

Circulating microRNAs in myasthenia gravis (MG)

Anna Rostedt Punga, MD, PhD¹ and Tanel Punga, PhD²

¹Department of Medical Sciences, Clinical Neurophysiology and ²Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden

ABSTRACT

One of the main difficulties in predicting the clinical course of myasthenia gravis (MG) is the heterogeneity of the disease, where disease progression differs greatly depending on the patient's subgroup. MG subgroups are classified according to the age of onset [early onset MG (EOMG; onset \leq 50 years) versus late-onset MG (LOMG; onset $>$ 50 years)]; the presence of a thymoma (thymoma associated MG); antibody subtype [acetylcholine receptor antibody seropositive (AChR+), muscle-specific tyrosine kinase antibody seropositive (MuSK+)]; or presence of autoantibodies against low-density lipoprotein receptor-related protein 4 (Lrp4) or agrin as well as clinical subtypes (ocular versus generalized MG). The diagnostic tests for MG, such as autoantibody titers, neurophysiological tests, and objective clinical fatigue scores, do not necessarily reflect disease progression. Hence, there is a great need for reliable, objective biomarkers in MG to follow the disease course and the individualized response to therapy toward personalized medicine. In this regard, circulating microRNAs (miRNAs) have emerged as promising potential biomarkers due to their accessibility in body fluids and unique profiles in different diseases, including autoimmune disorders. Several studies on circulating miRNAs in MG subtypes have revealed specific miRNA profiles in patient sera. In generalized AChR+ EOMG, miR-150-5p and miR-21-5p are the most elevated miRNAs, with lower levels observed upon treatment with immunosuppression and thymectomy. In AChR+ generalized LOMG, miR-150-5p, miR-21-5p, and miR-30e-5p levels are elevated and decreased by the clinical response after immunosuppression. In ocular MG, higher levels of miR-30e-5p discriminate patients who will later generalize from those remaining ocular. In contrast, in MuSK+ MG, the levels of the let-7 miRNA family members are elevated. Studies of circulating miRNA profiles in Lrp4 or agrin antibody seropositive MG are still lacking. This review summarizes the present knowledge of circulating miRNAs in different subgroups of MG.

Keywords: circulating microRNA, myasthenia gravis, miR-150-5p, miR-21-5p, miR-30e-5p, biomarker.

1. Introduction

Myasthenia gravis (MG) is an autoimmune neuromuscular disorder that causes fatigable skeletal muscle weakness. The global incidence and prevalence of MG are increasing in adults at all ages of onset, with an annual incidence of roughly 10–29 cases per million and a prevalence ranging from 100 to 350 cases per million people (1). MG is a heterogeneous disease with different subgroups based on serological status, age at onset, clinical phenotype, and association with thymic pathology. The serological subgroups include patients that have antibodies against the nicotinic acetylcholine receptor (AChR; ~85%), the muscle-specific tyrosine kinase (MuSK; ~7%), and lipodensity related protein 4 (Lrp4; ~1-2%) (2). Early-onset MG (EOMG) refers to patients with onset of the disease between ages 19–50 years and typically affects women with AChR antibody-positive (AChR+) MG and thymus hyperplasia. Late-onset MG, instead, is more common in men with atrophic thymus. Recently, a group of very-late-onset in the ages above 65 has been described (3). These subgroups can be further subdivided according to clinical weakness into MG affecting only the extraocular muscles, known as ocular MG (OMG), or MG affecting skeletal muscle groups outside the ocular area, called generalized MG (GMG). Most patients present with extraocular manifestations alone; however, up to 85% of patients develop the generalized disease within two years of symptom onset. Since MG patients can have different patterns of fatigable muscle weakness over time (4) and the disease is very heterogeneous and fluctuating, there is a strong need for prognostic biomarkers of MG progression and treatment outcome. Autoantibodies are valuable diagnostic biomarkers; however, autoantibody titers do not necessarily correlate with disease severity of treatment response (5). Circulating microRNAs (miRNAs) are easily measured in blood samples and are changed in different disease states (6). Therefore, they have also been suggested as potential prognostic biomarkers in MG (7, 8). This review summarizes the data on circulating miRNAs in MG.

2. Extracellular circulating microRNAs (miRNAs)

MiRNAs are short, endogenous non-coding RNA molecules that interact specifically with mRNAs. Due to their specific interaction with different mRNA molecules, they can control the stability and translation of mRNA. Indeed, miRNA interactions with various mRNAs have been shown to regulate critical cellular processes, including differentiation, proliferation, and apoptosis (9). It has been estimated that about 2300 true mature miRNAs regulate the expression of more than 60% of protein-coding genes. Altered miRNA expression is found in several disease states, including cancer, cardiovascular and autoimmune diseases (10–12). In addition to their intracellular accumulation, mature miRNAs are detectable outside the cells, in the extracellular space.

2.1. Circulating miRNA as potential biomarkers

Circulating miRNAs can be found in human body fluids, including plasma and serum. Notably, circulating miRNAs are stable and can withstand low pH and multiple freeze-thaw cycles (13). One of the reasons for this stability is that circulating miRNAs are embedded into membrane-enclosed extracellular vesicles, such as microvesicles and exosomes (14). Although the microvesicles and exosomes are structurally similar, they differ in size and cellular origin. Notably, both vesicles contain embedded miRNAs and are released from the cells under physiological and pathophysiological conditions (15).

Circulating miRNAs can be considered paracrine and endocrine signaling molecules that can alter gene expression on nearby and distant target cells (16). Furthermore, a correlation between circulating miRNA levels and disease status has highlighted these molecules as potential biomarkers for diagnosis and disease monitoring (17). Circulating miRNAs fulfill the requirements for a biomarker as they are specific, very stable, easily accessible in a minimally invasive manner, and their detection is cost-effective. The number of studies showing circulating miRNAs as potential biomarkers is constantly rising. Quantitative reverse transcription PCR (qRT-PCR) is often considered the standard method to evaluate miRNA expression profiles since this method is robust, easy to perform, and quick (18). However, normalization of the qRT-PCR data between different circulating miRNA samples is challenging due to the lack of a universal “housekeeping gene” (17). However, miR-191 is useful as a housekeeping gene for normalization purposes, both in serum and plasma miRNA studies since it is consistently detected in most patients (19-21). Given that most blood samples are stored as serum and there are more RNA degrading enzymes (RNases) present in plasma, miRNA profiles are often analyzed in serum.

2.2. Circulating miRNA profiles in MG subgroups

2.2.1. Acetylcholine receptor antibody seropositive (AChR+) early-onset MG (EOMG)

EOMG primarily affects women and is often associated with thymic hyperplasia. In AChR+ EOMG female GMG patients without immunosuppressive treatment, the serum levels of the immunomiRNAs miR-150-5p and miR-21-5p are elevated, whereas the miR-27a-3p level is reduced, compared to matched healthy control women (22) (**Figure 1**). Also, in sera from more heterogeneous clinical cohorts of male and female AChR+ and AChR- MG patients, miR-150-5p and miR-21-5p levels are elevated compared to healthy controls and patients with other autoimmune diseases, such as psoriasis and Addison’s disease. The levels of miR-150-5p and miR-21-5p are significantly lower in the sera from MG patients on immunosuppressive treatment than those who are immunosuppressive naïve (23).

Serum levels of miR-150-5p are reduced upon thymectomy in line with clinical improvement in AChR+ patients (22, 24). Longitudinal analysis of miR-150-5p and miR-21-5p in the prospective randomized control trial termed MGTX indicated that miR-150-5p levels decreased significantly two years after thymectomy, whereas no significant reduction was found in the group treated with prednisone (24). Further, rituximab treatment reduces the serum exosomal miR-150-5p levels in correlation with clinical MG scores and patients’ prednisone requirement (25). Intriguingly, serum miR-150-5p and miR-21-5p levels are also lowered after a 12-week physical exercise intervention in MG patients (26).

The aforementioned circulating miRNAs are not the only reported alterations in AChR+ MG patient biofluids. Another profiling of circulating miRNAs in different AChR+ MG patients [EOMG, LOMG and thymoma associated MG (TAMG)] sera revealed that at least seven miRNAs were downregulated (miR-15b, miR-122, miR-140-3p, miR-185, miR-192, miR-20b, miR-885-5p) compared with healthy controls (27). Nevertheless, in this study, miRNA differences were not found between treated and untreated MG patients (27). Two other studies confirmed lower serum levels of miR-20b in patients with TAMG (28, 29). Serum miR-20b was downregulated both in generalized and AChR+ ocular MG (OMG) patients, and miR-20b expression in generalized MG was much lower than that found in OMG (28). Furthermore, miR-20b levels increased after treatment with corticosteroids in this particular study (28).

2.2.2. Late-onset MG (LOMG)

In LOMG, most patients are male and often have thymus atrophy, in contrast to EOMG, which primarily affects women and is associated with thymic hyperplasia (30). Nevertheless, the majority of LOMG patients also are AChR+. Five miRNAs were found to be elevated in sera from LOMG patients with no immunosuppressive treatment: miR-106b-3p, miR-30e-5p, miR-223-5p, miR-140-5p, and miR-19b-3p (31) (**Figure 1**). To assess the prospective influence of these miRNAs in sera of immunosuppressive naïve generalized LOMG patients with immunosuppression, these miRNAs were longitudinally analyzed up to two years after the MG onset (31). Since 96% of these LOMG patients were AChR+, the previously found elevated miRNAs miR-21-5p and miR-150-5p (7) were also analyzed. After immunosuppression initiation, the steady decline in clinical MGC score at and after one-year follow-up in the LOMG cohort correlated with reduced levels of miR-150-5p, miR-21-5p, and miR-30e-5p (31). LOMG patients with generalized disease had higher miR-150-5p and miR-21-5p than those with purely ocular symptoms (31) (**Figure 1**).

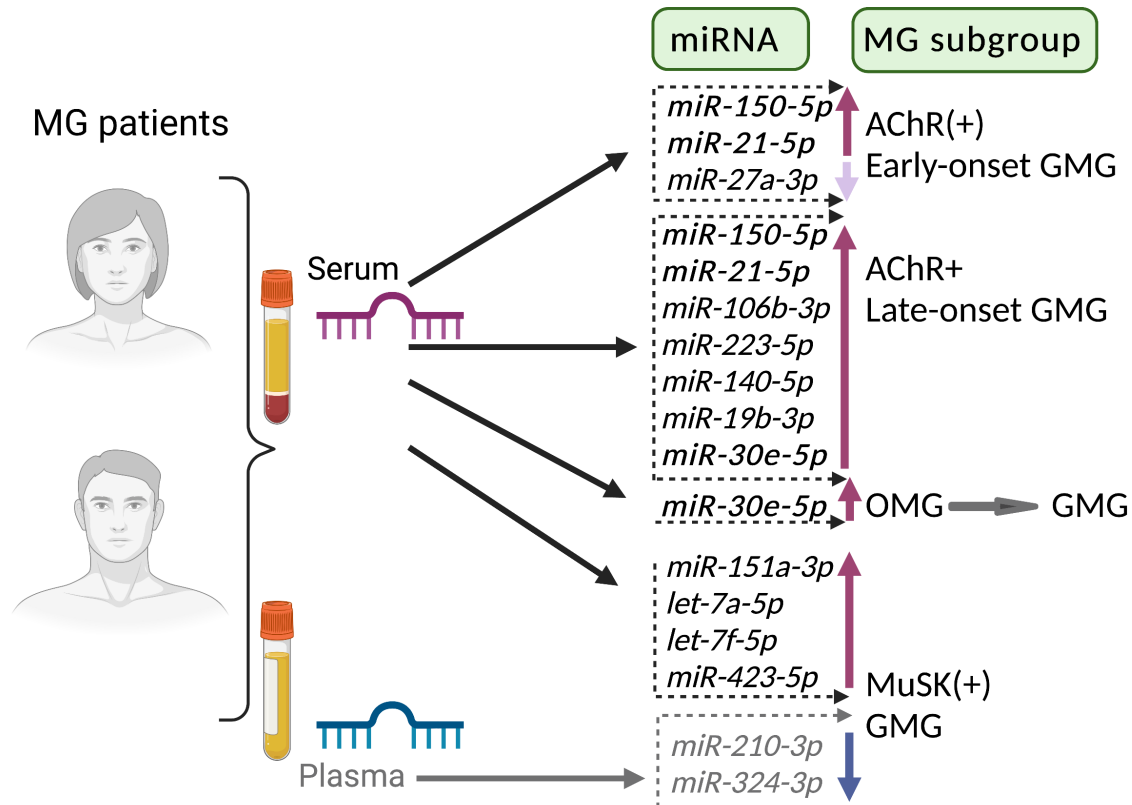


Figure 1. Summary of the circulating microRNAs in serum and plasma that are found associated with the different subgroups of myasthenia gravis. MicroRNAs highlighted in bold have been shown to be reduced upon thymectomy (*miR-150-5p*) or immunosuppression (*miR-150-5p*, *miR-21-5p* and *miR-30e-5p*). Arrows indicate increased or reduced levels of the miRNAs. MG, myasthenia gravis; GMG, generalized MG; OMG, ocular MG; AChR(+), acetylcholine receptor antibody seropositive; MuSK(+), muscle-specific tyrosine kinase antibody seropositive.

2.2.3 Ocular MG (OMG)

OMG is defined as clinical MG symptoms and signs only in the extraocular muscles, manifesting as ptosis and diplopia. There are no predictive markers for the risk of conversion from OMG to GMG; however, AChR+ MG patients are considered to have a higher risk for generalization than AChR antibody seronegative patients (32). Due to differences in some miRNAs in LOMG patients (31), one study aimed at determining whether serum miRNAs could be used as potential predictors of the generalization of OMG (33). For this purpose, 83 OMG serum samples (82 immunosuppression treatment naïve) were assayed within three months of OMG diagnosis and at a follow-up visit. The *miR-30e-5p* and *miR-150-5p* were significantly higher in patients who developed GMG than those who remained with OMG. Of these two miRNAs, *miR-30e-5p* has 96% sensitivity for differentiating OMG and GMG in all patients and 100% in LOMG patients (33) (Figure 1). Considering that treatment with corticosteroids could modify the progression of OMG to GMG (34) and that half of the OMG patients generalize within one year (35), predictive biomarkers would be helpful to tailor the

immunosuppressive treatment of individual OMG patients. This could, for example, imply initiating immunosuppressive therapy at an earlier stage if *miR-30e-5p* levels are higher.

2.2.4 Muscle-specific tyrosine kinase antibody seropositive (MuSK+) MG

MuSK+ MG is considered a more homogenous disease subtype that differs from AChR+ MG by having more bulbar symptoms, no thymic hyperplasia, and different treatment response (36). Therefore, it could be suspected that MuSK+ MG has a different profile of circulating miRNAs than AChR+ MG. In sera from MuSK+ MG patients, the profile of *miR-151a-3p*, *let-7a-5p*, *let-7f-5p*, and *miR-423-5p* are all increased compared to healthy matched control individuals (37).

As most blood samples are stored as serum, most studies have analyzed circulating miRNAs in serum; nevertheless, plasma concentrations of miRNAs cannot be presumed to be interchangeable (38). Analysis of the miRNA profile in the plasma of MuSK+ MG patients instead suggests lower values of two other miRNAs: *miR-210-3p* and *miR-324-3p* (20).

2.2.5. Unselected cohort of MG patients compared to other neuroimmune diseases.

Serum miR-30e-5p, miR-150-5p, and miR-21-5p levels correlate with clinical course in specific MG patient subgroups (**Figure 1**). In light of this, another study aimed at better characterizing these three miRNAs, regardless of the MG subgroup, shortly after MG onset and determining their sensitivity and specificity for MG diagnosis, as well as their predictive power for disease relapse (19). Serum levels of these miRNAs in 27 newly diagnosed MG patients were compared with 245 healthy individuals and 20 patients with non-MG neuroimmune diseases. Levels of miR-30e-5p and miR-150-5p significantly differed between MG patients and healthy controls; however, no difference was seen compared with patients affected by the other neuroimmune diseases (multiple sclerosis, Lambert-Eaton myasthenic syndrome, chronic inflammatory demyelinating polyneuropathy and inflammatory myelitis) (19). In all MG patients, miR-150-5p has a sensitivity of 85% and a specificity of 48%; higher values in EOMG with a sensitivity of 90% and specificity of 58% (19). This is in line with a previous study indicating high miR-150 levels are found in other autoimmune conditions, including multiple sclerosis. miR-30e-5p is more specific than sensitive for MG, with a sensitivity of 56% and specificity of 86% (19). Intriguingly, high levels of miR-30e-5p predicted MG relapse with a hazard ratio of 2.81 (19), in line with higher miR-30e-5p levels in those OMG patients who transitioned to GMG (21).

3. The link between circulating miRNAs in MG and intracellular pathophysiology

MiR-150-5p and miR-21-5p are so-called immuno-miRNAs and important regulators for developing and differentiating T cells (39). The effector organ in AChR+ EOMG, the thymus, is often characterized by hyperplasia with ectopic germinal centers consisting of infiltrating B cells (40, 41). MiR-150 is a marker of lymphocyte activation and regulates proliferation, apoptosis, and differentiation of natural killer (NK), T cells, and B cells (39, 42, 43) (44). MiR-150 expression is considerably higher in the germinal centers of the thymus of AChR+ EOMG patients compared to healthy controls (45). Further, miR-150 levels are lower in peripheral CD4+ T cells of AChR+ EOMG patients than in healthy controls. Thus, increased serum levels of miR-150-5p could result from released miR-150 from activated peripheral CD4+ T cells (45). One hypothesis is that miR-150 is regulated by its release into the extracellular space (46). There is a positive correlation between the B cell marker *CD19* mRNA and miR-150 expression in the thymus, which could implement an interaction between miR-150-5p and the CD19+ cells involved in the autoimmune response in MG (45). Furthermore, miR-150 treatment of PBMCs affects the main proto-oncogene MYB, and thus, miR-150 could play a role in EOMG both at the thymic level and in the periphery by modulating the expression of target

genes and peripheral cell survival (45). Expression of two pro-apoptotic genes targeted by miR-150: Tumor Protein 53 (P53) and Apoptosis Inducing Factor Mitochondria associated 2 (*AIFM2*), are also increased upon anti-miR-150-5p treatment (45).

The other immunomiRNAs, miR-21-5p, is highly expressed in T regulatory cells (39) and also associated with other autoimmune diseases such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (10, 47). MiR-21 is induced by several pro-inflammatory molecules, and can regulate the NF- κ B and NLRP3 pathways (48). NF- κ B activation promotes the hyper-expression of target genes involved in pro-inflammatory/stress-like responses, including pro-IL-1 β and pro-IL-18 (49). MiR-21 orchestrates the fine-tuning of the inflammatory response through direct and indirect activities on these pathways (48).

The third miRNA in AChR+ MG, miR-30e-5p, is somewhat contradictorily downregulated in EOMG (22) and upregulated in LOMG (31). Intriguingly, the low-density lipoprotein receptor-related protein 6 (LRP6), one of the critical co-receptors for Wnts (a family of genes that encode secretory glycoproteins), is a direct target of miR-30e (50). Thus, there is a potential role for miR-30e in regulating muscle homeostasis.

The let-7 miRNA family members have been extensively studied because of their broad functional role in various cellular processes, including neuronal development and embryogenesis (51, 52). The let-7 miRNAs stimulate the Toll-like receptor 7 (TLR7), thereby activating T cells (53). Further, the involvement of TLR7 in CD4+ T cells induces T cell unresponsiveness (54). Let-7a-5p and let-7f-5p are upregulated in PBMCs isolated from thymoma-associated MG patients (55), whereas let-7f-5p is instead downregulated in the thymus of AChR+ EOMG patients (56). Although a key role has been suggested for TLRs in thymic hyperplasia-associated EOMG, through abnormal activation of TLRs, the role of TLRs in MuSK+ MG remains to be defined (57).

Neither miR-210-3p nor miR-324-3p have previously been reported to be dysregulated in immune-mediated diseases. MiR-210-3p has been found to be dysregulated in several cancers (58), and miR-324-3p has been mentioned as a potential biomarker in osteoporosis (59).

4. Conclusion

In summary, circulating miRNAs could serve as potential biomarkers in MG and MG subgroups to monitor the disease course. miR-150-5p is highly sensitive but has low specificity for MG. In contrast, miR-30e-5p has the most significant potential as a predictive biomarker for the disease course in MG, regardless of the subgroup. Multicenter trials for validation of these miRNAs are needed.

Corresponding Author

Professor Anna Rostedt Punga, MD, PhD

anna.rostedt.punga@neuro.uu.se**References**

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