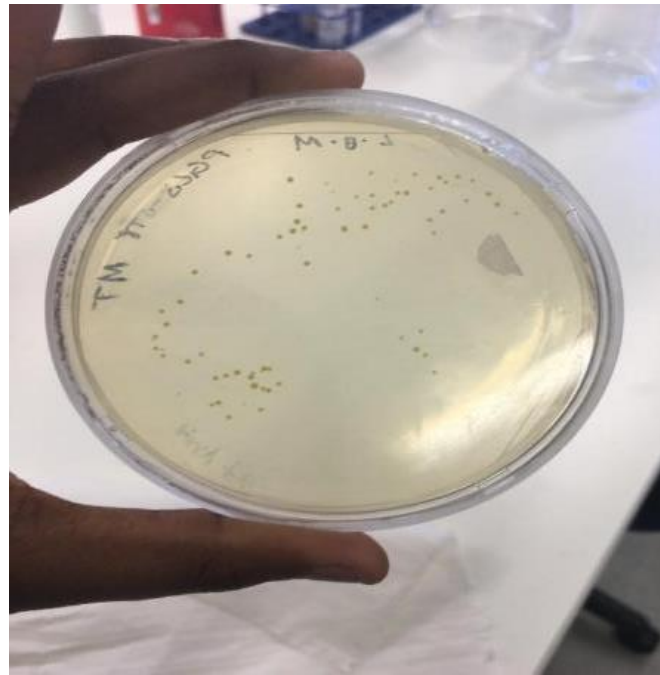


Figure 16: Ampicillin resistant bacterial colonies (containing mutant pGL3-ST8SIA1 plasmid) grown on LB agar. Following successful amplification, the mutated PCR product was DpnI treated and ligated. An aliquot of the ligation mixture was used to transform *E. coli*. The transformants were spread plated on ampicillin agar plates. The presence of these bacterial colonies suggests successful deletion and re-ligation of the mutant pGL3-ST8SIA1 plasmid.

Sequencing verification is essential to ensure that the nucleotide sequence of plasmid DNA is as expected, particularly to ensure the incorporation of the deletion in the correct position within the sequence without the introduction of other additional mutations. The sequencing confirmed successful incorporation of a CCC deletion at the correct position in the sequence (Figure 17 which shows the deletion marked with “X”).

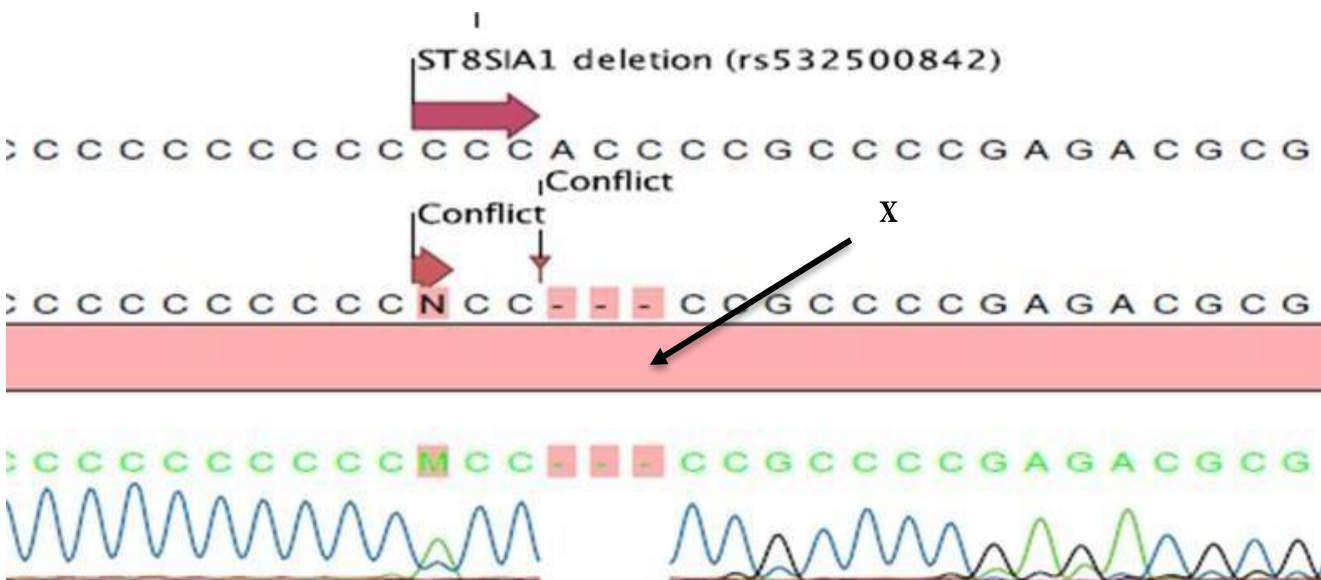


Figure 17: Following maxiprep of the transformants, the plasmid DNA was sequenced to verify the successful introduction of delCCC and to ensure no other additional mutations were found. The sequencing shows that a CCC deletion was successfully incorporated in the correct location (marked with “X”).

Since both the conformation and the size of plasmid DNA may affect transfection efficiency (Gibco, 2018), it was important to ensure that these plasmid properties were similar in the WT and MT pGL3-*ST8SIA1* plasmids. The conformation of plasmid DNA can be assessed on an agarose gel where the different conformations appear as bands of different sizes based on their migration properties. The agarose is a polymer which forms a mesh when made into a gel which can separate DNA on the basis of size under potential difference. There are 3 main plasmid conformations (supercoiled, open circular & linear conformations) of which supercoiled is the most naturally occurring. This form of DNA is the most compact so as to facilitate the tight packing within the cell. When current is applied to an agarose gel, this form migrates faster due to its small (more compact size) in comparison to the other plasmid DNA conformations. Transient transfection is most efficient with supercoiled plasmid DNA as its small, compact nature facilitates entry into cells. Figure 18 shows that the plasmid conformations of the WT and MT pGL3-*ST8SIA1* plasmids are similar, including a high proportion of fast-migrating supercoiled DNA, which suggests that any observed differences between WT and MT plasmid activity are not related to differences in transfection efficiency.

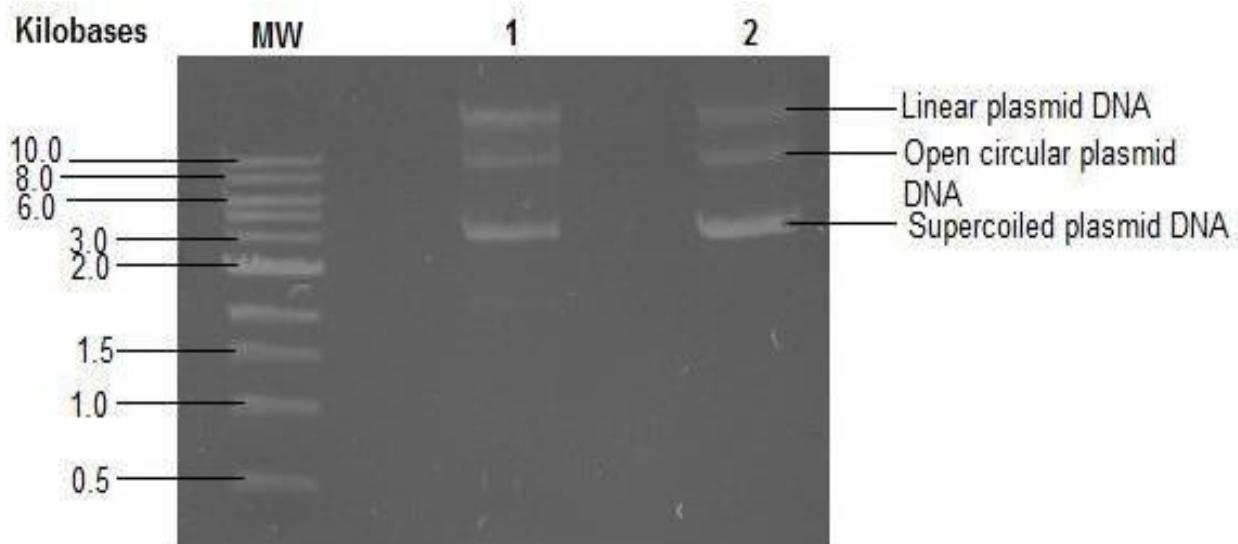


Figure 18: Agarose gel (0.5%) electrophoresis of pGL3-*ST8SIA1* WT and MT plasmids. The resultant gel includes a 1Kb NEB ladder as the molecular weight marker (lane MW) serving as a sizing reference, the WT plasmid (lane 1) and MT plasmid (lane 2). This image shows that the WT and MT plasmids have the same conformations.

4.3 Luciferase reporter assays

Luciferase assays were used to ascertain the functionality of the CCC deletion in the 5' UTR region of the *ST8SIA1* gene by comparing the relative luciferase activity of the WT pGL3-*ST8SIA1* versus MT pGL3-*ST8SIA1* constructs. The principle behind these experiments is illustrated in Figure 12 in the Methods section. In order to perform luciferase assays, it is necessary to deliver the promoter-reporter construct inside the cell where the cell's endogenous pool of transcription factors can initiate transcription and translation of the reporter gene. This process is called transfection and is achieved by various methods such as the chemical transfection methods (which is the method used in this study).

This method involves positively charged nucleic acid/chemical complexes attracted to the negatively charged cell membrane and passing through the cell membrane. The exact mechanism is unknown but is suspected to involve either phagocytosis or endocytosis. The transfection efficiency of chemical methods is largely dependent on factors such as nucleic acid/ chemical ratio, solution pH and cell membrane conditions. This process yields lower transfection efficiency when compared to other transfection methods and varies depending on the cell type (Kim and Eberwine, 2010).

4.3.1 Comparison of transfection reagents

The HT1080 cell line is reported in the literature to have good transfection efficiency while the C2C12 cell line is known to be difficult to transfect (Yamano, Dai and Moursi, 2010). Therefore, optimisation experiments were necessary to establish the optimal transfection protocol for this cell line. Optimisation experiments compared culture substrate (no substrate vs Matrigel (Corning) and transfection reagents (Xtreme gene vs Lipofectamine). C2C12 cells were grown in the presence and absence of Matrigel to ascertain whether the presence of Matrigel aids cell growth (to try and minimise cytotoxicity effects of lipofectamine) as well as to determine whether its addition (Matrigel) improves overall transfection in this cell line at two different transfection reagent (lipofectamine) doses (Figure 19).

When choosing a transfection reagent, it is essential to choose a reagent which yields good transfection efficiency without killing the cells (with low toxicity). Features of cytotoxicity in cultured cells include Cell rounding, which is indicative of lifting, dead floating cells, vacuolation, cell shrinkage as well as the formation of blebs on the cell surface etc. (Bustillo *et al.*, 2009). It was, therefore, these features of cytotoxicity that were used as markers and exclusion criteria when considering a transfection reagent. Figure 19 depicts the cells as observed under a light microscope at 40x objective magnification. Figure 19A depicts the C2C12 cells prior to transfection in the absence of Matrigel and Figure 19B shows the C2C12 cells in the presence of Matrigel also prior to transfection. There was no observable difference in terms of cell growth with the addition of Matrigel when viewed under a light microscope.

The cytotoxicity of Lipofectamine in C2C12 cells was evaluated by light microscopy following transfection for 24 hours at 2 different doses; 1.5µl and 3µl, with cells grown in the presence and absence of Matrigel (Figure 19). 24 hours post-transfection; the 3µl dose (Figure 16 C-D) resulted in more cell death compared to the 1.5µl dose (Figure 19 E-F) under a light microscope. For comparison, healthy, untransfected C2C12 cells are shown in figure 19A-B. At 24 hours there was no observable difference between the 1.5 and 3µl Lipofectamine doses in terms of cytotoxicity as there appeared to be similar in cell growth visually at both doses. As a result, the 1.5µl dose was therefore chosen as the best dose to use for subsequent experiments.

The transfection efficiency of X-tremeGENE (Merck) and Lipofectamine (Merck) was compared in the

C2C12 cell line to select a reagent with the best transfection efficiency. The C2C12 cells were transfected with Xtreme gene (3:1 DNA to Xtreme gene concentration) and 1.5µl Lipofectamine dose and transfection efficiency determined 24 hours post-transfection. Transfection efficiency was measured by comparing the fold luciferase expression of WT and MT plasmids relative to empty (pGL3 basic vector) using both Lipofectamine and XtremeGene as transfection reagents. A fold expression >1 was taken as the measure of transfection efficiency. Lipofectamine yielded better transfection efficiency in comparison to XtremeGene (Figure 20, $p= 0.003$) as a result was chosen as the transfection reagent to use for subsequent experiments.

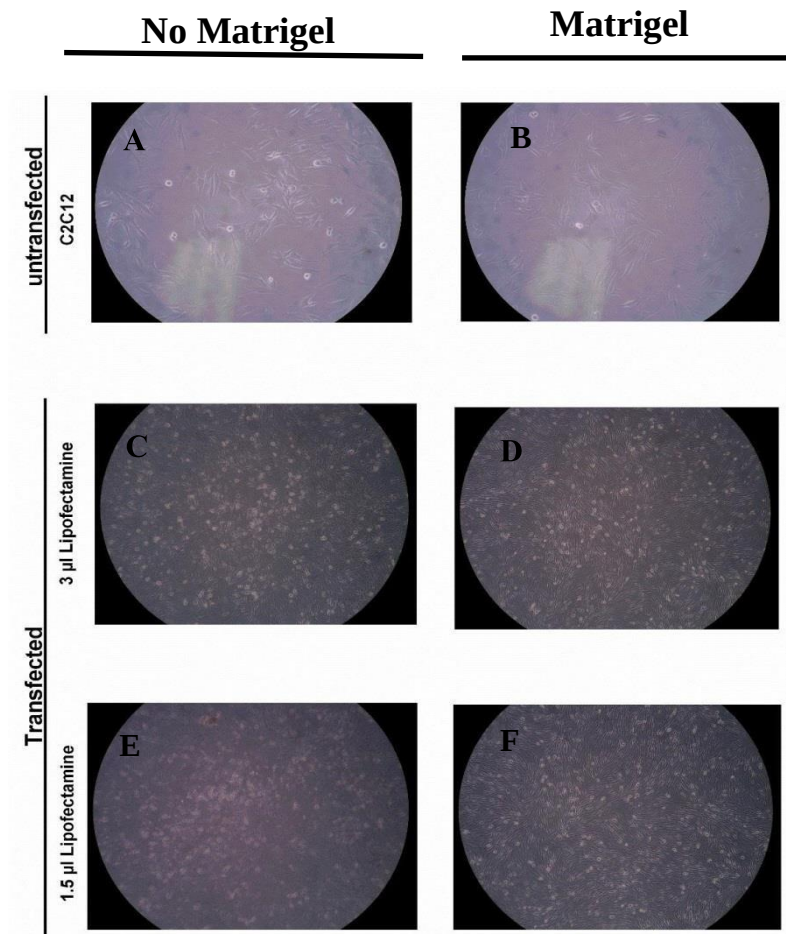


Figure 19: A composite of C2C12 cells treated for 24 hours with Lipofectamine at 1.5 and 3 µl doses grown both in the presence and absence of Matrigel and cell death observed by light microscopy at 40x objective magnification. The 1.5 µl dose was chosen as the working dose as there was less cell death observed under a light microscope at this dose.

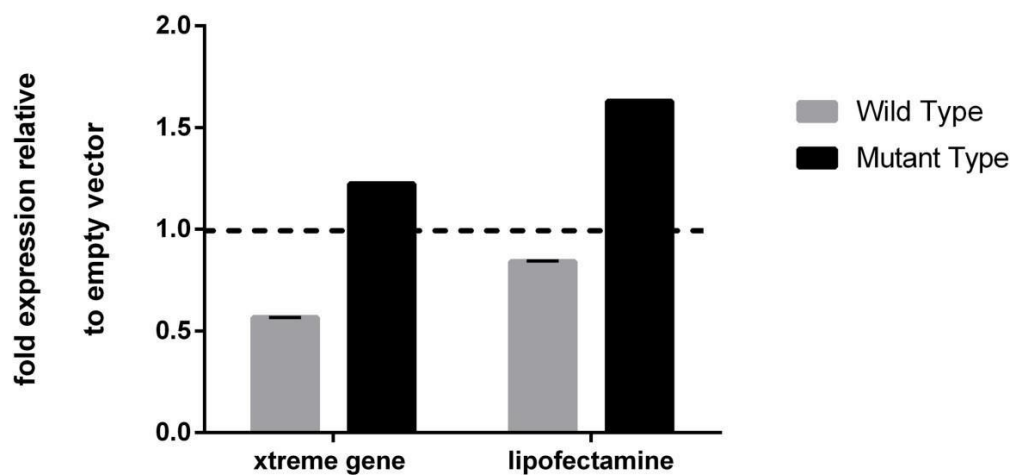


Figure 20: Comparison of XtremeGene and Lipofectamine transfection efficiency in C2C12 cells. WT and MT pGL3-ST8SIA1 were transfected into C2C12 cell line using both lipofectamine and XtremeGene transfection reagents. Fold expression of each vector plasmid relative to empty pGL3-basic vector was measured. TK-renilla was used as the control vector. This experiment is a representative experiment of two independent experiments. The transfection reagent for use was chosen upon which transfection reagent facilitated each vector producing luciferase activity of >1. The error bars are SD and were used as indicators of variation across the wells in this experiment.

4.3.2 Comparison of internal control vectors

Co-reporters are used in transient transfections to normalise the values of the experimental reporter gene for variations in transfection efficiency between experiments as well as to account for differences and/or errors in sample handling. It is therefore important to choose a stable co-reporter whose constitutive expression is not modulated by experimental factors which could result in either the upregulation or downregulation of the amount of luciferase enzyme produced. TK renilla and pCMV renilla internal control vectors were assessed and compared to determine which of the two control vectors would be used in subsequent luciferase assays. Fold expression relative to empty vector, at varying WT pGL3-*ST8SIA1* promoter concentrations was measured in HT1080 cell line in relation to the type of renilla used. The HT1080 cell line was chosen due to its ease of transfection. To render a co-reporter (control vector) as being stable we expect to see an increase in relative luciferase expression with increasing promoter dose (i.e. TK renilla, Figure 21). Based upon this criterion, TK renilla was thus chosen as the internal control vector as it showed a stable consistent trend of increase in relative luciferase activity with increase pGL3*ST8SIA1* promoter dose in the HT1080 cell line. In contrast, normalising to the pCMV renilla vector yielded erratic luciferase activity suggesting that this internal vector was not stable (Figure 21). The erratic activity observed with pCMV renilla could be attributed to this internal vector having a strong promoter as such competing with our promoter of interest (i.e. WT and MT pGL3-*ST8SIA1*) for transcription factor binding.

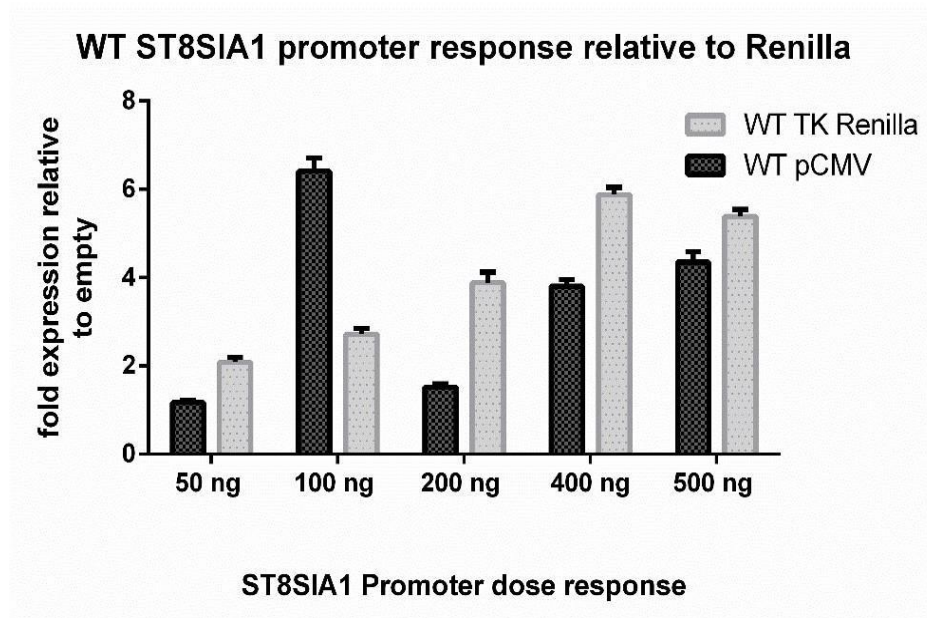


Figure 22: Basal *ST8SIA1* promoter activity in HT1080 cells in relation to the type of renilla used. Dose-response of basal *ST8SIA1* promoter in HT1080 cells was measured with TK Renilla and pCMV as the internal controls. This experiment represents n=1, the error bars are representative of standard deviation (SD) and where SD is used as an indicator of variation across wells. The luciferase activity was expressed compared to empty pGL3 basic vector activity.

After establishing optimal transfection protocols, both the wild type (WT) pGL3-*ST8SIA1* plasmid and mutant (MT) pGL3-*ST8SIA1* plasmids (containing the *ST8SIA1* CCC deletion introduced by site-directed mutagenesis) were transfected into HT1080 cells (a human fibrosarcoma cell line) and C2C12 cells (a mouse myoblast cell line). Cells described previously. Wild type and MT promoter activity were compared under basal conditions (i.e. untreated) and following treatment with MG sera from patients with OP- MG (see methods section 3.6). In these experiments, luciferase assays were performed in the presence of 5% MG sera for 24 hours to investigate its influence on both WT and MT pGL3-*ST8SIA1* promoter activity in C2C12 myoblasts. C2C12 cells were selected as the most relevant cell line for the MG SERA stimulation experiments since these cells are derived from muscle tissue and MG is a disorder in which the antibodies target the muscle endplate region.

4.3.3 Determining the amount of pGL3-*ST8SIA1* required for transfection experiments

Firstly, it was necessary to establish the optimal “dose” of the pGL3-*ST8SIA1* plasmid to use in the transfection experiments. This was determined empirically in the HT1080 cell line since it was easy to transfect. Dose curves (transfecting increasing amounts of plasmid DNA) for both the WT and MT promoter constructs were performed and their promoter activity was compared. Figure 23 shows that both the WT and MT promoters show a dose-dependent increase in promoter activity with increasing amounts of the transfected plasmid. However, the MT promoter showed significantly lower activity than the WT promoter for all doses tested ($p=0.001$). Although for both promoters, the maximal

promoter activity was observed at a plasmid dose of 500ng (≈ 6 -fold in WT and ≈ 4 -fold in MT compared to the empty vector) we elected to use a lower dose because the activity plateaus and to avoid exogenous DNA in the cell.

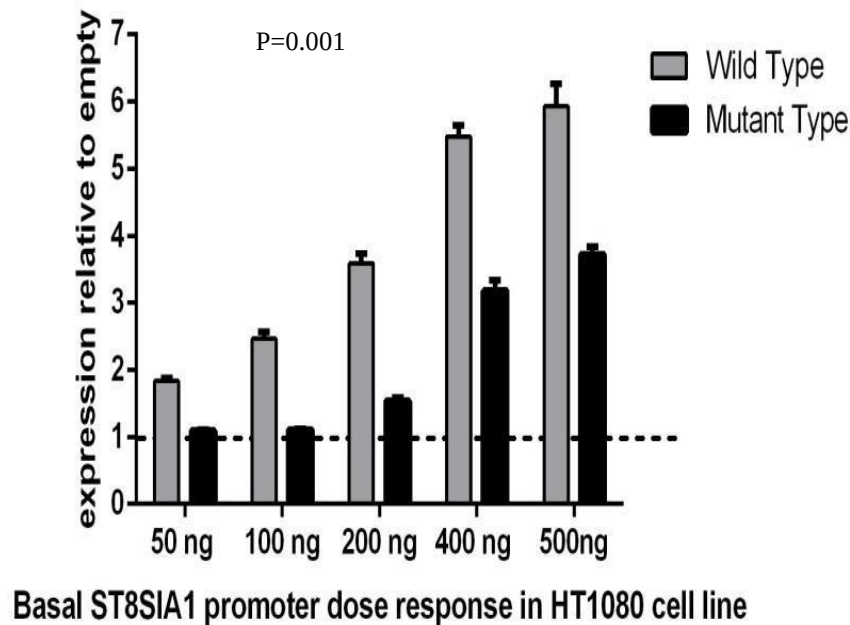


Figure 23: WT and MT ST8SIA1 dose-response of promoter constructs in HT0180 cells using transient transfection. WT and MT pGL3-ST8SIA1 were transfected into HT1080 cell line and Luciferase activity measured at varying promoter concentration within both. Luciferase activities are expressed compared to empty pGL3 basic vector activity. Experiments were performed in triplicate, the error bars are indicative of SEM. A paired student's t-test was performed comparing the relative luciferase activity between MT and WT pGL3-ST8SIA1.

4.3.4 WT & MT pGL3-ST8SIA1 activity in HT1080 cells

To confirm this difference in activity between the MT and WT promoters, luciferase assays were performed with 200ng and 400ng of the WT and MT plasmids in the HT1080 cell line. Similar to the results of the dose curves, the WT ST8SIA1 promoter activity was found to be ≈ 2 -fold higher compared to the MT promoter activity at both doses tested (200ng, $p = 0.031$; 400ng, $p = 0.003$) (Figure 24). This result suggests that the CCC deletion site in the ST8SIA1 promoter may be functional, at least in this fibroblast cell line. The lower basal promoter activity induced by the CCC deletion may either be attributed to the loss of a binding site for an activating transcription factor or the gain of a binding site for a repressing transcription factor.

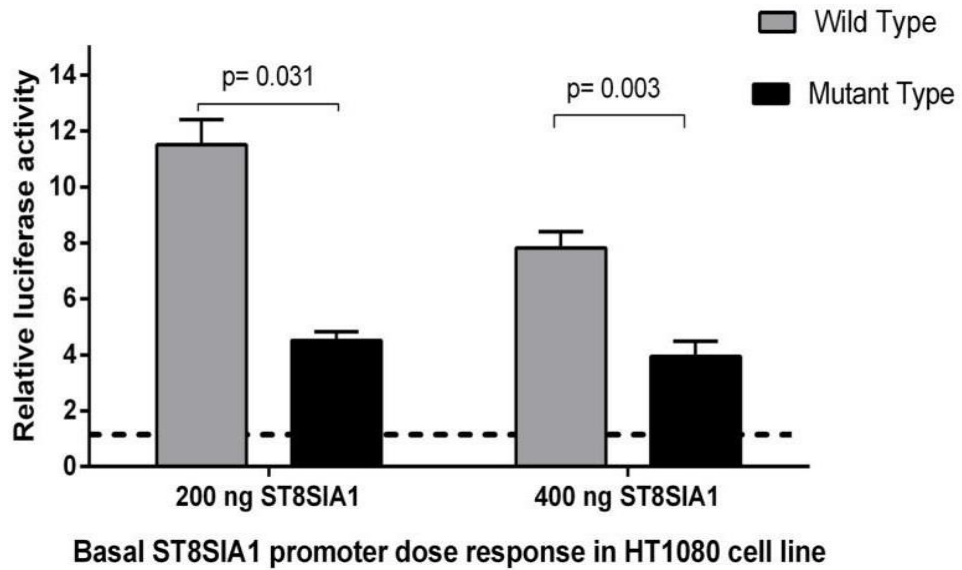


Figure 24: Relative fold expression of WT and MT ST8SIA1 basal promoter activity in HT1080 at concentrations of 200 and 400 ng promoter concentrations. WT and MT pGL3-ST8SIA1 were transfected into HT1080 cell line and Luciferase activity measured at 200 ng and 400 ng promoter concentrations within both. Luciferase activities are expressed compared to empty pGL3 basic vector activity. Both WT and MT transfection efficiencies were normalised through co-transfection with luciferase-expressing TK renilla plasmid as an internal control. Luciferase activities are expressed in comparison to pGL3 basic activity. This graph represents the pooled data from 3 biological repeats. Error bars show SEM. A paired student's t-test was performed comparing the Relative luciferase activity between MT and WT pGL3-ST8SIA1.

4.3.5 WT & MT pGL3-ST8SIA1 activity in C2C12 cells

Luciferase assays were next performed in C2C12 cells, in order to elucidate the functionality of the 5'UTR promoter variant in a muscle-specific cell context. The C2C12 myoblasts were transfected with 1000ng (this concentration was chosen based on previous experimental work done by Dr Nel in our research group where she found that this is the optimal DNA concentration for transfection for this cell line). The promoter activity of WT and MT pGL3-ST8SIA1 constructs were measured under basal conditions and following 5% MG sera treatment for 24 hours. Figure 25 shows that in contrast to the finding of lower basal promoter activity of the MT pGL3-ST8SIA1 construct in HT1080 cells, there was no significant difference in basal promoter activity between the WT and MT in C2C12 cells ($p=0.098$). In addition, MG sera treatment did not induce any significant differences compared to untreated cells in either WT or MT ST8SIA1 promoter activity ($p=0.20$ and $p=0.86$).

In conclusion, the luciferase assay using the ST8SIA1 promoter showed different results in the two cell lines tested. However, the fibroblast cell line that did show differences between the MT and WT plasmids had at least 10-fold higher luciferase activities compared the values in the C2C12 cells. This suggests that the results observed in the C2C12 cell line should be interpreted with caution given the technical difficulty with transfection.

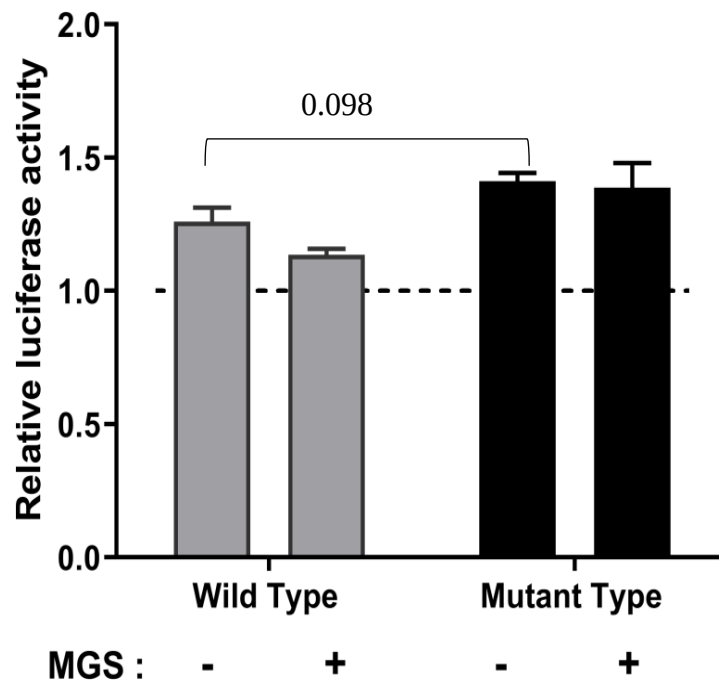


Figure 25: Relative fold expression of basal and MG sera stimulated WT and MT *ST8SIA1* promoter activity determined in the C2C12 cell line. WT and MT pGL3-*ST8SIA1* were transfected into C2C12 cell line and Luciferase activity measured at 1000ng promoter concentration within both. Luciferase activities are expressed compared to empty pGL3 basic vector activity. Experiments were performed in triplicate, the error bars are indicative of SEM. A paired student's t-test was performed comparing the Relative luciferase activity between MT and WT pGL3-*ST8SIA1*. TK renilla was used as internal control with normalisation to empty pGL3 basic vector. The error bars are indicative of SEM, n=3. Please note MGS= MG sera.

4.4 Quantitative PCR (qPCR)

We were interested in investigating the endogenous expression of *ST8SIA1* in patient-derived material to follow on the luciferase promoter work. Members in our group had previously completed promoter work to investigate the functionality of OP-MG susceptibility variants in two other genes (*IL6R* and *TGFB1*) (N. van Greunen Honours thesis work, Melissa Nel thesis work). This project provided the opportunity to investigate the endogenous expression of these genes in patient-derived material.

Orbital fibroblasts were opportunistically sampled from ocular alignment surgeries: 5 control patients (these were patients who had no MG and in extension no OP-MG) and 2 OP-MG patients. From these surgeries orbital tendon was obtained from which ocular fibroblasts were cultured and endogenous expression levels of *ST8SIA1*, *IL6R* and *TGFB1* were elucidated in untreated as well as in MG sera treated samples using qPCR. Although the “primary target” of MG-associated antibodies is the muscle endplate, we postulate that orbital derived fibroblasts may be informative as a surrogate tissue for the ocular environment as they differ from dermal fibroblasts.

These orbital fibroblasts were grown to confluency in 6cm dishes (each sample had an untreated, as well as a sera, treated plate); sera used in qPCR experiments are shown in Table 7. A schematic showing the possible tendon harvest site within EOM and the resultant cultured ocular fibroblasts are shown in Figure 26. In

addition to basal culture conditions, cells were stimulated with MG sera (homologous sera from two different MG patients) to examine the effect of MG sera on the endogenous expression of these genes.

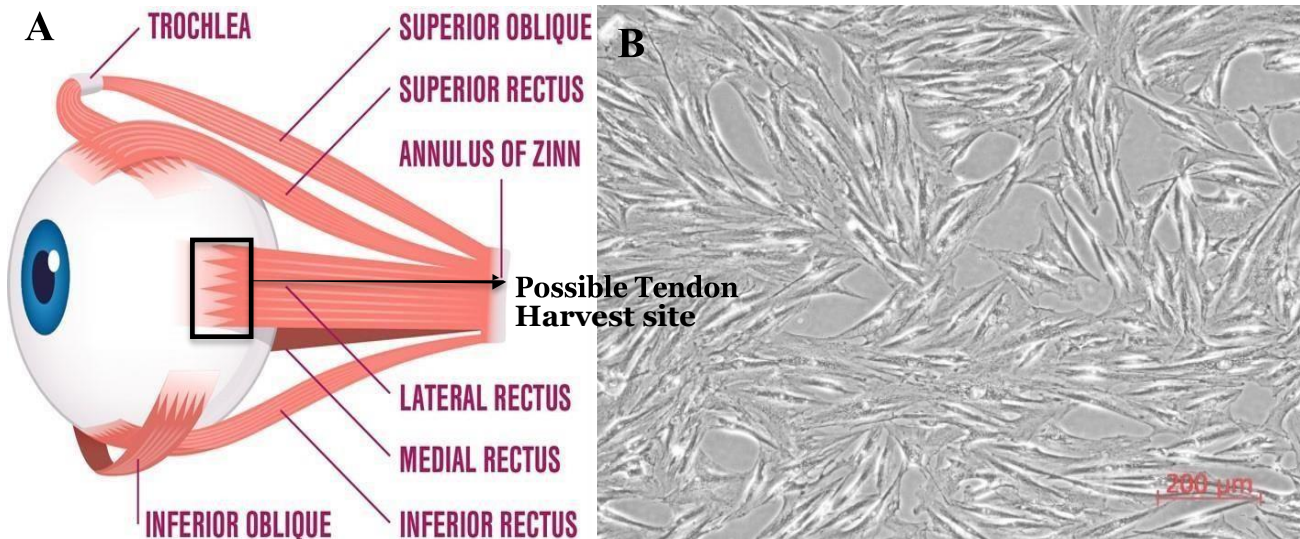


Figure 26: A schematic of extraocular muscles (A) and ocular tendon fibroblasts (B), as viewed under light microscopy after 24 hours at 40X objective (Myaware, 2018). The site from which tendon may have been harvested from is indicated by the rectangle, this is not the actual harvest site, but it has been highlighted for representative purposes.

The rationale for the use of MG sera as the stimulus to mimic MG conditions for the cellular environment is on the basis that MG sera contain immune-mediated cytokines in addition to pathogenic antibodies. As described in the methods the sera are derived from severely symptomatic MG patients but without any other illness such as infections. In order to assess whether an MG- specific stimulus will affect transcriptome in ocular fibroblasts, the fibroblast cultures were treated with MG sera and qPCR performed to assess whether MG sera induced changes in the gene expression of the above-mentioned genes.

4.4.1 Assessment of reference gene stability

Assessment of the stability of the reference gene is critical. Previously Dr Europa performed a preliminary expression array where 10 reference genes were studied in a single experiment ocular fibroblast from two individuals. From her work, *GUSB* and *RPLPO* appeared to be the most stable and I, therefore, validated the stability of these two genes in a larger experiment using ocular fibroblasts. We were interested in validating the stability of these genes following MG sera treatment to assess whether the expression the reference gene becomes influenced by MG sera which would make it unsuitable and unreliable for normalisation.

The stability of the reference gene was assessed by examination of which of the two reference genes in either OP-MG or control cases remained unaffected by the addition of MG sera across the different

samples (see Figure 27). For reference gene stability all the values for control as well as OP-MG ocular fibroblasts were combined, and the transcript levels examined in the presence and absence of sera using a t-test to assess different expression levels in untreated vs treated cells. *RPLPO* was found to be the most stable gene even in the presence of MG sera as there was no significant difference between the basal (untreated) and treated *RPLPO* expression levels across all the cell lines ($p=0.29$). In contrast, *GUSB* varied significantly ($p=0.007$) and therefore was not suitable as a reference gene for use in subsequent experiments (Figure 27). Note that Figure 27A shows a consistent reduction in *GUSB* expression following MG SERA treatment in almost all the samples.

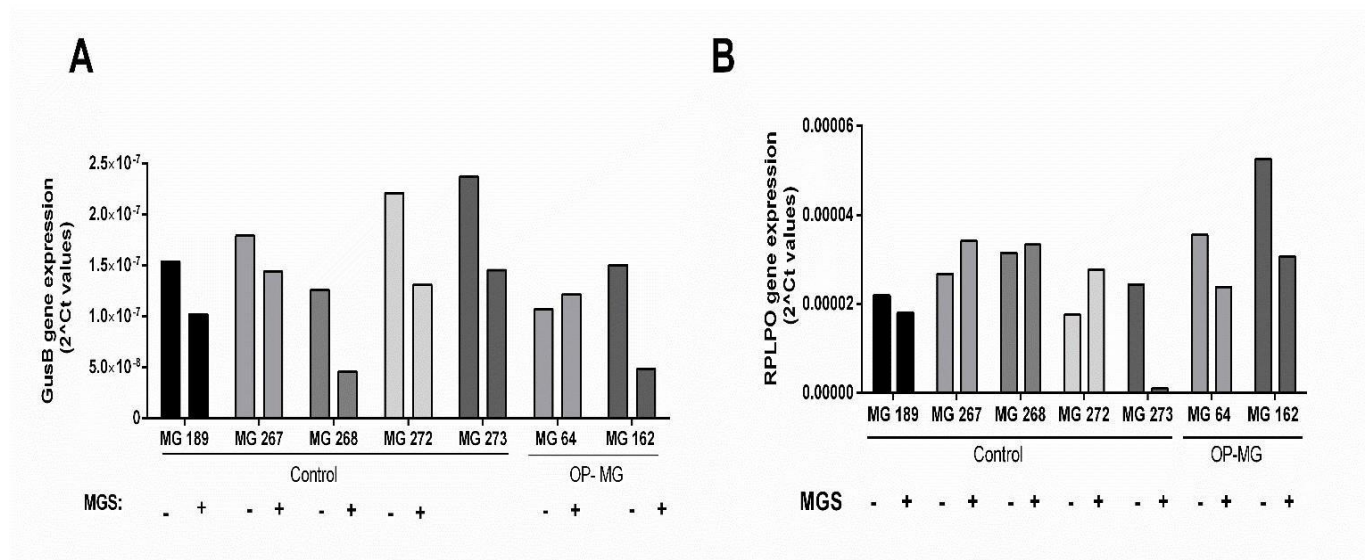


Figure 27: *RPLPO* is a suitable reference gene as MG sera treatment did not significantly alter its expression. *GUSB* (A) and *RPLPO* (B) were assessed as candidate reference genes in patient-derived ocular fibroblasts (n=5 controls, n=2 OP-MG). Gene expression levels for both candidate genes were measured using quantitative PCR (qPCR), comparing endogenous expression levels of both candidate genes in the presence and absence of 5% MG sera. Stability of each candidate reference genes was determined by performing a student's t-test comparing gene expression levels in basal (untreated) vs treated samples within each reference gene. Statistically, the significant value was defined as $p < 0.05$. (A) *RPLPO* $p=0.29$, (B) *GUSB* $p=0.007$. Please note MGS= MG sera.

4.4.2 Endogenous gene expression in ocular fibroblasts

The patient-derived ocular fibroblasts from control patients (n=5) and OP-MG patients (n=2) were grown to confluency in duplicate 6cm dishes; one dish was left untreated and the other was subjected to 5% MG sera treatment for 24 hours. These experiments were performed in duplicate and repeated at least 4 times using homologous sera from 3 different individuals. The cells were then lysed, and RNA was extracted using the HighPure RNA extraction kit (Roche) according to manufacturer's instruction. The RNA concentration was quantified using the Nanodrop 1000 v3.5.2 software (Inqaba Biotechnical Industries) and all A260/280 and A260/230, as well as the concentrations of the extracted RNA, was recorded. RNA was reverse transcribed to cDNA and the endogenous gene expression for *ST8SIA1*, *TGFB1* and *IL6R* was determined using qPCR.

4.4.3 Endogenous gene expression: *ST8SIA1*

The *ST8SIA1* expression levels in untreated and MG sera treated ocular fibroblasts were compared between OP-MG and control groups (Figure 28). There was a trend towards lower basal *ST8SIA1* expression in the OP-MG ocular fibroblasts when compared to the control ocular fibroblasts ($p=0.091$). Following treatment with MG sera, there was no change in *ST8SIA1* expression levels in control ocular fibroblasts ($p=0.352$) but *ST8SIA1* expression appeared to be downregulated in OP-MG samples though this was not statistically significant ($p=0.087$). Overall, when considering *ST8SIA1* expression in MG sera treated ocular fibroblasts, there was a lower expression in OP-MG compared to control samples ($p=0.024$). Although there is no genotyping data for the *ST8SIA1* CCC del variant after a previous attempt by the authors proved difficult as a result of the location of this variant in a repetitive GC rich area within the gene (Nel *et al.*, 2017). It is however interesting that the lower *ST8SIA1* transcripts in ocular fibroblasts derived from the OP-MG phenotype which may contain the *delCCC* are in keeping with the *ST8SIA1* promoter studies (refer results section, Figure 24) which showed lower promoter activity in MT *ST8SIA1* constructs.

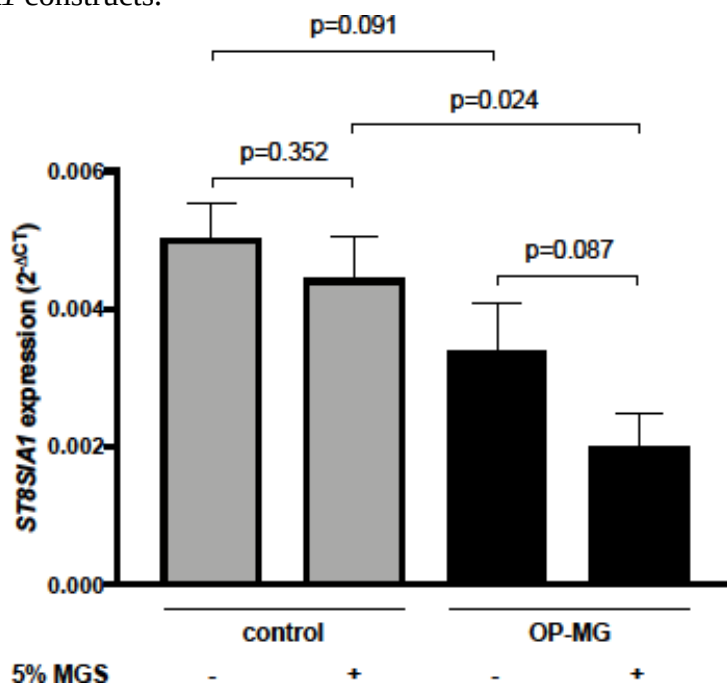


Figure 28: Comparison of endogenous *ST8SIA1* expression in OP-MG and control ocular fibroblasts under basal conditions and following treatment with MG sera. Endogenous *ST8SIA1* expression levels were measured in patient-derived ocular fibroblasts (n=5 control, n=2 OP-MG) by qPCR under basal conditions and following treatment with 5% MG sera for 24 hours. Expression levels are shown as $2^{-\Delta CT}$ following normalisation to *RPLP0* as a reference gene. These experiments were performed in duplicate and repeated 5 times using 3 different homologous sera from MG patients. Error bars represent SEM. Expression levels were compared using student's t-test: paired t-test comparing endogenous expression for control and OP-MG (basal vs 5% MG sera treated) and unpaired comparing endogenous expression between the OP-MG and controls (basal vs 5% MG sera treated). Please note MGS= MG sera.

4.4.4 Endogenous gene expression: *TGFB1*

Previously Nel et al. (2016) showed an African specific SNP in the promoter region of *TGFB1* (-387C>T) associated with OP-MG (Nel et al., 2017). Luciferase assays in HT1080 and C2C12 cell lines using promoter-reporter constructs containing the African specific *TGFB1* -387T allele had lower *TGFB1* transcriptional activity compared to the wild type allele (see Figure 3 in the introduction). Here we had the opportunity to assess endogenous *TGFB1* expression levels in untreated and 5% MG sera treated patient-derived ocular fibroblasts (Figure 29). We postulated that the orbital fibroblasts derived from OP-MG patients would also have lower *TGFB1* expression and one OP-MG patient whom we knew harboured the *TGFB1* -387T allele.

Overall there was no difference in *TGFB1* expression levels between OP-MG and control ocular fibroblasts under basal conditions ($p=0.323$) and following treatment with MG sera ($p=0.573$). Similarly, *TGFB1* expression levels were not significantly altered following MG sera treatment for either OP-MG ($p=0.927$) or control ocular fibroblasts ($p=0.167$).

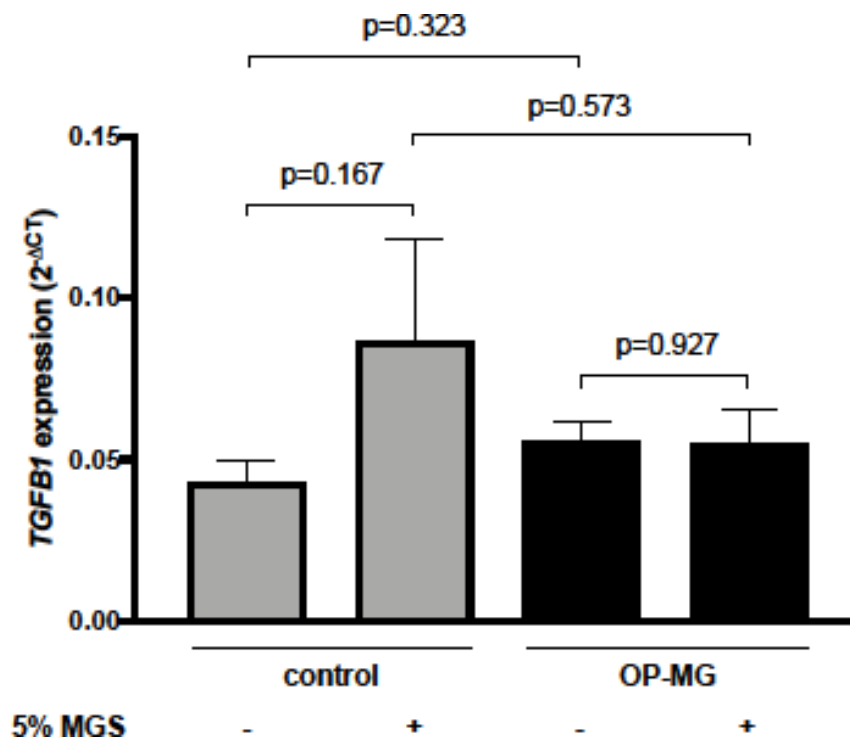


Figure 29: Comparison of endogenous *TGFB1* expression in OP-MG and control ocular fibroblasts under basal conditions and following treatment with MG sera. Endogenous *TGFB1* expression levels were measured in patient-derived ocular fibroblasts ($n=5$ controls, $n=2$ OP-MG) by qPCR under basal conditions and following treatment with 5% MG sera for 24 hours. Expression levels are shown as $2^{\Delta CT}$ following normalisation to *RPLPO* as a reference gene. These experiments were performed in duplicate and repeated 4 times using 3 different homologous sera from MG patients. Error bars represent SEM. Expression levels were compared using student's t-test: paired t-test comparing endogenous expression for control and OP-MG (basal vs 5% MG SERA treated) and unpaired comparing endogenous expression between the OP-MG and controls (basal vs 5% MG sera treated). Please note MGS= MG sera.

Genotyping data for only one control sample is known along with that of both OP-MG samples. The single control patient and one of the two OP-MG patients were homozygous wild type (*TGFB1* -387 C/C) and the remaining OP-MG patient was heterozygous for the variant (*TGFB1* -387 C/T). In order to determine whether the expression differences observed in the *TGFB1* promoter assays (based on the -387 genotype) predicts endogenous *TGFB1* expression in ocular fibroblasts, the endogenous *TGFB1* expression levels were determined and compared genotypically between the heterozygous *TGFB1* - 387 C/T OP-MG patient vs the homozygous *TGFB1* -387 C/C control patient (Figure 29). In this small sub-sample (n=2), there was no difference in basal *TGFB1* expression between the C/C sample and the C/T sample even when genotype was considered. Although the luciferase assays did not test the effect of MG sera on *TGFB1* promoter activity, here we found that there was a significant upregulation of *TGFB1* expression following MG sera in the control C/C genotype sample (p=0.010) which was not observed in the OP-MG C/T sample (p=0.943). It is not surprising that there is upregulation of *TGFB1* in wildtype as *TGFB1* is a gene which is involved in muscle response to injury and by adding sera we are mimicking the disease state within these cells and as such, we expected upregulation of this gene upon treatment with sera.

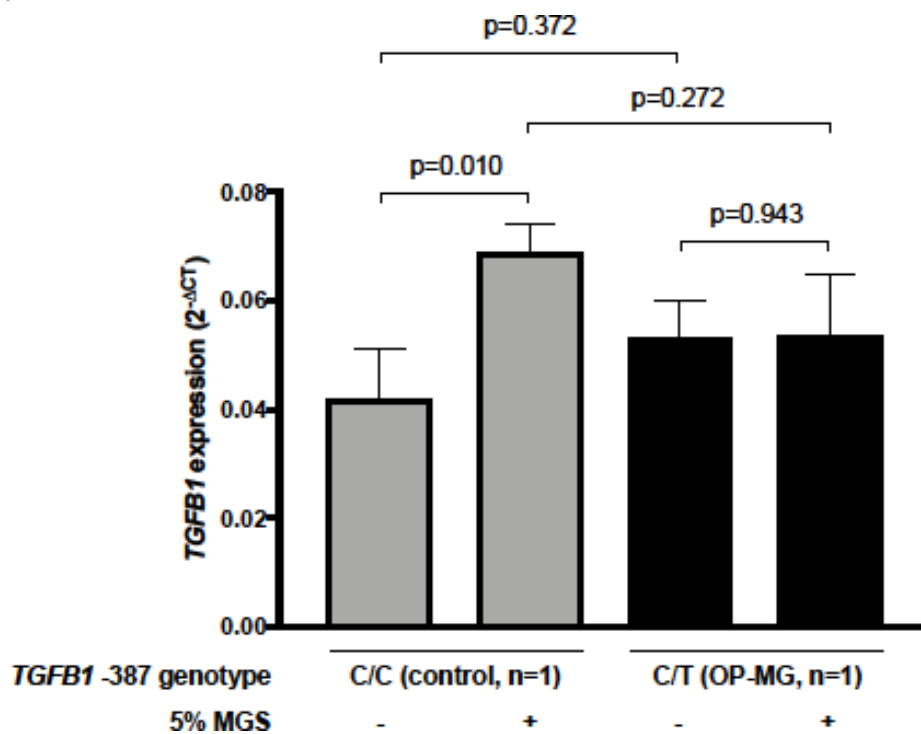


Figure 30: Comparison of endogenous *TGFB1* expression by -387C>T genotype in OP-MG and control ocular fibroblasts under basal conditions and following treatment with MG sera. Endogenous *TGFB1* expression levels were measured in patient-derived ocular fibroblasts (1 control with -387 C/C genotype and 1 OP-MG with -387 C/T genotype) by qPCR under basal conditions and following treatment with 5% MG SERA for 24 hours. Expression levels are shown as 2^{ΔCT} following normalisation to *RPLPO* as a reference gene. These experiments were performed in duplicate and repeated 4 times using 3 different homologous sera from MG patients. Error bars represent SEM. Expression levels were compared using student's t-test: paired t-test comparing endogenous expression for control and OP-MG (basal vs 5% MG sera treated) and unpaired comparing endogenous expression between the OP-MG and controls (basal vs 5% MG sera treated). Please note MGS= MG sera.

4.4.5 Endogenous gene expression: *IL6R*

Previously an *IL6R* c.*3043T>C variant was found to associate with OP-MG. Luciferase reporter assays using a pIS1-IL6-T reporter construct showed that the African specific *3043T>C allele in the *IL6R* 3'UTR altered *IL6R* expression (decreased in the HT1080 cell line and increased in the C2C12 cell line (see Introduction section, Figure 5). Since these assays were performed in transformed cell lines, we were interested to investigate whether endogenous *IL6R* expression levels differ between OP-MG and control ocular fibroblasts. *IL6R* expression in untreated and MG sera treated ocular fibroblasts were compared between OP-MG and control groups (Figure 31). There was no difference in *IL6R* expression levels between OP-MG and control ocular fibroblasts under basal conditions ($p=0.858$) and following treatment with MG sera ($p=0.272$). In OP-MG ocular fibroblasts, there was a trend towards lower *IL6R* expression following 5% MG sera treatment when compared to untreated cells ($p=0.083$) which was not observed in control ocular fibroblasts ($p=0.704$). The *IL6R* c.*3043 genotype is only known for the OP-MG samples (both T/C) which did not permit any meaningful comparison to be made by genotype.

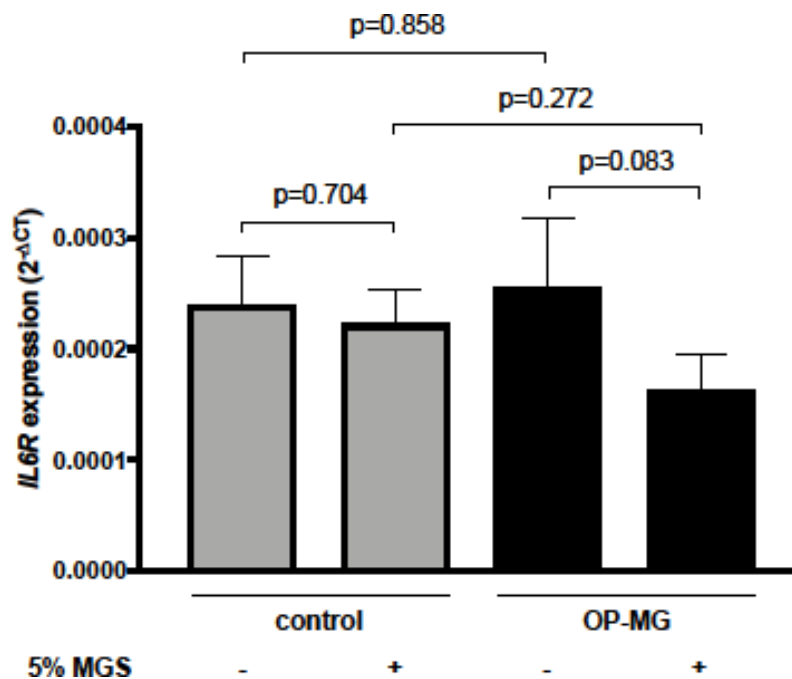


Figure 31: Comparison of endogenous *IL6R* expression by *IL6R* c.*3043 genotype in OP-MG and control ocular fibroblasts under basal conditions and following treatment with MG sera. Endogenous *IL6R* expression levels were measured in patient-derived ocular fibroblasts (n=5 controls, n=2 OP-MG) by qPCR under basal conditions and following treatment with 5% MG sera for 24 hours. Expression levels are shown as 2^{ΔCT} following normalisation to *RPLPO* as a reference gene. These experiments were performed in duplicate and repeated 4 times using 3 different homologous sera from MG patients. Error bars represent SEM. Expression levels were compared using student's t-test: paired t-test comparing endogenous expression for control and OP-MG (basal vs 5% MG sera treated) and unpaired comparing endogenous expression between the OP-MG and controls (basal vs 5% MG sera treated). Please note that MGS= MG sera.

5. DISCUSSION

This thesis describes an extension of previous work which identified OP-MG associated gene variants. The focus of this study was to elucidate the functional effect of gene variants which were identified by bioinformatics tools. The discovery of functional SNPs and gene variants by bioinformatics tools (i.e. *in silico*) is crucial; however, it is imperative to follow this up with functionality assays, such as the ones carried out in this study which include luciferase promoter-reporter assays and gene expression studies in patient-derived material. This allows for the determination of the biological relevance of these variants. In this study, we aimed to assess the functionality of a 5'UTR variant in the *ST8SIA1* gene, as no previous work had validated the effect of this variant in the context of myasthenia. We also wished to complete unfinished work previously undertaken by our group to assess the functionality of African specific variants in *TGFB1* and *IL6R*.

The *ST8SIA1*c.-477_-475 delCCC variant was introduced into a plasmid construct using site-directed mutagenesis and shown to be successful by diagnostic digest and Sanger sequencing. Wild type (WT) and mutant (MT) plasmids were transfected into HT0180 cells (human fibrosarcoma) and promoter activity compared under basal conditions, using luciferase assays. A two-fold decrease in basal promoter activity was observed for the MT plasmid. The lower activity in the HT1080 cells was suggested to be due to either the loss or gain of a transcription factor binding site. This is critical as the fundamental mechanisms of gene expression regulation occur at the transcription level via the binding of transcription factors to DNA sequences. There are identified cases whereby a mutation in the promoter regions causes the binding of a wrong transcription factor which in turn influences transcription levels. For example, it was shown experimentally that a point mutation T>C at 77 nucleotides upstream of the transcription starting site (TSS) of the δ -globin gene haemoglobin subunit gamma-1 changes the binding affinity of the transcription factor GATA-1 which in turn alters the expression levels of the gene (Laurila and Harri, 2009)(Laurila and Harri, 2009). The potential changes in promoter activity are consistent with alterations in transcription factor binding. Bioinformatics tools predicted that the CCC deletion would result in loss of binding sites for the transcription factors retinoid x receptor alpha (RXR α) and MYC-associated zinger finger protein (MAZ). Importantly, MAZ is important in the regulation of muscle-specific genes (Himeda, Ranish and Hauschka, 2008a), and MAZ transcripts are upregulated in skeletal muscle differentiation. Little is known, though, about the role of these transcription factors in fibroblasts and myasthenia gravis, as much of their characterisation has been in the context of cancer (Himeda, Ranish and Hauschka, 2008b).

The C2C12 mouse myoblast cell line was selected as the most relevant (muscle tissue source) for MG sera experiments. The WT and MT plasmids were transfected into these cells, and promoter activity

assessed under basal conditions and following MG sera treatment. The cells did not show a decrease in basal promoter activity observed in the HT1080 cells, and there were no observable effects of MG sera exposure. We encountered suboptimal transfection efficiency in the C2C12 cell line despite many attempts to try and optimise the transfection efficiency. This cell line is known for its difficulty to transfect (Auret *et al.*, 2014). Previous attempts by members within our group to transfect this cell line were subpar as such the transfection efficiencies observed in this study were an improvement when compared to assays previously performed within these cells.

The differences in the promoter activity between the two cell lines (HT1080 and C2C12) could be a result of cell-specific gene regulation, such as underlying differences in the available pool of transcription factors. In a tissue-specific complication such as OP-MG, a variant could be functional in one cell type however not in another (Aguet *et al.*, 2017). Transfection within the C2C12 cell line could be improved by coupling transfection with Electroporation. In electroporation, pulsed electrical fields are used to introduce DNA into the cell. Electroporation is said to work well with cell lines that are refractory to other techniques such as calcium phosphate-DNA co-precipitation. However, this technique is commonly used for cells which are in suspension and its efficiency in adhesive cell lines may differ (Potter, Weir and Leder, 1984) (Weaver and Chizmadzhev, 1996).

Endogenous gene expression for the three genes (*ST8SIA1*, *TGFB1* and *IL6R*) found to carry putative disease-associated variants by WES, were investigated in OP-MG patient-derived material in comparison to non-OP-MG patient-derived material. Our access to patient-derived material offered us a unique opportunity to draw conclusions, and to understand the functional effects of identified SNPs within the cell and, in extension, the body. Moreover, patient-derived sera were used in this study to mimic MG conditions and to assess the sera responsiveness of the genes of interest. While the exact constituents of the MG sera are not yet known, we hypothesised that it may contain disease-relevant cytokines. Previous studies have shown that primary human myoblast cultures exposure to MG sera resulted in morphological changes which correlated with disease severity, in contrast, to control sera (Luckman *et al.*, 2006). Our lab has previously validated that 5% MG sera can produce similar effects in both cell lines and patient-derived tissue (Melissa Nel, PhD Thesis, 2016).

Despite the small sample size in this study, endogenous *ST8SIA1* gene expression in patient-derived material was similar to the luciferase reporter assays in fibroblasts, showing lower *ST8SIA1* expression in OP-MG ocular fibroblasts following MG sera treatment. There was no response in the control ocular fibroblasts to MG sera. While we observed a potential alteration in promoter activity in patient-derived materials, we cannot directly relate the findings to the CCC deletion in the 5'UTR of *ST8SIA1* gene, within our samples. Genotyping of the *ST8SIA1 delCCC* failed due to the high GC content and repetitive nature of the flanking sequence (Nel *et al.*, 2017). As a result, we are not certain which of the control and OP-MG samples have the CCC deletion variant and whether the endogenous expression levels

correlate with either the presence or absence of this variant within our samples. Although there currently is no genotyping data for *ST8SIA1* variant, the previous WES study by Nel et al. (2017) reported that *ST8SIA1 delCCC* had a minor allele frequency of 0.82 in OP-MG cohort (n=11) and absent among South African population controls (SAHGP) (n=23)(Nel et al., 2017). This further supports that indeed the *ST8SIA1 delCCC* variant is an OP-MG associated gene variant.

Previously, an African specific SNP (-387C>T) in the *TGFB1* regulatory region was found to associate with OP-MG phenotype. It was found to be functional using promoter studies in HT1080 and C2C12 cells (Nel et al., 2016). We could not find a consistent difference in the endogenous expression levels of ocular fibroblasts between control and OP-MG cases. However, there was a difference in endogenous *TGFB1* expression levels in ocular fibroblasts in the -387C/T genotype: the significant upregulation of *TGFB1* expression following MG sera treatment in the control C/C genotype sample (p=0.010) was not observed in the OP-MG C/T sample.

The IL-6 pathway was identified as being relevant in MG (Maurer et al., 2015) and the African specific *IL-6R c.*3043T>C* was identified as an OP-MG associated variant (Nel et al., 2017). This SNP was predicted to result in the loss of miRNA binding sites (miR-338-3p and miR-364). These miRNAs were reported to be upregulated in sera of MG patients (Cao et al., 2015). We, therefore, expected that with the predicted loss of miRNA binding site there would be an increase in *IL6R* mRNA expression levels, within the OP-MG ocular fibroblasts containing the *c.*3043 T>C* SNP. Surprisingly, a trend towards lower *IL6R* expression following 5% MG sera treatment was observed when compared to untreated cells (p=0.083). Unfortunately, as both samples contained the *c.*3043T>C* SNP, we could not make any meaningful comparison to other genotypes. The previous WES study performed by Nel et al. (2017) found that *IL6RT>C* had a minor allele frequency (MAF) of 0.27 in OP-MG patients, in comparison to the South African population controls (SAHGP) which had MAF of 0.09, indicating its common frequency in OP-MG when compared to controls.

This study was not without challenges or limitations:

Firstly, inaccessibility of EOM makes the study of MG a challenge. This is illustrated in literature: the only work that has been published for human EOM was done on cadaver EOM samples. Moreover, consortiums such as the Genotype-tissue expression (GTEx) projects, which aim to characterise variation in gene expression levels across individuals and diverse tissue of the human body, do not possess RNA transcripts for EOM (Aguet et al., 2017). This not only highlights the issue with cell type availability (or lack therefore) but it also shines a light on the gap within our field which makes studying EOM related diseases a challenge.

Although EOM may not be easily available, ocular fibroblasts are a more easily culturable alternative that lies within the same microenvironment as EOM. That said, there is an additional lack of literature on ocular fibroblasts, and culturing these cells requires a significant amount of trial and error.

Secondly, the fact that the homologous sera used in this study are not screened creates variability, we do not know if at the time of harvesting the donor MG patients had other additional diseases or infections. Therefore, sera from control cohorts should be used for treatment along with the sera from the MG patients in order to draw more meaningful comparisons.

Thirdly, the small sample size of this study requires that the results be interpreted with appropriate caution. The small sample size is a challenge, especially when analysing data, illustrated by our limited OP-MG samples. Excluding and/or the determination of an outlier become difficult with small sample size. In the long run, we hope to get more OP-MG patient samples, to increase the certainty of our observations. Fourthly, we were unable to genotype some of the samples and had to rely only on phenotype to base our findings. We still need to perform genotyping on the control patients as there is no available genotype data for these patients.

Despite the limitations, my work makes an important contribution to the understanding of the OP-MG pathogenesis. The results show, for the first time, that *ST8SIA1*, part of the gangliosphingolipid pathway, is likely relevant in OP-MG pathogenesis. Additionally, the results confirm that IL6, part of the myokine pathway, plays a role in OP-MG, in keeping with previous reports in MG (Maurer *et al.*, 2015). Gangliosides are important in lipid raft stabilisation. Lipid rafts play a vital role in concentrating receptors such as AChR and IL-6R in membranes. These results, therefore, suggest that *ST8SIA1* is important in receptor stabilisation and muscle recovery. There is an observable trend of *ST8SIA1* repression following MG sera treatment in OP-MG but not control ocular fibroblasts. Although these results do not support our initial hypothesis, we hypothesise that lower *ST8SIA1* expression in response to MG sera has negative implications for extraocular muscle endplate repair and vulnerability to complement damage.

Future work should include genotyping of samples to draw a direct comparison between phenotypic characteristics in terms of genotype. DNA was extracted from a single ocular fibroblast, therefore, DNA extraction and genotyping still need to be carried out for the remaining control samples. Ideally, the variants which were investigated ought to be studied in EOM once this cell type becomes available.

Although OP-MG may not be life-threatening, the limitations it poses can prove disabling and distressing to patients. This study contributes to the better understanding of disease pathogenesis, in the context of patient-derived ocular fibroblasts, and will hopefully contribute towards the long-term aim of alleviating patient suffering.

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Appendix

Mycoplasma Test: Mounting Fluid

- 20 mM Citric acid
- 55 mM Na₂HPO₄·2H₂O
- 50% glycerol
- pH to 5.5 and store at 4°C

Phosphate Buffer Saline

- 80g NaCl
- 12.6g Na₂HPO₄ anhydrous
- 2g KCL
- 2.4g KH₂PO₄
- Dissolve in 800ml dH₂O (using a stirrer bar)
- pH to 7.4 with concentrated HCl
- Fill up to 1L and autoclave
- For use as 1xPBS dilute 100 ml 10x PBS+ 900ml dH₂O

Agarose Gel

- Measure 1 g of **agarose**.
- Mix **agarose** powder with 100 mL 1xTAE in a microwavable flask. ...
- Microwave for 1-3 min until the **agarose** is completely dissolved (but do not overboil the solution, as some of the buffer will evaporate and thus alter the final percentage of **agarose** in the **gel**).

Lauria Agar Plates

- Add the following to 800ml H₂O. 10g Bacto-tryptone. 5g yeast extract. 10g NaCl.
- Adjust pH to 7.5 with NaOH.
- Add 15g **agar**.
- Melt **agar** into solution in the microwave.
- Adjust volume to 1L with dH₂O.
- Sterilize by autoclaving.

Lauria Broth

- Add the following to 800ml H₂O
- 10g Bacto-tryptone.
- 5g yeast extract.
- 10g NaCl.
- Adjust pH to 7.5 with NaOH.
- Adjust volume to 1L with dH₂O
- Sterilize by autoclaving

Calcium Chloride

- 58g CaCl_2
- 1L H_2O
- Autoclave and store at 4°C

TE buffer

- 1M Tris-Cl (pH 8.0)
- 0.5M EDTA (pH 8.0)
- Distilled water to 100 ml