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PhD

PROGRAM IN TRANSLATIONAL AND MOLECULAR MEDICINE

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UNIVERSITY OF MILANO-BICOCCA
SCHOOL OF MEDICINE AND SCHOOL OF SCIENCE

Identification of a new molecular signature in peripheral blood mononuclear cells from patients affected by myasthenia gravis

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XXVIII CYCLE
ACADEMIC YEAR
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To my family:

"One never notices what has been done;
one can only see what remains to be done"

(Marie Curie, *Letter to her brother*, 1894)

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1.1. History of myasthenia gravis

Myasthenia gravis (MG) was the first disorder of the nervous system to be recognized as being antibody-mediated. The earliest description of the disease was probably in 1672 when the English physician and anatomist Thomas Willis, well-known for the 'Circle of Willis', reported the clinical case of a woman with a chronic paralysis of her tongue and limbs. "She speaks freely and readily enough for a while, but after a long period of speech…she is not able to speak a word and is as mute as a fish"; these were the words that he wrote to describe the symptoms of the woman in *De Anima Brutorum* [1]. It took more than two centuries later, in 1895, for the first modern description of the disease to be issued. The German psychiatric and neurologist Friedrich Jolly, described the condition of two young boys with muscle weakness that was exacerbated by activity and improved with rest. He coined the term *myasthenia gravis* using the Greek terms for muscle (*myo*) and weakness (*asthenia*) and adding the Latin word for severe (*gravis*) [2].

Jumping in the more recent history of MG, in 1934 the Scottish-born physician Mary Broadford Walker noted that MG resembled a curare-like poisoning syndrome and in a letter published to the *Lancet* she demonstrated the clinical effectiveness of an esterase that blocks acetylcholine, the physostigmine, in a woman who suffered from MG. Dr. Mary Walker was the first clinician to successfully try AChE inhibitors in MG (known as the 'Mary Walker effect'), providing an excellent contribution in MG research [3].

Later on, in 1936, the Nobel Prize-winner Sir Henry Hallet Dale discovered the physiological mechanisms of the nerve impulse and showed that neuromuscular transmission depended on the release of acetylcholine (ACh) from the motor nerve terminal into the synaptic space [4]. Moreover, in that year the American physician Alfred Blalock summarized in a case report the literature data available at that time concerning the presence of thymic defects in MG patients and performed a surgical removal of a tumour, localized within the thymus, in a teenage girl affected by MG. After three years from surgery, he reported a clinical improvement of patients' conditions, suggesting that thymectomy may be beneficial in tumour-associated MG [5].

Sixteen years later, another Nobel Prize-winner Sir Bernard Katz with the neuroscientist Paul Fatt recorded the small endplate potentials (EPPs) that occurred when a *quanta* of ACh was released from the nerve [6]. These discoveries became fundamental when Dan Elmqvist and colleagues showed that the depolarizing EPPs were strongly reduced in muscle biopsies of MG patients [7].

Up to the middle of the 20th century the number of myasthenic syndromes increased as well as the etiological and pathological descriptions. The most important hypothesis concerning the triggering mechanisms arose in 1960 by John Simpson. He proposed that MG was an autoimmune disease caused by antibodies (Abs) against a protein localized at the motor endplate of the muscle [8]. At that time, the protein receptor of ACh (acetylcholine receptor, AChR) had not been discovered. Nevertheless, in the early 60's and 70's, several studies showed that the snake-derived α-bungarotoxin irreversibly

blocked the neuromuscular transmission by competing with ACh for binding to AChR [9,10]. Fambrough *et al.* demonstrated that the neuromuscular junction (NMJ) of MG patients was characterized by a marked reduction of the number of AChRs compared to the muscle of healthy subjects [11]. Only in 1990 the complete sequence of the AChR was determined: AChR is a pentameric membrane protein consisting of $(\alpha)_2$, β, δ, and ε subunits in the adult form (the ε subunit replaced the γ subunit present in the foetal form) [12,13]. Each subunit comprises of an extracellular domain, four trans-membrane regions and a cytoplasmic loop [14].

Nowadays, MG is considered the prototype of antibody-mediated autoimmune disorders, thus Simpson's hypothesis was not too far from reality. MG satisfies a set of five criteria as proposed by Daniel Drachman in 2003 [15]:

1. *Autoantibodies are present in patients with the disease.* Anti-AChR Abs are found in the sera of more than 80% of MG patients, whereas in individuals without MG are absent [16];
2. *Antibodies interact with the target antigen.* The majority of AChR-specific Abs are directed against an extracellular portion of the α subunit of AChR known as the main immunogenic region [17];
3. *Passive transfer of antibodies reproduces features of disease*. Injections of immunoglobulins from MG patients' sera into experimental animals recapitulate the disease. Reduction of the neuromuscular transmission and the number of AChRs at the NMJ are observed [18];

4. *Immunization with antigen produces a model disease.* Active immunization of guinea pigs and rats with AChR, purified from the electric organ of *Electrophorus electricus* or *Torpedo californica*, induces experimental autoimmune MG with the production of autoantibodies against AChR [19];
5. *Reduction of antibody levels ameliorates the disease.* In MG patients, the removal of circulating Abs by plasma exchange give rise to clinical symptom improvements [20].

1.2. Epidemiology

MG is a relatively rare disorder with a prevalence estimated at 200 cases per million inhabitants in the United States and from 7.7 to 11.1 per 100,000 inhabitants in Europe [21,22]. Over the past forty years, MG prevalence underwent a 4-fold increase; this number will likely rise considering the improvements in diagnostic tests, effective therapies and longer life span. Incidence of MG may broadly differ from 1.7 to 21 per million people on geographical basis. MG is influenced by sex and age; before fifty years of age, the percentage of affected women represents 60-70% of all patients, whereas after the age of 50 the incidence is higher in men [21,23]. Childhood MG is infrequent (about 15%) in North America and Europe, whereas in China 50% of patients with ocular signs receives a diagnosis of MG before the age of 15 [21,23,24].

1.3. Pathogenesis of MG: the role of autoantibodies

MG is an autoimmune disease characterized by a defective neuromuscular transmission caused by autoantibodies against post-synaptic proteins (*e.g.* AChR) of the NMJ. The antibody-mediated mechanisms underlying MG pathogenesis are described below.

1.3.1. Architecture and function of the NMJ

NMJ development starts when a motor neuron reaches a myotube and begins to release a glycoprotein, named agrin, that binds to a trans-membrane protein, the low density lipoprotein receptor-related protein 4 (LRP4), which then makes contact with a postsynaptic tyrosine kinase, the muscle-specific kinase (MuSK) (Figure 1). This process leads to the activation of the MuSK-mediated signalling pathway that triggers AChR clustering and the folding of the postsynaptic membrane [25-28]. Hence, agrin, MuSK, and LRP4 are key molecules in the initiation of the synaptic contact between the motor neuron and the muscle fibre and in the maintenance of the specialized NMJ.

The mature NMJ consists of three components: i) the presynaptic motor nerve terminal where ACh is synthesized and released; ii) the synaptic space that contains AChE, and iii) the postsynaptic muscle membrane characterized by deep folds in which AChRs are closely packed on top of them (Figure 1) [29,30].

Neuromuscular transmission begins when the action potential from the cholinergic motor neuron reaches the presynaptic nerve terminal, this depolarizing current opens voltage-gated calcium

channels on the presynaptic membrane. Calcium influx triggers the exocytosis of synaptic vesicles carrying ACh. A *quanta* of ACh is released at every nerve impulse, it diffuses across the synaptic space and binds to AChRs on the postsynaptic membrane. AChE localized at the synaptic cleft can hydrolyse ACh terminating the synaptic transmission. Upon ACh binding, AChR opens leading to the influx of sodium and potassium into the muscle fibre that induces a local depolarization, the EPP, that consequently it activates voltage-gated sodium channels propagating the nerve transmission along the muscle. In normal NMJs, the EPP is higher than the safety threshold required to generate the action potential, whereas in MG the loss of AChRs results in a reduction of EPP amplitudes below the safety threshold causing the failure of the neuromuscular transmission [7,29,30].

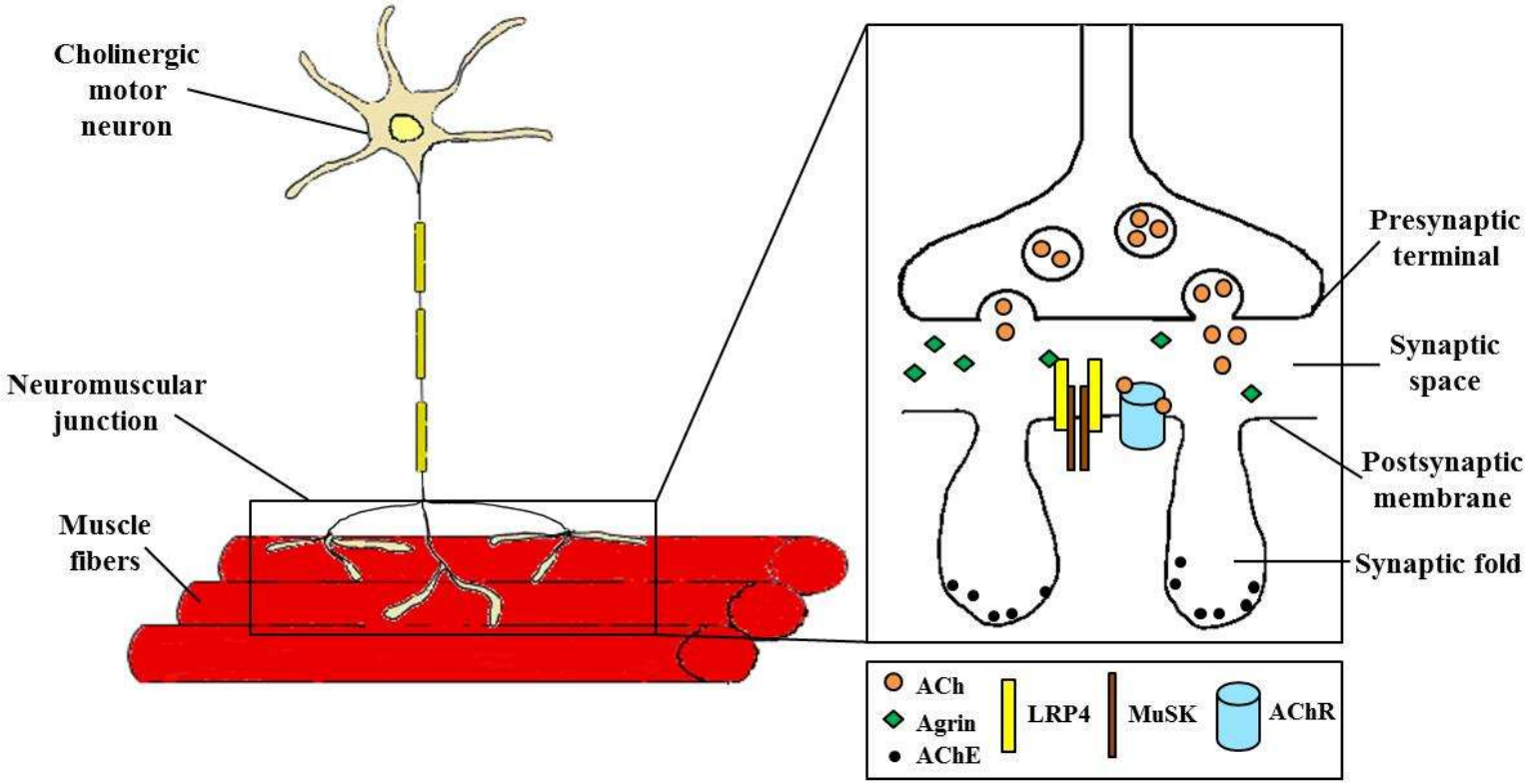

Figure 1. Simplified structure of the neuromuscular junction. The major constituents of the neuromuscular junction involved in MG are displayed. ACh: Acetylcholine; AChE: Acetylcholinesterase; LRP4: Low density lipoprotein receptor-related protein 4; MuSK: Muscle-specific kinase; AChR: Acetylcholine receptor.

1.3.2. Effector functions of anti-AChR antibodies

The impairment of the neuromuscular transmission in MG NMJs results from anti-AChR Abs action via three effector mechanisms: i) activation of complement; ii) increased degradation of AChRs, also known as antigenic modulation, and iii) blocking of ACh-binding sites (Figure 2).

Anti-AChR Abs have typically IgG1 or IgG3 isotypes thus are capable of activating the complement. The complement cascade leads to the formation of the membrane attack complex and the lysis of the muscle membrane [31]. The destruction of the postsynaptic membrane results in major morphological alterations characterized by the simplification of the membrane folding and the reduction of AChRs and voltage-gated sodium channels [32]. Moreover, antigenic modulation caused by anti-AChR Abs may contribute to the functional loss of AChRs. IgG have two antigen-binding sites thus may cross-link AChR molecules accelerating internalization and degradation of the receptors which are not adequately compensated by *de novo* protein synthesis; hence, reducing AChR availability [33]. Finally, anti-AChR Abs directly interfere with the binding of ACh to its receptor by blocking ACh-binding sites inducing of a loss of AChR function. These effector mechanisms of anti-AChR Abs cause the failure of neuromuscular transmission and give rise to myasthenic symptoms such as fatigue and muscle weakness (Figure 2) [30,34].

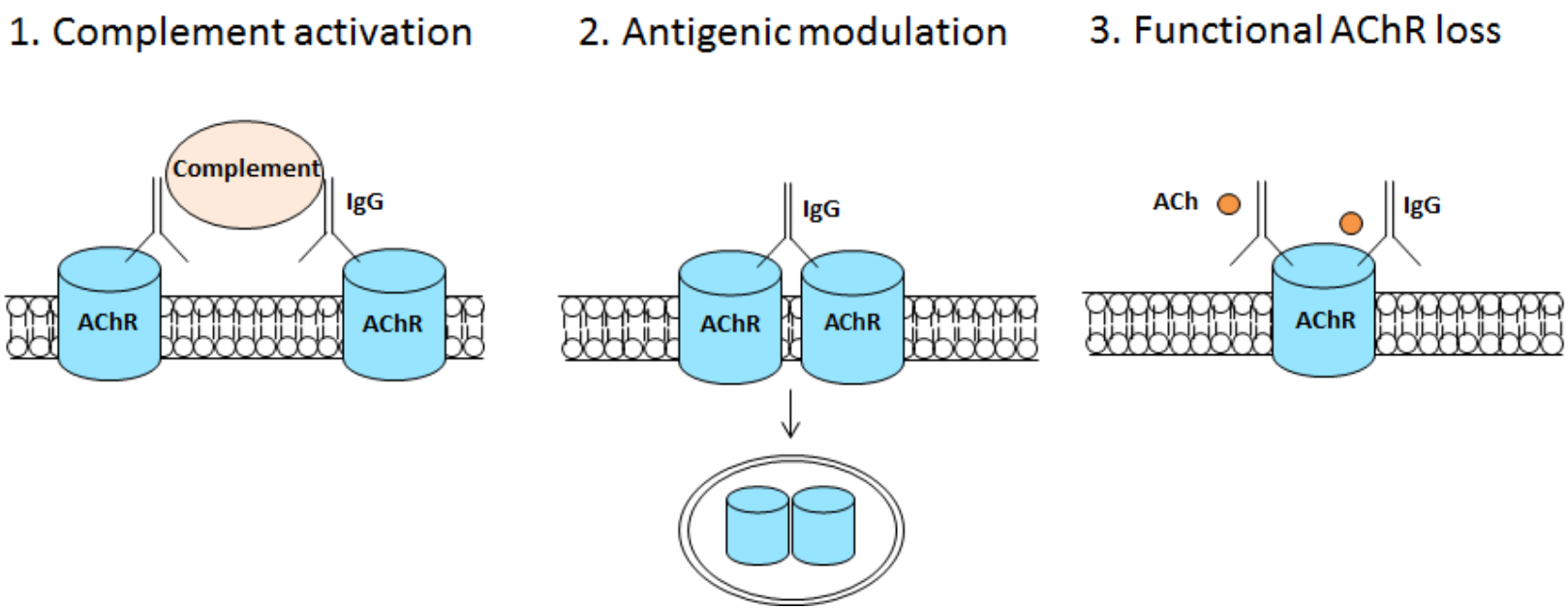

Figure 2. Three mechanisms of function of anti-AChR autoantibodies: (1) IgG autoantibodies bind and activate the complement; (2) Autoantibodies promote cross-linking and degradation of AChR; (3) Antibodies block ACh-binding site leading to AChR loss of function. AChR: Acetylcholine receptor; ACh: Acetylcholine; IgG: Immunoglobulin IgG isotype.

1.4. Clinical classification

The most recurrent primary manifestation of MG is ocular weakness displaying by ptosis and/or diplopia in almost 85% of patients; however, only 15% of them presents a pure ocular form during their clinical history. In the majority of patients within two years from MG onset, disease progresses in a generalized form characterized by bulbar signs including dysarthria, dysphagia, dysphonia, and respiratory muscle involvement in combination with axial and limb weakness [23,35]. Symptoms may be variable based on age of onset, autoantibody specificity, and thymic abnormalities allowing the classification of MG patients in several clinical subgroups.

The generalized form with AChR-positive Abs (AChR-MG) is present in more than 80% of patients and it can be classified into early-onset MG (EOMG) with a disease onset before the age of 50 and late-onset MG (LOMG) after 50 years of age. AChR-positive EOMG clinical subgroup is characterized by a female predominance and by thymic hyperplasia [36] and this is the most common hence best-investigated MG subgroup. On the other hand, LOMG is more frequent in men and is typically associated with thymoma, a tumour of thymic epithelial cells; this late-onset form manifests with severe symptoms such as bulbar muscle involvement [23,36-38].

Approximately 5% of MG patients, mainly women, have autoantibodies against MuSK, a tyrosine kinase involved in NMJ integrity. MuSK-positive MG patients (MuSK-MG) have a severe form with frequent bulbar and respiratory muscle involvement, whereas thymic alterations are uncommon [39-42]. Anti-MuSK Abs

have IgG4 isotype thus they are not able to activate the complement cascade [43]. Unlike AChR-MG, a correlation between antibody titre and disease severity has been reported in MuSK-MG patients [44,45].

The remaining 7-10% of patients lack anti-AChR or anti-MuSK Abs and are thus referred as ‘seronegative MG’ patients. In a variable proportion (12-50%) of these patients, the presence of IgG1 Abs against LRP4, an agrin receptor that activates MuSK and contributes to AChR clustering, has been recently discovered [46,47]. Moreover, about 50% of ‘seronegative MG’ patients have IgG1 autoantibodies to clustered AChR and have a clinical spectrum similar to AChR-MG [48]. The main features of MG clinical subgroups are summarized in Table 1.

Table 1. Classification of MG clinical subgroups.

	AChR	MuSK	LRP4	Clustered AChR
Percentage of patients	80-85%	5-8%	2-3%	~5%
Population features	EOMG: F>M LOMG: F<M	F>M	Unknown	Unknown
Antibody isotype	IgG1 and IgG3	IgG4	IgG1	IgG1
Role of complement	Yes	No	Likely	Likely
Correlation of Ab titer with disease severity	No	Yes	Unknown	Unknown
Thymic abnormalities	EOMG: hyperplasia LOMG: thymoma	No	Unknown	Likely similar to AChR-MG

Ab, antibody; AChR, acetylcholine receptor; EOMG, early-onset MG; F, female; LOMG, late-onset MG; LRP4, lipoprotein receptor-related protein 4; M, male; MG, myasthenia gravis; MuSK, muscle-specific kinase.

1.5. Etiological factors in MG

MG is a multifactorial disease and is probably linked to a combination of predisposing genetic factors and environmental agents.

1.5.1. Genetic susceptibility

Familial MG is extremely rare and few studies are available on twins or familial cases. Nevertheless, a study showed that the concordance rate in monozygotic twins was between 30 and 40%, whereas in dizygotic twins the frequency was about 4-5%, highlighting the role of a genetic susceptible background in MG predisposition [49].

The association between human leukocyte antigen (HLA) class I and II genes and MG is well established. A robust genetic association was observed between the HLA-A1-B8-DR3 haplotype and Caucasian EOMG patients [50]. The HLA-A1, HLA-B8, and HLA-DR3 alleles belong to the most frequent HLA haplotype in European populations, the '8.1 ancestral haplotype' (*i.e.* HLA-A1, C7, B8, C4AQ0, C4B1, DR3, and DQ2); this term was coined to define regions of extremely conserved genomic sequences with a common ancestor [51]. The 8.1 haplotype showed a high linkage disequilibrium, therefore the causal allele is not yet discovered. Among HLA class II genes, some findings highlighted the involvement of HLA-DQ alleles in MG. In particular, HLA-DQB1*0502 allele was reported to be associated with Italian MG patients [52] whereas, in Southeast Texas region, a new association between HLA-DQ loci and Caucasian American EOMG patients was found, supporting the role of territorial environmental factors in HLA susceptibility [53].

Non HLA-related genes were also found to be associated with MG. Polymorphisms on cytotoxic lymphocyte-associated protein-4 (CTLA4) [54], type II interferon (IFN-II), IL-10, IL-12 [55], and protein tyrosine phosphatase nonreceptor-22 (PTPN22) were observed in MG [56,57]. Of note, PTPN22 gene encodes for a tyrosine phosphatase and is mainly expressed in lymphoid tissues. A variant of this gene, localized in the promoter region, was found to be associated with a mild form of MG with low autoantibody titres in an Italian population, suggesting a role of this variant in MG susceptibility [58]. Of particular interest, Giraud *et al.* discovered a variant in the promoter of cholinergic receptor nicotinic alpha polypeptide 1 (CHRNA1) gene, encoding for the α subunit of AChR, that was associated with EOMG patients. The variant abolished CHRNA1 promoter activity in thymic epithelial cells thus controlling the expression of a self-antigen within the thymus, and influencing central tolerance maintenance [59]. Moreover, a genome-wide association study identified a variant of TNF-α-induced protein 3-interacting protein 1 (TNIP1) gene as a risk factor in EOMG patients. *TNIP1* is a signalling protein involved in inflammation and immune-related diseases however its exact role remains to be studied both in physiological and pathological conditions [60].

Despite the numerous studies, the mechanisms that link the genetic basis with disease pathology remain unknown and a comprehensive analysis between genes and environmental risk factors might be relevant in MG.

1.5.2. Gender influence

Autoimmune diseases show a gender bias. In MG, women prevalence was observed with a female/male ratio of 3:1 [61]. Sex hormones may play a key role in gender influence; oestrogen can modify both innate and adaptive immune responses by modulating cell proliferation, cytokine production, and antibody production [62,63]. It is uncertain whether sex hormones have an influence on AChR function. The up-regulated expression of oestrogen receptors observed in both peripheral blood cells and thymus of MG patients, may alter thymic microenvironment making it prone to oestrogen effect [64]. Further investigations are needed to better understand the role of sex hormones in MG.

1.5.3. Environmental risk factors

The contribution of genetics in MG predisposition is well known, however MG concordance in monozygotic twins was estimated to be up to 40% suggesting also the contribution of environmental factors in MG aetiology [49]. Drugs, pollution, microbioma changes, and infections are the main candidate factors [65]. In particular, pathogen exposure may be a major source of environmental triggering factors in autoimmunity [66]. Of note, the association between type 1 diabetes and coxsackievirus and cytomegalovirus was reported as well as between multiple sclerosis, rheumatoid arthritis and systemic lupus erythematous with Epstein-Barr virus [67]. However, clinical disease manifestations could occur weeks, months or years after pathogen clearance (known as the ‘hit-and-run’ hypothesis) therefore a link between viral infections and autoimmunity remains elusive [67].

Nevertheless, growing evidence supports the hypothesis of a contribution of viruses in autoimmune processes [68].

In the section 1.6.5. the role of viral infections in MG will be discussed.

1.6. Role of the thymus in MG

It is well accepted that the thymus plays a central role in MG and this organ is considered the key site of autosensitization process against AChR. Physiological features of thymic architecture and its pathological modifications in MG are reported below.

1.6.1. Anatomical, cellular, and functional organization

The thymus is a primary lymphoid organ of the lymphatic system localized in the thoracic cavity, above the heart. It is the prime site of T cell maturation and central tolerance induction. It is composed by two lobes, each of which can be histologically divided into an outer cortex and an inner medulla [69].

Thymic cortex consists of immature thymocytes (developing T cells) and cortical epithelial cells; it is the area in which the primary events of T cell maturation occur, including positive selection that promotes the selective survival of HLA-restricted T cells [69].

Thymic medulla consists instead of mature thymocytes, medullary thymic epithelial cells (mTECs), dendritic cells (DCs), and macrophages [69]. The medulla is the main thymic region in which self-reactive thymocytes undergo negative selection. In particular, autoimmune regulator (AIRE) transcription factor guides the

expression of self-antigens (*e.g.* insulin and AChR) in mTECs that, along with myoid cells, interact with developing T cells leading to the removal of autoreactive T cells [70-72]. Considering the critical role of the thymus in shaping the immune system, thymic anatomical and functional modifications may lead to the escape of autoreactive T cells from the thymus likely triggering autoimmune reactions. Unsurprisingly, pathological changes of the thymus are associated with MG. In particular, almost 80% of AChR-MG patients show thymic morphological alterations such as hyperplasia, very common in EOMG, and thymoma, frequently found in LOMG [36].

1.6.2. Thymoma

Thymoma is a slow-growing tumour of thymic epithelial cells and it is classified by the World Health Organization based on the histological properties of the neoplastic cells and lymphocyte content [73]. Type B2 thymoma (*i.e.* cortical thymoma) is most frequently associated with MG [74].

Anti-AChR autoantibodies were detected in all MG patients with thymoma, suggesting that neoplastic conversion of epithelial cells plays a crucial role in the development of autoimmunity against AChR [74,75]. In particular, several tolerogenic factors were defective in thymomas, such as the expression of AIRE, HLA class II molecules, and forkhead box P3 (FoxP3) transcription factor of regulatory T cells [76-78]. Moreover, thymomas were characterized by the absence or reduction of AChR-expressing myoid cells and by the disorganization of the thymic architecture [36,79,80]. In conclusion, thymomas display pathological features that may lead to

the break-down of self-tolerance and the initiation of anti-AChR autoimmune response.

1.6.3. Thymic hyperplasia

Thymic hyperplasia is characterized by the presence of infiltrating B cells in the thymic medulla or in the perivascular space. B-cell infiltrates can be organized into ectopic germinal centers (GCs) often surrounded by AChR-expressing myoid cells (follicular hyperplasia) or can be disseminated in the medulla (diffuse hyperplasia) [81]. B cells in thymic GCs undergo somatic hypermutation and antigen-driven selection increasing heterogeneity of B cell population [82]. Although the mechanisms that drive the infiltration of peripheral B cells into the thymus and their organization into follicle-like structures are not fully known, the overexpression of some chemokines, such as C-X-C motif chemokines 10 and 13 (CXCL10 and CXCL13) and chemokine ligand 19 (CCL19) were reported in MG thymus that may be responsible for the migration of B cells into the tissue [83-86]. Moreover, hyperplastic MG thymus showed active neo-angiogenesis characterized by the formation of abnormal lymphatic vessels and specialized post-capillary swellings, the high endothelial venules (HEVs), that expressed chemokines on their surface, including the T and B cell-chemoattractant CCL21 [83]. However, the mechanism responsible for the development of thymic ectopic HEVs remains unknown. Furthermore, SDF-1, also known as CXCL12, was demonstrated to be expressed by HEVs; this chemokine was responsible for the recruitment of DCs, macrophages, and B cells into the thymus [87]. Altogether, these results suggest that upon

chemokine stimulation the migration of peripheral B cells via active angiogenetic processes may contribute to the abnormal formation of GCs within the thymus.

1.6.4. Intra-thymic mechanisms of anti-AChR response

Many evidence supports the involvement of the thymus in AChR-MG pathogenesis:

1. As mentioned before, thymus showed marked pathological modifications in most AChR-MG patients (*e.g.* hyperplasia and thymoma) [36];

2. Correlation between autoantibody titres and severity of thymic hyperplasia occurred in MG patients [88];

3. Transplantation of thymic fragments of MG patients into immunodeficient host mice transferred a self-reactive microenvironment and human anti-AChR Abs were detected 1 to 2 weeks after transplantation [89].

4. Thymectomy resulted in a complete stable remission in a high proportion of AChR-MG patients [90];

5. The anti-AChR antibody titre reduction was inversely correlated with the number of GC B cells removed after thymectomy [91];

6. Inside the thymus are all the elements required to trigger and maintain autoimmunity, including AChR-expressing thymic epithelial cells (TECs), myoid cells [92-95], DCs [69], AChR-

autoreactive T cells, and plasma cells producing anti-AChR antibodies [92-94].

The mechanisms by which all these cellular components could trigger autoimmune processes within the thymus are described below.

Thymic myoid cells express all AChR subunits, as well as the functional receptor [96-98]. Poëa-Guyon *et al*. reported that thymic myoid cells increased the expression of AChR subunits upon proinflammatory cytokine stimulation (*i.e.* IFN-II), suggesting that an inflammatory environment within the thymus could enhance AChR expression and contribute to initiation of an autoimmune response directed to AChR [95]. Although thymic myoid cells do not express HLA class II molecules, hence they do not directly present antigens to HLA class II-restricted T cells, it was shown that AChR fragments released by myoid cells were internalized by DCs and likely cross-presented to AChR-specific T cells [99,100]. This hypothesis was supported by the evidence that in thymic hyperplasia myoid cells and DCs were frequently found together [101]. Moreover, MG thymus displayed complement deposition on epithelial cells and myoid cells, localized nearby GCs [102]. Long-term complement-mediated lysis of thymic myoid cells could increase AChR fragment availability for DCs, as well as GC formation, thus creating a vicious circle that could be responsible for establishing a self-maintaining anti-AChR autoreactivity within the thymus [103].

With regards to TECs, they express α, β, and ε AChR subunits, as well as HLA class II molecules [104]. Like myoid cells, upon IFN-II stimulation TECs increased AChR subunit expression thus they are

thought to be involved in intra-thymic anti-AChR autosensitization process in MG [95]. As previously described, mTECs are key cells involved in central tolerance by expressing self-antigens through AIRE transcription factor. In human mTECs, a study showed that AIRE modulated the transcriptional levels of CHRNA1, encoding the AChR α subunit, thus possibly controlling the balance between self-tolerance and autoimmunity [59].

With regards to T cell involvement in intra-thymic anti-AChR autosensitization, AChR-specific $CD4^+$ T cells were observed in peripheral blood and in the thymus of MG patients [92,93,105], and anti-CD4 monoclonal antibody administration ameliorated MG symptoms, thus suggesting that autoreactive $CD4^+$ T cells may be involved in autoimmune mechanisms in MG [106].

Back in 1989, Sisely and colleagues demonstrated that the proliferation of peripheral blood T cells was enhanced in MG patients in response to AChR [107]. One year later and more recently in 2005, several epitopes localized in the α subunit of AChR were identified as crucial for the anti-AChR $CD4^+$ T cell sensitization, suggesting that in MG T cell response is heterogeneous [108,109]. Moreover, in peripheral blood cells of MG patients, $CD8^+$ T cell depletion resulted in a higher anti-AChR Ab secretion than healthy subjects, suggesting a regulatory function of suppressor $CD8^+$ T cells [110,111].

With regards to B cell role in intra-thymic mechanisms of anti-AChR response, Guigou and co-workers demonstrated that MG thymuses contained polyclonal B cells. In addition, several evidence demonstrated that thymic B cells were activated *in vivo* and underwent

isotype switching [112,113]. Moreover, Vincent *et al*. observed that thymic fragments of MG patients spontaneously produced anti-AChR antibodies and also showed that thymic cell suspensions were enriched in long-lived terminally-differentiated plasma cells, suggesting that MG thymus is characterized by the presence of locally-activated AChR-specific B cells [114-116].

1.6.5. Inflammation and innate immunity activation

The inflammatory environment in MG thymus is characterized by the overexpression of several chemokines, cytokines, (*e.g.* CCL21, CXCL10, and CXCL13), and IFN-inducible genes [83-87,95]. Of note, IFN imprinting has a key role in intra-thymic anti-AChR autosensitization processes [95].

The contribution of IFNs (type I, II, and III) in MG is discussed below [117].

Type I IFN family comprises of 12 IFN-α subtypes, one IFN-β, in addition to IFN-δ, -ε, -κ, -τ, and -ω. The role of IFN-I is not well investigated neither in animal models nor in humans, likely due to IFN-I family complexity, although all its members have antiviral properties [117]. Nevertheless, evidence suggested the involvement of IFN-I in MG. Of interest, it was shown that human MG thymuses displayed an increased expression of IFN-β that was also involved in α-AChR overexpression in TECs [118].

With regards to IFN-II, it was shown that IFN-II (or IFN-γ) or IFN-II-receptor knock-out mice were resistant to experimental autoimmune myasthenia gravis (EAMG) and showed a reduction of

anti-AChR Ab levels, suggesting that IFN-II has a pathogenic role in anti-AChR antibody-mediated immune response in EAMG [119,120]. As mentioned before, IFN-II increased the expression of AChR subunits, especially α subunit, in TECs and myoid cell cultures [95].

IFN-III family was described more recently [117], therefore its role in MG has not been significantly described, yet.

Altogether, these findings suggest that the inflammatory status of MG thymus, characterized by IFN up-regulation, could modify intra-thymic AChR expression thus influencing central tolerance maintenance and subsequently triggering autoimmune reactions. The observation that the antiviral IFN-I is overexpressed in MG thymus suggests a possible link between pathogen infection and MG. The molecular players that may be involved in this link are discussed below.

The pivotal role of innate immune response in autoimmune diseases as well as the interconnection between the innate and adaptive immune systems are currently emerging [121]. In innate immunity, Toll-like receptors (TLRs) are pattern-recognition receptors mainly expressed by macrophages and DCs that have a key role in the recognition of conserved pathogen-associated molecules and in the activation of inflammatory and antiviral innate immune responses, to successfully enrol the defence against pathogens [121]. TLRs can also promote the maturation of antigen-presenting cells and the secretion of IFN-I and other inflammatory cytokines consequently leading to the activation of adaptive immune cells. It is therefore possible that TLR-mediated activation of autoreactive T cells could contribute to autoimmunity development [121,122]. Recently, Wang and colleagues

showed that in peripheral blood cells of MG patients the expression levels of TLRs were dysregulated (*e.g.* TLR4, TLR3, and TLR9). In particular, TLR9 mRNA levels were increased and were also associated with clinical severity, highlighting the possible role of TLR-mediated pathways in the pathogenesis of MG [123]. In addition, MG thymuses showed increased expression levels of TLR4 as well as the presence of TLR4 activators (*e.g.* cytomegalovirus, herpes zoster, and enterovirus) and a persistent poliovirus infection, supporting the idea that, in MG thymus, the dysregulation of TLR4-mediated innate immune response to pathogens could sustain a chronic inflammation thus triggering autoreactivity [124,125]. Moreover, Cufi *et al*. demonstrated that a TLR3-agonist, the double-stranded RNA/polyinosinic–polycytidylic acid, specifically increased the expression of AChR α subunit in human TECs by the release of IFN-β; TLR3 and IFN-β expression levels were also up-regulated [118]. In summary, TLR activation, inflammation, and IFN-I production are key features of MG thymus and highlight the contribution of innate immunity in the intra-thymic anti-AChR autosensitization. However, the origin of this dysregulated innate immunity activation in MG thymus is still unknown.

Epstein-Barr virus (EBV), a widely diffused herpes virus, is associated with the triggering of several autoimmune diseases including multiple sclerosis, systemic lupus erythematosus, and rheumatoid arthritis [126]. In multiple sclerosis patients, active lesions of the brain showed signs of EBV latency and activation of TLR3-mediated innate immune responses with the overexpression of IFN-α that may contribute to neuroinflammation [127]. EBV also encodes

double-stranded RNA-like molecules that can activate TLR3 signalling leading to IFN-I production [128]. The presence of EBV latent and lytic transcripts and proteins in B cells and plasma cells were found in hyperplastic MG thymuses, indicating EBV persistence and reactivation [129,130]. Since EBV is able to infect and immortalize B cells [126], it may trigger a self-sustaining autoreactivity within MG thymus by transforming autoreactive B cells. These findings support the hypothesis that EBV alone, or in combination with other pathogens, could be a plausible source of innate immunity activation and TLR signalling dysregulation in MG thymuses that may sustain autoimmune processes. Further studies are needed to better investigate the underlined mechanisms.

1.7. Alteration of immunoregulatory mechanisms in MG

The mechanisms of immunoregulation are impaired in MG patients, thus contributing to disease pathogenesis. In particular, in hyperplastic MG thymuses, the number of $CD4^+CD25^+$ regulatory T (Treg) cells was strongly defective, as well as the expression levels of the transcription factor Foxp3, demonstrating a severe functional loss of thymic Treg cells [131]. Moreover, in MG thymuses and in peripheral blood cells the suppressive function of Treg cells was defective [132,133]. In MG thymus, Treg cells also showed an overexpression of Th17- (*i.e.* IL-17A, IL-17F, IL-21, IL-22, and IL-26) and Th1-related cytokines (*i.e.* IFN-γ and TNF-α) [132,134]. Studies addressing the role of Th17 cells in MG are limited. Nevertheless, in peripheral blood cells, an increase in Th17 cells and Th17-related

cytokines was found in thymoma-associated MG patients [135]. In addition, the pro-inflammatory IL-17 was increased in the serum of MG patients and it was correlated with anti-AChR antibody titres, supporting the pathogenic role of IL-17 in MG [136].

To conclude, at the peripheral level, inflammation and suppressive activity of Treg are altered, resulting in impaired immunoregulatory mechanisms of peripheral self-tolerance that may contribute to the development of autoimmune processes.

1.8. Treatments

The ultimate aim of MG therapy is to reach a complete stable remission defined as no clinical signs without treatment for at least one year. Therapeutic strategies include three different lines: i) symptomatic therapies, ii) immunomodulating therapies, and iii) thymectomy [137].

1.8.1. Symptomatic therapies

Oral AChE inhibitors are the first-line treatment in MG. They do not interfere with disease progression, but they act at the symptomatic level by increasing the amount of ACh at the NMJ, therefore improving neuromuscular transmission. The effect of AChE inhibitors can last few hours hence they have a short-term benefit range [137]. Side effects are primarily due to cholinergic stimulation of muscarinic AChRs and include gut hyper motility, hyperhidrosis, bronchial secretion, and bradycardia, frequently observed in older patients [137]. Pyridostigmine bromide is the most frequently used AChE inhibitor. Other AChE inhibitors include neostigmine, that is less

effective and cause muscarinic adverse effects more often than pyridostigmine bromide, and edrophonium that has an extremely short half-life and it is mainly used for diagnostic purposes [137].

1.8.2. Immunomodulating therapies

Immunomodulating therapies interfere with the pathological processes underlying MG.

Oral steroids are recommended as first-choice drug when immunosuppression is needed [138]. They are typically used in a chronic-based regimen hence the risk of severe adverse effects is high; osteoporosis, hypertension, glaucoma, gastrointestinal irritation, and diabetes mellitus are just some of the possible side effects [137]. Prednisone is the most regularly used steroid due to its strong immunosuppressive action [139].

Other immunosuppressive drugs, such as azathioprine and cyclosporin A, require a longer time to achieve clinical improvements as compared to steroids. They are used as alternative treatment when steroids are contraindicated.

Azathioprine is a purine analogue and strongly inhibits proliferating T and B cells and may cause bone marrow and liver toxicity [138,140]. With regards to cyclosporine A, it inhibits IL-2 production by $CD4^+$ T helper cells and it may induce hypertension and renal toxicity.

Plasma exchange (PE) and intravenous immunoglobulins (IVIg) are short-term immunomodulating therapies that interfere with

autoantibody effects and are commonly used to achieve rapid clinical improvement in disease exacerbation. Plasma exchange temporarily decreases the amount of circulating autoantibodies and their beneficial effects can last up to 5 weeks [141,142]. Chronic PE treatments are limited in case of poor vascular access and they can also reduce coagulation factors leading to bleeding predisposition [143].

IVIg therapy shows symptom improvements within 2 weeks and have a similar efficacy as compared to PE [137]. The mechanisms of action are unknown, although it has been proposed that the effect of IVIg could be attributed to the interaction with Fcγ receptors expressed on immune cells thus hypothetically can modulate the immune response [144].

1.8.3. Thymectomy

Thymectomy modifies the natural history of the disease and it is mandatory in patients with thymoma, whereas it is a therapeutic option in non-thymomatous AChR-MG patients with generalized MG and with a disease onset before the age of 50 in order to increase the chance of remission [138,145,146]. Unfortunately, randomized clinical trials indicating the advantages of thymectomy are not available [137].

In conclusion, a clinically relevant aim in MG treatment relies on the development of drugs selectively targeting the key immunopathological steps of the disease without altering the immune system. Ideal therapeutic strategies require high efficacy, minimal side effects, and optimal schedules.

New molecular studies that exploit high throughput sequencing techniques could improve our knowledge of the dysregulated molecules involved in disease pathogenesis and they may represent a biological source for the discovery of novel immunological therapies. Further investigations are therefore needed to improve patients' quality of life.

1.9. Scope of the thesis

In our studies we aim at better investigate the possible mechanisms that link the intra-thymic MG pathogenesis with the self-reactive processes that occur in the muscle through the peripheral vascular system.

Chapter 2 contains a manuscript submitted for publication describing the identification of a novel molecular signature associated with infection and inflammation pathways in peripheral blood cells of AChR-EOMG patients.

Chapter 3 contains a paper showing TLR7 and TLR9 overexpression in MG thymuses characterized by an active EBV infection. Our overall findings indicated that dysregulation of the EBV-driven TLR7- and TLR9-mediated innate immune response may favour inflammation and autoreactivity in the thymus of MG patients.

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Chapter 2.

A novel infection- and inflammation-associated molecular signature in peripheral blood of myasthenia gravis patients

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Myasthenia gravis; peripheral blood mononuclear cells; whole-transcriptome sequencing; inflammation; viral infection

Abbreviations:

AChR: Acetylcholine receptor

AChR-EOMG: Acetylcholine receptor-early onset AChR-MG

AZA: Azathioprine

IPA: Ingenuity Pathway Analysis

lincRNAs: Long non-coding intergenic RNAs

lncRNAs: Long non-coding RNAs

MG: Myasthenia gravis

miRNAs: microRNAs:

pre-miRNAs: miRNA precursors:

NMJ: Neuromuscular junction

PBMCs: Peripheral blood mononuclear cells

RNA-seq: RNA-sequencing

Summary

Myasthenia gravis (MG) is a T-cell dependent autoimmune disorder of the neuromuscular junction, characterised by muscle weakness and fatigability. Autoimmunity is thought to initiate in the thymus of acetylcholine receptor (AChR)-positive MG patients; however, the molecular mechanisms linking intra-thymic MG pathogenesis with autoreactivity *via* the circulation to the muscle target organ are poorly understood. Using whole-transcriptome sequencing, we compared the transcriptional profile of peripheral blood mononuclear cells from AChR-early onset MG (AChR-EOMG) patients with healthy controls: 178 coding transcripts and 229 long non-coding RNAs, including 11 pre-miRNAs, were differentially expressed. Among the 178 coding transcripts, 128 were annotated of which 17% were associated with the 'infectious disease' functional category and 46% with 'inflammatory disease' and 'inflammatory response-associated' categories. Validation of selected transcripts by qPCR indicated that of the infectious disease-related transcripts, *ETF1*, *NFKB2*, *PLK3*, and *PPP1R15A* were upregulated, whereas *CLC* and *IL4* were downregulated in AChR-EOMG patients; in the 'inflammatory' categories, *ABCA1*, *FUS*, and *RELB* were upregulated, suggesting a

contribution of these molecules to immunological dysfunctions in MG. Data selection and validation were also based on predicted microRNA-mRNA interactions. We found that miR-612, miR-3654, and miR-3651 were increased, whereas miR-612-putative *AKAp12* and *HRH4* targets and the miR-3651-putative *CRISP3* target were downregulated in AChR-EOMG, also suggesting altered immunoregulation. Our findings reveal a novel peripheral molecular signature in AChR-EOMG, reflecting a critical involvement of inflammatory mechanisms in disease pathogenesis. Further investigations into the molecules identified here may improve our understanding of AChR-EOMG molecular basis, with a possible future impact on its therapy.

Introduction

Myasthenia gravis (MG), a T cell-dependent, B cell-mediated autoimmune disorder affecting the neuromuscular junction (NMJ), is characterised by the presence of autoantibodies against post-synaptic membrane proteins of striated skeletal muscle. These autoantibodies promote autoimmunity by disrupting neuromuscular transmission, resulting in clinical symptoms such as muscle weakness and abnormal fatigability [1]. Antibodies directed against the acetylcholine receptor (AChR) of the post-synaptic NMJ are detected in approximately 80% of patients [2], but other autoimmune targets, including the muscle kinase receptor and lipoprotein receptor-related protein 4, have also been observed in a variable proportion of seronegative patients [3-5]. Several lines of evidence support the contribution of the thymus in the pathogenesis of MG. This lymphoid organ is commonly recognised as the main site of autosensitisation in most AChR-positive MG (AChR-MG) patients. These patients show histological and functional thymic abnormalities, including hyperplasia and thymoma, and in a high proportion of cases, thymectomy results in symptomatic improvement [6-8].

MG is a chronic although treatable disease; therapeutic strategies take three different lines: i) symptomatic therapies consisting of the use of

acetylcholinesterase inhibitors, such as pyridostigmine bromide; ii) immunosuppressive treatments, such as azathioprine (AZA) and steroids; and iii) alteration of the natural history of the disease (*e.g.* thymectomy) [9]. However, in a substantial proportion of cases, these treatments do not lead to complete stable remission [10] and may give rise to severe side effects, highlighting the need for more specific and effective therapies. More detailed characterisation of the immunological and molecular alterations leading to autoimmunity development and perpetuation would be helpful to guide this process.

Growing evidence points to the presence of a chronic inflammatory state in the MG thymus, likely due to persistent viral replications, which could alter innate immune responses and lead to the breakdown of self-tolerance, triggering autoimmune reactions [11-14]. Increased expression and production of cytokines in peripheral blood cells is also observed, further supporting a contribution of inflammation to MG pathogenesis [15,16]. However, a comprehensive understanding of the immunological alterations occurring in the periphery of MG patients, and the link between thymic inflammation and muscle autoreactivity is lacking.

Recent developments in sequencing technology are opening the door to comprehensive and high-throughput approaches for understanding

the molecular basis of disease, including in autoimmunity. RNA-sequencing (RNA-seq) has improved the sensitivity for detection of novel (dysregulated) coding transcripts, non-coding transcripts, and splicing variants [17]. This technology has been used to detect novel long non-coding RNAs (lncRNAs) that are associated with immune-related functions in psoriatic skin, suggesting an involvement of these lncRNAs in the pathogenesis of psoriasis [18]. Recently, next-generation sequencing was used to identify a number of novel disease-associated genes and isoforms from the transcriptomes of synovial fibroblasts of rheumatoid arthritis patients, contributing to better understand of the molecular mechanisms of disease [19].

Here, we exploit whole-transcriptome sequencing to study the transcriptional profile of peripheral blood mononuclear cells of early-onset (≤ 50 years old) AChR-positive MG (AChR-EOMG) patients, who represent the most studied MG clinical subgroup [20]. We selected a clinically well-defined and homogeneous cohort of patients to reduce the impact of external confounding factors with the aim of identifying a molecular signature of disease that could be used to aid understanding of the development of peripheral autoimmunity in MG.

Materials and methods

Patients and specimen collection

The study included nineteen MG patients (14 females, 5 males, mean age at blood collection ± SD: 41.21 ± 12.81 years old) and 12 age- and sex-matched healthy controls (8 females, 4 males, mean age ± SD: 38.42 ± 9.99 years old) with no autoimmune diseases or signs of infection. Written informed consent was obtained from each patient and control. The study was approved by the Ethics Committee of the Neurological Institute 'Carlo Besta'. MG patients were selected based on the following criteria: early-onset (≤50 years old) of symptoms, positivity for anti-AChR antibodies, and treatment regimen. At the time of blood collection, 13 AChR-EOMG patients were untreated or treated only with acetylcholinesterase inhibitors, and 6 patients were treated with AZA and/or acetylcholinesterase inhibitors; 7 patients were thymectomised (hereinafter referred as post-thymectomy), whereas the remaining 12 did not undergo thymic removal (hereinafter referred as pre-thymectomy) before sampling. Among the pre-thymectomy patients, two patients (MG1 and MG3) had thymoma diagnosed by computed tomography. Of the post-thymectomy patients, four (MG7, MG8, MG9, and MG10) had thymic hyperplasia, two had a normally involuted thymus (MG11 and MG19), and one

(MG18) had thymoma. For the post-thymectomy patients, the time interval range between thymectomy and blood collection was <11 years, except for MG11 (25 years). Unfortunately, pre- and post-thymectomy samples from the same patients were not available. None of the patients presented signs of infections at time of blood collection with the exception of one patient (MG11) who had chronic hepatitis C. Recent infections were reported for MG12 who had H1N1 influenza A virus infection two months before sampling and MG1 who had mononucleosis approximately 6 months before sampling (two months before MG onset). Patients' clinical characteristics are summarised in Table 1 with an indication of the experimental analyses performed. Whole blood was collected in EDTA-supplemented tubes from patients and controls, and peripheral blood mononuclear cells (PBMCs) were then isolated using Lymphoprep (Axis-Shield, Dundee, Scotland) according to the manufacturer's recommendations. PBMCs were frozen in FBS containing 10% DMSO (Euroclone, Milan, Italy) and stored in liquid nitrogen until use.

RNA isolation

Total RNA was extracted from PBMCs using the mirVana mRNA extraction kit (Thermo Fisher Scientific, Waltham, MA, USA)

following the manufacturer's instructions. RNA quality was assessed using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and RNA integrity number (RIN) was calculated for each sample (mean ± SD: 7.68 ± 1.49). RNA was then quantified using Quant-iT RiboGreen RNA Reagent (Thermo Fisher Scientific).

Whole-transcriptome sequencing

Total RNA from 11 AChR-EOMG patients (9 females, 2 males, mean age ± SD: 37.1 ± 8.8 years old) and 6 healthy controls (4 females, 2 males, mean age ± SD: 36.2 ± 8.6 years old) (discovery cohort) was analysed by whole-transcriptome sequencing (Table 1). cDNA libraries were prepared from 100 ng of total RNA using the TruSeq Stranded Total RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. All libraries were subjected to a 2 × 51-cycle run on an Illumina HiSeq 2000 (Illumina). The total reads produced from each sample were between 34,700,444 and 52,053,472, with a mean across all samples of 43,498,491 reads.

Mapping against long non-coding intergenic RNAs and micro RNA precursors

Precursors of human micro RNAs (miRNA) were downloaded from miRBase (version 20) (www.mirbase.org). Long non-coding intergenic

RNAs (lincRNAs) sequences were retrieved from BioMart - Ensembl (01-07-2013) (www.ensembl.org) [21], searching only for mature lincRNAs in the *Homo sapiens* Hg19 reference genome (www.genome.ucsc.edu). MiRNA precursors and lincRNAs were combined in a unique dataset and used to create a reference for STAR aligner [22]. For this analysis, because we also used the miRNA precursors, we mapped only R1 of the pair-reads.

Differential expression analysis and functional data annotation

R software (www.R-project.org) [23] was employed for RNA-seq data elaboration using the DESeq Bioconductor package [24]. Data were further analysed by QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, CA, USA, www.qiagen.com/ingenuity). Ingenuity Knowledge Base was exploited to study functional category distribution of differentially expressed transcripts. Predicted microRNA-mRNA interactions were analysed by IPA TargetScan algorithm [25].

Spotfire software (TIBCO Software Inc., Palo Alto, CA, USA) was used to generate heat maps of the dysregulated molecules identified.

NanoString validation

Validation of selected differentially expressed transcripts was performed in 17 AChR-EOMG patients (12 females, 5 males, mean age ± SD: 42.5 ± 12.9 years old) and 12 healthy controls (8 females, 4

males, mean age ± SD: 38.4 ± 10.0 years old) (validation cohort), using the NanoString nCounter system (NanoString Technologies, Seattle, WA, USA) [26] (Table 1). nCounter Elements TagSets were used according to the manufacturer's instructions. Capture and reporter tags were hybridised to 100 ng of total RNA and incubated at 67 °C for 18 h. After hybridisation, transcript-tag complexes were applied to the nCounter Preparation Station. Data were collected using the nCounter Digital Analyzer. Human β-actin (*ACTB*) (Entrez Gene ID: 60) and ornithine decarboxylase antizyme 1 (*OAZ1*) (Entrez Gene ID: 4946) were used as endogenous genes. Spike-in RNA positive and negative controls were also included. Data were log 2-transformed after being normalised in three steps. First, raw counts were corrected against the spike-in RNA positive controls; then, data were background-subtracted using the spike-in RNA negative control threshold, which was set as the mean plus two standard deviations. Finally, a normalisation factor was calculated from the geometric mean of the endogenous genes and applied to the background-subtracted data [27].

Reverse transcription and qPCR

Validation of selected differentially expressed miRNAs was performed in 17 AChR-EOMG patients (12 females, 5 males, mean age ± SD: 42.5 ± 12.9 years old) and 12 healthy controls (8 females, 4 males, mean age ± SD: 38.4 ± 10.0 years old) by qPCR (Table 1). Total RNA (100 ng) were reverse transcribed using the Universal cDNA synthesis kit II (Exiqon, Vedbaek, Denmark) following the manufacturer's recommendations. A quality control UniSp6 RNA spike-in was included. cDNA was diluted 5-fold and amplified in duplicate using ExiLENT SYBR Green master mix along with pre-designed or custom microRNA PCR primer sets (All Exiqon products). *SNORD48* was used as an endogenous control (Exiqon). A no-template control, performed without any cDNA template, was included in all experiments. qPCR was carried out in 96-well plates using the CFX96 Real-time PCR detection system (BIO-RAD, Hercules, CA, USA) with one step at 95 °C for 10 min and 40 cycles of 10 sec at 95 °C and 1 min at 60 °C. Levels of the target transcripts were expressed as relative values ($2^{-\Delta Ct} \times 1{,}000$) normalised against *SNORD48*. A list of the miRNA primer sets used is reported in Supplementary Table S1.

Statistical analyses

Data distribution was tested using the Kolmogorov-Smirnov test. Normally distributed data were analysed using the Student's *t* test or one-way ANOVA, followed by Bonferroni *post-hoc* testing. Non-parametric data were analysed using the Mann-Whitney test for the comparison of two groups. MiRNA-mRNA correlation analysis was performed using the parametric Pearson correlation test. *P* values < 0.05 were considered statistically significant. GraphPad Prism v5.0 (La Jolla, CA, USA) was used for data elaboration and statistical analyses.

Results

Peripheral blood transcriptome is altered in AChR-EOMG patients.

To identify possible molecules involved in AChR-EOMG pathogenesis in peripheral blood cells, we analysed the transcriptional profile of PBMC from a clinically homogeneous group of AChR-EOMG patients by whole-transcriptome sequencing (n = 11); age- and sex-matched healthy donors were used as controls (n = 6). Results showed that 178 coding transcripts and 229 lncRNAs, including 11 microRNA precursors (pre-miRNAs), were differentially expressed between AChR-EOMG patients and healthy controls (Fig. 1). No difference was observed between pre- and post-thymectomy patients (data not shown). Among the differentially expressed genes and pre-miRNAs, 128 transcripts and 9 pre-miRNAs were annotated by IPA software (Supporting information, Tables S2 and S3).

Infection- and inflammation-associated transcriptional patterns are dysregulated in AChR-EOMG patient PBMCs.

From the above-described discovery phase, several differentially expressed genes and miRNAs were selected for validation following

two main criteria based on Ingenuity Knowledge Base functional enrichments: i) genes significantly enriched for biological functional categories as ‘inflammatory disease’, ‘inflammatory response’, and ‘infectious disease’ (Supporting information, Table S4) and ii) predicted miRNA-mRNA interactions. Several studies from our and other’s groups have provided data showing a contribution of pathogen infections (*e.g.* Epstein-Barr virus) and chronic inflammation in the intra-thymic pathogenesis of AChR-MG [28-30]. Based on these observations, we considered whether the inflammatory signature of the MG thymus might be reflected at the PBMC level. In line with this hypothesis, we found that 17% (22 out of 128) of the differentially expressed genes identified by RNA-seq belonged to the ‘infectious disease’ category and 46% (59 out of 128) were associated with ‘inflammatory disease’ and ‘inflammatory response’ categories (Supporting information, Table S4). Among the ‘infectious disease-related’ transcripts, we selected for validation *ADRA2B*, *CLC*, *ETF1*, *IL4*, *NFKB2*, *PLK3*, *POLR2A*, and *PPP1R15A*, of which *ADRA2B*, *CLC*, *IL4*, *NFKB2*, and *PPP1R15A* were also included in the ‘inflammatory disease’ and/or ‘inflammatory response’ categories, together with *ABCA1*, *FUS*, and *RELB* (Supporting information, Tables S2 and S4). The expression of these transcripts was analysed in

PBMC from the validation cohort of patients and controls using NanoString technology. We found that *CLC* and *IL4* mRNA levels were significantly downregulated in AChR-EOMG patients (mean ± SEM: 2.27 ± 0.52 and 0.39 ± 0.17, respectively) compared to healthy controls (mean ± SEM: 8.63 ± 0.20 and 4.23 ± 0.26, respectively) (Fig. 2a). On the contrary, *NFKB2*, *PLK3*, *PPP1R15A*, and *ETF1* transcript levels were significantly upregulated in AChR-EOMG samples (mean ± SEM: 9.23 ± 0.16, 8.72 ± 0.15, 10.32 ± 0.14, and 8.65 ± 0.09, respectively) *versus* healthy controls (mean ± SEM: 8.64 ± 0.06, 7.68 ± 0.08, 9.39 ± 0.09, 8.16 ± 0.06, respectively) (Fig. 2a). *ADRA2B* and *POLR2A* mRNA levels were not altered (Supporting information, Fig. S1). Moreover, mRNA levels of the inflammatory-associated transcripts *ABCA1*, *FUS*, and *RELB* mRNA levels were significantly upregulated in AChR-EOMG PBMC (mean ± SEM: 7.09 ± 0.17, 9.43 ± 0.10 and 8.38 ± 0.15, respectively) *versus* healthy controls (mean ± SEM: 6.42 ± 0.16, 8.86 ± 0.08, 7.53 ± 0.12, respectively) (Fig. 2b). Gene expression data were also analysed by grouping the patients based on thymectomy or drug treatment at time of blood collection. Transcriptional levels were similar between pre- and post-thymectomy AChR-EOMG patients and between AZA-treated or acetylcholinesterase inhibitor regimen-based patients,

indicating that thymectomy or immunosuppressive treatment with AZA did not influence their expression levels in this patient cohort (Supporting information, Figs. S2 and S3).

MiR-612, miR-3654, miR-3651, and pre-miR-3651 are upregulated in PBMC of AChR-EOMG patients.

Of the identified miRNA precursors, pre-miR-612, pre-miR-1299, and pre-miR-3651 (Supporting information, Table S3) were selected for validation along with their predicted target transcripts annotated by IPA TargetScan (*i.e.* pre-miR-612: *ABCA1*, *AKAp12*, *FUS*, *HRH4*; pre-mir-1299: *ETF1*; pre-miR-3651: *CRISP3*). We also combined, in an IPA network analysis, the differentially expressed pre-miRNAs and the differentially spliced variants, and found that pre-miR-3654 interacted with the differentially spliced *NR3C1* gene, which was functionally connected with some differentially expressed transcripts (*i.e. JUN, NFKB complex,* and *Ap1*) (data not shown). Hence, we added pre-miR-3654 into the validation panel. qPCR validation was performed in PBMC of the validation cohort using mature- and precursor-specific miRNA probes. Our data showed that miR-612 and miR-3654 were significantly upregulated in AChR-EOMG patients (mean ± SEM: 0.25 ± 0.09 and 0.107 ± 0.029, respectively) compared

to healthy controls (mean ± SEM: 0.0010 ± 0.0005 and 0.017 ± 0.0052, respectively) (Fig. 3a-b). Pre-miR-612, pre-miR-3654, and pre-miR-1299 were not detected. However, pre-miR-3651 and miR-3651 were both significantly upregulated in AChR-EOMG patients (mean ± SEM: 23.78 ± 1.67 and 36.71 ± 2.96, respectively) *versus* healthy controls (mean ± SEM: 9.07 ± 1.06 and 14.28 ± 2.16, respectively) (Fig. 3c-d). MiR-1299 expression was not altered in AChR-EOMG patients (Fig. 3e). No difference in miRNA expression levels was detected between pre- and post-thymectomy patients and between untreated and AZA- or acetylcholinesterase inhibitor-treated patients (Supporting information, Fig. S4).

Identification of dysregulated putative miRNA-mRNA target pairs in PBMC of AChR-EOMG patients.

Selected predicted target transcripts of dysregulated miRNAs were analysed in the validation cohort using NanoString technology. Among the miR-612-predicted target transcripts, *ABCA1* and *FUS* were significantly upregulated in AChR-EOMG PBMCs, as described above. In contrast, *AKAp12* and *HRH4* transcriptional levels were significantly downregulated in patients (mean ± SEM: 0.69 ± 0.33 and

3.72 ± 0.14, respectively) *versus* healthy controls (mean ± SEM: 5.28 ± 0.16 and 4.92 ± 0.12, respectively) (Fig. 4). Moreover, miR-3651-predicted target *CRISP3* mRNA levels were downregulated in AChR-EOMG patients (mean ± SEM: 0.034 ± 0.034 *versus* 3.59 ± 0.62) (Fig. 4). Transcriptional profiles of all these predicted dysregulated miRNA-target transcripts did not differ between pre- and post-thymectomy patients or between untreated and AZA- or acetylcholinesterase inhibitor-treated patients (Supporting information, Fig. S5). We searched for the presence of anti-correlation between dysregulated miR-612 and miR-3651, and their predicted target transcripts, indicative of a direct relationship. An anti-correlation trend was detected between miR-612 and *AKAp12* and *HRH4* transcripts (Pearson test, r = -0.81 and r = -0.75, respectively; $p < 0.001$) (Supporting information, Fig. S6). We did not find significant anti-correlation trends between the other predicted miRNA/mRNA target transcript pairs (*i.e.* miR-612/*ABCA1*; miR-612/*FUS*; miR-3651/*CRISP3*), suggesting that alternative post-transcriptional mechanisms or different miRNAs might modulate the expression of these transcripts (data not shown). Taken together, our results identify a molecular signature in PBMCs associated with AChR-EOMG characterised by a profile of 16 dysregulated molecules

involved in inflammatory or infectious disease-related immune responses (Fig. 5).

Discussion

The clinical features of myasthenia gravis vary widely, exhibiting high heterogeneity that manifests in a variable autoantibody status, age of onset, and thymic histology, suggesting different pathological mechanisms among different disease subgroups. Although AChR-EOMG is the most frequent and better-studied MG clinical subgroup, the underlying pathogenic mechanisms are not clearly defined. A putative pathogenic model for AChR-MG has been proposed, in which persistent intra-thymic pathogen infections in genetically susceptible individuals may lead to dysregulated innate immune responses and long-term inflammation, favouring autosensitisation and autoimmunity [9,10,12,29]. An active inflammatory state has also been observed at the peripheral level; AChR-MG patients are characterised by increased serum levels of pro-inflammatory cytokines (*e.g.* IL-17 and IL-32α), as well as a persistent clonal expansion of $CD4^+$ T helper cells [31-33]. However, the exact molecular alterations leading to the development and maintenance of the autoimmune process in AChR-EOMG patients are unknown.

The development of next-generation sequencing technologies, such as whole-transcriptome sequencing, allows comprehensive and accurate analysis of the differential regulation of both coding and non-coding

transcripts in disease, providing new insights into the transcriptional regulation of disease mechanisms. Here, whole-transcriptome analysis was applied for the first time to study the transcriptional profile of peripheral blood cells from AChR-EOMG patients with the aim of identifying dysregulated molecules that may underlie pathogenic mechanisms. We identified 128 coding transcripts and 229 lncRNAs, including 9 miRNA precursors that were differentially expressed in PBMCs of AChR-EOMG patients compared to healthy controls. We focused our attention on annotated coding transcripts and miRNAs.

Consistent with the hypothesis of a contribution of infection and inflammation to AChR-MG, RNA-seq of AChR-EOMG patients identified a significant enrichment of differentially expressed genes in the peripheral transcriptome associated with 'infectious disease', 'inflammatory disease', and 'inflammatory response' functional categories. Gene expression analysis of selected transcripts in the validation cohort revealed that AChR-EOMG PBMCs were characterised by dysregulated 'infectious-associated' *CLC*, *ETF1*, *IL4*, *NFKB2*, *PLK3*, and *PPP1R15A* transcripts and 'inflammatory-associated' *ABCA1*, *FUS*, and *RELB* molecules. Among the patients included in our study, only one had a chronic pathogen infection at blood collection, indicating that the molecular signature we identified

was not due to occasional infection events but is of pathological relevance to AChR-EOMG. In addition, the expression levels of the dysregulated 'infectious-' and 'inflammatory-associated' transcripts in AChR-EOMG patients were not normalised by surgical removal of the thymus and/or by AZA-based immunosuppressive treatment. More than 50% of patients (11 out of 19) had active symptomatic disease in terms of clinical score, suggesting that the molecules identified here are involved in persistent pathological mechanisms occurring in peripheral blood cells and are not a direct target of the above-mentioned therapies.

The involvement of the 'infectious-associated' *CLC*, *ETF1*, *NFKB2*, *PLK3*, and *PPP1R15A* and 'inflammatory-associated' *ABCA1*, *FUS*, and *RELB* transcripts has not previously been described in MG pathogenesis, therefore the role of these molecules should be investigated in future studies.

Transcripts of the multi-functional Th2 cytokine *IL4*, previously shown to be involved in MG pathogenesis [34], was identified within the 'infectious disease-' and 'inflammatory-associated' signature that was downregulated in the PBMC of AChR-EOMG patients. Several studies performed in the experimental MG mouse model suggested that *Il4* has a protective function and *Il4*-mediated regulatory

mechanisms contribute to prevent an autoimmune reaction against AChR [35,36]. Our findings strengthen the role of *IL4* in AChR-EOMG, and strongly support the idea that the disruption of its protective action via downregulation of the *IL4* transcript may favour MG maintenance.

Among the 'infection-related' transcripts, the upregulation of *PPP1R15A* in the peripheral blood of AChR-EOMG patients was of particular interest. *PPP1R15A*, also known as *GADD34*, is a DNA damage-inducible protein that it is required for innate immune Toll-like receptor (TLR) 3-dependent production of cytokines, such as IFN-β, and has been shown to have a role in the regulation of Chikungunya virus infection in mice [37]. Recently, Cufi *et al.* demonstrated that TLR3-mediated double-stranded RNA signalling increased thymic expression of the AChR α subunit through the release of IFN-β, favouring the production of anti-AChR antibodies [11]. Thus, the upregulation of *PPP1R15A* observed here in AChR-EOMG patients may be relevant to the perturbed inflammatory state and to the sustained autoimmune response in peripheral immune cells.

MiRNAs perform a regulatory function in the majority of biological processes and their dysregulation has been reported in many autoimmune diseases, such as rheumatoid arthritis and systemic lupus

erythematosus [38,39]. Studies investigating the role of miRNAs in MG pathogenesis are still limited. Recently, Cao Y *et al*. observed that dysregulated miRNA-gene interactions are involved in pathways that might be relevant to MG pathogenesis, such as miR-29a/b and genes associated with immune-related pathways (*e.g.* the MAPK signalling pathway) [40]. Moreover, increased levels of circulating miR-21-5p and miR-150-5p have been identified specifically in MG patients compared with healthy donors and patients with other autoimmune diseases (*i.e.* Addison's disease, Crohn's disease, and psoriasis), identifying them as possible biological markers of MG [41,42]. In our dataset, the above-mentioned miRNAs were not identified; this is possibly because the RNA-seq method that we used targeted long intracellular transcripts. However, we were able to detect other dysregulated pre-miRNAs, which could be validated by qPCR. Specifically, we identified a pattern of upregulated miRNAs (*i.e.* miR-612, miR-3654, miR-3651, and pre-miR-3651) associated with AChR-EOMG. The majority of the dysregulated miRNAs identified here have been discovered only recently, thus little is known about their biological roles. In particular, miR-612 and miR-3651 have been studied solely in cancer in which they were found to suppress tumour growth and progression [43-46]. Our findings highlight the

involvement of novel dysregulated miRNAs in AChR-EOMG that might alter regulatory transcriptional mechanisms relevant for disease pathogenesis. MiR-612-predicted target *HRH4* and *AKAp1*2 transcripts were downregulated in AChR-EOMG patients. Interestingly, anti-correlation analysis revealed a potentially direct relationship between miR-612, and *HRH4* and *AKAp12* mRNA targets. Specifically, *HRH4* is expressed in several immune cell types and is involved in the release of chemotactic cytokines (*e.g.* IL-6 and CCL2), indicative of an immunomodulatory role [47]. Indeed the involvement of *HRH4* has been supposed in several inflammatory diseases including multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus [47-49].

Gene polymorphisms *AKAp12* were recently identified as genetic risk factors for MG [50]. In addition, autoantibodies against AKAp12 were observed in EOMG patients more than two decades ago, although the role of AKAp12 as an autoantigen in MG remains elusive [51]. A possible role of *AKAp12* in autoimmune/inflammatory processes is supported by the observation that the rodent orthologue of human *AKAp12,* Scr-suppressed C kinase substrate, was upregulated during the pathogenesis of experimental autoimmune encephalomyelitis, inducing the production of TNF-α in astrocytes and thus contributing

to disease exacerbation [52,53]. Interestingly, TNF-α production is significantly increased in the MG thymus and is suspected to play a role in regulatory mechanisms by influencing the suppressive activity of regulatory T cells [54,55].

CRISP3 is present in the secretory granules of neutrophils involved in inflammatory reactions associated with infection, thus *CRISP3* may also play a role in the innate immune response, although its biological function is unknown [56,57]. We did not investigate the implications of its altered expression in the present work.

Using a comprehensive and unbiased whole-transcriptome sequencing approach allowed us to identify 16 dysregulated molecules in PBMC of a clinically homogeneous cohort of AChR-EOMG patients. Further studies will be conducted in other clinical MG subgroups in order to evaluate the specificity of the molecular expression. The novel molecular signature identified here are likely to reflect pathogenic mechanisms occurring in the peripheral blood cells of AChR-EOMG patients, implicating 'infectious-' and 'inflammatory-associated' molecules as key factors for disease progression. Combining our findings on PBMCs with what is known from the literature, it is tempting to speculate that, in the context of predisposing genetic factors and triggering event(s) (*e.g.* pathogen infection), the

establishment of a chronic inflammatory state may contribute to sustain the autoimmune process [9,13,14].

Our findings provide new evidence on the gene expression dynamics associated with AChR-EOMG and represent a valuable data source for further investigations into the molecular basis of MG pathogenesis that could be applied towards the development of novel therapeutic strategies.

Author's contribution

FZ, LM, RAC, RM, PB, PC, and CB conceived and designed the study, and wrote the manuscript. CB performed the experiments and data analysis. JL carried out RNA-seq experiment. RAC performed RNA-seq data analysis. SKG helped with the experiments. FB participated in the design of the study. RM, CA, SB, and GC enrolled patients and evaluated clinical parameters. EF processed biological samples. FA carried out clinical diagnostic tests.

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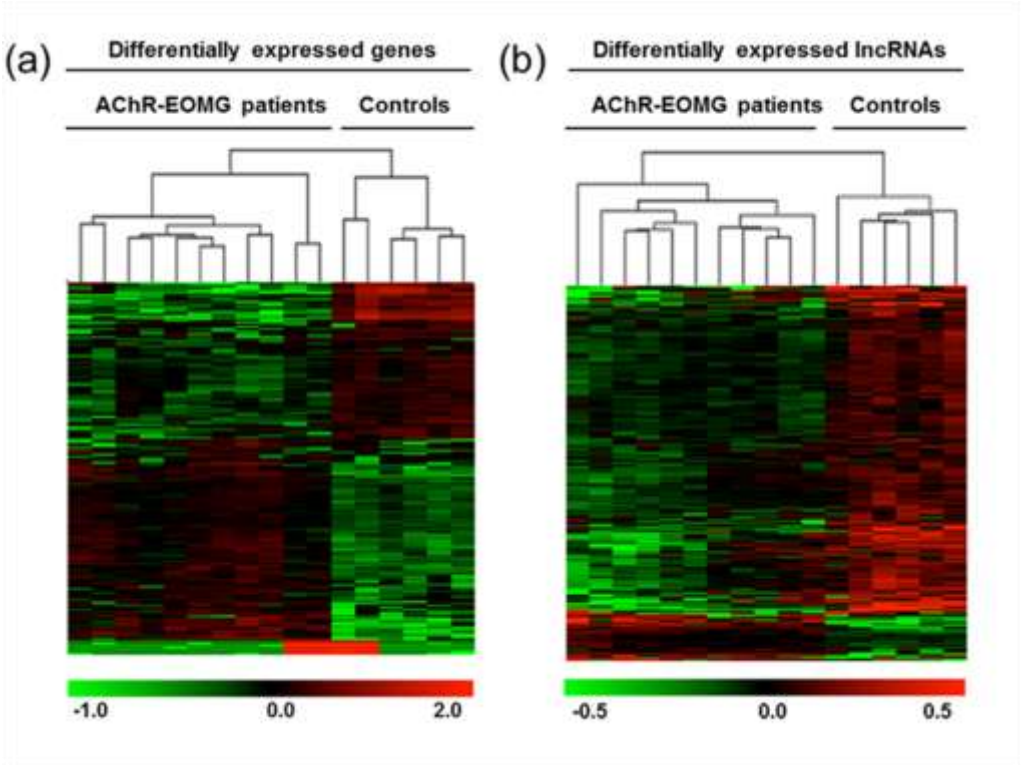

Figure 1. Heat maps of (a) dysregulated genes and (b) long non-coding RNAs (lncRNAs) in AChR-EOMG patients (n = 11) compared to healthy controls (n = 6) Hierarchical clustering was performed using Euclidean distance and average clustering on mean gene-centred data. Data are expressed as log2 fold change. Red indicates upregulation and green indicates downregulation.

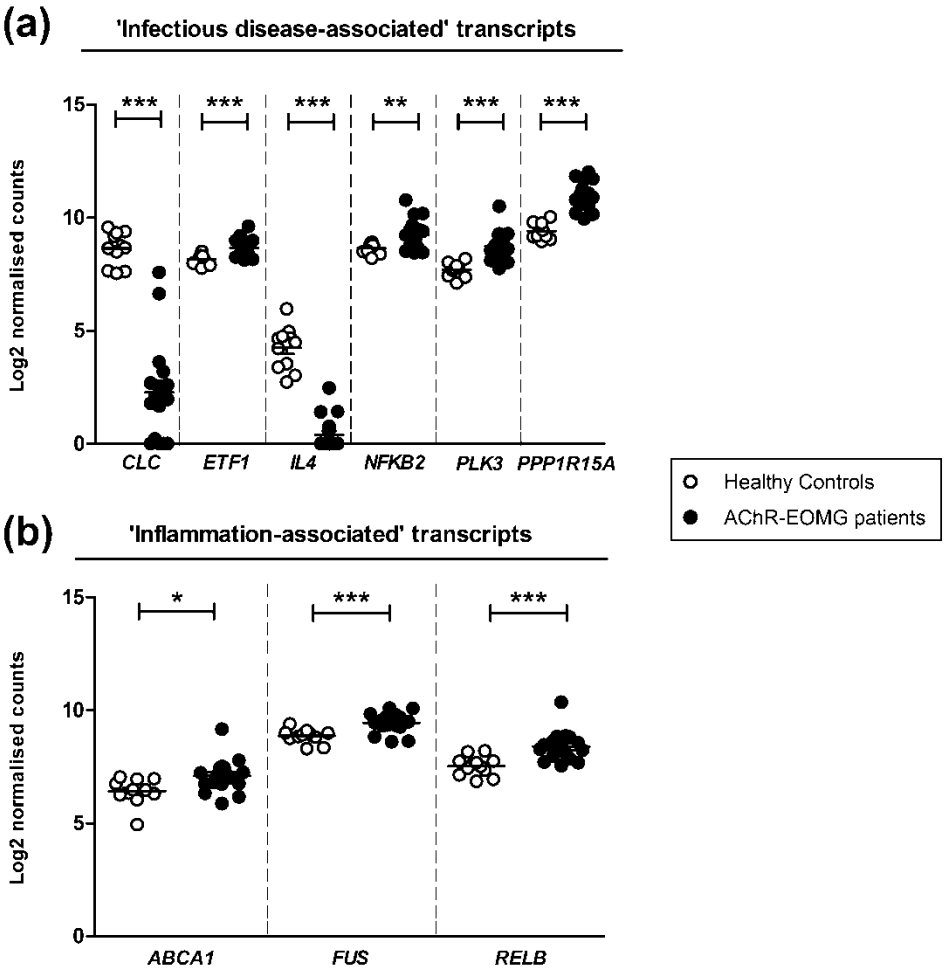

Figure 2. Molecular signature in PBMCs of the validation cohorts

(a) Expression levels of 'infectious disease-associated' transcripts: *CLC*, *ETF1*, *IL4*, *NFKB2*, *PLK3*, *PPP1R15A*. (b) Expression levels of 'inflammation-associated' transcripts: *ABCA1*, *FUS*, and *RELB*. PBMC from 12 healthy controls (open circle) and 17 AChR-EOMG patients (closed circle) were analysed by NanoString technology. Results are represented as scatter dot plots and they are expressed as

log2 normalised counts towards endogenous genes *OAZ1* and *ACTB*. Mean values ± SEM are shown. *P* values were assessed by Student's *t* test for all genes, except for *IL4* for which Mann-Whitney test was applied. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

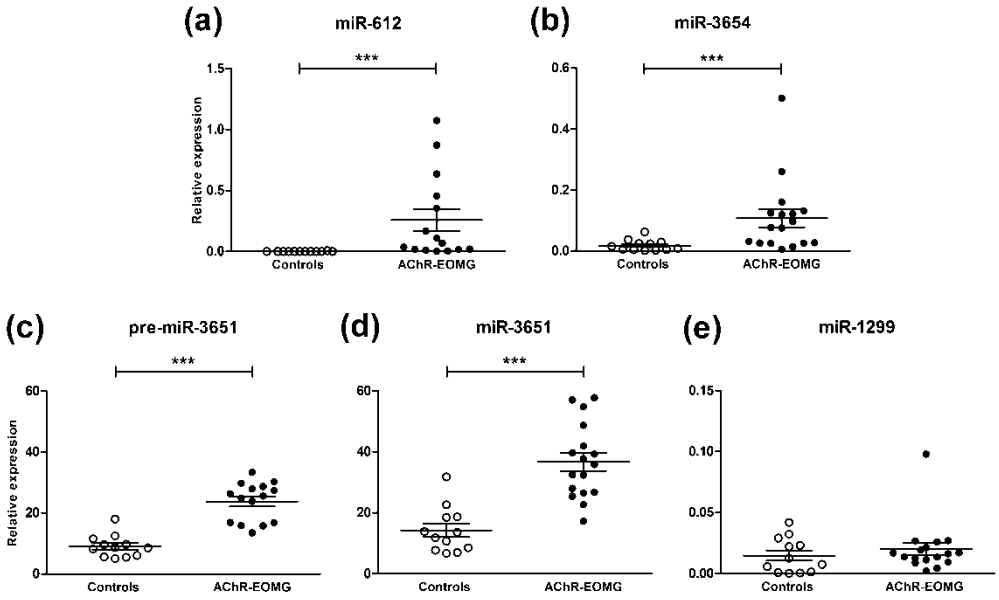

Figure 3. Validation of differentially expressed miRNAs

qPCR analysis of (a) miR-612, (b) miR-3654, (c) pre-miR-3651, (d) miR-3651, and (e) miR-1299 in PBMC from 12 healthy controls (open circle) and 17 AChR-EOMG patients (closed circle). Due to RNA scarcity, miR-612 and pre-miR-3651 were analysed in PBMC of 15 AChR-EOMG patients. Results are shown as scatter dot plots and reported as relative expression ($2^{-\Delta Ct} \times 1{,}000$) normalised towards the housekeeping gene *SNORD48*. Mean values ± SEM are reported. *P* values were assessed by Mann Whitney test for data shown in (a), (b), and (e) graphs; Student's *t* test was applied for data shown in (c) and (d) graphs. ***$P < 0.001$.

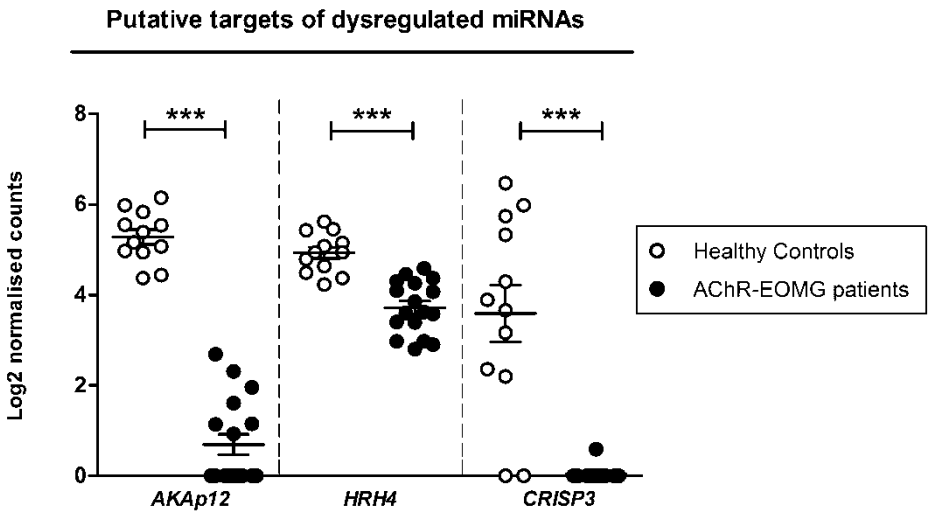

Figure 4. Validation of the putative target transcripts of dysregulated miRNAs NanoString analysis of miR-612-putative *AKAp12* and *HRH4* target transcripts and miR-3651-putative *CRISP3* target transcripts in PBMC from 12 healthy controls (open circle) and 17 AChR-EOMG patients (closed circle). Results are represented as scatter dot plots and expressed as log2 normalised counts towards the endogenous genes, *OAZ1* and *ACTB*. Mean values ± SEM are shown. *P* values were assessed by Mann Whitney test for *AKAp12* and *CRISP3* and by Student's *t* test for *HRH4*. ***$P < 0.001$.

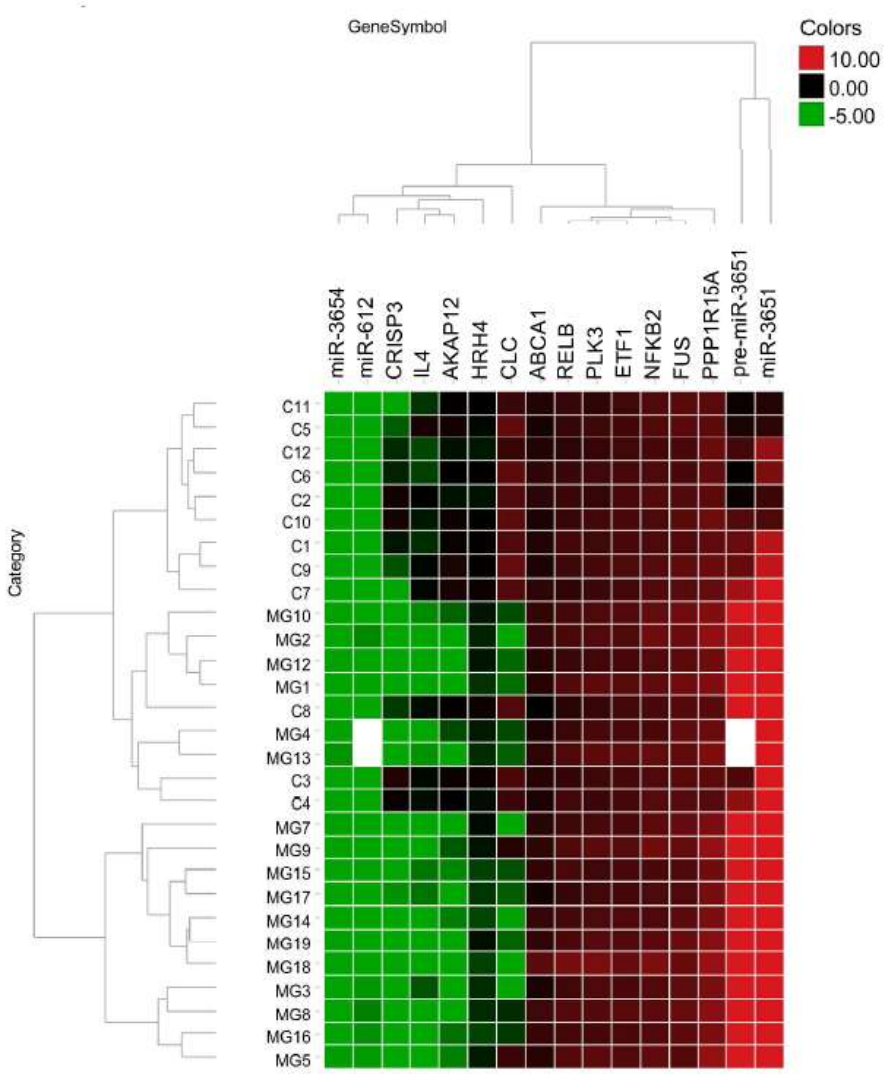

Figure 5. Heat map of the 16 dysregulated molecules in 17 AChR-EOMG patients (MG) and 12 healthy controls (C)

Hierarchical clustering was performed using Euclidean distance. For each molecule of each individual, data are expressed as log2 ratio of the expression levels against the mean of all subjects. Average values are shown. Red indicates upregulation and green indicates downregulation.

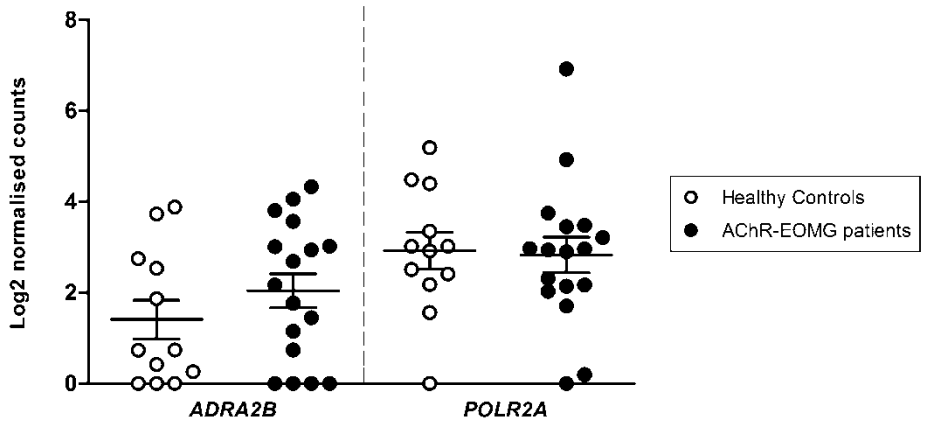

Figure S1. Expression levels of *ADRA2B* and *POLR2A* transcripts in the validation cohorts

NanoString analysis of *ADRA2B* and *POLR2A* mRNA levels in PBMCs from healthy controls (n = 12) (open circle) and AChR-EOMG patients (n = 17) (closed circle) were unchanged between the two groups. Results are shown as scatter dot plots and expressed as log2 normalised counts towards endogenous genes *OAZ1* and *ACTB*. Mean values ± SEM are reported.

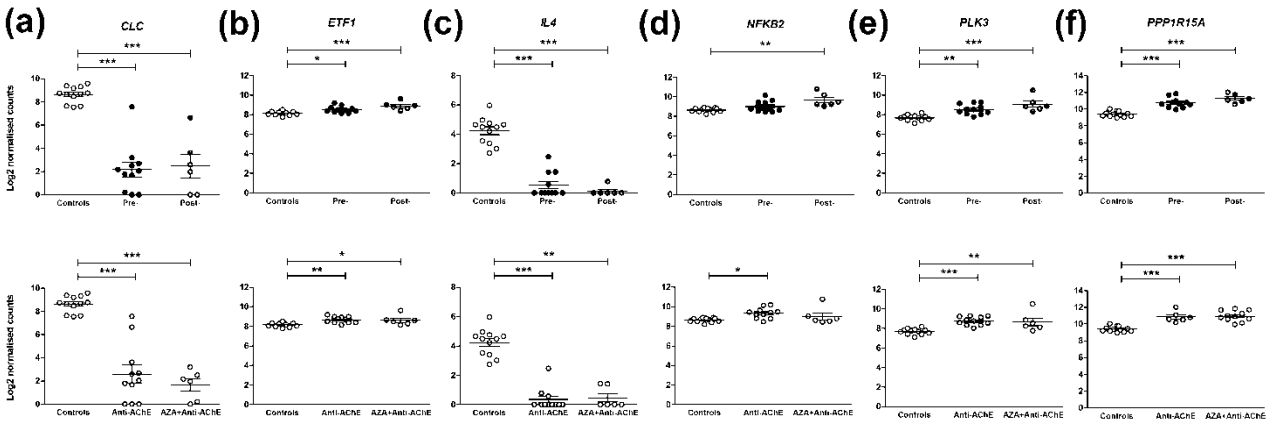

Figure S2. 'Infectious disease-associated' dysregulated transcripts in PBMC from AChR-EOMG patients based on thymectomy (top panels) or drug treatment (bottom panels). NanoString analysis of (a) *CLC*, (b) *ETF1*, (c) *IL4*, (d) *NFKB2*, (e) *PLK3*, and (f) *PPP1R15A* transcripts. Pre (patients not undergoing thymectomy; n = 11); post (patients thymectomised; n = 6); anti-AChE (patients treated with acetylcholinesterase inhibitors; n = 11); AZA + Anti-AChE (patients treated with azathioprine (AZA) only or in combination with anti-AChE; n = 6). Healthy controls (Controls) were included (n = 12). Results are represented as scatter dot plots and expressed as log2 normalised counts towards the endogenous genes, *OAZ1* and *ACTB*. Mean values ± SEM are shown. *P* values were assessed by one-way ANOVA followed by Bonferroni *post-hoc* test. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

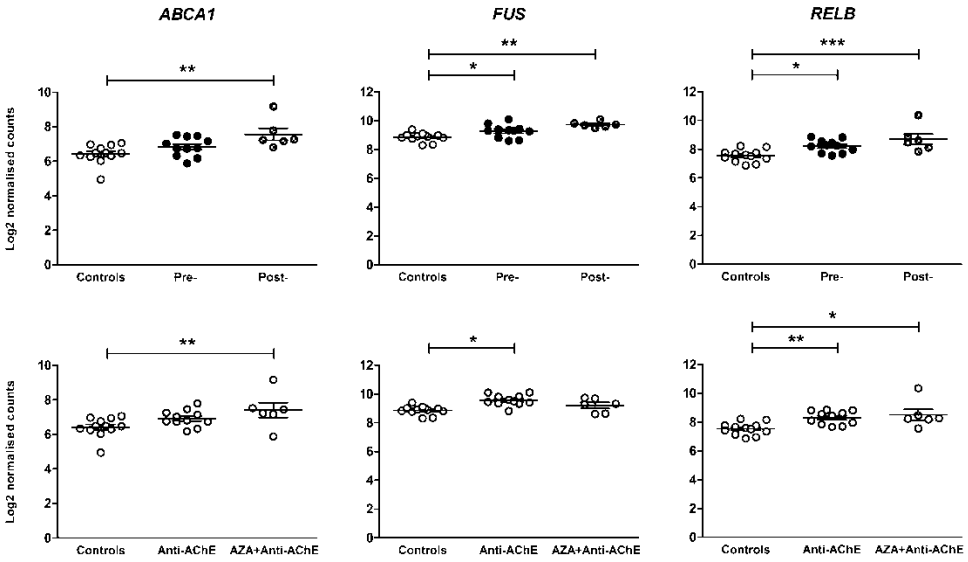

Figure S3. 'Inflammatory-associated' dysregulated transcripts in PBMCs of AChR-EOMG patients grouped based on thymectomy (top panels) or drug treatment (bottom panels) NanoString analysis of (a) *ABCA1*, (b) *FUS*, and (c) *RELB* transcripts. Pre (patients not undergoing thymectomy; n = 11); post (patients thymectomised; n = 6); anti-AChE (patients treated with acetylcholinesterase inhibitors; n = 11); AZA + Anti-AChE (patients treated with azathioprine (AZA) only or in combination with anti-AChE; n = 6). Healthy controls (Controls) were included (n = 12). Results are represented as scatter dot plots and expressed as log2 normalised counts towards the endogenous genes, *OAZ1* and *ACTB*. Mean values ± SEM are shown.

P values were assessed by one-way ANOVA followed by Bonferroni *post-hoc* test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

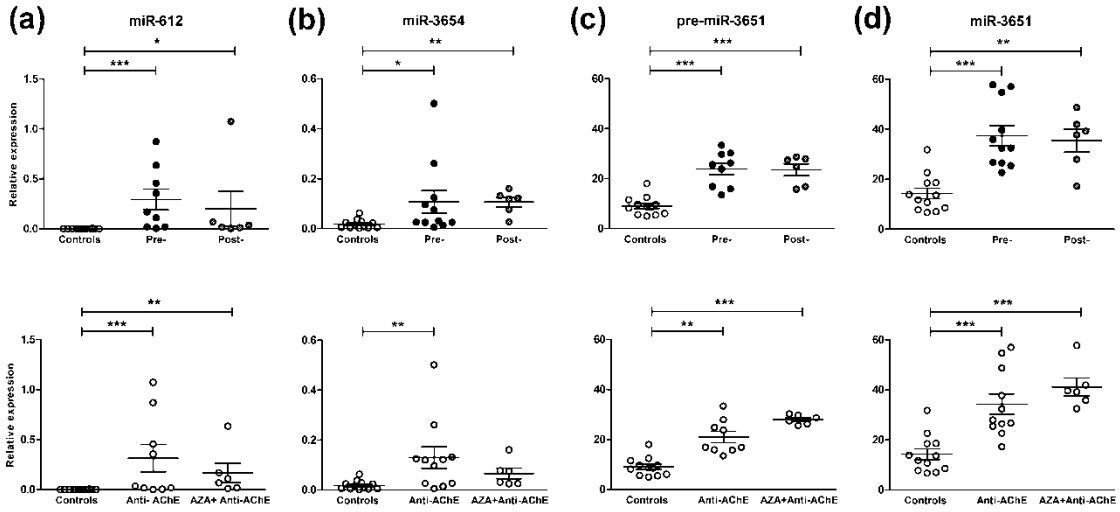

Figure S4. qPCR analysis of the differentially expressed (a) miR-612, (b) miR-3654, (c) pre-miR-3651 and (d) miR-3651 in PBMCs of AChR-EOMG patients based on thymectomy (top panels) or drug treatment (bottom panels)

Pre (patients not undergoing thymectomy; n = 11); post (patients thymectomised; n = 6); anti-AChE (patients treated with acetylcholinesterase inhibitors; n = 11); AZA + Anti-AChE (patients treated with azathioprine (AZA) only or in combination with anti-AChE; n = 6). Healthy controls (Controls) were included (n = 12). For miR-612 and pre-miR-3651 9 pre-thymectomy and 9 anti-AChE-treated EOMG patients were analysed. Results are shown as scatter dot plots and reported as relative expression ($2^{-\Delta Ct} \times 1{,}000$) normalized towards the housekeeping gene *SNORD48*. Mean values ± SEM are

reported. *P* values were assessed by one-way ANOVA followed by Bonferroni *post-hoc* test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

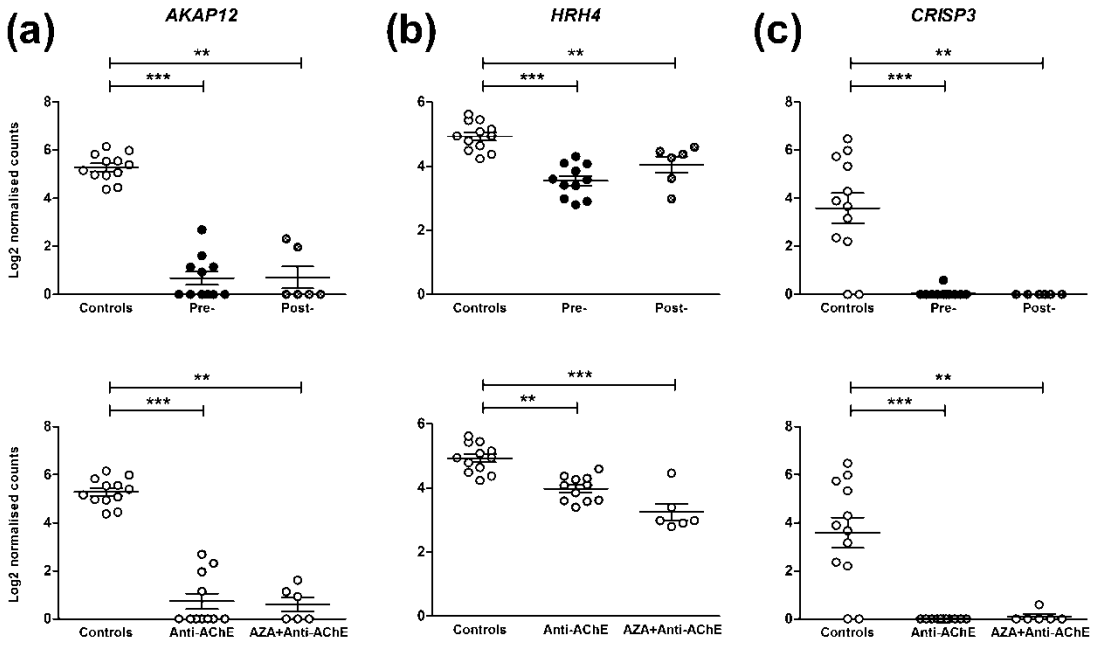

Figure S5. NanoString analysis of putative targets of dysregulated miRNAs in PBMCs of AChR-EOMG patients grouped based on thymectomy (top panels) and drug treatment (bottom panels)

(a-b) *AKAp12* and *HRH4* transcripts are miR-612-putative targets and (c) *CRISP3* transcript is an miR-3651-putative target. Pre (patients not undergoing thymectomy; n = 11); post (patients thymectomised; n = 6); anti-AChE (patients treated with acetylcholinesterase inhibitors; n = 11); AZA + Anti-AChE (patients treated with azathioprine (AZA) only or in combination with anti-AChE; n = 6). Healthy controls (Controls) were included (n = 12). Results are represented as scatter dot plots and expressed as log2 normalised counts towards the endogenous genes, *OAZ1* and *ACTB*. Mean values ± SEM are shown.

P values were assessed by one-way ANOVA followed by Bonferroni *post-hoc* test. $^{**}P < 0.01$; $^{***}P < 0.001$.

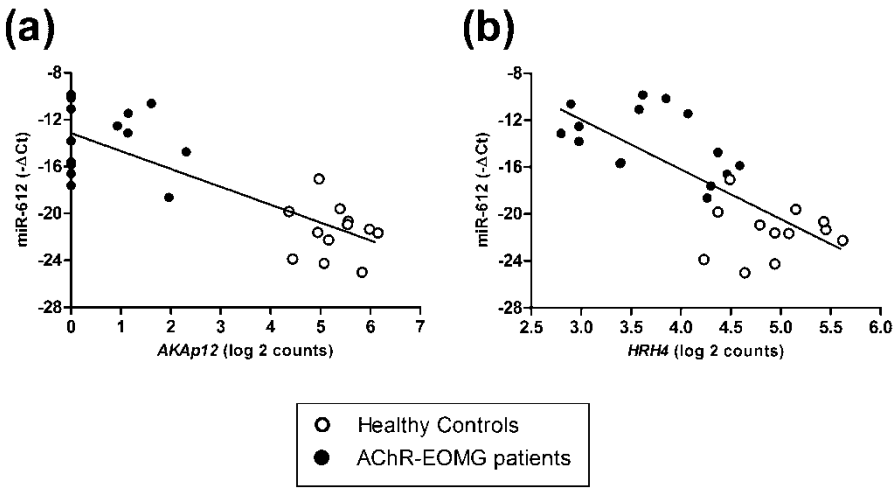

Figure S6. Anti-correlation analysis of the expression levels between miR-612 and its putative target transcripts in PBMCs from 14 AChR-EOMG patients (closed circle) and 12 healthy controls (open circle)

(a) Anti-correlation analysis between the expression levels of miR-612 and *AKAp12* target transcripts (Pearson test, r = -0.81; $P < 0.001$). (b) Anti-correlation analysis between the expression levels of miR-612 and *HRH4* target transcripts (Pearson test, r = -0.75; $P < 0.001$). Results are reported as –ΔCt values for miR-612 expression levels, and as log 2 normalised counts towards the endogenous genes, *OAZ1* and *ACTB,* for *AKAp12* and *HRH4* expression levels.

Table 1. Clinical features of patients with early onset myasthenia gravis and controls and type of analysis performed on their biological samples.

Sample Name	Sex	Age at bleeding (years)	Osserman at onset	Osserman at bleeding	Condition	Therapy at bleeding	RNA-seq	Nano-String	qPCR
MG1	F	43	2A	2B	Pre[a]	Anti-AChE	✓	✓	✓
MG2	F	38	1	2A	Pre	Anti-AChE	✓	✓	✓
MG3	M	41	1	2B	Pre[a]	Anti-AChE	✓	✓	✓
MG4	M	46	2A	2B	Pre	Anti-AChE	✓	✓	✓
MG5	F	28	2A	2A	Pre	Anti-AChE	✓	✓	✓
MG6	F	28	2A	PR	Pre	Anti-AChE	✓	-	-
MG7	F	33	2B	2A	Post[b]	Anti-AChE	✓	✓	✓
MG8	F	28	1	PR	Post[b]	Anti-AChE	✓	✓	✓
MG9	F	35	1	R	Post[b]	None	✓	✓	✓
MG10	F	56	2B	2B	Post[b]	None	✓	✓	✓
MG11	F	32	1	2B	Post[c]	Anti-AChE	✓	-	-
MG12	F	36	2B	2B	Pre	None	-	✓	✓
MG13	F	34	2A	PR	Pre	Anti-AChE	-	✓	✓
MG14	M	66	2B	PR	Pre	AZA	-	✓	✓
MG15	F	32	2B	PR	Pre	AZA+Anti-AChE	-	✓	✓
MG16	M	65	2B	2B	Pre	AZA	-	✓	✓
MG17	F	53	3	2A	Pre	AZA+Anti-AChE	-	✓	✓
MG18	M	29	2A	PR	Post[d]	AZA+Anti-AChE	-	✓	✓
MG19	F	60	2B	PR	Post[c]	AZA	-	✓	✓
C1	M	37	n.a.	n.a.	n.a.	n.a.	✓	✓	✓
C2	F	26	n.a.	n.a.	n.a.	n.a.	✓	✓	✓
C3	F	33	n.a.	n.a.	n.a.	n.a.	✓	✓	✓
C4	M	48	n.a.	n.a.	n.a.	n.a.	✓	✓	✓
C5	F	44	n.a.	n.a.	n.a.	n.a.	✓	✓	✓
C6	F	29	n.a.	n.a.	n.a.	n.a.	✓	✓	✓
C7	F	43	n.a.	n.a.	n.a.	n.a.	-	✓	✓
C8	M	55	n.a.	n.a.	n.a.	n.a.	-	✓	✓
C9	F	27	n.a.	n.a.	n.a.	n.a.	-	✓	✓
C10	F	32	n.a.	n.a.	n.a.	n.a.	-	✓	✓
C11	F	53	n.a.	n.a.	n.a.	n.a.	-	✓	✓
C12	M	34	n.a.	n.a.	n.a.	n.a.	-	✓	✓

MG = myasthenia gravis; C = healthy controls; F = female; M = male; Pre = bleeding pre-thymectomy; Post = bleeding post-thymectomy; Anti-AChE = acetylcholinesterase inhibitor; AZA= azathioprine; n.a. = not available; PR = Pharmacological remission; R = Remission.

[a]Pre-thymectomy patients who had thymoma at the time of blood collection, diagnosed by computed tomography.

[b]Post-thymectomy patients who had thymic hyperplasia.

[c]Post-thymectomy patients who had normally involuted thymus.

[d]Post-thymectomy patients who had thymoma.

Supplementary Table S1. List of miRNA primer sets and their corresponding accession numbers in miRBase (version 20).

miRNA	Type	Design ID	Accession Number
hsa-miR-612	Pre-designed	None	MIMAT0003280
hsa-miR-3654	Custom	252871-1	MIMAT0018074
hsa-miR-1299	Custom	252875-1	MIMAT0005887
hsa-miR-3651	Custom	252867-1	MIMAT0018071
pre-hsa-miR-612	Custom	301783	MI0003625
pre-hsa-miR-3654	Custom	301781	MI0016054
pre-hsa-miR-1299	Custom	301782	MI0006359
pre-hsa-miR-3651	Custom	301780	MI0016051

Supplementary Table S2. List of the differentially expressed genes in PBMCs from AChR-EOMG patients (pre- and post-thymectomy) compared to healthy controls (ctrl) annotated by Ingenuity Pathway Analysis with their corresponding gene identification numbers in Entrez database along with their main biological features and level of expression reported as log2.

Entrez Gene ID	Symbol	Entrez Gene Name	Location	Type(s)	log2(pre/ctrl)	log2(post/ctrl)	log2(pre/ctrl)-log2(post/ctrl)
8284	KDM5D	lysine (K)-specific demethylase 5D	Nucleus	other	-0.094	-3.834	3.740
8653	DDX3Y	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, Y-linked	Cytoplasm	enzyme	-0.405	-4.141	3.736
8287	USP9Y	ubiquitin specific peptidase 9, Y-linked	Cytoplasm	peptidase	-0.158	-3.724	3.566
9086	EIF1AY	eukaryotic translation initiation factor 1A, Y-linked	Cytoplasm	translation regulator	-0.155	-3.006	2.851
150197	LOC150197	uncharacterized LOC150197	unknown	other	-0.736	-2.266	1.530
391322	LOC391322	D-dopachrome tautomerase-like	unknown	other	-1.176	-2.649	1.473
3849	KRT2	keratin 2	Cytoplasm	other	-1.794	-3.041	1.247
728358	DEFA1 (includes others)	defensin, alpha 1	Cytoplasm	other	-5.120	-6.158	1.038
1178	CLC	Charcot-Leyden crystal protein	Cytoplasm	enzyme	-6.624	-7.377	0.753
6286	S100P	S100 calcium binding protein P	Cytoplasm	other	-1.194	-1.877	0.683
401024	FSIP2	fibrous sheath interacting protein 2	Cytoplasm	other	-1.270	-1.944	0.674
3067	HDC	histidine decarboxylase	Cytoplasm	enzyme	-4.948	-5.586	0.638
221981	THSD7A	thrombospondin type I domain containing 7A	unknown	other	-3.053	-3.575	0.522
554202	MIR31HG	MIR31 host gene (non-protein coding)	unknown	other	-2.212	-2.730	0.518
168537	GIMAP7	GTPase IMAP family member 7	unknown	other	-1.256	-1.740	0.484
51330	TNFRSF12A	tumor necrosis factor receptor superfamily member 12A	Plasma Membrane	transmembrane receptor	3.346	2.867	0.479
3301	DNAJA1	DnaJ (Hsp40) homolog subfamily A member 1	Nucleus	other	3.177	2.712	0.465
6947	TCN1	transcobalamin I (vitamin B12 binding protein R binder family)	Cytoplasm	transporter	-5.969	-6.410	0.441
1359	CPA3	carboxypeptidase A3 (mast cell)	Extracellular Space	peptidase	-5.312	-5.748	0.436
221061	FAM171A1	family with sequence similarity 171 member A1	unknown	other	-1.076	-1.500	0.424
59340	HRH4	histamine receptor H4	Plasma Membrane	G-protein coupled receptor	-3.354	-3.748	0.394
4665	NAB2	NGFI-A binding protein 2 (EGR1 binding protein 2)	Nucleus	transcription regulator	1.766	1.423	0.343
677772	SCARNA6	small Cajal body-specific RNA 6	unknown	other	-1.039	-1.352	0.313

5357	PLS1	plastin 1	Cytoplasm	other	-1.131	-1.436	0.305
10663	CXCR6	chemokine (C-X-C motif) receptor 6	Plasma Membrane	G-protein coupled receptor	-0.928	-1.229	0.301
51761	ATP8A2	ATPase aminophospholipid transporter class I type 8A member 2	Plasma Membrane	transporter	-1.577	-1.867	0.290
7846	TUBA1A	tubulin alpha 1a	Cytoplasm	other	0.919	0.636	0.283
2624	GATA2	GATA binding protein 2	Nucleus	transcription regulator	-2.556	-2.812	0.256
57002	YAE1D1	Yae1 domain containing 1	unknown	other	-1.197	-1.451	0.254
2334	AFF2	AF4/FMR2 family member 2	Nucleus	other	-0.785	-1.030	0.245
55303	GIMAP4	GTPase IMAP family member 4	Nucleus	other	-1.456	-1.696	0.240
3725	JUN	jun proto-oncogene	Nucleus	transcription regulator	2.953	2.713	0.240
2206	MS4A2	membrane-spanning 4-domains subfamily A member 2	Plasma Membrane	transmembrane receptor	-4.649	-4.865	0.216
677767	SCARNA7	small Cajal body-specific RNA 7	unknown	other	-1.163	-1.352	0.189
3397	ID1	inhibitor of DNA binding 1 dominant negative helix-loop-helix protein	Nucleus	transcription regulator	3.351	3.169	0.182
10321	CRISP3	cysteine-rich secretory protein 3	Extracellular Space	other	-5.971	-6.107	0.136
643246	MAP1LC3B2	microtubule-associated protein 1 light chain 3 beta 2	unknown	other	1.636	1.505	0.131
81532	MOB2	MOB kinase activator 2	Nucleus	other	2.859	2.774	0.085
339804	C2orf74	chromosome 2 open reading frame 74	unknown	other	-1.223	-1.303	0.080
677775	SCARNA5	small Cajal body-specific RNA 5	unknown	other	-1.287	-1.353	0.066
1316	KLF6	Kruppel-like factor 6	Nucleus	transcription regulator	1.277	1.218	0.059
415116	PIM3	pim-3 oncogene	unknown	kinase	1.633	1.575	0.058
6164	RPL34	ribosomal protein L34	Cytoplasm	other	-0.873	-0.931	0.058
10209	EIF1	eukaryotic translation initiation factor 1	unknown	translation regulator	1.147	1.114	0.033
9479	MAPK8IP1	mitogen-activated protein kinase 8 interacting protein 1	Cytoplasm	other	1.418	1.387	0.031
51678	MPP6	membrane protein. palmitoylated 6 (MAGUK p55 subfamily member 6)	Plasma Membrane	kinase	-0.995	-1.014	0.019
55888	ZKSCAN7	zinc finger with KRAB and SCAN domains 7	Nucleus	transcription regulator	-1.241	-1.248	0.007
642446	TRIM64/TRIM64B	tripartite motif containing 64	unknown	other	-2.282	-2.282	0.000
6790	AURKA	aurora kinase A	Nucleus	kinase	-1.131	-1.123	-0.008

401466	C8orf59	chromosome 8 open reading frame 59	unknown	other	-1.014	-0.980	-0.034
23760	PITPNB	phosphatidylinositol transfer protein beta	Cytoplasm	transporter	1.750	1.803	-0.053
64651	CSRNP1	cysteine-serine-rich nuclear protein 1	Nucleus	transcription regulator	1.907	1.971	-0.064
283417	DPY19L2	dpy-19-like 2 (C. elegans)	unknown	other	-1.624	-1.559	-0.065
9590	AKAP12	A kinase (PRKA) anchor protein 12	Cytoplasm	transporter	-3.382	-3.311	-0.071
7280	TUBB2A	tubulin beta 2A class IIa	Cytoplasm	other	3.155	3.230	-0.075
4335	MNT	MNT MAX dimerization protein	Nucleus	transcription regulator	1.204	1.287	-0.083
160760	PPTC7	PTC7 protein phosphatase homolog (S. cerevisiae)	Cytoplasm	phosphatase	0.969	1.052	-0.083
10240	MRPS31	mitochondrial ribosomal protein S31	Cytoplasm	other	-1.097	-1.008	-0.089
152100	CMC1	COX assembly mitochondrial protein 1 homolog (S. cerevisiae)	Cytoplasm	other	-1.051	-0.958	-0.093
58484	NLRC4	NLR family CARD domain containing 4	Cytoplasm	other	-1.417	-1.322	-0.095
6385	SDC4	syndecan 4	Plasma Membrane	other	1.310	1.411	-0.101
767	CA8	carbonic anhydrase VIII	Cytoplasm	enzyme	-2.379	-2.273	-0.106
4097	MAFG	v-maf musculoaponeurotic fibrosarcoma oncogene homolog G (avian)	Nucleus	transcription regulator	1.190	1.300	-0.110
79896	THNSL1	threonine synthase-like 1 (S. cerevisiae)	unknown	kinase	-1.288	-1.178	-0.110
55017	C14orf119	chromosome 14 open reading frame 119	unknown	other	-1.151	-1.034	-0.117
9518	GDF15	growth differentiation factor 15	Extracellular Space	growth factor	3.075	3.194	-0.119
84957	RELT	RELT tumor necrosis factor receptor	Plasma Membrane	transmembrane receptor	1.147	1.268	-0.121
84275	SLC25A33	solute carrier family 25 (pyrimidine nucleotide carrier) member 33	Cytoplasm	other	1.314	1.437	-0.123
5430	POLR2A	polymerase (RNA) II (DNA directed) polypeptide A 220kDa	Nucleus	enzyme	1.163	1.293	-0.130
112817	HOGA1	4-hydroxy-2-oxoglutarate aldolase 1	Cytoplasm	enzyme	1.013	1.150	-0.137
221143	N6AMT2	N-6 adenine-specific DNA methyltransferase 2 (putative)	unknown	enzyme	-1.380	-1.242	-0.138
339318	ZNF181	zinc finger protein 181	unknown	other	-1.116	-0.978	-0.138
23645	PPP1R15A	protein phosphatase 1 regulatory subunit 15A	Cytoplasm	other	1.876	2.020	-0.144
2521	FUS	fused in sarcoma	Nucleus	transcription regulator	0.958	1.114	-0.156

51307	FAM53C	family with sequence similarity 53 member C	unknown	other	0.974	1.134	-0.160
90525	SHF	Src homology 2 domain containing F	unknown	other	2.527	2.691	-0.164
29950	SERTAD1	SERTA domain containing 1	Nucleus	transcription regulator	1.947	2.115	-0.168
200558	APLF	aprataxin and PNKP like factor	Cytoplasm	enzyme	-1.093	-0.923	-0.170
50807	ASAP1	ArfGAP with SH3 domain ankyrin repeat and PH domain 1	Plasma Membrane	other	1.678	1.854	-0.176
51296	SLC15A3	solute carrier family 15 member 3	Cytoplasm	transporter	0.945	1.135	-0.190
2354	FOSB	FBJ murine osteosarcoma viral oncogene homolog B	Nucleus	transcription regulator	2.195	2.388	-0.193
80321	CEP70	centrosomal protein 70kDa	Cytoplasm	other	-1.559	-1.356	-0.203
2107	ETF1	eukaryotic translation termination factor 1	Cytoplasm	translation regulator	0.755	0.961	-0.206
157983	C9orf66	chromosome 9 open reading frame 66	unknown	other	1.224	1.431	-0.207
10383	TUBB4B	tubulin beta 4B class IVb	Cytoplasm	other	1.459	1.680	-0.221
3565	IL4	interleukin 4	Extracellular Space	cytokine	-3.571	-3.349	-0.222
83694	RPS6KL1	ribosomal protein S6 kinase-like 1	unknown	kinase	1.161	1.386	-0.225
1263	PLK3	polo-like kinase 3	Nucleus	kinase	1.417	1.644	-0.227
80726	KIAA1683	KIAA1683	Cytoplasm	other	1.682	1.911	-0.229
3475	IFRD1	interferon-related developmental regulator 1	Nucleus	other	0.815	1.052	-0.237
51278	IER5	immediate early response 5	unknown	other	1.606	1.858	-0.252
5153	PDE1B	phosphodiesterase 1B calmodulin-dependent	Cytoplasm	enzyme	0.974	1.257	-0.283
5971	RELB	v-rel reticuloendotheliosis viral oncogene homolog B	Nucleus	transcription regulator	0.811	1.121	-0.310
78990	OTUB2	OTU domain ubiquitin aldehyde binding 2	unknown	enzyme	1.067	1.384	-0.317
4791	NFKB2	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	Nucleus	transcription regulator	0.923	1.242	-0.319
4317	MMP8	matrix metallopeptidase 8 (neutrophil collagenase)	Extracellular Space	peptidase	-5.784	-5.456	-0.328
1119	CHKA	choline kinase alpha	Cytoplasm	kinase	0.787	1.138	-0.351
79369	B3GNT4	UDP-GlcNAc:betaGal beta-1.3-N-acetylglucosaminyltransferase 4	Plasma Membrane	enzyme	1.273	1.629	-0.356
90525	SHF	Src homology 2 domain containing F	unknown	other	1.353	1.718	-0.365

100303749	LOC100303749	LSM3 homolog U6 small nuclear RNA associated (S. cerevisiae) pseudogene	unknown	other	1.085	1.454	-0.369
22822	PHLDA1	pleckstrin homology-like domain family A member 1	Cytoplasm	other	0.732	1.117	-0.385
54507	ADAMTSL4	ADAMTS-like 4	Extracellular Space	other	0.808	1.200	-0.392
10089	KCNK7	potassium channel subfamily K member 7	Plasma Membrane	ion channel	2.299	2.704	-0.405
4794	NFKBIE	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor epsilon	Nucleus	transcription regulator	1.454	1.863	-0.409
7288	TULP2	tubby like protein 2	unknown	enzyme	2.854	3.271	-0.417
8835	SOCS2	suppressor of cytokine signaling 2	Cytoplasm	other	-1.224	-0.784	-0.440
22898	DENND3	DENN/MADD domain containing 3	unknown	other	0.604	1.063	-0.459
140687	GCNT7	glucosaminyl (N-acetyl) transferase family member 7	unknown	other	1.387	1.878	-0.491
441951	ZFAS1	ZNFX1 antisense RNA 1	unknown	other	1.006	1.516	-0.510
27338	UBE2S	ubiquitin-conjugating enzyme E2S	Nucleus	enzyme	1.469	1.986	-0.517
149478	BTBD19	BTB (POZ) domain containing 19	unknown	other	2.158	2.682	-0.524
3680	ITGA9	integrin alpha 9	Plasma Membrane	other	-1.702	-1.170	-0.532
54972	TMEM132A	transmembrane protein 132A	Cytoplasm	other	1.212	1.774	-0.562
932	MS4A3	membrane-spanning 4-domains subfamily A member 3 (hematopoietic cell-specific)	Plasma Membrane	other	-5.750	-5.151	-0.599
284723	SLC25A34	solute carrier family 25 member 34	Cytoplasm	other	1.048	1.652	-0.604
9283	GPR37L1	G protein-coupled receptor 37 like 1	Plasma Membrane	G-protein coupled receptor	2.249	2.854	-0.605
57662	CAMSAP3	calmodulin regulated spectrin-associated protein family member 3	Cytoplasm	other	1.424	2.035	-0.611
8061	FOSL1	FOS-like antigen 1	Nucleus	transcription regulator	3.324	3.952	-0.628
55350	VNN3	vanin 3	Extracellular Space	enzyme	-2.291	-1.621	-0.670
147906	DACT3	dapper antagonist of beta-catenin homolog 3 (Xenopus laevis)	unknown	other	2.222	2.893	-0.671
5798	PTPRN	protein tyrosine phosphatase receptor type N	Plasma Membrane	phosphatase	1.748	2.429	-0.681
100500826	MIR3909	microRNA 3909	Cytoplasm	microRNA	2.231	3.080	-0.849
11107	PRDM5	PR domain containing 5	Nucleus	other	-2.636	-1.776	-0.860
728084	LOC728084	uncharacterized LOC728084	unknown	other	-3.854	-2.978	-0.876
1119	CHKA	choline kinase alpha	Cytoplasm	kinase	1.257	2.253	-0.996

285758	LOC285758	uncharacterized LOC285758	unknown	other	1.685	2.815	-1.130
19	ABCA1	ATP-binding cassette sub-family A (ABC1) member 1	Plasma Membrane	transporter	1.204	2.565	-1.361
151	ADRA2B	adrenoceptor alpha 2B	Plasma Membrane	G-protein coupled receptor	1.911	3.520	-1.609

Supplementary Table S3. List of long non-coding RNAs expressed as normalized counts for library size and quantile normalization between samples in PBMCs from AChR-EOMG patients (pre- and post-thymectomy) and healthy controls (ctrl).

	Mean pre-	Mean post-	Mean Ctrl	pre	pre	pre	pre	pre	pre	post	post	post	post	post	ctrl	ctrl	ctrl	ctrl	ctrl	ctrl
ENSG00000245466|71075515|14|71108015|lincRNA	5.75	5.75	5.64	5.75	5.75	5.75	5.75	5.75	5.75	5.75	5.75	5.75	5.75	5.75	5.75	5.41	5.75	5.46	5.75	5.75
ENSG00000188825|41447213|17|41466567|lincRNA	5.02	5.00	5.50	5.03	5.03	5.03	4.88	5.09	5.03	5.09	4.91	4.97	5.18	4.95	5.28	5.75	5.41	5.75	5.57	5.28
ENSG00000260682|81993524|16|81996298|lincRNA	5.46	5.44	5.38	5.57	5.46	5.41	5.41	5.46	5.46	5.46	5.46	5.46	5.57	5.28	5.46	5.09	5.57	5.09	5.46	5.57
ENSG00000253143|71383347|8|71392858|lincRNA	5.35	5.37	5.17	5.34	5.34	5.34	5.34	5.34	5.41	5.34	5.34	5.34	5.34	5.46	5.41	4.95	5.34	4.95	4.95	5.46
ENSG00000251562|65265233|11|65273940|lincRNA	5.41	5.41	5.16	5.41	5.41	5.46	5.46	5.41	5.34	5.41	5.41	5.41	5.41	5.41	5.34	4.97	4.95	4.97	5.34	5.41
ENSG00000269900|35657748|9|35658015|lincRNA	4.97	4.71	4.90	5.09	5.09	4.61	4.82	5.03	5.18	4.97	4.82	4.67	4.97	4.37	4.82	4.88	4.88	4.82	4.82	5.18
ENSG00000259380|38431796|15|38519088|lincRNA	4.38	4.41	4.49	4.43	4.41	4.35	4.33	4.35	4.39	4.37	4.43	4.37	4.41	4.43	4.67	4.45	4.45	4.45	4.47	4.45
ENSG00000250312|124386|4|157779|lincRNA	4.39	4.33	4.45	4.41	4.45	4.27	4.45	4.45	4.29	4.41	4.37	4.30	4.37	4.29	4.47	4.43	4.47	4.56	4.41	4.37
ENSG00000228463|227615|1|267253|lincRNA	4.16	4.13	4.30	4.22	4.18	4.10	4.20	4.18	4.06	4.18	4.13	4.11	4.17	4.11	4.30	4.27	4.33	4.39	4.27	4.23
ENSG00000259198|40604816|15|40608835|lincRNA	4.04	3.99	4.16	4.16	4.08	3.91	4.08	4.12	3.86	4.08	3.98	4.01	4.04	3.91	4.16	4.13	4.22	4.21	4.16	4.11
ENSG00000263753|5232875|18|5246507|lincRNA	4.04	4.00	4.12	4.06	4.06	4.01	4.06	4.08	3.99	4.06	4.01	3.98	4.01	4.01	4.13	4.07	4.16	4.18	4.12	4.07
ENSG00000265091|5232875|18|5238525|lincRNA	3.80	3.68	4.04	3.89	3.90	3.68	3.87	3.91	3.54	3.84	3.71	3.69	3.74	3.57	3.97	3.97	4.18	4.20	4.04	3.86
ENSG00000198468|213025450|1|213031430|lincRNA	3.75	3.72	3.92	3.82	3.77	3.69	3.77	3.78	3.66	3.77	3.72	3.72	3.72	3.71	3.87	3.91	3.94	4.01	3.92	3.86
ENSG00000262477|29992145|18|29993199|lincRNA	3.77	3.71	3.91	3.88	3.85	3.69	3.84	3.86	3.51	3.82	3.72	3.72	3.79	3.62	3.93	3.86	3.99	3.94	3.91	3.85
ENSG00000224810|182031812|1|182038388|lincRNA	3.75	3.76	3.90	3.83	3.79	3.72	3.74	3.72	3.71	3.79	3.78	3.75	3.81	3.69	3.96	3.94	3.87	3.89	3.93	3.81
ENSG00000256193|128399917|12|128436097|lincRNA	3.74	3.73	3.88	3.79	3.79	3.71	3.73	3.74	3.71	3.79	3.74	3.74	3.80	3.62	3.89	3.92	3.83	3.88	3.88	3.90
ENSG00000234718|7778165|7|7782204|lincRNA	3.57	3.49	3.86	3.72	3.67	3.36	3.70	3.68	3.30	3.64	3.54	3.46	3.57	3.39	3.87	3.83	3.94	3.97	3.83	3.72
ENSG00000249731|1968208|	3.67	3.67	3.84	3.72	3.70	3.63	3.63	3.64	3.70	3.67	3.68	3.65	3.69	3.66	3.94	3.83	3.78	3.83	3.84	3.79

5|1969127|lincRNA																				
ENSG00000240567|161144215|3|161166030|lincRNA	3.56	3.46	3.81	3.69	3.68	3.37	3.68	3.67	3.26	3.62	3.51	3.44	3.52	3.36	3.79	3.72	3.89	3.95	3.79	3.73
ENSG00000197332|11750591|19|11797382|lincRNA	3.62	3.59	3.80	3.64	3.66	3.59	3.68	3.65	3.51	3.66	3.63	3.63	3.63	3.47	3.79	3.80	3.80	3.91	3.81	3.69
ENSG00000247363|69068151|12|69081535|lincRNA	3.52	3.45	3.79	3.68	3.58	3.35	3.64	3.62	3.25	3.56	3.48	3.46	3.49	3.35	3.78	3.75	3.85	3.93	3.78	3.68
ENSG00000224074|24137760|3|24144729|lincRNA	3.58	3.54	3.79	3.69	3.67	3.44	3.67	3.63	3.38	3.63	3.56	3.51	3.62	3.49	3.85	3.78	3.84	3.84	3.77	3.68
ENSG00000269888|161146900|3|161147401|lincRNA	3.52	3.39	3.79	3.65	3.64	3.36	3.63	3.60	3.27	3.56	3.44	3.37	3.46	3.30	3.80	3.72	3.88	3.93	3.77	3.65
ENSG00000261211|6680542|6|6683866|lincRNA	3.83	3.85	3.77	3.81	3.82	3.84	3.82	3.83	3.87	3.83	3.82	3.84	3.83	3.89	3.76	3.77	3.77	3.76	3.77	3.77
ENSG00000264247|72259010|18|72265744|lincRNA	3.59	3.56	3.71	3.62	3.61	3.57	3.59	3.60	3.53	3.60	3.57	3.57	3.60	3.51	3.77	3.68	3.69	3.73	3.74	3.64
ENSG00000230105|56644824|X|56650891|lincRNA	3.52	3.52	3.67	3.58	3.55	3.49	3.47	3.48	3.54	3.53	3.54	3.51	3.56	3.48	3.76	3.70	3.63	3.66	3.68	3.61
ENSG00000253103|112111190|8|112248432|lincRNA	3.44	3.48	3.67	3.52	3.47	3.41	3.40	3.39	3.47	3.45	3.47	3.46	3.47	3.50	3.80	3.65	3.68	3.69	3.67	3.53
ENSG00000179136|12453099|17|12546171|lincRNA	3.51	3.51	3.65	3.57	3.54	3.50	3.51	3.50	3.43	3.53	3.53	3.52	3.56	3.43	3.71	3.69	3.61	3.65	3.71	3.54
ENSG00000249409|121560245|4|121571951|lincRNA	3.46	3.47	3.63	3.53	3.49	3.43	3.37	3.44	3.49	3.47	3.50	3.49	3.50	3.39	3.72	3.63	3.65	3.54	3.66	3.56
ENSG00000230606|98081083|2|98095220|lincRNA	3.45	3.42	3.61	3.50	3.53	3.37	3.46	3.46	3.39	3.43	3.44	3.39	3.46	3.38	3.68	3.69	3.58	3.61	3.63	3.50
ENSG00000251339|59646791|4|59849870|lincRNA	3.36	3.37	3.57	3.42	3.38	3.32	3.31	3.36	3.36	3.36	3.39	3.35	3.37	3.39	3.72	3.51	3.51	3.59	3.60	3.49
ENSG00000248434|135988388|4|136018813|lincRNA	3.40	3.38	3.54	3.41	3.42	3.40	3.42	3.37	3.38	3.41	3.38	3.39	3.41	3.33	3.57	3.55	3.48	3.56	3.55	3.54
ENSG00000253424|157683145|5|157685444|lincRNA	3.38	3.37	3.52	3.45	3.40	3.35	3.34	3.34	3.41	3.39	3.38	3.37	3.40	3.31	3.62	3.53	3.43	3.51	3.54	3.52
ENSG00000249484|151998525|5|152603103|lincRNA	3.57	3.57	3.48	3.54	3.55	3.57	3.56	3.55	3.62	3.54	3.55	3.56	3.53	3.65	3.47	3.49	3.47	3.50	3.47	3.48
ENSG00000259234|79484049|15|79576287|lincRNA	3.22	3.15	3.45	3.28	3.23	3.17	3.27	3.32	3.04	3.27	3.19	3.22	3.22	2.94	3.46	3.42	3.54	3.55	3.40	3.32
ENSG00000232931|96472866|2|96481963|lincRNA	3.27	3.25	3.44	3.33	3.31	3.25	3.24	3.24	3.22	3.30	3.28	3.22	3.31	3.19	3.55	3.47	3.39	3.43	3.47	3.34
ENSG00000251152|11470874|4|11479820|lincRNA	3.29	3.27	3.44	3.35	3.32	3.27	3.27	3.29	3.23	3.30	3.30	3.27	3.33	3.19	3.50	3.47	3.40	3.46	3.50	3.31
ENSG00000231422|25401987|10|25450165|lincRNA	3.50	3.51	3.42	3.49	3.48	3.50	3.50	3.49	3.54	3.47	3.48	3.50	3.48	3.57	3.41	3.44	3.41	3.41	3.43	3.43

ENSG00000233757|95873283|2|95881351|lincRNA	3.17	3.10	3.41	3.19	3.21	3.07	3.31	3.22	3.00	3.26	3.21	3.11	3.16	2.92	3.44	3.42	3.41	3.54	3.43	3.24
ENSG00000260975|52231924|16|52261868|lncRNA	3.18	3.20	3.38	3.27	3.22	3.15	3.12	3.15	3.21	3.19	3.20	3.20	3.21	3.20	3.49	3.34	3.42	3.38	3.40	3.27
ENSG00000256218|5475214|12|5476940|lincRNA	3.21	3.21	3.36	3.25	3.25	3.18	3.21	3.22	3.17	3.26	3.24	3.22	3.25	3.12	3.41	3.35	3.31	3.38	3.34	3.35
ENSG00000230448|13850656|2|14541087|lincRNA	3.19	3.19	3.34	3.25	3.21	3.16	3.18	3.18	3.14	3.22	3.19	3.21	3.24	3.13	3.43	3.41	3.28	3.34	3.39	3.22
ENSG00000234810|56046710|1|56200675|lincRNA	3.15	3.15	3.34	3.20	3.14	3.11	3.14	3.16	3.16	3.19	3.19	3.15	3.18	3.09	3.38	3.30	3.27	3.41	3.37	3.33
hsa-mir-4426	2.96	2.85	3.34	3.10	3.02	2.73	3.07	3.04	2.80	2.97	2.84	2.80	2.98	2.79	3.37	3.43	3.48	3.42	3.26	3.06
ENSG00000237943|6622381|10|6627641|lincRNA	2.92	2.78	3.30	3.14	3.20	2.65	3.04	2.99	2.52	3.09	2.96	2.54	2.77	2.86	3.34	3.36	3.31	3.29	3.20	3.28
ENSG00000213793|53418449|19|53426723|lncRNA	3.08	3.02	3.27	3.10	3.10	3.01	3.13	3.11	3.03	3.11	3.05	3.01	3.06	2.97	3.25	3.32	3.27	3.41	3.25	3.13
ENSG00000257931|19932012|14|19972292|lncRNA	2.80	2.83	3.24	2.88	2.85	2.71	2.78	2.79	2.80	2.80	2.80	2.73	2.82	2.98	3.47	3.09	3.35	3.42	3.19	2.94
ENSG00000223579|20632023|22|20661407|lncRNA	3.10	3.08	3.24	3.10	3.10	3.08	3.09	3.09	3.12	3.06	3.07	3.09	3.12	3.03	3.29	3.22	3.22	3.25	3.23	3.21
ENSG00000258038|29299450|14|29437606|lncRNA	3.27	3.25	3.18	3.25	3.26	3.27	3.26	3.28	3.32	3.25	3.21	3.25	3.27	3.28	3.09	3.16	3.16	3.16	3.20	3.27
ENSG00000231028|135818489|6|136037193|lincRNA	2.98	2.98	3.16	3.05	3.03	2.99	2.99	2.97	2.85	3.02	3.00	2.95	3.03	2.95	3.23	3.14	3.20	3.19	3.17	3.02
ENSG00000234864|43191483|10|43201901|lncRNA	2.98	3.00	3.15	3.03	3.02	2.97	2.95	2.99	2.95	2.99	3.01	3.01	3.05	2.95	3.25	3.20	3.09	3.16	3.16	3.07
ENSG00000261770|28248092|19|28251757|lncRNA	2.97	2.98	3.15	2.96	2.98	3.01	3.00	2.96	2.92	3.01	2.99	2.96	2.97	3.00	3.26	3.12	3.10	3.21	3.17	3.05
ENSG00000236268|83439565|1|83451891|lncRNA	3.02	2.98	3.15	3.02	3.04	3.03	3.06	3.08	2.86	3.01	2.97	2.99	3.04	2.93	3.12	3.10	3.16	3.30	3.15	3.06
ENSG00000228623|115759495|9|115774507|lincRNA	2.92	2.85	3.15	2.98	2.95	2.82	3.08	2.96	2.75	3.00	2.95	2.85	2.87	2.71	3.20	3.10	3.17	3.24	3.18	3.00
ENSG00000224559|132394598|2|132407188|lincRNA	2.98	3.01	3.14	3.02	3.02	2.97	2.92	2.98	2.95	3.02	3.03	2.97	3.03	3.00	3.25	3.16	3.12	3.15	3.16	2.98
ENSG00000231189|224363491|2|224369329|lincRNA	3.02	3.00	3.14	3.04	3.05	3.01	3.00	3.02	2.98	3.04	2.99	3.00	3.03	2.96	3.18	3.10	3.13	3.17	3.17	3.08
ENSG00000231367|38633861|2|38742882|lincRNA	3.22	3.20	3.13	3.17	3.23	3.21	3.23	3.22	3.23	3.17	3.18	3.20	3.18	3.26	3.17	3.15	3.15	3.14	3.08	3.07
ENSG00000260359|95024040|15|95027181|lncRNA	2.94	2.93	3.10	2.95	2.98	2.92	2.94	2.95	2.89	2.96	2.93	2.91	3.00	2.89	3.13	3.12	3.04	3.13	3.15	3.05
ENSG00000206142|21655279|22|21679331|lncRNA	2.94	2.94	3.10	2.95	2.97	2.94	2.91	2.94	2.95	2.92	2.94	2.95	2.94	2.92	3.19	2.99	3.08	3.19	3.11	3.03

ENSG00000268416|20929729|19|20938056|lncRNA	2.91	2.85	3.07	2.94	2.94	2.82	2.91	2.95	2.88	2.99	2.87	2.81	2.85	2.85	3.13	3.05	3.02	3.08	3.09	3.05
ENSG00000268899|20929729|HSCHR19_1_CTG3|20938056|lincRNA	2.91	2.85	3.07	2.94	2.94	2.82	2.91	2.95	2.88	2.99	2.87	2.81	2.85	2.85	3.13	3.05	3.02	3.08	3.09	3.05
ENSG00000270074|12270336|8|12271882|lincRNA	2.88	2.80	3.02	2.93	2.96	2.74	2.96	2.96	2.72	2.94	2.83	2.80	2.82	2.75	3.05	3.01	3.02	3.02	3.04	3.00
ENSG00000251301|68726668|12|68845443|lincRNA	2.75	2.81	3.00	2.74	2.81	2.81	2.69	2.79	2.69	2.92	2.84	2.75	2.88	2.75	2.99	3.05	3.10	2.99	2.97	2.92
ENSG00000248975|30421603|14|30766249|lincRNA	2.88	2.86	2.99	2.92	2.87	2.85	2.87	2.85	2.93	2.87	2.86	2.87	2.83	2.88	3.07	3.00	2.96	2.96	2.98	2.99
ENSG00000237658|168433452|1|168464882|lincRNA	2.84	2.85	2.97	2.87	2.83	2.83	2.78	2.80	2.90	2.83	2.85	2.84	2.88	2.84	3.05	2.96	2.90	2.97	2.93	2.99
ENSG00000232388|18548064|20|18550207|lincRNA	2.71	2.66	2.97	2.80	2.76	2.66	2.75	2.77	2.49	2.75	2.69	2.72	2.70	2.51	2.82	2.91	3.04	3.17	3.03	2.82
ENSG00000237851|143109260|6|143115223|lincRNA	2.81	2.82	2.95	2.78	2.83	2.87	2.82	2.82	2.74	2.84	2.81	2.81	2.81	2.83	3.03	2.94	2.94	3.02	2.95	2.82
ENSG00000267164|16015366|19|16018044|lincRNA	2.79	2.81	2.94	2.84	2.82	2.80	2.73	2.78	2.77	2.80	2.84	2.84	2.83	2.71	3.06	2.95	2.91	2.91	3.01	2.81
ENSG00000233178|71010609|9|71021416|lincRNA	2.77	2.74	2.93	2.81	2.83	2.77	2.79	2.83	2.57	2.82	2.75	2.71	2.80	2.69	2.99	2.93	2.91	3.03	2.92	2.83
ENSG00000265220|71010609|HG962_PATCH|71021416|lincRNA	2.77	2.74	2.93	2.81	2.83	2.77	2.79	2.83	2.57	2.82	2.75	2.71	2.80	2.69	2.99	2.93	2.91	3.03	2.92	2.83
hsa-mir-3654	2.76	2.67	2.92	2.89	2.80	2.59	2.83	2.88	2.57	2.82	2.65	2.66	2.72	2.63	2.92	2.77	3.06	2.98	2.91	2.87
ENSG00000218510|22351681|1|22357716|lincRNA	2.69	2.47	2.89	2.68	2.55	2.72	2.88	2.70	2.63	2.66	2.62	2.52	2.68	2.07	2.97	2.92	2.84	2.96	2.91	2.74
ENSG00000249867|28724162|11|29085368|lincRNA	2.74	2.74	2.88	2.76	2.77	2.75	2.74	2.73	2.72	2.72	2.76	2.79	2.76	2.66	2.92	2.91	2.83	2.94	2.90	2.78
ENSG00000267104|58039724|17|58096413|lincRNA	2.77	2.69	2.88	2.80	2.75	2.69	2.84	2.83	2.68	2.81	2.73	2.77	2.67	2.60	2.83	2.87	2.93	2.92	2.90	2.82
ENSG00000259104|36605314|14|36645674|lincRNA	2.73	2.72	2.86	2.73	2.72	2.74	2.76	2.73	2.70	2.73	2.74	2.73	2.78	2.65	2.88	2.84	2.77	2.88	2.86	2.91
ENSG00000259758|141530255|8|141539600|lincRNA	2.76	2.69	2.84	2.85	2.77	2.60	2.80	2.81	2.71	2.78	2.64	2.71	2.75	2.66	2.89	2.77	2.87	2.78	2.91	2.84
ENSG00000259347|67278699|15|67351591|lincRNA	2.74	2.67	2.84	2.82	2.75	2.73	2.84	2.78	2.56	2.74	2.70	2.71	2.67	2.60	2.86	2.76	2.88	2.89	2.80	2.84
ENSG00000225868|38314362|19|38345855|lincRNA	2.92	2.92	2.81	2.91	2.90	2.93	2.87	2.94	2.97	2.88	2.88	2.93	2.90	2.98	2.80	2.88	2.75	2.76	2.84	2.83
ENSG00000259929|18027046|16|18245452|lincRNA	2.67	2.64	2.81	2.70	2.69	2.61	2.69	2.72	2.61	2.69	2.67	2.63	2.64	2.61	2.85	2.78	2.78	2.95	2.79	2.70
ENSG00000249932|12470426	2.60	2.66	2.80	2.64	2.67	2.66	2.59	2.59	2.46	2.65	2.66	2.68	2.69	2.60	2.85	2.82	2.74	2.88	2.88	2.62

1|5|124704842|lincRNA																				
ENSG00000261804|53407405|16|53418657|lincRNA	2.66	2.69	2.78	2.73	2.68	2.67	2.63	2.63	2.61	2.72	2.71	2.71	2.71	2.63	2.76	2.84	2.76	2.82	2.82	2.68
ENSG00000268544|149575345|1|149575767|lincRNA	2.44	2.30	2.73	2.61	2.50	2.22	2.60	2.47	2.27	2.51	2.45	1.96	2.32	2.45	2.85	2.75	2.81	2.75	2.59	2.64
hsa-mir-1282	2.56	2.56	2.72	2.62	2.54	2.60	2.50	2.64	2.44	2.52	2.57	2.64	2.58	2.47	2.67	2.71	2.83	2.72	2.62	2.75
ENSG00000253288|138821687|8|139095813|lincRNA	2.59	2.56	2.71	2.62	2.63	2.54	2.57	2.58	2.60	2.56	2.55	2.55	2.57	2.59	2.76	2.68	2.64	2.75	2.73	2.70
ENSG00000225725|7812536|8|7866277|lincRNA	2.56	2.54	2.69	2.58	2.62	2.52	2.56	2.52	2.58	2.62	2.53	2.53	2.54	2.54	2.73	2.70	2.66	2.61	2.71	2.72
ENSG00000228422|11781111|20|11851363|lincRNA	2.83	2.81	2.68	2.79	2.79	2.82	2.82	2.82	2.92	2.79	2.80	2.77	2.79	2.87	2.72	2.68	2.69	2.59	2.69	2.69
ENSG00000228294|19670480|14|19686002|lincRNA	2.56	2.55	2.67	2.61	2.53	2.49	2.60	2.57	2.53	2.57	2.61	2.61	2.59	2.39	2.71	2.64	2.68	2.67	2.74	2.59
hsa-mir-6516	1.92	1.82	2.67	1.97	1.96	1.83	1.94	1.92	1.88	1.93	1.74	1.83	1.85	1.85	2.26	3.02	2.92	3.06	2.70	2.05
ENSG00000259820|135804263|8|135810515|lincRNA	2.52	2.54	2.66	2.48	2.56	2.51	2.51	2.52	2.53	2.50	2.54	2.55	2.57	2.50	2.69	2.67	2.65	2.68	2.66	2.63
ENSG00000215394|19889386|14|19904890|lincRNA	2.55	2.54	2.66	2.60	2.53	2.47	2.59	2.56	2.52	2.56	2.60	2.61	2.59	2.36	2.70	2.63	2.66	2.65	2.71	2.59
ENSG00000206062|90562780|X|90563877|lincRNA	2.50	2.52	2.65	2.53	2.52	2.52	2.53	2.51	2.41	2.52	2.48	2.48	2.53	2.57	2.73	2.63	2.62	2.66	2.68	2.56
ENSG00000238217|200472791|2|200523855|lincRNA	2.52	2.53	2.65	2.52	2.54	2.54	2.57	2.47	2.49	2.56	2.57	2.53	2.58	2.46	2.61	2.72	2.61	2.67	2.64	2.62
ENSG00000266985|34046474|19|34048376|lincRNA	2.52	2.50	2.64	2.55	2.54	2.54	2.52	2.52	2.48	2.51	2.52	2.54	2.51	2.42	2.66	2.64	2.58	2.67	2.67	2.63
ENSG00000256039|10705962|12|10710648|lincRNA	2.09	2.11	2.64	2.38	2.00	1.78	2.35	2.48	1.52	2.22	2.12	1.79	2.20	2.34	2.83	2.68	2.73	2.70	2.18	2.72
ENSG00000259093|63589751|14|63594932|lincRNA	2.44	2.34	2.64	2.42	2.39	2.32	2.64	2.54	2.33	2.50	2.42	2.35	2.45	2.15	2.63	2.59	2.73	2.71	2.58	2.58
ENSG00000227456|35552978|21|35562220|lincRNA	2.49	2.50	2.61	2.54	2.53	2.50	2.49	2.43	2.48	2.52	2.49	2.52	2.52	2.47	2.68	2.57	2.53	2.64	2.62	2.61
ENSG00000261742|65318402|16|65610203|lincRNA	2.70	2.76	2.60	2.68	2.68	2.70	2.65	2.70	2.77	2.75	2.74	2.74	2.73	2.82	2.58	2.60	2.59	2.61	2.58	2.62
ENSG00000189295|17134956|22|17175538|lincRNA	2.45	2.37	2.60	2.46	2.48	2.42	2.50	2.52	2.35	2.37	2.37	2.37	2.44	2.30	2.55	2.57	2.69	2.71	2.61	2.45
ENSG00000270171|7753184|1|7754904|lincRNA	2.49	2.50	2.60	2.52	2.50	2.50	2.51	2.46	2.46	2.55	2.53	2.47	2.51	2.51	2.67	2.54	2.58	2.56	2.58	2.64
ENSG00000254606|28538379|11|28540888|lincRNA	2.81	2.85	2.59	2.74	2.80	2.88	2.78	2.80	2.88	2.79	2.86	2.90	2.81	2.82	2.53	2.47	2.61	2.56	2.65	2.74
ENSG00000260322|80580628|1|80582603|lincRNA	2.42	2.41	2.57	2.47	2.42	2.44	2.46	2.37	2.37	2.43	2.41	2.41	2.45	2.38	2.65	2.57	2.54	2.63	2.50	2.55

ENSG00000253557|18942502|8|19116979|lincRNA	2.72	2.70	2.57	2.68	2.66	2.76	2.72	2.68	2.80	2.66	2.68	2.68	2.67	2.77	2.58	2.61	2.56	2.55	2.50	2.63
ENSG00000244342|62936105|3|63110738|lincRNA	2.44	2.41	2.57	2.48	2.47	2.37	2.33	2.57	2.39	2.52	2.32	2.37	2.46	2.48	2.59	2.63	2.57	2.52	2.54	2.55
ENSG00000234773|12305830|19|12348582|lincRNA	2.41	2.30	2.57	2.32	2.44	2.30	2.56	2.44	2.40	2.43	2.40	2.36	2.35	2.10	2.39	2.57	2.62	2.73	2.53	2.57
ENSG00000248408|80584915|4|80617991|lincRNA	2.47	2.47	2.57	2.43	2.49	2.52	2.50	2.50	2.40	2.57	2.44	2.47	2.51	2.48	2.63	2.47	2.61	2.61	2.63	2.45
ENSG00000259976|114033348|3|114035026|lincRNA	2.42	2.39	2.56	2.44	2.45	2.41	2.42	2.42	2.41	2.37	2.46	2.38	2.43	2.28	2.60	2.69	2.52	2.56	2.53	2.47
ENSG00000255565|15933002|12|15935054|lincRNA	2.44	2.39	2.55	2.52	2.51	2.43	2.47	2.47	2.24	2.50	2.43	2.31	2.49	2.34	2.60	2.49	2.56	2.56	2.56	2.56
ENSG00000267259|26559616|17|26562307|lincRNA	2.37	2.31	2.53	2.44	2.38	2.28	2.34	2.44	2.35	2.43	2.33	2.26	2.34	2.28	2.58	2.53	2.43	2.59	2.52	2.51
ENSG00000227066|20480831|1|20487061|lincRNA	2.40	2.31	2.52	2.48	2.42	2.30	2.30	2.46	2.42	2.51	2.35	2.31	2.35	2.22	2.53	2.61	2.42	2.46	2.57	2.51
ENSG00000260834|65224876|16|65268817|lincRNA	2.36	2.41	2.52	2.38	2.35	2.34	2.36	2.32	2.42	2.31	2.37	2.37	2.40	2.50	2.64	2.42	2.50	2.59	2.48	2.47
ENSG00000253880|5901875|8|6115058|lincRNA	2.42	2.40	2.52	2.40	2.45	2.44	2.46	2.34	2.43	2.36	2.35	2.33	2.41	2.49	2.59	2.53	2.55	2.50	2.45	2.47
ENSG00000260804|217081768|2|217084915|lincRNA	2.30	2.18	2.50	2.40	2.32	2.17	2.38	2.35	2.18	2.41	2.19	2.19	2.15	2.20	2.56	2.45	2.44	2.52	2.52	2.48
ENSG00000259129|48234157|14|48264295|lincRNA	2.36	2.37	2.49	2.42	2.38	2.37	2.22	2.37	2.41	2.35	2.37	2.41	2.37	2.33	2.56	2.54	2.39	2.52	2.47	2.49
ENSG00000268471|153457416|4|153460415|lincRNA	2.17	2.00	2.48	2.29	2.36	1.90	2.28	2.36	1.85	2.34	1.87	1.94	1.98	2.21	2.59	2.49	2.60	2.40	2.52	2.25
ENSG00000228536|219583023|1|219585283|lincRNA	2.36	2.35	2.47	2.39	2.35	2.41	2.39	2.39	2.24	2.35	2.34	2.35	2.38	2.34	2.51	2.47	2.54	2.49	2.41	2.43
ENSG00000253317|73108569|8|73114504|lincRNA	2.32	2.37	2.47	2.37	2.39	2.31	2.25	2.27	2.35	2.36	2.36	2.33	2.38	2.39	2.52	2.50	2.39	2.53	2.38	2.49
ENSG00000240241|78316090|3|78343881|lincRNA	2.39	2.28	2.45	2.32	2.40	2.34	2.42	2.51	2.34	2.41	2.17	2.29	2.39	2.30	2.43	2.48	2.53	2.41	2.40	2.46
ENSG00000261480|28964523|15|28983577|lincRNA	2.30	2.17	2.44	2.34	2.48	2.13	2.42	2.31	2.12	2.44	2.28	2.01	2.19	2.19	2.48	2.40	2.52	2.46	2.43	2.36
ENSG00000249174|18965970|5|19142455|lincRNA	2.35	2.29	2.44	2.27	2.34	2.43	2.43	2.29	2.32	2.40	2.30	2.25	2.36	2.25	2.48	2.51	2.46	2.44	2.35	2.41
ENSG00000251185|76194278|4|76286392|lincRNA	2.25	2.31	2.39	2.33	2.24	2.22	2.16	2.23	2.33	2.19	2.29	2.27	2.30	2.37	2.51	2.31	2.37	2.46	2.34	2.35
ENSG00000255433|56615954|11|56645554|lincRNA	2.30	2.22	2.36	2.37	2.20	2.33	2.40	2.28	2.26	2.36	2.22	2.15	2.30	2.20	2.46	2.35	2.35	2.23	2.40	2.37
ENSG00000263059|56627028	2.30	2.22	2.36	2.37	2.20	2.33	2.40	2.28	2.26	2.36	2.22	2.15	2.30	2.20	2.46	2.35	2.35	2.23	2.40	2.37

|HG151_NOVEL_TEST|56656628|lincRNA																				
ENSG00000225870|11174818 5|13|11175544 4|lincRNA	2.24	2.26	2.36	2.24	2.22	2.24	2.21	2.23	2.28	2.24	2.26	2.25	2.25	2.27	2.42	2.26	2.28	2.48	2.38	2.33
ENSG00000251292|23724885 |4|23735202|lincRNA	2.18	2.21	2.36	2.13	2.21	2.19	2.17	2.24	2.17	2.12	2.21	2.20	2.17	2.24	2.40	2.23	2.36	2.54	2.39	2.22
ENSG00000262491|69062740 |HSCHR5_1_CTG1|69064595 |lincRNA	2.20	1.97	2.35	2.23	2.19	2.09	2.27	2.33	2.12	2.27	2.12	1.84	2.08	1.82	2.32	2.44	2.46	2.38	2.37	2.13
ENSG00000224914|89102493 |10|89117639|lincRNA	2.28	2.21	2.35	2.31	2.22	2.24	2.27	2.31	2.34	2.25	2.25	2.27	2.18	2.16	2.38	2.40	2.34	2.41	2.18	2.38
hsa-mir-3651	1.99	1.76	2.34	2.10	2.10	1.98	1.75	2.07	1.95	2.04	1.73	1.78	1.83	1.71	2.03	2.51	2.58	2.60	2.27	2.03
ENSG00000251048|18489685 |4|18491131|lincRNA	2.22	2.20	2.32	2.29	2.24	2.13	2.11	2.23	2.32	2.20	2.26	2.16	2.21	2.18	2.39	2.32	2.23	2.30	2.28	2.39
ENSG00000261364|10889528 5|3|108896212| lincRNA	2.22	2.23	2.32	2.21	2.14	2.35	2.19	2.26	2.18	2.22	2.23	2.22	2.26	2.21	2.42	2.24	2.37	2.32	2.24	2.32
ENSG00000197462|12555792 5|7|125573886| lincRNA	2.14	2.14	2.31	2.02	2.15	2.32	2.11	2.21	2.07	2.14	2.10	2.17	2.11	2.21	2.41	2.17	2.31	2.49	2.29	2.21
ENSG00000237954|95820945 |1|95846556|lincRNA	2.20	2.18	2.31	2.29	2.18	2.20	2.15	2.17	2.22	2.17	2.23	2.06	2.24	2.19	2.37	2.21	2.17	2.43	2.38	2.30
ENSG00000260777|26590660 |17|26593395|lincRNA	2.18	2.15	2.30	2.26	2.20	2.18	2.26	2.11	2.06	2.20	2.15	2.13	2.19	2.11	2.41	2.32	2.31	2.32	2.29	2.18
ENSG00000265217|63273326 |18|63319289|lincRNA	2.23	2.21	2.30	2.16	2.23	2.32	2.26	2.21	2.22	2.22	2.14	2.20	2.20	2.29	2.32	2.26	2.34	2.34	2.24	2.30
ENSG00000225298|29094698 |21|29123552|lincRNA	2.16	2.16	2.29	2.07	2.23	2.07	2.13	2.22	2.24	2.18	2.14	2.24	2.20	2.06	2.35	2.26	2.21	2.24	2.43	2.26
ENSG00000224017|41141202 |7|41173105|lincRNA	2.11	2.15	2.29	2.09	2.17	2.09	2.07	2.08	2.13	2.17	2.14	2.10	2.15	2.22	2.39	2.35	2.23	2.34	2.20	2.20
ENSG00000270189|53198279 |X|53200096|lincRNA	2.14	2.15	2.28	2.24	2.15	2.08	2.12	2.12	2.17	2.19	2.18	2.14	2.13	2.13	2.27	2.26	2.25	2.24	2.37	2.27
ENSG00000271073|52912097 |HG1433_PATCH|52913914|lincRNA	2.14	2.15	2.28	2.24	2.15	2.08	2.12	2.12	2.17	2.19	2.18	2.14	2.13	2.13	2.27	2.26	2.25	2.24	2.37	2.27
ENSG00000266490|29119390 |17|29119851|lincRNA	2.08	1.96	2.27	2.12	1.99	2.00	2.18	2.25	1.92	2.13	1.99	2.01	2.05	1.81	2.16	2.31	2.27	2.35	2.26	2.25
ENSG00000231876|26329428 |16|26345749|lincRNA	2.42	2.48	2.26	2.32	2.42	2.42	2.44	2.39	2.50	2.37	2.51	2.46	2.36	2.58	2.22	2.23	2.24	2.29	2.31	2.29
ENSG00000235576|7865932| 2|7870836|lincRNA	1.81	1.56	2.26	1.83	1.93	1.66	2.13	1.90	1.43	2.02	1.62	1.56	1.75	1.30	2.35	2.47	2.32	2.34	2.12	1.98
ENSG00000178440|51732523 |10|51741813|lincRNA	2.18	2.13	2.26	2.17	2.24	2.15	2.30	2.06	2.18	2.09	2.04	2.24	2.17	2.05	2.31	2.27	2.35	2.25	2.31	2.07
ENSG00000269646|53426635	2.16	2.11	2.26	2.02	2.06	2.29	2.23	2.29	2.05	2.15	2.09	2.16	2.20	1.99	2.30	2.21	2.29	2.29	2.27	2.18

|19|53427328|lincRNA																				
ENSG00000251372|139230865|4|139345498|lincRNA	2.14	2.13	2.25	2.10	2.19	2.17	2.19	2.09	2.13	2.07	2.16	2.16	2.17	2.05	2.29	2.32	2.22	2.31	2.24	2.14
ENSG00000267924|24000401|19|24057503|lincRNA	2.18	2.06	2.25	2.20	2.25	2.14	2.19	2.24	2.06	2.17	2.07	2.05	2.13	1.98	2.17	2.28	2.41	2.27	2.26	2.10
ENSG00000230133|24180403|20|24205224|lincRNA	2.10	2.05	2.24	2.07	2.02	2.25	2.22	1.99	2.03	2.21	2.03	2.00	2.05	2.11	2.32	2.34	2.21	2.21	2.15	2.21
ENSG00000237002|36727544|10|36731320|lincRNA	2.02	2.06	2.23	2.15	2.18	1.84	2.09	1.97	1.87	1.99	2.15	2.02	2.19	1.89	2.21	2.17	2.28	2.34	2.32	2.07
ENSG00000255087|126936897|11|126971212|lincRNA	2.12	2.14	2.23	2.14	2.18	2.20	2.04	2.07	2.10	2.13	2.19	2.15	2.14	2.05	2.29	2.20	2.26	2.23	2.28	2.12
ENSG00000255727|15154767|12|15159617|lincRNA	2.10	2.18	2.22	2.18	2.12	2.09	2.05	2.01	2.15	2.13	2.17	2.13	2.16	2.27	2.28	2.19	2.11	2.24	2.30	2.18
ENSG00000233215|23381263|21|23470778|lincRNA	2.05	2.04	2.20	2.04	2.12	2.06	1.98	2.01	2.07	2.07	2.07	2.05	2.05	2.00	2.31	2.24	2.11	2.22	2.19	2.14
ENSG00000251376|54350256|5|54350968|lincRNA	2.09	2.09	2.19	2.09	2.14	2.11	2.06	2.02	2.11	2.15	2.08	2.05	2.08	2.15	2.22	2.09	2.23	2.24	2.18	2.21
ENSG00000268279|14178644|3|14189548|lincRNA	2.06	2.06	2.17	2.12	1.99	1.98	2.17	2.15	1.95	2.11	2.15	2.12	2.05	1.92	2.17	2.14	2.34	2.09	2.13	2.15
ENSG00000258332|74432866|12|74439608|lincRNA	2.03	2.05	2.15	2.02	1.99	1.91	2.13	2.03	2.11	2.01	2.10	2.00	2.09	1.99	2.26	2.13	2.05	2.23	2.12	2.12
ENSG00000250269|124718552|5|124719148|lincRNA	1.88	1.76	2.15	1.98	1.83	1.84	2.07	1.96	1.60	2.02	1.79	1.65	1.97	1.61	2.24	2.26	2.12	2.23	2.09	1.93
ENSG00000170161|66553273|9|66555928|lincRNA	1.98	1.77	2.14	1.83	1.91	1.93	2.15	2.13	1.90	2.08	1.94	1.94	1.89	1.30	2.04	2.10	2.31	2.04	2.23	2.12
ENSG00000260517|29127448|16|29229181|lincRNA	2.03	2.00	2.14	2.08	1.96	2.00	2.08	2.05	1.99	2.08	2.04	2.06	2.04	1.87	2.01	2.06	2.13	2.24	2.19	2.19
ENSG00000254266|79338338|8|79470738|lincRNA	2.06	1.97	2.12	1.99	2.13	1.97	2.10	2.11	2.06	2.02	2.06	1.92	1.99	1.92	2.10	2.22	2.14	2.19	2.10	1.99
ENSG00000255455|130736149|11|130740142|lincRNA	2.05	1.94	2.12	2.14	2.11	1.90	2.06	2.10	2.00	2.10	1.90	1.85	2.01	2.02	2.20	2.02	2.22	2.12	2.10	2.06
ENSG00000231177|10326103|3|10327430|lincRNA	1.97	1.89	2.12	2.05	2.03	1.95	1.95	1.91	1.95	2.10	1.93	1.96	1.83	1.82	2.09	2.14	2.26	2.06	2.00	2.15
ENSG00000270012|49129517|X|49132231|lincRNA	1.88	1.71	2.10	1.96	1.92	1.72	2.00	1.92	1.75	1.85	1.82	1.60	1.79	1.64	2.16	2.06	2.18	2.10	2.04	2.04
ENSG00000270129|49132453|HG1436_HG1432_PATCH|49135167|lincRNA	1.88	1.71	2.10	1.96	1.92	1.72	2.00	1.92	1.75	1.85	1.82	1.60	1.79	1.64	2.16	2.06	2.18	2.10	2.04	2.04
ENSG00000251527|66724479|4|66725922|lincRNA	2.00	1.98	2.07	2.03	2.01	1.84	2.04	1.97	2.10	1.95	1.99	1.93	2.02	1.98	2.07	2.09	2.03	1.99	2.03	2.21
ENSG00000237857|98177116	1.92	1.80	2.06	1.95	1.97	1.87	1.98	1.92	1.81	1.95	1.88	1.84	1.94	1.56	2.13	2.07	2.12	2.18	1.98	1.88

|9|98189078|lincRNA																				
ENSG00000224750|92213927|10|92262725|lincRNA	1.86	1.88	2.05	1.91	1.95	1.92	1.71	1.79	1.88	1.97	1.93	1.89	1.84	1.87	2.12	1.93	2.02	2.23	2.04	1.94
ENSG00000250945|127964585|4|127998917|lincRNA	1.93	1.88	2.04	2.01	1.95	1.90	1.90	1.84	1.98	1.92	1.97	1.80	1.93	1.84	2.11	2.10	1.89	2.14	2.06	1.96
ENSG00000259812|6628186|10|6628882|lincRNA	1.77	1.63	2.04	1.90	2.03	1.50	2.00	2.00	1.16	1.84	1.87	1.32	1.43	1.88	2.03	2.19	1.87	1.97	2.17	2.00
ENSG00000258028|29406095|14|29497969|lincRNA	1.87	1.97	2.02	1.93	1.93	1.83	1.82	1.72	2.00	1.89	1.94	1.90	1.87	2.15	2.01	1.97	2.04	2.06	2.07	1.98
ENSG00000228857|114588626|2|114600933|lincRNA	1.56	1.25	2.01	1.72	1.61	1.30	1.50	1.65	1.57	1.53	1.17	0.98	1.50	1.37	1.95	2.19	2.14	2.27	1.93	1.58
ENSG00000256440|25984350|12|25987116|lincRNA	2.24	2.23	2.01	2.19	2.16	2.28	2.33	2.17	2.34	2.17	2.19	2.23	2.23	2.25	2.00	1.91	2.01	2.01	2.03	2.07
hsa-mir-1299	1.73	1.63	1.95	1.99	1.89	1.34	1.31	1.99	1.85	1.61	1.56	1.64	1.69	1.61	1.95	2.10	1.70	1.90	1.84	2.21
ENSG00000267194|67547499|17|67550002|lincRNA	1.72	1.58	1.94	1.89	1.76	1.43	1.78	1.85	1.63	1.78	1.45	1.65	1.59	1.62	1.88	1.99	1.95	2.07	2.01	1.75
ENSG00000245685|190701490|4|190861426|lincRNA	1.73	1.82	1.94	1.70	1.62	1.72	1.71	1.85	1.76	1.89	1.82	1.86	1.82	1.80	1.91	2.16	2.00	1.85	1.93	1.80
ENSG00000254488|23200175|Y|23206610|lincRNA	1.54	1.61	1.92	1.41	1.59	1.49	1.54	1.57	1.66	1.58	1.42	1.58	1.64	1.80	1.99	1.63	2.01	2.22	1.90	1.81
ENSG00000269729|46667150|19|46683904|lincRNA	1.87	1.79	1.92	1.85	1.89	1.77	1.91	1.88	1.92	1.90	1.76	1.74	1.80	1.87	1.94	1.91	1.98	1.84	1.90	1.96
ENSG00000236537|158703295|6|158733390|lincRNA	1.90	1.69	1.91	1.94	1.93	1.80	1.97	1.99	1.78	1.81	1.76	1.56	1.77	1.66	2.13	1.76	1.97	1.83	2.01	1.76
ENSG00000204837|41961615|9|42019580|lincRNA	1.62	1.37	1.91	1.51	1.47	1.64	1.75	1.87	1.50	1.82	1.48	1.49	1.42	1.10	1.83	1.90	2.06	1.91	1.90	1.86
ENSG00000237357|44401766|9|44404440|lincRNA	1.76	1.67	1.91	1.72	1.82	1.70	1.79	1.87	1.66	1.85	1.74	1.69	1.55	1.68	1.89	2.00	2.08	1.81	1.96	1.69
ENSG00000259959|47842139|4|47846356|lincRNA	1.81	1.67	1.91	1.84	1.81	1.57	1.99	1.88	1.78	1.82	1.81	1.71	1.63	1.52	1.87	1.97	1.94	1.99	1.96	1.69
ENSG00000270297|75239964|6|75443995|lincRNA	1.73	1.69	1.87	1.74	1.74	1.77	1.63	1.77	1.75	1.68	1.78	1.70	1.71	1.58	1.96	1.82	1.88	1.80	1.97	1.82
ENSG00000226007|46843638|9|46846025|lincRNA	1.75	1.62	1.85	1.72	1.80	1.66	1.81	1.86	1.64	1.84	1.69	1.65	1.47	1.68	1.86	1.90	2.00	1.80	1.94	1.62
ENSG00000261589|53246184|17|53250993|lincRNA	1.71	1.67	1.84	1.82	1.72	1.59	1.66	1.78	1.71	1.74	1.61	1.72	1.68	1.66	1.79	1.97	1.80	1.84	1.85	1.82
ENSG00000225948|6660678|10|6667308|lincRNA	1.49	1.43	1.84	1.56	1.54	1.22	1.78	1.47	1.38	1.51	1.36	1.27	1.40	1.69	1.82	2.02	1.78	1.71	2.00	1.71
ENSG00000258800|64065592|14|64065966|lincRNA	1.48	1.41	1.82	1.47	1.68	1.10	1.70	1.52	1.39	1.45	1.44	1.30	1.45	1.47	1.86	1.78	1.89	1.88	1.76	1.78
ENSG00000261122|34977639	1.09	1.28	1.81	0.95	1.17	1.20	1.09	1.11	1.04	1.10	1.17	1.02	0.97	1.95	2.07	1.30	1.93	2.18	1.92	1.47

|16|34990886|lincRNA																				
ENSG00000237877|186897618|2|186947960|lincRNA	1.40	1.45	1.79	1.55	1.33	0.93	1.44	1.54	1.64	1.52	1.74	1.45	1.45	1.16	1.76	1.84	1.70	1.84	1.95	1.62
ENSG00000237253|148598398|1|148599531|lincRNA	1.43	1.29	1.78	1.51	1.46	1.12	1.70	1.54	1.27	1.36	1.34	1.13	1.55	1.16	1.76	1.84	1.80	1.88	1.73	1.65
ENSG00000263398|145104538|HG1287_PATCH|145105671|lincRNA	1.43	1.29	1.78	1.51	1.46	1.12	1.70	1.54	1.27	1.36	1.34	1.13	1.55	1.16	1.76	1.84	1.80	1.88	1.73	1.65
ENSG00000237343|148760504|1|148761583|lincRNA	1.36	1.27	1.77	1.43	1.48	1.10	1.64	1.38	1.10	1.29	1.25	1.09	1.58	1.16	1.72	1.88	1.79	1.85	1.75	1.62
ENSG00000258476|94406338|15|94421585|lincRNA	1.65	1.74	1.73	1.67	1.54	1.66	1.64	1.76	1.63	1.67	1.71	1.77	1.68	1.80	1.64	1.47	1.86	1.74	1.88	1.81
ENSG00000246084|97925153|14|98047938|lincRNA	1.50	1.25	1.71	1.70	1.50	1.16	1.68	1.63	1.34	1.47	1.39	0.98	1.13	1.49	1.72	1.67	1.60	1.74	1.63	1.88
ENSG00000224023|127389005|10|127408135|lincRNA	1.51	1.39	1.69	1.53	1.63	1.55	1.72	1.70	0.93	1.42	1.31	1.32	1.44	1.49	1.91	1.51	1.71	1.87	1.85	1.32
ENSG00000244345|72553957|3|72599703|lincRNA	1.50	1.60	1.67	1.61	1.64	1.54	1.27	1.40	1.55	1.63	1.62	1.73	1.59	1.45	1.60	1.63	1.76	1.73	1.51	1.80
ENSG00000232080|32685782|6|32686612|lincRNA	1.49	1.49	1.64	1.71	1.40	1.45	1.31	1.54	1.50	1.38	1.25	1.58	1.52	1.62	1.55	1.55	1.79	1.62	1.75	1.59
ENSG00000269942|103331776|13|103332308|lincRNA	1.49	1.39	1.63	1.60	1.54	1.30	1.38	1.64	1.47	1.29	1.50	1.05	1.47	1.52	1.72	1.61	1.70	1.57	1.51	1.66
ENSG00000236140|148246959|1|148248104|lincRNA	1.31	1.08	1.61	1.37	1.43	0.93	1.50	1.45	1.20	1.25	1.14	1.17	1.52	0.51	1.62	1.69	1.54	1.64	1.62	1.54
ENSG00000265318|145973025|HG1287_PATCH|145974170|lincRNA	1.31	1.08	1.61	1.37	1.43	0.93	1.50	1.45	1.20	1.25	1.14	1.17	1.52	0.51	1.62	1.69	1.54	1.64	1.62	1.54
ENSG00000226791|99378401|2|99388543|lincRNA	1.49	1.26	1.60	1.49	1.61	1.32	1.72	1.49	1.32	1.50	1.35	1.20	1.22	1.25	1.64	1.64	1.40	1.49	1.71	1.71
ENSG00000261220|134585426|8|134586104|lincRNA	1.24	1.32	1.57	1.30	1.30	1.36	1.18	1.32	1.01	1.45	1.20	1.30	1.31	1.45	1.62	1.58	1.52	1.62	1.49	1.56
ENSG00000255418|231864461|11|23224707|lincRNA	1.16	1.18	1.52	1.22	1.25	1.12	1.27	1.04	1.05	1.30	1.08	1.32	1.22	1.10	1.74	1.47	1.59	1.47	1.52	1.34
ENSG00000254302|34032404|8|34042413|lincRNA	1.23	1.43	1.51	1.29	1.35	1.22	0.84	1.18	1.47	1.29	1.30	1.40	1.47	1.56	1.54	1.40	1.49	1.49	1.66	1.49
ENSG00000250889|74343544|5|74348668|lincRNA	1.14	0.99	1.50	1.35	1.57	0.72	1.14	1.36	0.71	1.18	1.17	0.92	1.36	0.51	1.30	1.24	1.79	1.52	1.56	1.58
ENSG00000255989|74213653|2|74220867|lincRNA	1.83	1.92	1.46	1.82	1.75	1.91	1.78	1.84	1.90	1.81	2.03	2.00	1.82	1.81	1.36	1.58	1.45	1.45	1.34	1.58
ENSG00000229628|46030504|7|46040497|lincRNA	1.28	1.15	1.46	1.24	1.33	1.39	1.50	1.15	1.10	1.28	1.39	1.15	1.10	0.94	1.58	1.30	1.57	1.47	1.54	1.30
ENSG00000244063|11458696	0.86	0.85	1.46	1.03	1.07	0.97	1.27	0.00	0.85	0.85	0.98	0.71	0.87	0.84	1.52	1.47	1.47	1.77	1.45	1.06

7|2|114588696|lincRNA																				
ENSG00000246363|89404458|12|89413456|lincRNA	0.68	0.90	1.44	0.82	0.65	1.20	0.84	0.00	0.59	1.12	1.08	1.20	1.09	0.23	1.36	1.18	1.64	1.64	1.39	1.45
ENSG00000260837|8861264|2|8862816|lincRNA	1.11	0.93	1.43	1.39	1.02	1.12	1.18	1.30	0.65	1.16	0.92	1.15	1.13	0.51	1.54	1.36	1.27	1.43	1.55	1.43
ENSG00000267381|37978901|19|37979554|lincRNA	1.11	1.22	1.41	1.15	1.25	0.97	1.31	1.15	0.85	0.99	1.38	1.05	1.20	1.25	1.45	1.47	1.61	1.50	1.48	0.93
ENSG00000270195|1714548|4|1715349|lincRNA	1.26	1.12	1.40	1.40	1.20	0.97	1.31	1.47	1.23	1.23	1.27	0.92	1.15	1.16	1.45	1.40	1.61	1.45	1.09	1.42
ENSG00000231682|235353234|2|235363239|lincRNA	1.12	0.86	1.36	0.85	1.34	0.83	1.22	1.47	1.01	0.92	1.08	0.79	0.61	0.94	1.25	1.55	1.25	1.06	1.47	1.58
ENSG00000235304|39164210|X|39186616|lincRNA	1.20	1.22	1.36	1.12	1.11	1.14	1.41	1.34	1.10	1.30	1.14	1.32	1.17	1.25	1.30	1.24	1.52	1.34	1.52	1.23
hsa-mir-548f-2	1.05	1.17	1.32	1.15	1.04	1.08	1.02	0.92	1.09	1.02	1.11	1.23	1.30	1.03	1.30	1.18	1.35	1.43	1.43	1.20
ENSG00000270079|2287163|19|2287942|lincRNA	1.63	1.77	1.31	1.64	1.68	1.76	1.52	1.71	1.48	1.74	1.68	1.77	1.78	1.85	1.42	1.30	1.25	1.20	1.13	1.54
ENSG00000267290|176907|19|177913|lincRNA	1.05	0.72	1.30	1.15	1.02	1.18	0.72	1.08	1.14	0.95	0.94	0.85	1.10	0.00	1.36	1.55	1.28	1.39	1.20	1.02
ENSG00000270091|19799903|17|19800600|lincRNA	0.94	0.95	1.29	1.23	0.51	1.10	1.09	1.00	0.71	1.30	0.94	0.98	1.03	0.84	1.30	1.12	1.43	1.20	1.16	1.53
ENSG00000241679|142842204|3|142850735|lincRNA	0.95	1.26	1.29	0.72	1.35	1.14	0.55	1.08	0.85	1.25	1.04	1.13	1.36	1.49	1.25	0.82	1.25	1.27	1.49	1.64
ENSG00000259237|53408562|15|53421895|lincRNA	0.91	1.02	1.27	0.98	0.91	0.93	0.55	1.08	1.04	0.88	1.11	1.02	0.84	1.10	1.16	1.36	1.14	1.34	1.31	1.32
ENSG00000204583|133179736|12|133180307|lincRNA	0.89	0.98	1.27	0.98	1.07	1.08	0.72	0.79	0.71	1.43	1.04	0.92	0.92	1.03	1.30	1.36	1.49	1.41	1.24	0.80
ENSG00000261043|76051842|15|76054746|lincRNA	1.44	1.93	1.17	1.06	1.57	1.61	1.68	1.47	1.23	1.40	1.57	2.07	1.93	2.17	1.10	1.30	1.05	1.20	1.16	1.20
hsa-mir-612	1.65	1.78	1.02	1.58	1.58	1.98	1.54	1.60	1.62	1.69	1.64	1.92	1.65	1.93	0.11	0.66	1.22	1.30	1.47	1.36
ENSG00000236066|113550876|1|113589677|lincRNA	0.57	0.31	0.88	0.32	0.65	0.15	0.55	0.61	1.18	0.13	0.35	0.79	0.11	0.00	0.48	1.12	0.61	1.01	0.86	1.20
ENSG00000230731|44808254|13|44817328|lincRNA	0.21	0.23	0.87	0.00	0.11	0.50	0.55	0.00	0.11	0.13	0.58	0.00	0.11	0.23	1.04	0.43	0.70	1.06	0.71	1.30
ENSG00000257156|89441964|12|89703330|lincRNA	0.02	0.03	0.79	0.00	0.00	0.00	0.00	0.00	0.11	0.00	0.00	0.00	0.11	0.00	0.11	0.82	1.10	1.30	0.71	0.71
hsa-mir-5194	1.23	1.46	0.79	1.15	0.93	1.43	1.38	1.24	1.23	1.32	1.51	1.51	1.17	1.66	0.69	0.82	0.04	1.01	0.97	1.20
ENSG00000251377|153128573|4|153147022|lincRNA	0.21	0.00	0.67	0.32	0.11	0.00	0.00	0.00	0.82	0.13	0.00	0.00	0.00	0.00	0.69	0.66	0.77	0.72	0.71	0.50
hsa-mir-4440	1.27	1.33	0.67	1.30	1.18	1.36	1.14	1.32	1.30	1.45	1.36	1.30	0.94	1.71	0.00	1.04	0.61	0.92	0.71	0.71
hsa-mir-6753	1.31	1.34	0.66	1.19	1.30	1.45	1.50	1.30	1.14	1.10	1.22	1.39	1.24	1.52	0.92	0.82	0.36	0.56	0.71	0.61
ENSG00000238129|26171625|20|26174582|lincRNA	0.00	0.26	0.50	0.00	0.00	0.00	0.00	0.00	0.00	0.13	0.00	0.00	0.00	1.03	0.69	0.66	0.61	0.72	0.00	0.31

ENSG00000260303|146973756|4|146977914|lincRNA	0.13	0.00	0.49	0.00	0.00	0.50	0.30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.69	0.82	0.51	0.56	0.29	0.05
ENSG00000230710|40755946|13|40763165|lincRNA	0.00	0.00	0.41	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.11	0.66	0.77	0.06	0.04	0.80
ENSG00000254197|142288327|8|142302961|lincRNA	0.67	1.04	0.12	0.78	0.00	0.83	1.02	0.61	0.77	0.61	0.94	1.35	0.87	1.03	0.00	0.00	0.36	0.00	0.04	0.31

Supplementary Table S4. Classification of the 128 differentially expressed genes in PBMCs from AChR-EOMG patients annotated by Ingenuity Pathway Analysis based on functional enrichments.

Category	p-value	Molecules
Dermatological Diseases and Conditions	4.25E-07-9.38E-03	JUN,DEFA1 (includes others),TCN1,MS4A2,TUBB2A,TUBA1A,RELB,HRH4,S100P,PITPNB,ID1,MNT,AURKA,IL4,ABCA1,VNN3,ADRA2B,SOCS2,EIF1,TUBB4B,KRT2,MMP8,FOSL1,ETF1,NLRC4
Inflammatory Disease	4.25E-07-5.82E-03	JUN,DEFA1 (includes others),TCN1,MS4A2,TUBB2A,TUBA1A,CPA3,HDC,RELB,HRH4,S100P,PITPNB,ID1,CLC,MNT,IL4,ABCA1,SOCS2,ADRA2B,EIF1,ITGA9,TUBB4B,NFKB2,EIF1AY,PPP1R15A,MMP8,KDM5D,TNFRSF12A,NLRC4
Inflammatory Response	4.25E-07-9.33E-03	JUN,GATA2,DEFA1 (includes others),MS4A2,TUBB2A,CXCR6,TUBA1A,CPA3,HDC,C8orf59,RELB,HRH4,MAFG,PITPNB,ID1,GDF15,MNT,FUS,IL4,ABCA1,SOCS2,EIF1,ITGA9,TUBB4B,NFKB2,MMP8,TNFRSF12A,FOSL1,NLRC4,OTUB2
Cardiovascular Disease	5.95E-07-8.7E-03	JUN,DNAJA1,GDF15,KLF6,ABCA1,IL4,ADRA2B,TUBB4B,TUBB2A,CXCR6,TUBA1A,MMP8,PPP1R15A,FOSB,TNFRSF12A,SDC4,S100P,PIM3
Respiratory Disease	1.76E-06-5.82E-03	JUN,DEFA1 (includes others),ID1,AURKA,POLR2A,CLC,IFRD1,IL4,ADRA2B,ITGA9,TUBB4B,TCN1,MS4A2,TUBB2A,EIF1AY,AKAP12,TUBA1A,MAPK8IP1,PLS1,MMP8,KDM5D,S100P
Immunological Disease	2.03E-06-9.38E-03	JUN,GATA2,DEFA1 (includes others),TCN1,MS4A2,TUBB2A,TUBA1A,CPA3,HDC,RELB,HRH4,PITPNB,ID1,AURKA,FUS,MNT,IL4,ABCA1,ADRA2B,SOCS2,EIF1,ITGA9,TUBB4B,NFKB2,RPL34,PPP1R15A,TNFRSF12A
Infectious Diseases	3.17E-06-5.22E-03	JUN,DNAJA1,DEFA1 (includes others),POLR2A,PTPRN,CLC,IL4,SOCS2,ADRA2B,TMEM132A,TUBB4B,TCN1,TUBB2A,PLK3,NFKB2,CXCR6,EIF1AY,TUBA1A,PPP1R15A,KDM5D,ETF1,S100P
Cancer	4.77E-06-9.38E-03	JUN,GATA2,ASAP1,DNAJA1,POLR2A,PRDM5,CHKA,NFKBIE,TCN1,MS4A2,TUBB2A,UBE2S,CXCR6,AKAP12,TUBA1A,CPA3,MAPK8IP1,PLS1,RELB,HRH4,DACT3,FOSB,S100P,PIM3,ID1,GDF15,AURKA,MNT,KLF6,FUS,SERTAD1,ABCA1,IL4,ADRA2B,SOCS2,EIF1,ITGA9,TUBB4B,PHLDA1,NFKB2,FAM171A1,MMP8RPL34,KDM5D,TNFRSF12A,FOSL1
Organismal Injury and Abnormalities	4.77E-06-9.38E-03	JUN,GATA2,ASAP1,DNAJA1,POLR2A,PRDM5,CHKA,NFKBIE,TCN1,MS4A2,TUBB2A,UBE2S,CXCR6,AKAP12,TUBA1A,CPA3,MAPK8IP1,PLS1,RELB,HRH4,DACT3,FOSB,S100P,PIM3,ID1,GDF15,AURKA,MNT,KLF6,FUS,SERTAD1,ABCA1,IL4,ADRA2B,SOCS2,EIF1,ITGA9,TUBB4B,PHLDA1,NFKB2,FAM171A1,MMP8RPL34,PPP1R15A,KDM5D,TNFRSF12A,FOSL1
Reproductive System Disease	4.77E-06-8.99E-03	JUN,ASAP1,POLR2A,PRDM5,TCN1,MS4A2,TUBB2A,UBE2S,TUBA1A,AKAP12,CPA3,MAPK8IP1,RELB,HRH4,DACT3,FOSB,S100P,GDF15,ID1,KLF6,AURKA,IL4,ABCA1,ADRA2B,SOCS2,EIF1,ITGA9,TUBB4B,FAM171A1,MMP8,FOSL1
Endocrine	1.51E-05-	JUN,ID1,GDF15,KLF6,ADRA2B,CHKA,TUBB4B,TCN1,TUBB2A,TU

System Disorders	8.08E-03	BA1A,FAM171A1,MAPK8IP1,S100P, PIM3
Neurological Disease	4.78E-05-9.08E-03	JUN,DNAJA1,IFRD1,TUBB2A,TUBA1A,HDC,RELB,HRH4,FOSB,S100P,PIM3,ID1,FUS,PTPRN,SERTAD1, ABCA1,PDE1B,IL4,ADRA2B,ITGA9,TUBB4B,NFKB2,PLK3,IER5,CA8,MMP8,SDC4,TNFRSF12A
Hematological Disease	4.82E-05-9.38E-03	JUN,GATA2,ID1,GDF15,AURKA,MNT,FUS,IL4,SOCS2,NFKBIE,TUBB4B,TCN1,NFKB2,TUBB2A,TUBA1A, CPA3,MAPK8IP1,RELB,RPL34,PPP1R15A
Renal and Urological Disease	4.82E-05-7.42E-03	TUBB2A,JUN,TUBA1A,MAPK8IP1,KLF6,AURKA,ABCA1,RPL34,FOSL1,TUBB4B,TCN1,S100P
Gastrointestinal Disease	7.36E-05-6.7E-03	JUN,DNAJA1,ID1,GDF15,FUS,KLF6,AURKA,SERTAD1,IL4,ADRA2B,ITGA9,TUBB4B,TCN1,TUBB2A, PHLDA1,UBE2S,TUBA1A,CPA3,MAPK8IP1,RELB,DACT3,KDM5D,S100P,PIM3
Ophthalmic Disease	7.36E-05-1.78E-03	TUBB2A,TUBA1A,ADRA2B,TUBB4B
Hereditary Disorder	1.06E-04-5.64E-03	TUBB2A,GATA2,TUBA1A,FUS,IFRD1,IL4,MMP8,TUBB4B
Connective Tissue Disorders	1.26E-04-5.22E-03	TUBB2A,JUN,TUBA1A,IL4,TUBB4B
Skeletal and Muscular Disorders	1.26E-04-8.78E-03	TUBB2A,JUN,TUBA1A,GDF15,IL4,ADRA2B,TUBB4B,PIM3
Developmental Disorder	6.14E-04-5.22E-03	TUBB2A,JUN,TUBA1A,CA8,TUBB4B
Metabolic Disease	6.14E-04-5.22E-03	TUBB2A,TUBA1A,ABCA1,TUBB4B
Psychological Disorders	1.78E-03-4.03E-03	TUBB2A,JUN,TUBA1A,DNAJA1,IER5,RELB,PDE1B,IL4,FOSB,ADRA2B,SDC4,TUBB4B
Hepatic System Disease	5.22E-03-5.22E-03	JUN
Tumor Morphology	5.22E-03-5.64E-03	JUN,GDF15,IL4,FOSL1
Antimicrobial Response	6.66E-03-6.66E-03	CPA3,DEFA1 (includes others),C8orf59,IL4,FOSL1,NLRC4,OTUB2

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Chapter 3

Increased expression of Toll-like receptors 7 and 9 in myasthenia gravis thymus characterized by active Epstein-Barr virus infection

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Abbreviations:

MG, myasthenia gravis; EBV, Epstein-Barr virus; TLR, toll-like receptor; GC, germinal center; IFN, interferon; AChR, acetylcholine receptor; EBER, EBV-encoded small RNA; LCM, laser-capture microdissection; IRF8, interferon regulatory factor 8; LMP1, Epstein–Barr virus latent membrane protein 1; DAPI, 4',6-diamidino-2-phenylindole, dihydrochloride; TEC, thymic epithelial cells; pDC, plasmacytoid dendritic cell; mDC, myeloid dendritic cell; SLE, systemic lupus erythematosus; RA, rheumatoid arthritis; RIG-I, retinoic acid inducible gene-I.

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ABSTRACT

Considerable data implicate the thymus as the main site of autosensitization to the acetylcholine receptor in myasthenia gravis (MG), a B-cell-mediated autoimmune disease affecting the neuromuscular junction. We recently demonstrated an active Epstein-Barr virus (EBV) infection in the thymus of MG patients, suggesting that EBV might contribute to the onset or maintenance of the autoimmune response within MG thymus, because of its ability to activate and immortalize autoreactive B cells. EBV has been reported to elicit and modulate Toll-like receptor (TLR) 7- and TLR9-mediated innate immune responses, which are known to favor B-cell dysfunction and autoimmunity. Aim of this study was to investigate whether EBV infection is associated with altered expression of TLR7 and TLR9 in MG thymus. By real-time PCR, we found that TLR7 and TLR9 mRNA levels were significantly higher in EBV-positive MG compared to EBV-negative normal thymuses. By confocal microscopy, high expression levels of TLR7 and TLR9 proteins were observed in B cells and plasma cells of MG thymic germinal centers (GCs) and lymphoid infiltrates, where the two receptors co-localized with EBV antigens. An increased frequency of Ki67-positive

proliferating B cells was found in MG thymuses, where we also detected proliferating cells expressing TLR7, TLR9 and EBV antigens, thus supporting the idea that EBV-associated TLR7/9 signaling may promote abnormal B-cell activation and proliferation. Along with B cells and plasma cells, thymic epithelium, plasmacytoid dendritic cells and macrophages exhibited

enhanced TLR7 and TLR9 expression in MG thymus; TLR7 was also increased in thymic myeloid dendritic cells and its transcriptional levels positively correlated with those of interferon (IFN)-β. We suggested that TLR7/9 signaling may be involved in antiviral type I IFN production and long-term inflammation in EBV-infected MG thymuses. Our overall findings indicate that EBV-driven TLR7- and TLR9-mediated innate immune responses may participate in the intra-thymic pathogenesis of MG.

KEYWORDS:

Myasthenia gravis; Thymus; Epstein-Barr virus; Toll-like receptor 7; Toll-like receptor 9

1. Introduction

Myasthenia gravis (MG) is an autoimmune disorder characterized by fluctuating muscle weakness and fatigability, resulting from the production of autoantibodies against neuromuscular junction (NMJ) components. In most patients (>80%) the target of the autoimmune reaction is the postsynaptic acetylcholine receptor (AChR); less frequently, targets of autoimmunity are the muscle specific kinase receptor or the low-density lipoprotein receptor–related protein 4 [1].

Several lines of evidence support the involvement of the thymus in the pathogenesis of AChR-MG [1]. In most AChR-positive MG patients thymus exhibits pathological changes, including hyperplasia, which is the most common alteration in early-onset (MG onset <50 years of age) patients, and thymoma, which occurs most frequently in late-onset patients (>50 years) [2,3]. Thymic hyperplasia is characterized by expanded perivascular spaces containing B-cell infiltrates, that can be organized into germinal centers (GCs) forming follicles (follicular hyperplasia) or distributed throughout the thymic medulla (diffuse hyperplasia or thymitis) [2,4,5]. Autoreactive T cells and activated B cells producing autoantibodies can be isolated from hyperplastic MG

thymus, indicating that the anti-AChR autoimmune reaction develops, and probably arises, within this organ [6–8].

Pathogen infections are suspected to play a role in autoimmune diseases through the induction of dysregulated Toll-like receptor (TLR)-mediated innate immune responses, which can lead to inflammation, general activation of the adaptive immune system and autosensitization [9,10]. Growing evidence of chronic inflammation and TLR3 and 4 activation in MG thymus strongly supports the hypothesis that, in the context of a genetic susceptible background, persistent or dysregulated innate immune responses to an unknown "danger signal", such as a pathogen infection, might contribute to the intra-thymic MG etiology [11–14]. Among pathogens, Epstein-Barr virus (EBV), a human γ herpesvirus that infects most (90-95%) of the world population, is one of the main candidates suspected to play a role in initiation or exacerbation of autoimmune diseases, due to its ability to promote abnormal activation and survival of B cells, and to disrupt critical B-cell tolerance checkpoints [15,16]. Recently, we showed an active EBV infection in hyperplastic (both follicular and diffuse) and involuted MG thymuses, but not in normal control thymuses [17,18], suggesting that EBV could contribute to onset or

perpetuation of autoimmunity within the thymus of MG patients. Experimental evidence showed that latent EBV proteins can interfere with normal B-cell functions through mechanisms which include an increased B-cell sensitivity to TLR stimulation [19]. Indeed, along with antigen binding to the B-cell receptor and CD40 stimulation, TLR stimuli mediated by TLR7 and TLR9 provide additional co-stimulatory signals for proliferation and maturation of B cells, including autoreactive B cells [20,21]. TLR7 and TLR9 are intracellular endosomal-lysosomal receptors able to recognize viral single-stranded RNA (ssRNA) and unmethylated 2′-deoxyribo(cytidine-phosphateguanosine) (CpG) bacterial or viral DNA, respectively [22]. In vitro and in vivo studies suggested that loss of TLR7 and TLR9 signaling regulation can lead to autoimmunity, due to the ability of these receptors to stimulate B-cell activation and autoantibody production [23]. A crosstalk between EBV, TLR7 and TLR9 can be hypothesized: i) EBV can alter the expression of the two receptors in B cells [24]; ii) EBV itself can elicit TLR7- and TLR9-mediated signaling [25,26]; iii) TLR7 and TLR9 signaling pathways have a “super-additive” effect on the EBV-driven B-cell activation and transformation process [27].

In this study, we investigated the potential association of EBV infection with altered expression of TLR7 and TLR9 in MG thymuses. Our data demonstrate a significant contribution of the signaling pathways mediated by the two receptors to the intra-thymic pathogenesis of MG.

Material and Methods

2.1 Thymus specimens

The study included 15 thymuses with follicular hyperplasia, 11 thymuses with diffuse hyperplasia and 10 involuted thymuses obtained from 29 female and 7 male MG patients (mean age at disease onset: 27.2 ± 9.6 years), who underwent thymectomy (mean age at thymic surgery: 29.6 ± 9.7 years). The study was approved by the Ethic Committee of the Neurological Institute 'Carlo Besta', and each patient provided written informed consent for thymectomy and use of thymus for research purposes.

Histological classification of thymuses was performed at the Department of Pathological Anatomy, Azienda Ospedaliera Bolognini (Seriate, Bergamo). Patients' clinical characteristics are summarized in Table 1. All MG thymuses included in this study resulted positive for intra-thymic EBV infection in our previous studies [17,18]. As controls, we examined 10 non-pathological thymuses from patients undergoing cardiovascular surgery (4 females and 6 males; mean age at surgery: 24.6 ± 13.9); all were tested for the presence of EBV DNA

and EBV-encoded small RNA 1 (EBER1), as previously described [17,18], and resulted EBV-negative.

For each thymus, some fragments were fixed in 10% formalin for histopathological classification; other fragments were snap-frozen and stored at -80 °C for immunohistochemistry and molecular analyses.

2.2 Laser-capture microdissection (LCM)

Six snap-frozen MG hyperplastic thymuses were subjected to LCM of GCs using a Nikon Eclipse TE2000-S microscope (Nikon GMBH, Germany), equipped with a laser microdissector CellCut (MMI). For each thymus, six to ten 15-μm thick serial sections were mounted on membrane slides for LCM, stained by 50% hematoxylin and fixed in RNase-free 75%-100% ethanol. Sections before and after these series were stained for CD20, a B-cell marker, to identify GCs, as described below. From each MG thymic sample, at least 20 GCs (from consecutive serial sections) were microdissected and pooled in a single cap; sections devoid of microdissected GCs were collected in separate caps. Whole sections from 5 control thymuses were collected as controls. The isolated tissue fragments of each series were

incubated in lysis buffer (RNeasy Micro kit, Qiagen, Valencia, CA) at 37 °C for 1 hour and centrifuged at 800 X g for 5 minutes; lysates were then stored at -80 °C until use.

2.3 Reverse transcription and real-time PCR

Total RNA was extracted from snap-frozen thymic fragments using the TRIzol method (Thermo Fischer Scientific, Waltham, MA), and from LCM samples using the RNeasy Micro kit (Qiagen), according to the manufacturers' instructions. DNase I-treated RNA samples were retrotranscribed using Superscript VILO cDNA synthesis kit and amplified in duplicates using predesigned functionally tested Taqman gene expression assays specific for TLR7, TLR9, interferon regulatory factor 8 (IRF8), interferon (IFN)-β, MX1, and retinoic acid inducible gene-I (RIG-I) (Thermo Fischer Scientific). Human GAPDH was stably expressed in both control and MG thymic tissues and was used as endogenous control (Thermo Fischer Scientific). Transcriptional levels of the target genes were expressed as relative values ($2^{-\Delta Ct}$ x 100) normalized with GAPDH.

2.4 Immunohistochemistry (IHC)

IHC was performed on 6-μm thick sections of snap-frozen thymic tissues from 4 follicular hyperplastic, 4 diffuse hyperplastic, 4 involuted MG thymuses and 4 control thymuses. Sections were fixed in 4% PFA for 10 minutes, incubated in 1.5% hydrogen peroxide in methanol for 15 minutes, to eliminate endogenous peroxidase activity, and in 5% bovine serum albumin for 1 hour, to block non-specific binding sites. Then, sections were immunostained with primary antibodies specific for the B-cell marker CD20 (1:300; clone L26, Dako, Glostrup, Denmark) and the plasma cell marker CD138 (1:50, clone MI15, Dako). Secondary labelling was performed with HRP Anti-Mouse antibody (Dako) followed by incubation with 3,3' -Diaminobenzidine (DAB; Dako) and hematoxylin counterstaining. Negative controls included IgG isotype controls. Images were digitally acquired with the Aperio ScanScope system and visualized using the ImageScope v11.2.0.780 software (Aperio, Nikon). For each thymus, a whole histological section (at least 10 mm^2) was analysed. The content of B cells in MG and control thymic sections was expressed as percentage of thymic area occupied by CD20-positive single cells, infiltrates and GCs (if present). Moreover, CD138-

positive cells were counted in MG and normal thymic sections and their number was normalized to the total medullary surface analysed, expressed as mm^2.

2.5 Double immunofluorescence

Six-μm thick serial sections of snap-frozen thymic tissues from 5 follicular hyperplastic, 5 diffuse hyperplastic, 5 involuted MG thymuses and 4 control thymuses were fixed in 4% PFA for 10 minutes, incubated in cold methanol for 10 minutes and in 5% bovine serum albumin for 1 hour; then, they were immunostained over-night at 4°C with combinations of primary antibodies against: TLR7 (1:50, Santa Cruz, Dallas, TX); TLR9 (1:20, Santa Cruz); CD20 (Dako; or ready-to-use, Abcam, Cambridge, UK); CD138 (Dako); cytokeratin (CK) (1:100, Dako), a marker of thymic epithelial cells (TECs); BDCA2 (1:20, Miltenyi Biotec, Bologna, Italy), a marker of plasmacytoid dendritic cells (pDCs); CD11c (1:20, BD Biosciences, Milan, Italy), a marker of myeloid dendritic cells; CD68, a marker of macrophages (1:50, Dako); the T-cell markers CD4 (1:10, Dako) and CD8 (1:10, Dako); the proliferation-associated marker Ki67 (1:100,

Dako; or 1:200 Lab Vision, Waltham, MA USA); the EBV latent marker LMP1 (ready-to-use, clone CS 1-4, Dako), and the EBV lytic marker BZLF1 (1:10, Lifespan Biosciences Inc., Seattle, WA). Sections were then incubated for 1 hour with a mixture of Cy2-conjugated goat anti-mouse IgG and Cy3-conjugated goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Baltimore Pike, West Grove, PA); nuclei were stained with 4',6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI; Thermo Fischer Scientific). As negative controls, primary antibodies were replaced with isotype-specific IgGs (Dako). Fluorescence images were captured by the C1 laser scanning confocal microscope system (Nikon) and at least 4 representative 60X fields per section were analyzed using Image J software (version 1.43u). CD20/TLR7, CD20/TLR9, CD138/TLR7, and CD138/TLR9 double positive cells were counted in sections of 12 MG (4 for each thymic pathology) and 4 normal thymuses. CD20/TLR7 and CD20/TLR9 double positive cells were also counted in at least 3 GCs of 5 follicular hyperplastic MG thymuses. CD20/Ki67, TLR7/Ki67 and TLR9/Ki67 double positive cells were counted in sections of 10 hyperplastic (5 follicular and 5 diffuse), 3 involuted MG thymuses and 5 normal thymuses. Immunoreactivity for TLR7 and TLR9 of CK-positive TECs, CD11c-positive mDCs,

BDCA2-positive pDCs, CD68-positive macrophages, CD4-positive T cells, and CD8-positive T cells was evaluated in sections of 9 MG (3 for each thymic pathology) and 3 normal thymuses by three evaluators (P.C., S.M., and C.B.) independently, and graded using the following semi-quantitative scoring system: - = no staining; ± = very weak positivity or rare positive cells; + = weak or sporadic; ++ = moderate or frequent; +++ = strong or extensive.

2.6 Statistical analysis

Data distribution was tested via Shapiro-Wilk test: normally distributed data ($p>0.05$) were analyzed via ANOVA, followed by multiple comparisons of means with Tukey post-hoc test; non-parametric data ($p<0.05$) were analyzed by Kruskal-Wallis test with Bonferroni post-hoc test for multiple comparisons, or by Mann-Whitney test for comparison of two groups, as indicated in figure legends. Differences were considered statistically significant when the p values were <0.05. A generalized linear model was used to evaluate the relationship between TLR7 (or TLR9) mRNA levels and the relative proportion of B cells in MG thymuses, and between TLR7 (or

TLR9) mRNA levels and the number of GCs per cm^2 in follicular hyperplastic MG thymuses. The non-parametric Spearman correlation test was applied to search for a possible positive or negative correlation between INF-β and TLR7 (or TLR9) mRNA levels in MG thymuses and controls. All the analyses were performed with R statistical software (version 3.0.2.) (www.r-statistics.org).

3. Results

3.1 TLR7 and TLR9 mRNA levels are increased in EBV-positive MG thymuses

To verify whether TLR7 and TLR9 transcriptional levels were altered in EBV-infected MG thymuses, we performed quantitative real-time PCR in 36 MG thymuses, positive for latent and lytic EBV markers, and 10 normal control thymuses, all negative for the presence of EBV DNA and EBER1 [17,18]. We found that TLR7 and TLR9 mRNA levels were significantly increased in all the MG thymic subgroups analyzed compared to the control group (Fig. 1A), suggesting a possible activation of these receptors in EBV-infected MG thymuses. Of note, relative expression values (± SEM) of TLR7 were significantly higher than those of TLR9 (5.19 ± 0.85 *versus* 1.05 ± 0.21 in MG thymuses, Mann-Whitney U test: $p<0.0001$; 0.62 ± 0.08 *versus* 0.21 ± 0.11 in normal thymuses, Mann-Whitney U test: $p<0.01$)*;* this was particularly true for MG thymuses, where the marked difference between TLR7 and TLR9 mRNA levels (TLR7/TLR9 mRNA level ratio: 4.94 in MG thymuses *versus* 2.95 in normal thymuses) suggested a stronger activation of TLR7 compared to TLR9 in the pathological tissues examined.

TLR7 and TLR9 mRNA levels did not differ between MG patients treated with corticosteroids, patients untreated or treated only with cholinesterase inhibitors before thymectomy (Fig. 1B), indicating that immunosuppression has not effect on TLR7 and TLR9 expression in MG thymus.

3.2 TLR7 and TLR9 are overexpressed in B cells and plasma cells of MG thymuses and co-localize with EBV antigens

In our previous study, we showed the presence of EBV-positive B cells and plasma cells in MG thymuses, but not in normal thymuses, and also observed that MG B-cell medullary infiltrates and ectopic GCs were the main sites of EBV persistence and reactivation [17].

Here, we first identified B cells and plasma cells in the thymic medulla of healthy donors and MG patients by immunohistochemistry, and confirmed the previous evidence that MG thymuses, especially hyperplastic, are characterized by an higher proportion of CD20-positive B cells (organized or not in GCs), as well as CD138-positive plasma cells, compared to normal thymuses (Fig. 2A). Then, B cells and plasma cells were analyzed for the expression of TLR7 and TLR9 by double immunofluorescence. In normal thymuses, the percentage of B cells and plasma cells expressing the

two receptors was lower compared to MG thymuses (Fig. 2B and C) and positivity for TLR7 and TLR9, when detected, was weak (Fig. 3A and B). In all the MG thymuses investigated, signals of positivity for the two receptors were detected in most B cells and plasma cells, irrespective of thymic pathology (Fig. 3C and D, and Supplementary Fig. 1). Specifically, TLR7 and TLR9 were expressed in most GC CD20-positive B cells (74.3% ± 6.2 for TLR7; 68.5% ± 10.5 for TLR9) of follicular hyperplastic MG thymuses (Fig. 3C), as well as in a high proportion of infiltrating B cells not organized in GCs in follicular hyperplastic (Supplementary Fig. 1A), diffuse hyperplastic (Fig. 3C) and involuted MG thymuses (Supplementary Fig. 1B). For each MG thymic subgroup, especially that of follicular hyperplastic MG thymuses, all the tissues investigated showed the presence of numerous plasma cells highly expressing TLR7 and TLR9 (Fig. 2C; Fig. 3D; and Supplementary Fig. 1C).

To determine whether TLR7 and TLR9 over-expression in MG thymuses was merely due to the increased number of B cells, we searched for a relationship between the mRNA levels of the two receptors and the relative proportion of B cells. No correlation was found between TLR7 or TLR9 mRNA levels and the B-cell content present in MG thymic sections (generalized linear model, $p>0.05$) or

with the number of GCs per cm^2 in follicular hyperplastic MG thymuses (generalized linear model, $p>0.05$). These results suggested that TLR7 and TLR9 up-regulation in the thymus of MG patients was not directly linked to B-cell infiltration, but it could represent the response to an immune/inflammatory stimulus, such as EBV. By double immunofluorescence we observed co-localization of the EBV latent protein LMP1 and the lytic protein BZLF1 with TLR7 and TLR9 in MG thymuses (Fig. 4). Indeed, cells positive for LMP1 were detected in the thymic medulla of all the MG thymuses analyzed, and most of them was positive for TLR7 and TLR9 (Fig. 4C); in the same pathological tissues, scattered BZLF1-positive cells expressing the two receptors were also found (Fig. 4D), thus suggesting up-regulation of TLR7 and TLR9 in latently and lytically EBV-infected cells within MG thymuses. No positivity for the two EBV proteins analyzed was observed in normal thymuses (Fig. 4A and B), confirming the absence of EBV-infected cells in non-pathological thymic tissues.

3.3 Increased frequency of proliferating B cells in MG thymuses and colocalization of the proliferation-associated Ki67 antigen with TLR7, TLR9 and EBV latent protein LMP1.

Since TLR7 and TLR9 signaling has been reported to favor proliferation of B cells and increase EBV-driven B-cell proliferation and activation processes [20,21,27], we investigated whether MG thymuses were characterized by an increased frequency of proliferating B cells compared to normal thymuses, and whether TLR7, TLR9 and EBV antigens co-localized with the proliferating cells. We found a significant increased proportion of CD20-positive B cells expressing the proliferation-associated Ki67 antigen in MG compared to normal thymuses (Fig. 5A, D and G). In hyperplastic MG thymuses, Ki67/CD20 double positive proliferating B cells were mainly found at the edge of GCs or were randomly distributed in B-cell infiltrates of thymic medulla (Fig. 5D). Interestingly, in normal thymuses Ki67/TLR7 and Ki67/TLR9 double positive cells were rarely detected (Fig. 5B); by contrast, in MG thymuses Ki67-positive cells expressing TLR7 and TLR9 were frequently observed in medullary areas containing GCs and B-cell infiltrates (Fig. 5E), suggesting that they were proliferating B cells; the percentage of these cells was significantly higher in the MG tissues compared to controls (Fig. 5H). In addition, in MG thymuses, but not in normal thymuses, we detected LMP1-positive cells that were also positive for Ki67,

thereby indicating the presence of proliferating EBV-infected B cells in MG pathological thymic tissues (Fig. 5C and F).

3.4 TLR7 and TLR9 expression in MG thymic cell populations: a potential role in antiviral type I IFN production

As described above, TLR7 and TLR9 transcriptional levels did not correlate with the number of GCs in follicular hyperplastic MG thymuses, suggesting that their increase was also due to other cell types out of GCs. We therefore compared TLR7 and TLR9 mRNA levels in whole sections from normal thymuses, thymic GC-free sections from follicular hyperplastic MG thymuses and the corresponding microdissected GCs. To control GC microdissection quality and efficiency, IRF8 gene was analyzed in the same samples as marker of GC B cells [28,29]. As expected, IRF8 mRNA levels were significantly higher in microdissected GCs compared to whole thymic sections from adult controls and sections of follicular hyperplastic MG thymuses devoid of GCs (Fig. 6A). In pathological thymuses, both GC-free whole sections and microdissected GCs showed TLR7 and TLR9 expression levels significantly higher than those of whole sections from normal thymuses (Fig. 6A). These results, combined with data showing absence of correlation between

TLR7/9 mRNA levels and B cell content, indicated that TLR7 and TLR9 over-expression in MG thymuses likely involved not only B cells and plasma cells but also other thymic cell populations. By double immunofluorescence, we investigated TLR7 and TLR9 expression in CK-positive TECs, BDCA2-positive pDCs, CD11c-positive mDCs, CD68-positive macrophages, CD4- and CD8-positive T cells (Figs. 6 B–E, 7, and Supplementary Fig. 2). We observed a strong over-expression of TLR7 and TLR9 in thymic epithelium, pDCs and macrophages of all MG thymuses, irrespective of thymic histology, compared to controls, in terms of both number of positive cells and intensity of the immunostaining (Table 2; Fig. 6B to E; Fig. 7B-D, and Supplementary Fig. 2A, B and D). As regard to mDCs, immunostaining for TLR7 was weak in mDCs of normal thymuses, whereas this receptor, but not TLR9, was expressed in a high proportion of mDCs of all the MG thymuses investigated (Table 2; Fig. 7A and C, and Supplementary Fig. 2C). CD4- and CD8-positive T cells did not show marked differences for the expression of TLR7 and TLR9 between MG and normal thymic tissues (Table 2 and Supplementary Fig. 3).

Since TLR7 and TLR9 signaling is known to induce type I IFNs in response to viral infections, including EBV infection [22,30], we

hypothesized a possible relationship between the increased expression of the two receptors in MG thymic cell populations and the IFN signature which is known to characterize the thymus of MG patients [1,3]. We observed that IFN-β was overexpressed in the MG thymic tissues, along with type I IFN-stimulated genes, including MX1, which encodes an interferon-induced GTP-binding protein [31], and RIG-I, encoding a protein involved in viral double-stranded RNA recognition and antiviral immunity [32] (Supplementary Fig. 4). Interestingly, IFN-β mRNA levels positively correlated with those of TLR7 (Spearman test, $r=0.90$; $p<0.01$), but not of TLR9 (Spearman test, $r=0.45$; $p>0.05$) (Supplementary Fig. 4), suggesting that TLR7 signals might induce IFN-β in EBV-positive MG thymuses, or that IFN-β production in the inflamed MG thymuses might stimulate over-expression of TLR7 and other type I IFN-inducible genes, such as MX1 and RIG-I. In normal thymuses, IFN-β mRNA levels did not correlate with those of TLR7 (Spearman test, $r=0.80$; $p>0.05$) or TLR9 (Spearman test, $r=-0.10$; $p>0.05$).

4. Discussion

Viral infections and innate immune signaling by TLRs are suspected to play a critical role in the pathogenesis of many autoimmune diseases, particularly those characterized by chronic inflammation and antiviral IFN signature. Dysregulation of TLR-mediated innate immune responses is indeed suspected to favor development and perpetuation of autoimmunity by inducing abnormal activation of immune system cells in genetically susceptible individuals [9,10].

The thymus is considered the main site of onset and maintenance of autoimmunity in MG patients with anti-AChR antibodies. Growing data suggested a significant contribution of the innate immunity pathways in inducing intra-thymic immune dysregulation and autosensitization in MG patients, since TLR (i.e. TLR3, TLR4) activation and chronic inflammation, accompanied by type I IFN production, have been shown to be key features of MG thymus [3,11,13,33]. However, the origin of abnormal innate immune activation in this organ is still unknown.

EBV, a leading candidate in triggering several autoimmune diseases, is a biologically plausible source for endogenous innate immune activation, as it can establish a life-long infection in human cells with

persistent virus production following reactivation [16]. We recently showed the presence of EBV latent and lytic transcripts and proteins in B cells and plasma cells of hyperplastic (both follicular and diffuse) and involuted MG thymuses, thus suggesting that EBV persistence and reactivation might contribute to the intra-thymic pathogenesis of MG [17,18]. Herein, we hypothesize that EBV infection might trigger pathogenic TLR-mediated innate immune responses within MG thymuses.

TLR7 and TLR9 are two innate immune receptors whose expression and signaling pathways can be elicited or modulated by EBV. EBV gene expression may be a source of the single-stranded RNA that normally activates TLR7 signaling, whereas EBV genome, containing CpG motifs, may stimulate TLR9 [24–26]. These receptors have been implicated in the pathogenesis of some EBV-associated autoimmune diseases, such as systemic lupus erythematosus (SLE) [23,34], rheumatoid arthritis (RA) [35–37], and multiple sclerosis [38,39], but their potential role in MG has never been explored.

In this study, we found that TLR7 and TLR9 transcriptional levels were significantly increased in EBV-positive MG compared to EBV-negative control thymuses (Fig. 1A), suggesting that TLR7/9

signaling may be activated in the EBV-infected thymus of MG patients. It has been reported that EBV can modulate TLR7 and TLR9 expression in B cells, enhancing the ability of these cells to respond to TLR7/9 ligands [19,24,27]. We therefore checked whether TLR7 and TLR9 protein levels were also altered in B cells and plasma cells of MG thymuses, which previously resulted infected by EBV [17,18]. Interestingly, a higher proportion of B cells and plasma cells expressing the two receptors was found in MG thymuses compared to controls. Moreover, in MG samples, TLR7 and TLR9 transcriptional levels did not correlate with the B-cell content measured in the thymus sections, suggesting that their increase could be the response to a pathogen stimulus, such as EBV. Indeed, in pathological MG thymic tissues we detected latently (LMP1-positive) and lytically (BZLF1-positive) EBV-infected cells that expressed TLR7 and TLR9 (Fig. 4). In our previous study, all the MG thymuses investigated showed similar expression patterns of EBV latent and lytic markers, irrespective of the thymic histology [17]. Here, we observed that both hyperplastic (follicular and diffuse) and involuted thymuses were characterized by increased TLR7 and TLR9 mRNA levels, as well as over-expression of the two receptors in B cells and plasma cells expressing EBV antigens, thus suggesting that TLR7 and TLR9

signaling pathways may be activated via EBV in pathological thymic tissues of MG patients.

Of note, TLR7-positive B cells, both those forming GCs (in follicular hyperplastic thymuses) and those not organized in GCs (in all the three thymic pathologies), were more frequent than B cells expressing TLR9, suggesting a stronger activation of TLR7 than TLR9 in the MG thymic B-cell compartment. In vitro studies showed that expression of TLR7, and its downstream signaling mediators, is rapidly induced in B cells after EBV infection and that the TLR7-dependent pathway favors the EBV ability to establish a persistent infection [24]. Thus, the wide TLR7 expression we observed in MG thymic B cells could well be explained by the persistence of EBV in MG thymus.

TLR7- and TLR9-mediated pathways have been reported to provide B-cell activation stimuli and increase proliferation, survival and maturation of B cells, including those autoreactive, having a super-additive effect on the EBV-driven B-cell transformation process [19,20,27]. We hence hypothesized that elevated TLR7/9 signature in EBV-infected MG thymuses might be associated with abnormal B-cell activation and proliferation. Accordingly, we observed an increased frequency of proliferating B cells in MG thymuses

compared to controls, as well as an increased frequency of proliferating cells expressing TLR7 and TLR9, which were mainly detected in B-cell medullary infiltrates or at the edge of GCs (Fig. 5). In MG tissues, but not in controls, proliferating EBV-infected cells were detected (Fig. 5), thus suggesting that EBV-associated TLR7/9-mediated innate immune responses could induce abnormal B-cell proliferation. TLR7/9 signaling has been reported to significantly affect B-cell differentiation, GC formation and autoantibody production [40,41]. Our results strongly support the idea that TLR7 and TLR9 increase in B cells and plasma cells of MG thymuses could have a pathogenic significance, either promoting or propagating adaptive autoimmune responses. Thus, B-cell dysfunction via TLR7 and TLR9 might be a candidate mechanism, to be deeply explored, linking EBV with MG pathogenesis.

TLR7 and TLR9 signaling pathways may have distinct outcomes depending on the cell types expressing them. They are critically involved not only in the activation of B cells, but also in dendritic cell activation and cytokine induction, particularly type I IFNs [42]. In this regard, DC subsets show distinct TLR7 and TLR9 expression patterns, since pDCs express both receptors whereas mDCs express only TLR7

[43]. Our data demonstrated that TLR7 and TLR9 are overexpressed in medullary TECs, pDCs and macrophages of EBV-infected MG thymuses (both hyperplastic and involuted) compared to controls, whereas TLR7, but not TLR9, was also overexpressed in MG mDCs (Table 2; Fig. 6, 7 and Supplementary Fig. 2). Since TLR7 and TLR9 are key transducers of type I IFNs during infection with most viruses [22,30,44], our observations suggested that TLR7/9 signaling pathways might lead to abnormal secretion of type I IFNs during active EBV infection. Interestingly, we found that the transcriptional levels of TLR7 positively correlated with those of IFN-β (Supplementary Fig. 4), supporting the idea that TLR7 signaling might contribute to IFN-β production in EBV-positive MG thymuses. Furthermore, since TLR7 gene is inducible by type I IFNs [45–47], the TLR7 over-expression might be induced by IFN-β released in the inflamed MG thymic tissues. Indeed, gene expression levels of type I IFN-induced genes, including MX1 and RIG-I, are increased in MG thymuses (Supplementary Fig. 4), thus reinforcing the central role of IFN-β in the intra-thymic MG pathogenesis [3].

Of note, in MG thymuses TLR7 was more intensely and broadly expressed than TLR9: i) TLR7 mRNA levels were higher (around 5-

fold) than those of TLR9; ii) a higher proportion of diffuse infiltrating B cells expressed TLR7 than TLR9; iii) the percentage of B cells expressing TLR7 in GCs of follicular hyperplastic MG thymuses was higher than that expressing TLR9; iv) TLR7, but not, TLR9 was expressed in CD11c+ mDCs. A pathogenic role has been postulated for TLR7 in SLE, because up-regulated TLR7 expression and increased TLR7-dependent B-cell activation was associated with enhanced disease in SLE murine models [34,48]. Moreover, TLR7 is suspected to play a role in RA: it was overexpressed in EBV-infected synovia of RA patients and co-localized with type I IFNs, suggesting that EBV molecules may favor a pro-inflammatory environment via TLR7, leading to disturbed balance between tolerance and autoreactivity [35,36]. Similarly, we can hypothesize that EBV-driven TLR7 hyper-activation may favor or sustain harmful inflammatory responses in MG thymus, promoting or enhancing autoimmunity; antiviral type I interferon production in the inflammatory thymic milieu might in turn contribute to TLR7 over-expression, thus creating a pathogenic vicious circle.

To conclude, our overall data indicate for the first time that TLR7 and TLR9-mediated innate immune responses to EBV infection may

contribute to immunological alterations promoting or perpetuating autoimmunity in the thymus of MG patients.

5. Conclusions

In parallel with the accumulation of data on TLR involvement in autoimmunity, interest in therapeutic manipulation of these innate immune components is rapidly growing. Compounds able to modulate TLR7/9 signaling are among the main drug candidates in the development of innovative treatments for both autoimmune and inflammatory diseases; some of them have already been shown strong efficacy in animal models of some diseases, including SLE, RA and MS, and their use in clinical trials is increasing [39,49–51]. Our study provides new insights into the possible involvement of TLR7 and TLR9 in the pathogenesis of MG, paving the way to a novel hypothesis that TLR7/9-dependent innate immune responses to EBV might contribute to B-cell tolerance disruption, long-term inflammation and maintenance of the autoimmune response in the thymus of MG patients. Verification of this hypothesis and elucidation of the exact molecular mechanisms underlying the postulated intra-thymic cross-talk between EBV infection, TLR7/9 signaling and autoimmunity in MG, may represent an important starting point in the

realization of novel therapeutic approaches targeting these innate immune pathways.

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Disclosures

Conflict of interest: none.

Table 1. Summary of the main clinical characteristics of MG patients included in the study

	MG THYMIC PATHOLOGY		
	Follicular hyperplasia (n=15)	Diffuse hyperplasia (n=11)	Involuted thymus (n=10)
Sex (F:M)	13:2	9:2	7:3
Age at onset (years, mean ± SD)	25.7± 8.2[a]	26.3± 9.8[a]	29.9 ± 10.3[a]
Age at surgery (years, mean ± SD)	27.5 ± 9.4	28.9 ± 9.8	32.6 ± 10.8
Number of AChR-positive patients	12/15[b]	8/10[b,c]	9/10[b]
Number of corticosteroid-treated patients[d]	8/14[e]	8/11	10/10

[a]Information on age at onset was not available in two of the fifteen patients with follicular hyperplasia, one of the eleven patients with diffuse hyperplasia and one of the ten patients with involuted thymus;

[b]AChR-negative patients were also negative for the presence of anti-MuSK autoantibodies in serum;

[c]Information on autoantibody presence in serum was not available in one of the eleven patients with diffuse hyperplasia;

[d]Patients not treated with corticosteroids were untreated or treated only with cholinesterase inhibitors before thymectomy

[e]Data on therapy before thymectomy were missing in one of the fifteen patients with follicular hyperplasia.

Table 2. Immunoreactivity for TLR7 and TLR9 in thymic cell populations of normal and MG thymuses

	TLR7				TLR9			
	Normal thymus	**Follicular hyperplasia**	**Diffuse hyperplasia**	**Involuted thymus**	**Normal thymus**	**Follicular hyperplasia**	**Diffuse hyperplasia**	**Involuted thymus**
CK+ TECs	+	+++	+++	+++	+	+++	+++	++
BDCA2+ pDCs	++	+++	+++	+++	++	+++	+++	+++
CD11c+ mDCs	+	+++	++	++	-	±	-	±
CD68+ macrophages	±	++	+++	++	±	++	+	++
CD4+ T cells	++	++	++	++	++	++	++	++
CD8+ T cells	++	++	++	+	++	++	++	+

CK: cytokeratin; TECs: thymic epithelial cells; pDCs: plasmacytoid dendritic cells; mDCs: myeloid dendritic cells. Immunostaining for TLR7 and TLR9 was graded from – to +++ according to these criteria: - = no staining; ± = very weak positivity or very rare positive cells; + = weak or sporadic; ++ = moderate or frequent; +++ = strong or extensive.

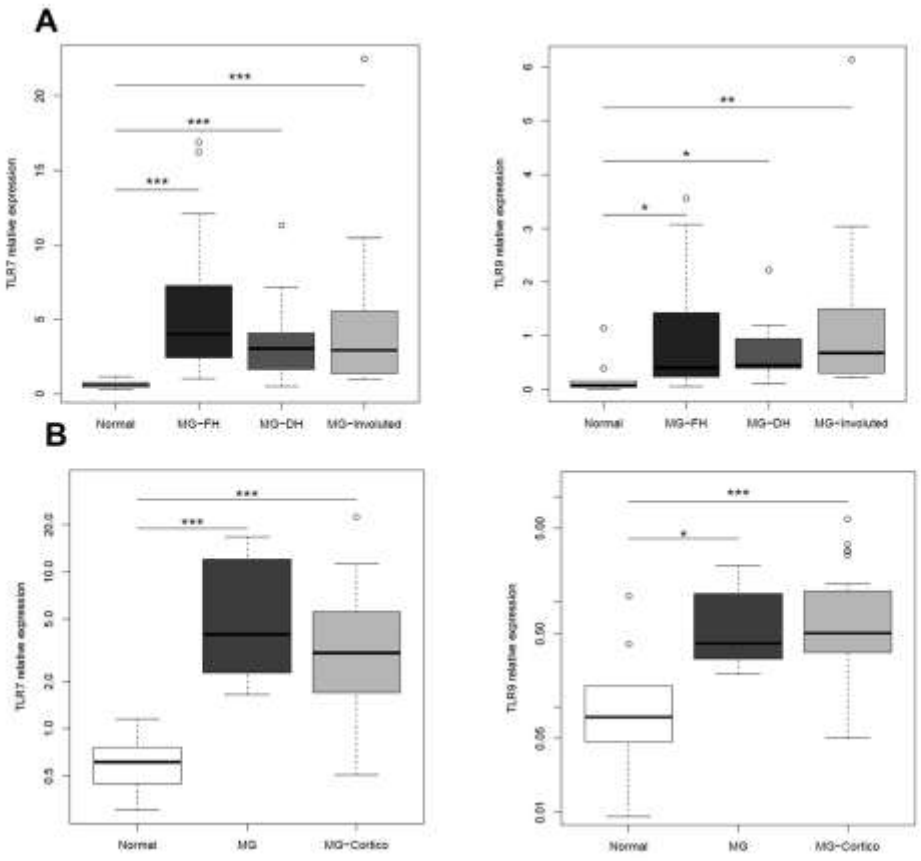


Figure 1

Fig. 1. Increased TLR7 and TLR9 transcriptional levels in MG thymuses. (A) Real-time PCR analysis to assess TLR7 and TLR9 transcriptional levels in 10 thymuses from adult healthy donors (Normal) and 36 MG thymuses, including 15 thymuses with follicular hyperplasia (MG-FH), 11 thymuses with diffuse hyperplasia (MG-DH) and 10 involuted thymuses (MG-Involuted). (B) Comparison of

TLR7 and TLR9 mRNA levels in thymuses from healthy donors (Normal, n=10) and MG patients classified as untreated (MG, n=9) and corticosteroid-treated (MG-Cortico, n=26), on the basis of the therapeutic treatment before thymecomy. In A and B boxplots, mRNA levels were expressed as relative values ($2^{-\Delta ct}$x100) normalized towards the housekeeping gene GAPDH; dark horizontal lines represent means, with the box representing the 25^{th} and 75^{th} percentiles, the whiskers the 5^{th} and 95^{th} percentiles, and the dots the outliers. *P* values were assessed by Kruskal-Wallis test with Bonferroni post-hoc test. *p<0.05; **p<0.01; ***p<0.001.

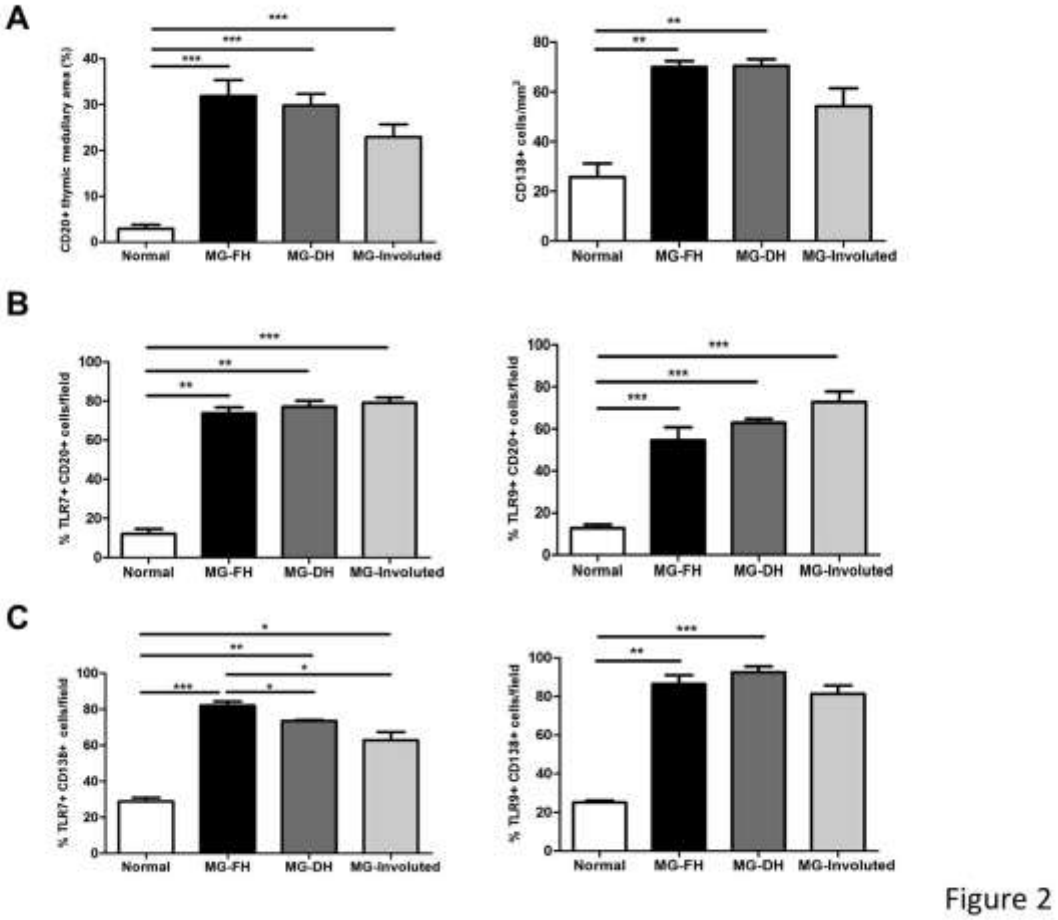


Fig. 2. Increased proportion of B cells and plasma cells expressing TLR7 and TLR9 in MG thymuses. (A) Content of CD20-positive B cells and CD138-positive plasma cells in thymic sections from normal (Normal, n=4) and MG thymuses with follicular hyperplasia (MG-FH, n=4), diffuse hyperplasia (MG-DH, n=4) and thymic involution (MG-Involuted, n=4) determined after immuonohistochemistry for CD20 and CD138. For each sample, a whole thymic section stained for CD20 or CD138 was analyzed using Aperio ImageScope v11.2.0.780 software (Aperio, Nikon). In the left graph, bars represent the mean

percentage (± SEM) of thymic section surface occupied by CD20-positive cells (present as single positive cells, aggregates or GCs) in each sample group. In the right graph, bars correspond to the mean number (± SEM) of CD138-positive cells per thymic medullary area (mm^2) obtained in each sample group. (B) and (C) TLR7/CD20 (B, left graph), TLR9/CD20 (B, right graph), TLR7/CD138 (C, left graph) and TLR9/CD138 (C, right graph) double positive cells were counted in 6 adjacent fields per section in 4 normal and 12 MG thymuses (4 for each pathological group). Bars in the graphs correspond to the mean percentage (± SEM) of CD20-positive B cells (B) or CD138-positive plasma cells (C) expressing TLR7 (left graphs) or TLR9 (right graphs) estimated in relation to the total number of CD20-positive or CD138-positive cells per field in each sample group. P values were assessed by Kruskal-Wallis test with Bonferroni post-hoc test for data shown in (A) and (C) graphs, and in the (B) left graph; for data shown in the (B) right graph, ANOVA followed by multiple comparisons of means with Tukey post-hoc test was used. *$p<0.05$; **$p<0.01$; ***$p<0.001$.

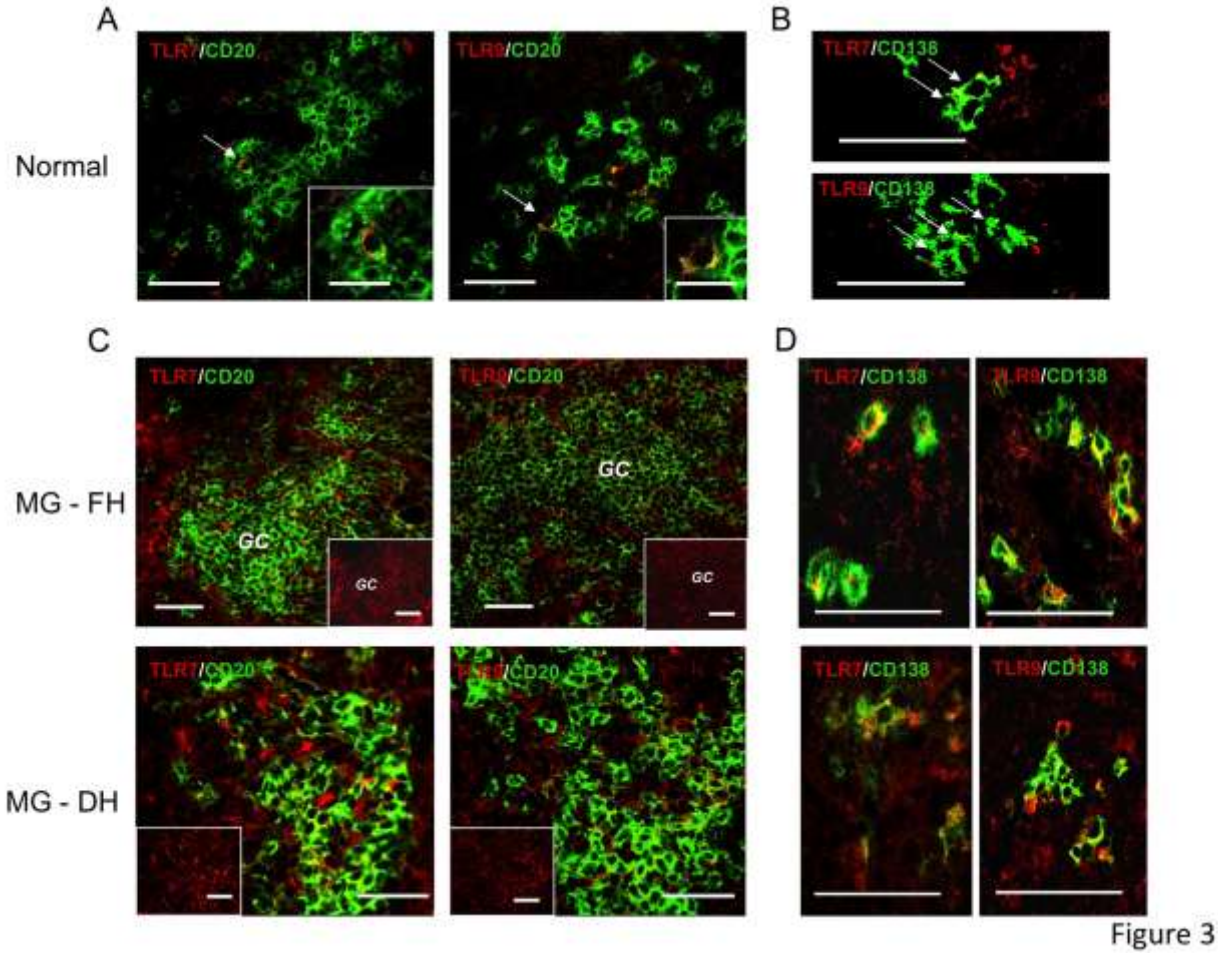


Figure 3

Fig. 3. Increased TLR7 and TLR9 expression in B cells and plasma cells of MG thymuses. (A) Double immunofluorescence staining of normal thymuses (Normal) for the B-cell marker CD20 (green) and TLR7 (red, left panel) or TLR9 (red, right panel). The insets display enlargements of the double positive cells shown in the main panels. (B) Sections of normal thymuses co-labeled for CD138 (green), a marker of plasma cells, and TLR7 (red, top panel) or TLR9 (red, low panel). The arrows in (A) and (B) indicate double positive cells. (C)

and (D) Sections of follicular (MG-FH) and diffuse (MG-DH) hyperplastic MG thymuses co-labeled for TLR7 (red, left panels) or TLR9 (red, right panels) in combination with CD20 (green, C panels) or CD138 (green, D panels). GC: germinal center. In the C insets, the same thymic areas of the main panels are displayed to reveal only TLR7 or TLR9 positivity. Magnification bars in the main panels: 50 μm; magnification bars in the insets: 20 μm (A) and 50 μm (C).

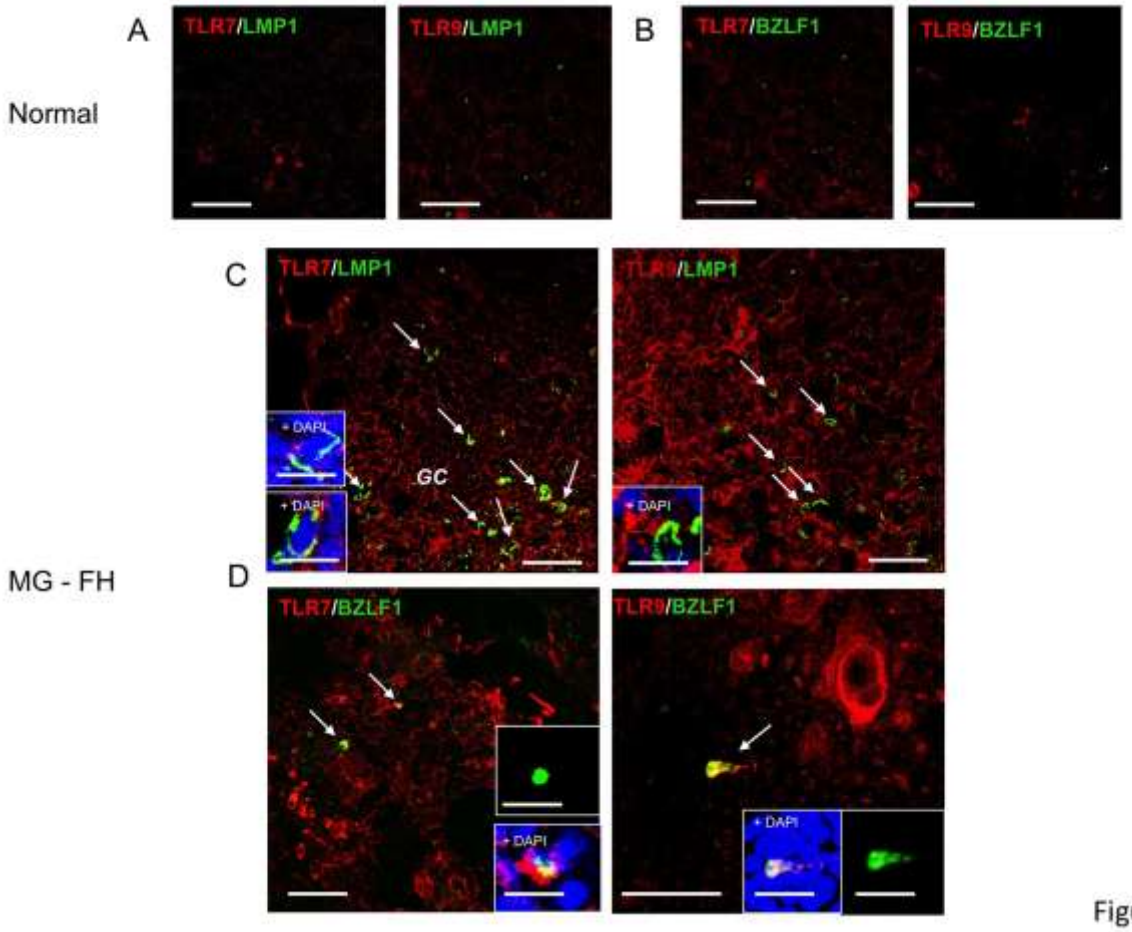


Fig. 4. Detection of EBV-infected cells positive for TLR7 and TLR9 in MG thymuses. (A) and (B) Sections from normal thymuses (Normal) co-labeled for TLR7 (red, A and B right panels) or TLR9 (red, A and B left panels) in combination with the EBV latency LMP1 (green, A panels) and the EBV lytic BZLF1 (green, B panels) proteins. (C) and (D) Sections from follicular hyperplastic MG thymuses (MG-FH) co-labeled for TLR7 (red, C and D left panels) or TLR9 (red, C and D right panels) in combination with LMP1 (green,

C panels) and BZLF1 (green, D panels). The arrows indicate double positive cells; the insets in the C and D panels display enlargement of the double positive cells to show DAPI staining (blue) or the nuclear BZLF1 positivity (green). GC: germinal center. Magnification bars in the main panels: 50 μm; magnification bars in the insets: 20 μm.

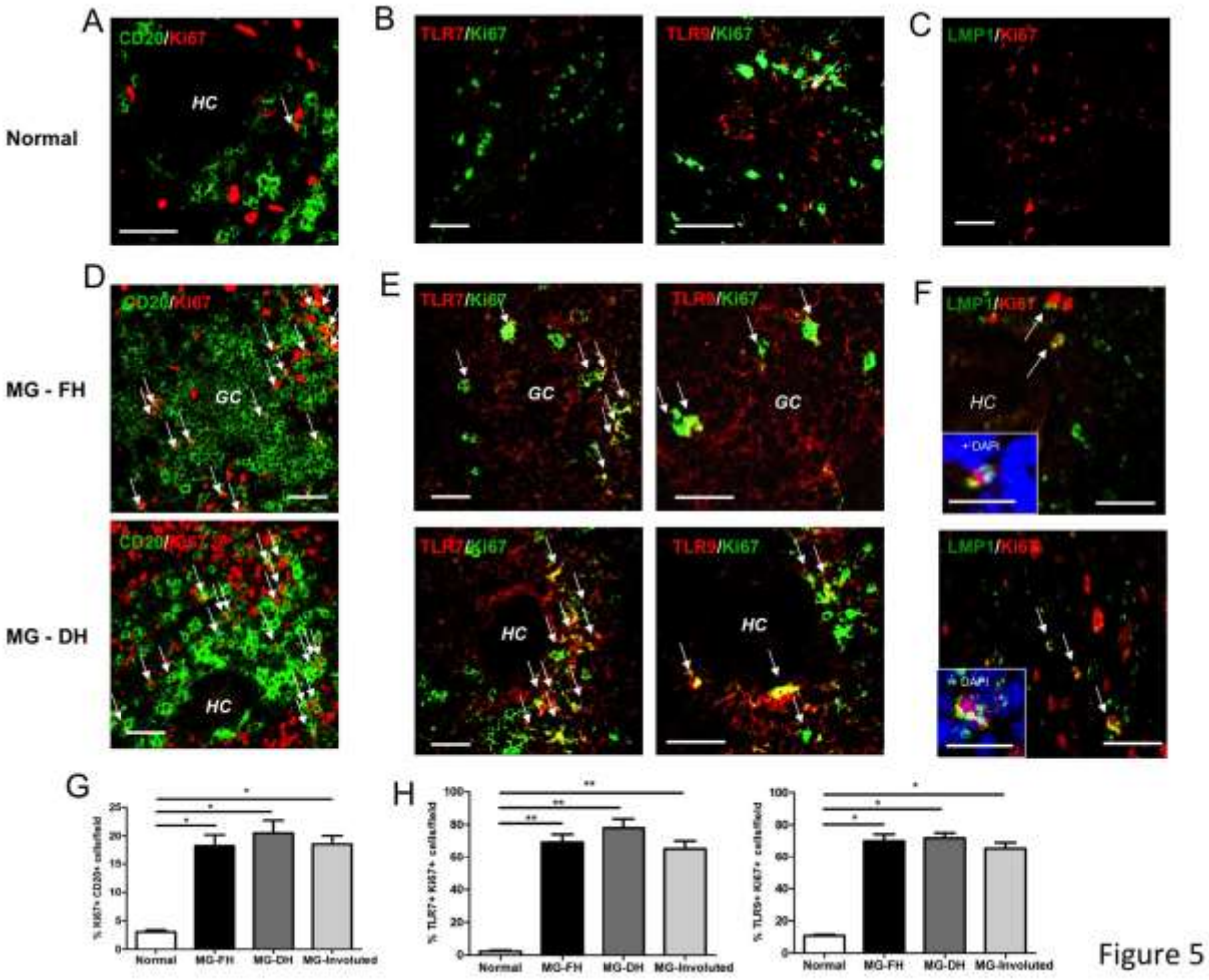


Figure 5

Fig. 5 Increased frequency of proliferating B cells in MG thymuses and co-localization of the proliferation-associated Ki67 marker with TLR7, TLR9 and EBV antigens. (A) to (C) Double immunofluorescence staining of normal thymuses (Normal) for the proliferation-associated Ki67 antigen (red in A and C panels; green in B panels) combined with the B cell marker CD20 (green, A panel), TLR7 (red, B left panel), TLR9 (red, B right panel), and the EBV latency protein LMP1 (green, C panel). (D) to (F) Double

immunofluorescence staining of follicular (MG-FH) and diffuse (MG-DH) hyperplastic MG thymuses for Ki67 (red in D and F panels; green in E panels) combined with CD20 (green, D panels), TLR7 (red, E left panels), TLR9 (red, E right panels), and the EBV latency protein LMP1 (green, F panels). The arrows show double positive cells. The insets in (F) panels show cells of the main panels (arrows) co-labeled for LMP1 (green), Ki67 (red) and DAPI (blu). Images on involuted thymus stainings are not shown. HC: Hassall's corpuscles; GC: germinal center. Magnification bars in the main panels: 50 □m; magnification bars in the insets: 20 μm. (G) and (H) CD20/Ki67 (G), TLR7/Ki67 (H left graph) and TLR9/Ki67 (H right graph) double positive cells were counted in 4 adjacent field per section in 5 normal, 5 MG-FH, 5 MG-DH, and 3 MG involuted thymuses. Bars in the (G) graph correspond to the mean percentage (± SEM) of CD20-positive B cells that were also positive for Ki67 per field in each sample group; bars in the (H) graphs correspond to the mean percentage (± SEM) of Ki67-positive cells expressing TLR7 (left graph) or TLR9 (right graph) per field in each sample group. P values were assessed by Kruskal-Wallis test with Bonferroni post-hoc test; *$p<0.05$; **$p<0.01$.

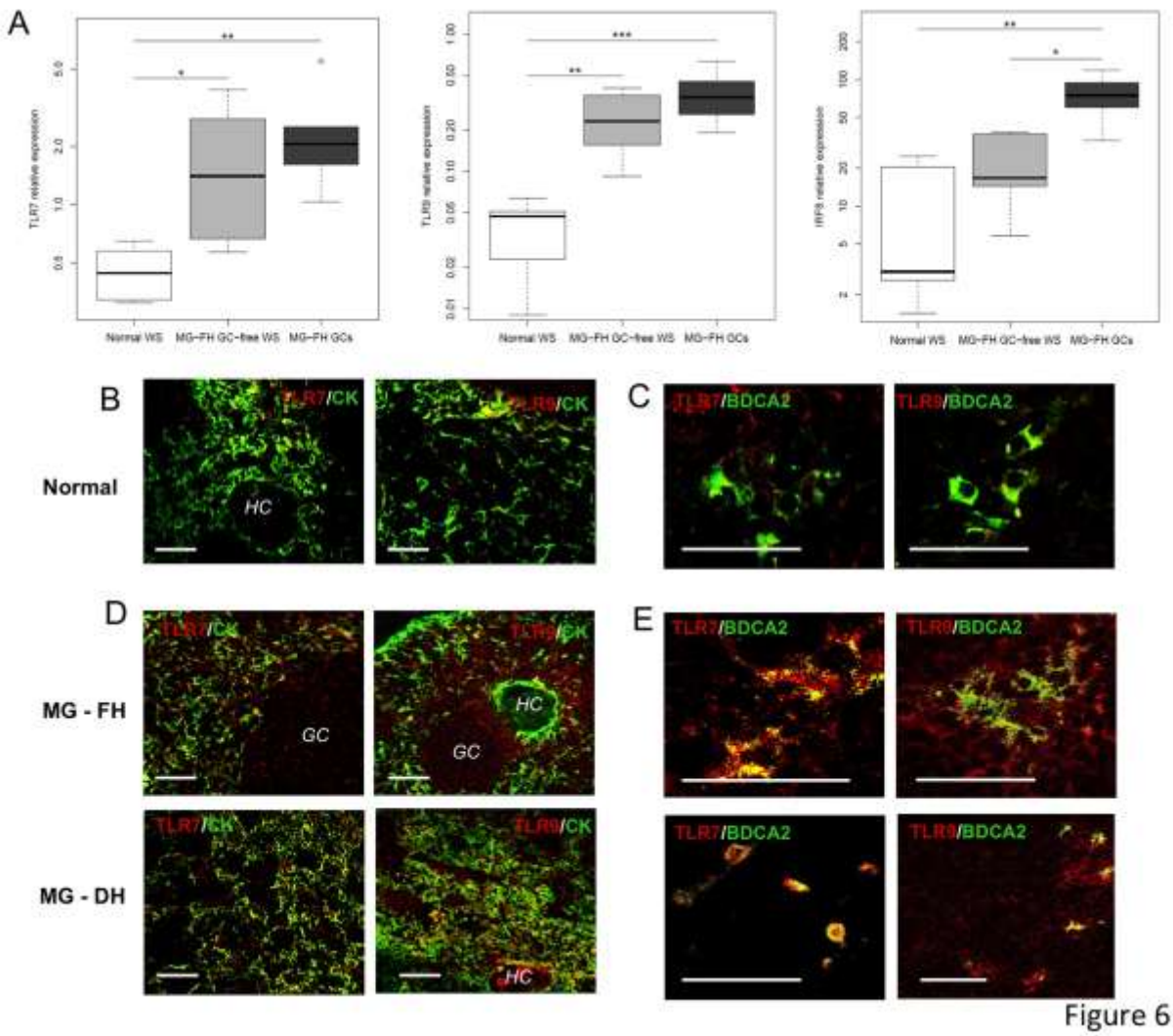


Figure 6

Fig. 6. Increased TLR7 and TLR9 mRNA levels in MG thymic sections devoid of germinal centers (GCs) and over-expression of the two receptors in MG thymic epithelium and plasmacytoid dendritic cells (pDCs). (A) Real-time PCR analysis to assess TLR7, TLR9 and interferon regulatory factor 8 (IRF8) transcriptional levels in whole sections from normal thymuses (Normal WS, n=6), thymic GCs-free sections (MG-FH GC-free WS) from follicular hyperplastic MG

thymuses (n=6) and the corresponding laser capture microdissected GCs (MG-FH GCs). IRF8 gene was analyzed as marker of GC B cells. In the boxplots, mRNA levels of the three genes were expressed as relative values ($2^{-\Delta ct}$x100) normalized towards the housekeeping gene GAPDH; dark horizontal lines represent means, with the box representing the 25^{th} and 75^{th} percentiles, the whiskers the 5^{th} and 95^{th} percentiles, and the dot an outlier. *P* values were assessed by Kruskal-Wallis test with Bonferroni post-hoc test. *p<0.05; **p<0.01; ***p<0.001. (B) to (E) Sections from normal thymuses (Normal) (B and C), follicular (MG-FH) and diffuse (MG-DH) hyperplastic MG (D and E) thymuses co-labeled for TLR7 (red, left panels) or TLR9 (red, right panels) in combination with cytokeratin (CK) (green, B and D panels), marker of epithelial cells, and BDCA2 (green, C and E panels), marker of pDCs. HC: Hassall's corpuscles; GC: germinal center. Magnification bars: 50 μm.

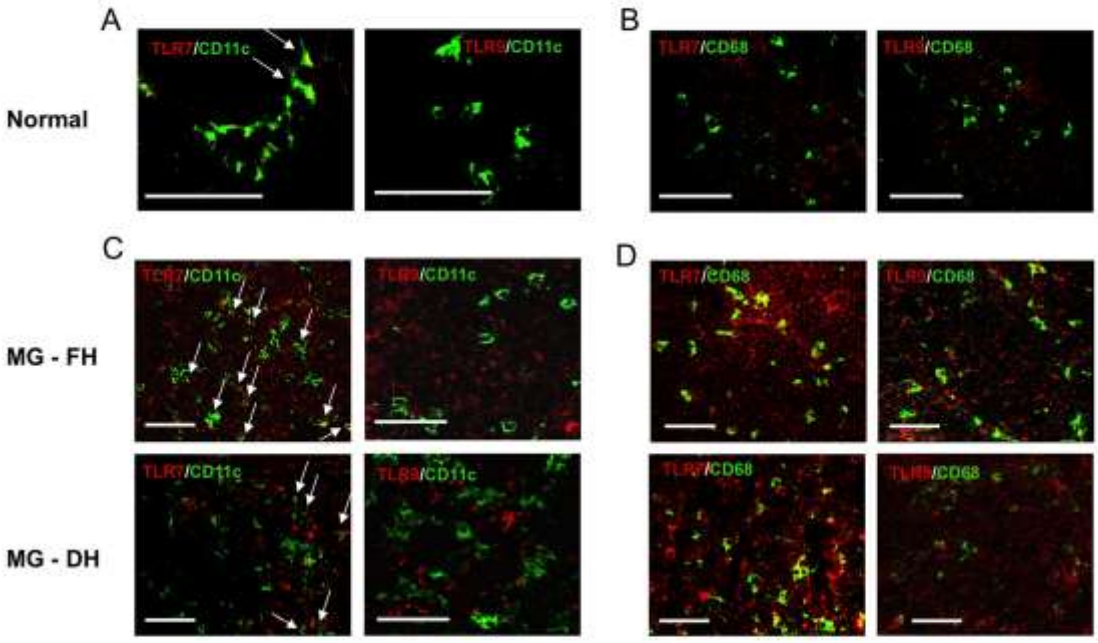
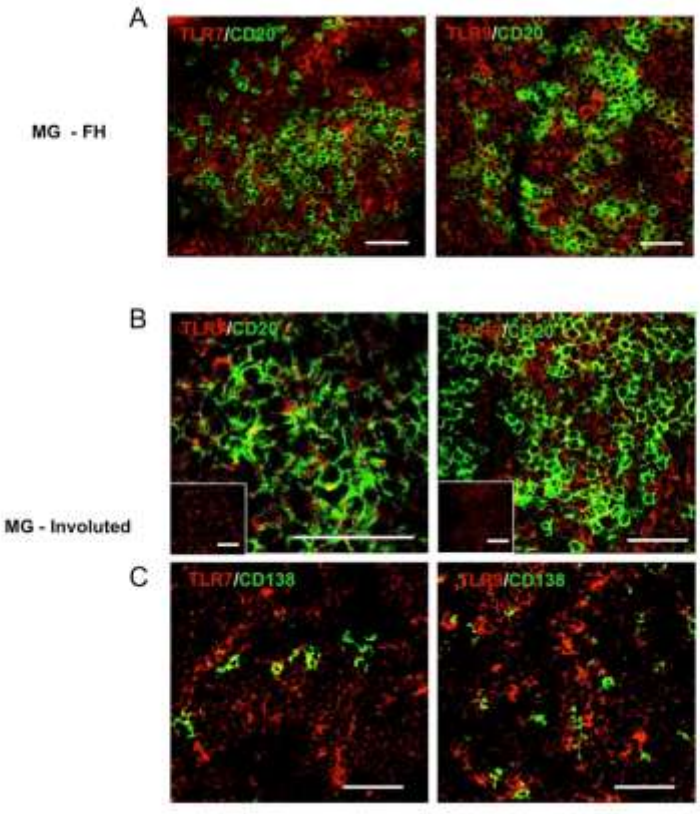


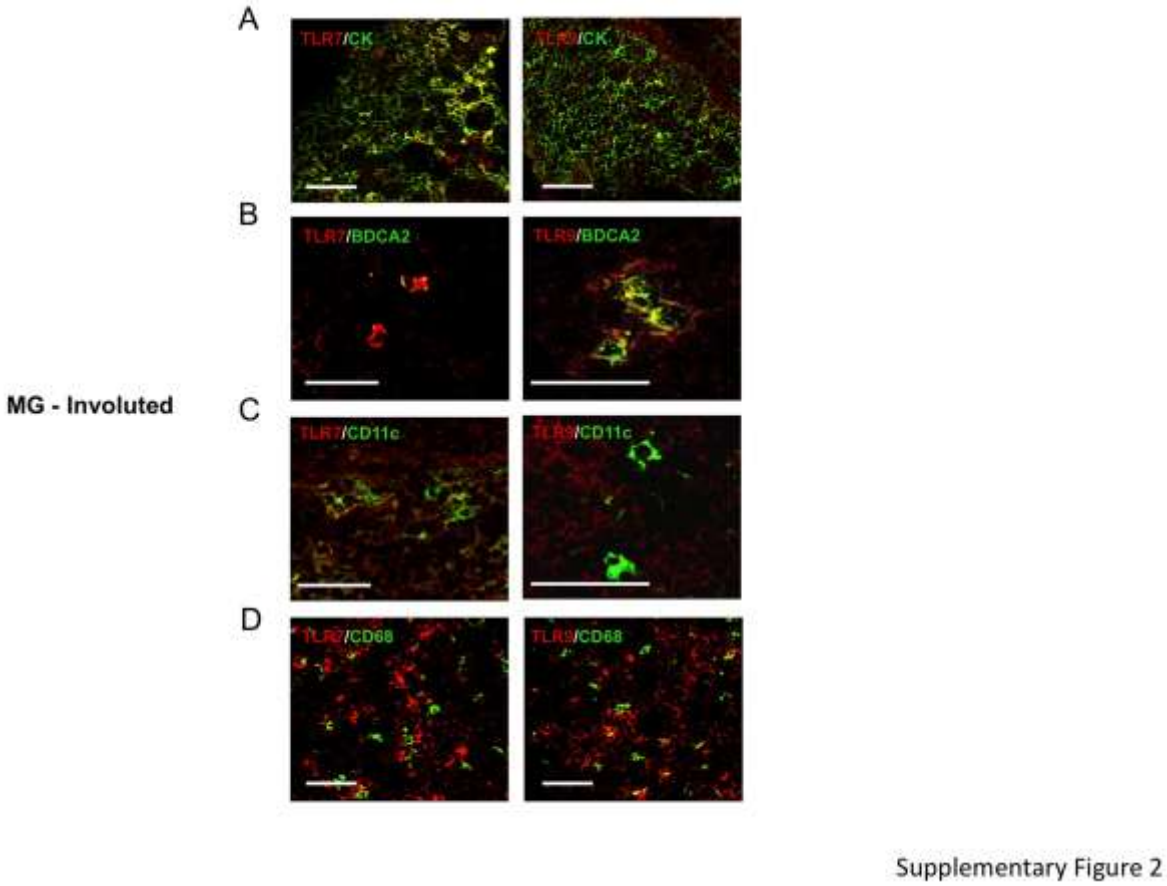
Figure 7

Fig. 7. Expression of TLR7 and TLR9 in myeloid dendritic cells (mDCs) and macrophages of normal and myasthenic thymuses. Sections from normal thymuses (Normal) (A and B), follicular (MG-FH) and diffuse (MG-DH) hyperplastic MG (C and D) thymuses co-labeled for TLR7 (red, left panels) or TLR9 (red, right panels) in combination with CD11c (green, A and C panels), marker of mDCs, and CD68 (green, B and D panels), marker of macrophages. Magnification bars: 50 μm.

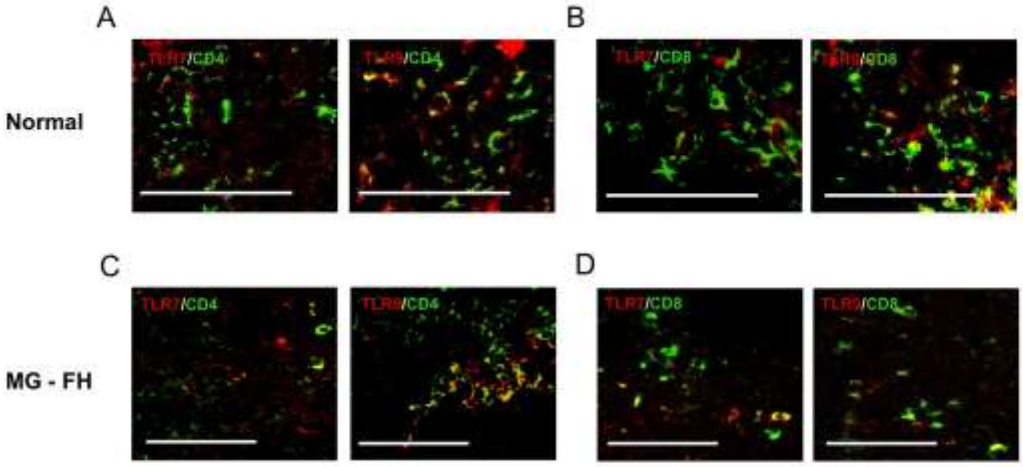


Supplementary Figure 1. Increased expression of TLR7 and TLR9 in B-cell lymphoid infiltrates of follicular hyperplastic MG thymuses, and in B cells and plasma cells of involuted MG thymuses. (A) Double immunofluorescence staining of follicular hyperplastic MG thymuses (MG-FH) for the B-cell marker CD20 (green) and TLR7 (red, left panel) or TLR9 (red, right panel). The two panels show

medullary areas of MG-FH thymuses characterized by the presence of B-cell infiltrates not organized into germinal centers. (B) and (C) Sections from involuted MG thymuses (MG-Involuted) co-labeled for TLR7 (red, left panels) or TLR9 (red, right panels) combined with CD20 (green, B panels) and CD138 (green, C panels). In the (B) insets, the same thymic areas of the main panels are displayed to reveal only TLR7 or TLR9 positivity. Magnification bars in the main panels and insets: 50 μm.

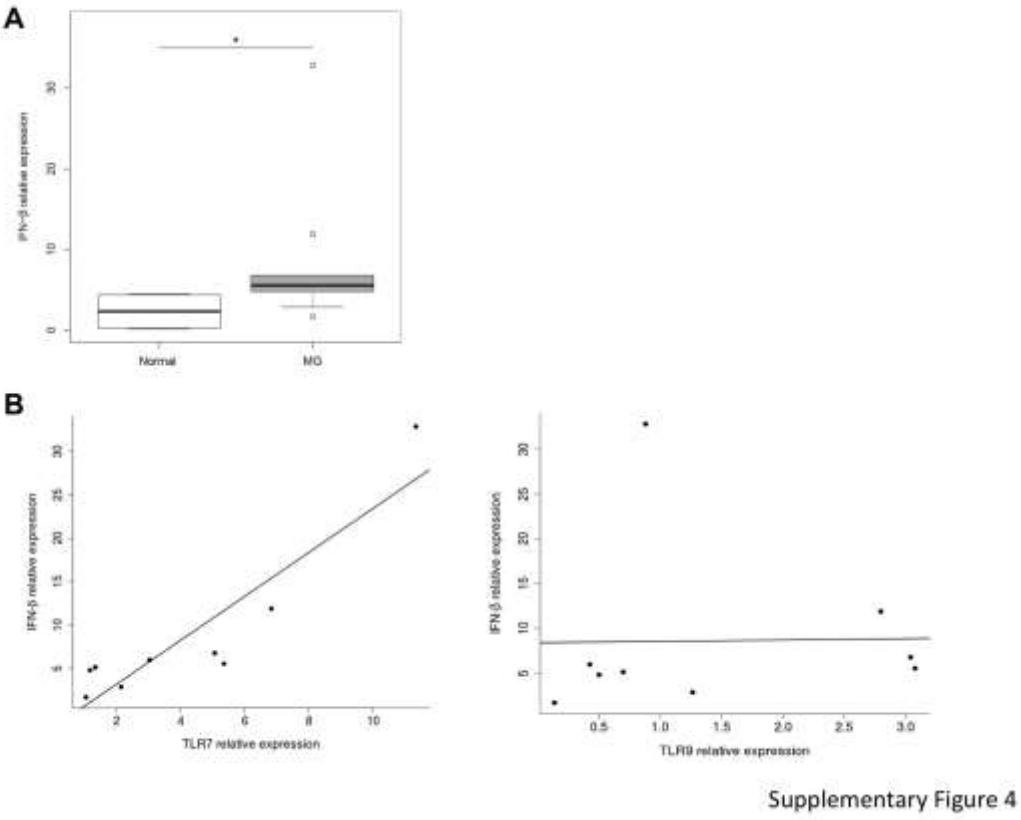


Supplementary Figure 2. Expression of TLR7 and TLR9 in epithelial cells, plasmacytoid dendritic cells (pDCs), myeloid dendritic cells (mDCs) and macrophages of involuted MG thymuses. Sections of involuted MG thymuses (MG-Involuted) co-labeled for TLR7 (red, left panels) or TLR9 (red, right panels) combined with: cytokeratin (CK) (green, A panels), marker of epithelial cells; BDCA2 (green, B panels), marker of pDCs; CD11c (green, C panels), marker of myeloid dendritic cells; and CD68 (green, D panels), marker of macrophages. Magnification bars: 50 μm.



Supplementary Figure 3

Supplementary Figure 3. Expression of TLR7 and TLR9 in T cells of normal and MG thymuses. Sections of (A and B) normal thymuses (Normal) and (C and D) follicular hyperplastic MG thymuses (MG-FH) co-labeled for TLR7 (red; left panels) or TLR9 (red; right panels) combined with CD4 (green, A and C panels) and CD8 (green, B and D panels). Magnification bars: 50 μm.



Supplementary Figure 4. Putative relationship between TLR7 over-expression and IFN-β induction in MG thymuses. (A) Real-time PCR to assess transcriptional levels of IFN-β, MX1 and RIG-I in normal (Normal, n=5) and MG thymuses (n=9). In the boxplots, mRNA levels were expressed as relative values ($2^{-\Delta ct}$x100) normalized towards the housekeeping gene GAPDH; dark horizontal lines represent means, with the box representing the 25th and 75th percentiles, the whiskers the 5th and 95th percentiles, and the dots the outliers. *P* values were assessed by Mann-Whitney test. *p<0.05;

***$p<0.001$. (B) Positive correlation between TLR7 and IFN-β mRNA levels (Spearman test, $r=0.90$; $p<0.01$), but not between TLR9 and IFN-β mRNA levels (Spearman test, $r=0.45$; $p>0.05$), in MG thymuses.

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Chapter 4

4.1. Summary and Conclusions

MG is a rare autoimmune disorder of the NMJ, mainly characterized by muscle weakness. In approximately 80% of MG patients the target of the autoimmune response is the AChR. AChR-MG is the best-investigated MG clinical subgroup [1,2].

Growing evidence supports the role of the thymus in AChR-MG pathogenesis. Within the thymus there are all the cellular components essential to initiate and sustain an anti-AChR autoimmune response, including AChR-expressing TECs and myoid cells, DCs, self-reactive anti-AChR T cells, and antibody-secreting plasma cells [3-7]. Moreover, TLR activation (*e.g.* TLR3, TLR4, TLR7, and TLR9) and chronic inflammation are key features of MG thymuses contributing to the intra-thymic autosensitization to AChR [8-12]. The exact mechanisms underlying AChR-MG pathogenesis are not entirely defined. A comprehensive analysis of the molecular alterations that occur in the peripheral vascular system could provide a link between the intra-thymic pathology and the muscle autoimmune processes.

Whole-transcriptome sequencing (total RNA-sequencing) is an high-throughput strategy that might be useful to fill this gap [13]. Recently, this technology has been applied in autoimmune disorders, such as rheumatoid arthritis and psoriasis, helping to better understand the molecular basis of the diseases [14,15].

In our study, whole-transcriptome sequencing was applied to study the peripheral transcriptional profile in a clinically-defined

cohort of AChR-EOMG patients and age- and gender-matched healthy donors. In peripheral blood cells, we identified 128 coding transcripts and 229 long non-coding RNAs, including 9 microRNA precursors, as differentially expressed between AChR-EOMG patients and healthy controls. The dysregulated coding transcripts were significantly enriched in ‘infectious-disease’, ‘inflammatory disease’ and ‘inflammatory response’ functional categories, suggesting that ‘infection-’ and ‘inflammatory-associated’ molecular factors are key hallmarks of AChR-EOMG peripheral blood cells. Validation of selected transcripts highlighted the role of the ‘infection-related’ *PPP1R15A* transcript in MG pathogenesis, found to be up-regulated in AChR-EOMG patients. *PPP1R15A* was of particular interest since it has been reported that it is necessary for the TLR3-mediated IFN-β production in the control mechanisms of Chikungunya virus infection in mice [16]. Cufi and colleagues recently showed that the expression of AChR α subunit in MG thymuses increased upon TLR3 activation via the release of IFN-β, sustaining an anti-AChR antibody production [9]. Therefore, the up-regulation of *PPP1R15A* in peripheral blood cells of AChR-EOMG patients might have a role in the perturbation of the inflammatory status and in the autoimmune response in periphery.

MicroRNAs (miRNAs) are small non-coding RNAs that bind to the 3’ untranslated region of target mRNAs mainly inducing post-transcriptional silencing of gene expression [17]. The alteration of miRNAs has been reported in several autoimmune disorders, including rheumatoid arthritis, psoriasis, and systemic lupus erythematosus [18-21]. Investigations exploring miRNAs in MG are limited. In our study, we identified and validated dysregulated

miRNAs (*i.e.* miR-612, miR-3654, miR-3651, and pre-miR-3651) associated with AChR-EOMG that have never been described before. However, further studies addressing their regulatory mechanisms of gene expression and their role in AChR-EOMG pathogenesis should be performed. Nevertheless, bioinformatics analysis showed that the differentially expressed *HRH4* transcript is one putative target of the dysregulated miR-612 and also anti-correlation analysis revealed a potentially direct connection between miR-612 and *HRH4*. Of interest, *HRH4* has an immunomodulatory function and it is also involved in many inflammatory-associated diseases such as rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis [22-24].

In summary, our data reveal a new 'infection-' and 'inflammatory-related' molecular signature in peripheral blood cells from AChR-EOMG patients. Although further investigations are required to better understand the functional role of the dysregulated molecules here identified, our data highlight their possible implications in disease pathogenesis.

These findings are in line with our recently published data regarding the contribution of EBV infection in the alteration of TLR-mediated immune responses in MG thymuses [12]. We found that TLR7 and TLR9 expression levels were increased in B cells and plasma cells of MG versus normal thymuses and co-localized with EBV latent and lytic proteins [12]. Since EBV may provide TLR7 (*e.g.* single-stranded RNAs) and TLR9 ligands (*e.g.* CpG motif-containing EBV genome) [25-27], we suggested that EBV may activate TLR7- and TLR9-mediated signalling pathways in MG

thymuses. Indeed, we observed that IFN-β was increased in EBV-infected MG thymuses along with type I IFN-inducible genes (*i.e.* MX1 and RIG-I), and TLR7 mRNA levels positively correlated with those of IFN-β, supporting the hypothesis that EBV-driven TLR7 signalling might contribute to IFN-β secretion in MG thymuses. To conclude, we hypothesised that EBV-mediated TLR7/9 abnormal activation, in combination with antiviral type I IFN production, may favour inflammatory responses thus contributing to intra-thymic MG pathogenesis.

Taken together, our findings suggested that in the context of a genetic susceptible background, triggering event(s), such as EBV infection, may alter innate immune responses favouring the establishment of a chronic inflammatory state within MG thymus. This scenario might be reflected and perpetuated in the peripheral vascular system that our data revealed to be characterized by altered 'infection' and 'inflammatory-associated' molecules, which in turn may sustain the autoimmune process.

4.2. Future perspectives

MG is a treatable disease, although the therapies, that are currently used, do not lead to a complete stable remission in all patients and may induce severe side effects. A better investigation into the molecular mechanisms of disease pathogenesis may allow the development of more effective targeted therapies [28,29]. Our studies helped to gain knowledge regarding the immunological and molecular alterations that occur in the thymus and in the peripheral blood cells of MG patients. Although further studies are needed to elucidate the

exact molecular mechanisms linked to MG pathogenesis, our data may represent an important source towards the development of new target-specific therapeutic approaches for MG, providing a possible valuable application from bench-to-bedside in translational medicine.

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Publications

Increased expression of Toll-like receptors 7 and 9 in myasthenia gravis thymus characterized by active Epstein-Barr virus infection

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