Multiple sclerosis and chemotherapy

Multiple sclerosis (MS) is a demyelinating, autoimmune and inflammatory disease that affects the central nervous system (CNS) and is one of the leading causes of disability in young adults worldwide. There are different drugs for the treatment of MS that aim to modify the natural course of the disease and thus slow its progression. This group of drugs is known as disease modifying therapy (DMT). To evaluate the effectiveness of these drugs, mainly clinical and MRI parameters are used. In recent years, different studies have emerged that propose the use of biomarkers to improve the evaluation of the effectiveness of DMT and to provide individualized therapy. MicroRNAs (miRNAs) are part of these biomarkers that have been proposed both for their wide availability in different tissues and body fluids and for their possible involvement in different biological processes. In this review we briefly discuss the mechanisms of action of different DMT available for the treatment of MS, as well as recent findings on the expression levels of different miRNAs in MS patients under treatment with DMT.

Multiple sclerosis and chemotherapy

MS is a demyelinating, autoimmune and inflammatory disease affecting the CNS, affecting 50–300 individuals out of every 100,000. Currently, approximately 2–3 million people worldwide are affected by MS (Thompson et al., 2018).

Autoimmune diseases (AD) can be classified according to their etiology:

- Mutations in genes such as FOXP3: in this type of AD, autoreactive T lymphocytes are present and trigger diseases such as Sjögren’s syndrome and vasculitis (Schwartz et al., 2020).
- Antibody-mediated: these are dominated by high levels of antibodies, such as antinuclear antibodies that react against nuclear and cytoplasmic components, as in systemic lupus erythematosus (Pisetsky and Lipsky, 2020).
- Granulomatous inflammation: chronic inflammation occurs due to infection or an external agent such as silicon oxide that allows the increase of activated macrophages that interact with T lymphocytes, which occurs in diseases such as Wegener’s granulomatosis and sarcoidosis (Beijer et al., 2017).
- Autoinflammatory: in which alterations of the innate immune system predominate, leading to an overproduction of interleukins that eventually leads to increased activity in cells of the adaptive immune system, as occurs in Behcet’s syndrome (Lecese et al., 2019).

According to different studies, the etiology of MS could be found within the immune system or within the central nervous system. During lymphocyte maturation in the thymus, most of the autoreactive T lymphocytes are eliminated; however, this process is imperfect, and some of these may be released. Under normal conditions, immune tolerance mechanisms keep these cells under control. If there is reduced function of regulatory T lymphocytes (Tregs) and/or increased resistance of B and T lymphocytes to suppressive mechanisms, self-reactive B and T lymphocytes may become activated and become aggressive effector cells for CNS cells (Dendrou et al., 2015). There are several risk factors that contribute to these alterations of the immune system. Among the main ones are: the presence of the HLA-DRB1*15:01 allele, which triples the risk of developing MS (Hollenbach and Oksenberg, 2015), obesity, which has a causal relationship with MS (Gianfrancesco et al., 2017) and recently Bjornevik et al., (2022) demonstrated that there is a causal relationship between Epstein-Barr virus infection and the prevalence of MS. Once activated, CD8+ T cells, Th1 and Th17 cells, B cells and innate immune cells can infiltrate the CNS by crossing the blood-brain barrier (BBB). The BBB is a dynamic structure composed of a layer of endothelial cells, the endothelial basal lamina, and the perivascular space. The latter is populated with perivascular macrophages and podocytes.
which prevent the passage of cells circulating in the blood into the CNS. During MS there is an alteration of the BBB, which allows hematopoietic cells to enter the CNS. T and B lymphocyte infiltrates are typical and may persist in the subarachnoid space as ‘tertiary follicles’ for several months. The composition of the immune cell infiltrate and its interactions with CNS cells initiate an inflammatory process that itself may cause demyelination, leading to axonal damage (Prinz and Priller, 2017). On the other hand, the alterations produced by the infiltration of immune system cells may facilitate the development of alterations in the central nervous system that may also lead to axonal damage. Mutations in the mitochondrial DNA may occur associated to the generation of high levels of reactive oxygen species facilitating the demyelination process. Furthermore, iron may be released in the demyelinating areas contributing to oxidative stress (Baecher-Allan et al., 2018). Regardless of the origin of MS, this disease is characterized by the presence of inflammation, gliosis and demyelination, resulting in the clinical presentation of the disease (Dendrou et al., 2015).

According to the clinical presentation, MS is classified into 3 types (Sand, 2015):

- Remitting-recurrent (RRMS): the most common form (85 % of patients present with this form of the disease at onset), it is characterized by the presence of acute neurological events followed by phases of recovery.
- Secondary progressive (SPMS): approximately 50 % of patients who debut with RRMS will have this type of MS after 10–20 years, remissions become infrequent, and patients present with gradual neurological deterioration.
- Primary progressive (PPMS): there is presence of neurological symptoms and progressive deterioration with absence of remissions since the onset of MS. Only 15 % of patients present this type of MS.

Currently, there is no treatment that allows complete remission of MS. According to their efficiency in preventing relapses and slowing disease progression, there are three groups of drugs: those of high effectiveness which comprise ocrelizumab, ofatumumab, natalizumab, alemtuzumab, and mitoxantrone; those of moderate effectiveness which include fingolimod, dimethyl fumarate, siponimod, ozanimod, and cladribine; and finally those of modest effectiveness which encompass teriflunomide, interferon beta, PEG-IFN-β, and glatiramer. In patients diagnosed with RRMS, the guidelines of both the American Academy of Neurology (Rae-Grant et al., 2018) and the European Academy of Neurology (Montalban et al., 2018) recommend initiating treatment with any DMT that is available considering the patient’s prognosis and the safety profile of the drug. For patients with SPMS they recommend assessing the risk of MS reactivation and advise discontinuation of treatment in patients with no clinical activity, no active MRI lesions, and advanced disability. In PPMS the treatment recommended by both guidelines is ocrelizumab.

The effectiveness of DMT in each patient is assessed by the number of annual relapses, the absence of new lesions on MRI and the patient’s tolerance to the drug. Although in recent years the use of various biomarkers has been proposed for monitoring the response to DMT, they are not yet routinely used. Such biomarkers are: neutralizing antibodies against interferon beta (Hurtado-Guerrero et al., 2017), neutralizing antibodies against natalizumab (Khoy et al., 2020), and neurofilament light chain (Kapoor et al., 2020). The purpose of these biomarkers would be to offer individualized therapy and thus stop disease progression as early as possible in MS patients. One type of biomarkers that may also be useful in the evaluation of the response to DMT are microRNAs (miRNAs).

MicroRNAs

miRNAs are small non-coding RNAs (about 20 nucleotides) that regulate mRNA stability and/or post-transcriptional synthesis of proteins and thus gene expression (Harris et al., 2017). miRNAs can be encoded by independent genes, but they can also be processed from introns and 3’ untranslated regions of mRNAs. The formation of miRNAs starts in the nucleus, with the help of RNA polymerase II the precursors of miRNAs known as primary miRNAs which have a variable length of hundreds of nucleotides are transcribed. Subsequently an RNase III called Drosha processes them and forms a hairpin structure of 60–70 nucleotides called pre-miRNAs, which, with the help of exportin-5 are transported to the cytoplasm where they are in turn processed by RNase III Dicer to become mature miRNAs. They are bound together with the argonaute protein to the RNA silencing complex (RISC). This complex is ultimately responsible for mRNA degradation or translation inhibition (Tsitkou and Lindsay, 2009; Tufekci et al., 2011). The miRNAs are packaged in microvesicles, exosomes or apoptotic bodies, which allows their identification in different organs and body fluids (Jagot et al., 2016). This set of extracellular vesicles may play an important role in intercellular communication, both locally and systemically, as they may be internalized by close-by as well as distant recipient cells (Dolcetti et al., 2020). Fig. 1 outlines the generation and types of microvesicles involved in the transport of miRNAs.

There are different methodologies for the analysis of miRNAs such as real-time PCR, microarrays, Northern-blot, and sequencing. Currently, the most used method for the study of miRNA expression is real-time PCR, which is a highly sensitive and specific method. To identify changes in expression levels, however, it is necessary to normalize the data, thus gene expression levels considered to be constitutively expressed, i.e., that are at least constant under the conditions to be compared, are used. Unfortunately, there is until now no consensus as to which are the best reference genes to obtain an adequate relative quantification to allow comparative studies between different samples. Ideally, they should be RNAs of a similar size to miRNAs, of comparable stability and without the existence of pseudogenes in the genome. Nuclear RNAs such as RNU1-4, RNU6-2, SNORD43 and SNORD44 are usually used but they have low stability and are susceptible to rapid degradation. Synthetic miRNAs that are added to the sample prior to the RNA extraction process are also used, but because they are exogenous molecules, they are not suitable for comparing miRNA levels, so they are mainly used to evaluate the yield of RNA extraction. It is preferable to use miRNAs as reference genes, although the choice of the most suitable miRNAs for the population to be studied is not straightforward and could be based on erroneous assumptions (Donati and Ciu, 2019; Rice et al., 2020)
example, mice deficient in miR-146a develop more severe EAE, in different cells of the immune system has been demonstrated. For (Chen et al., 2020), while miR-146a functions primarily as an immune-cell associated antigen-4 (CTLA-4) and promotes dendritic cell maturation by suppressing c-Fos, SHIP1, KPC1 and SOCS1 proteins (Talebi et al., 2020), while miR-146a functions primarily as an immunosuppressor and acts as a negative regulator of myeloid cells and both CD4+ and CD8+ T lymphocytes, and promotes the function of Tregs (Li et al., 2017).

In tests performed in the experimental autoimmune encephalomyelitis (EAE) animal model, used to study MS, the effect of miRNAs on different cells of the immune system has been demonstrated. For example, mice deficient in miR-146a develop more severe EAE, in addition, they have significantly higher levels of Th17 lymphocytes than controls (Li et al., 2017). Furthermore, in vivo silencing of miR-326 results in fewer Th17 lymphocytes and mild EAE (Du et al., 2009). Th17 lymphocytes are among the "proinflammatory" lymphocytes due to their production of IL-17, which in the human body results in chronic tissue inflammation (Yasuda et al., 2019).

Transl et al., (2021) report that in mice with EAE there is an increase of miR-20a levels in splenocytes and lymph node cells, and in their in vitro study they demonstrated that miR-20a suppression promotes the differentiation of CD4+ T lymphocytes into Tregs. Tregs are an indispensable component of the immune system for the maintenance of self-tolerance, prevent inflammation in lymphoid organs and contribute to homeostasis not only by controlling inflammatory processes but also in the production of growth factors (Sakaguchi et al., 2020). Talebi et al., (2017) reported that miR-142-5p and miR-142-3p which originate from the precursor miR-142 were increased in the spinal cord of EAE mice. In their in vitro studies they showed miR-142 expression in both T lymphocytes and macrophages and determined that transforming growth factor receptor beta 1 (TGFBR1) is a target of miR-142a-3p. Transforming growth factor beta 1 (TGF-β) is a cytokine with pleiotropic functions that include the regulation of inflammation, as well as the survival, growth, and differentiation of many cell types, such as Tregs and Th1 lymphocytes.

Many miRNAs have been studied in MS patients in both peripheral blood and cerebrospinal fluid (CSF). The samples evaluated are mainly

---

### Table 1

**Effect of disease-modifying therapy on levels of miRNAs.**

| miRNA     | Sample type       | Reference gene | Drug              | Treatment effect on MicroRNA levels                                                                 | Reference                  |
|-----------|-------------------|----------------|-------------------|--------------------------------------------------------------------------------------------------|---------------------------|
| -15b      | Serum             | cel-miR-39     | Fingolimod        | Raises expression levels after the sixth month of treatment                                        | (Fenoglio et al., 2016)   |
| 23a       |                   |                |                   |                                                                                                   |                           |
| 223       |                   |                |                   |                                                                                                   |                           |
| 145       | Blood             | unisp6 RNA     | IFN-β-1a          | Decreases expression levels                                                                         | (Eshbon et al., 2016)     |
| 20a       |                   |                |                   |                                                                                                   |                           |
| 18a       | RN6B              |                | Natalizumab       | Increased levels one year after initiation of treatment                                             | (Ingwersen et al., 2014)  |
| 20b       |                   |                |                   |                                                                                                   |                           |
| 29a       |                   |                |                   |                                                                                                   |                           |
| 103       |                   |                |                   |                                                                                                   |                           |
| 326       |                   |                |                   |                                                                                                   |                           |
| 125a-5p   | Plasma            | cel-miR-39     | Dimethyl fumarate | Decreased expression levels one year after treatment initiation                                     | (Giuliani et al., 2021)   |
| 146a-5p   |                   |                |                   |                                                                                                   |                           |
| 155       |                   |                |                   |                                                                                                   |                           |
| 34a-5p    | RN6B              |                | Fingolimod        | Higher levels compared to patients who do not respond to treatment                                 | (Eftekharian et al., 2019) |
| 204-5p    |                   |                | Fingolimod        | Lower levels compared to patients who do not respond to treatment                                  |                           |
| 142-3p    | PBMC              | RN6B           | Glatiramer acetate| Decrease compared to untreated patients                                                            | (Waschbich et al., 2011)  |
| 146a      |                   |                |                   |                                                                                                   |                           |
| 26a       |                   |                | RN6-6 P, SNORD68  | Natalizumab                                                                                       | (Mannelli et al., 2016)   |
| 155       |                   |                |                   |                                                                                                   |                           |
| let-7b    | Exosomes/ Serum   | cel-miR-39     | IFN-β-1b          | Decrease compared to untreated patients                                                            | (Manna et al., 2018)      |
| 15b-3p    |                   |                |                   |                                                                                                   |                           |
| 19b-3p    |                   |                |                   |                                                                                                   |                           |
| 23a-3p    |                   |                |                   |                                                                                                   |                           |
| 26a-5p    |                   |                |                   |                                                                                                   |                           |
| 122-5p    |                   |                |                   |                                                                                                   |                           |
| 142-3p    |                   |                |                   |                                                                                                   |                           |
| 146a-5p   |                   |                |                   |                                                                                                   |                           |
| 215-5p    |                   |                |                   |                                                                                                   |                           |
| 223-3p    |                   |                |                   |                                                                                                   |                           |
| 320b      |                   |                |                   |                                                                                                   |                           |
| 320d      |                   |                |                   |                                                                                                   |                           |
| 451a      |                   |                |                   |                                                                                                   |                           |
| 486-5p    |                   |                |                   |                                                                                                   |                           |
| 22-3p     |                   |                |                   |                                                                                                   |                           |
| 660-5p    |                   |                |                   |                                                                                                   |                           |
| 126       | CD4 +             | RN44           | Natalizumab       | Decreased expression of miRNAs one year after the start of treatment                                | (Meira et al., 2014)      |
| 150       | CSF               | cel-miR-39, cel-miR-54, cel-miR-238 | Natalizumab | Decreased expression of miRNAs one year after the start of treatment                                | (Bergman et al., 2016)   |
plasma, serum, peripheral blood mononuclear cells (PBMC), CSF and T cells. The main advantage of peripheral blood samples is that they are not as invasive as other sample types such as CSF or synovial fluid. It is preferable to use plasma rather than serum for the measurement of miRNAs, since during the clotting process platelets may release microvesicles containing miRNAs that may interfere with the analysis (Chen et al., 2021). Zheleznjakova et al. (2021) performed an analysis of miRNAs in PBMC, plasma, CSF cells, and cell-free CSF from MS patients. They found that most of the miRNAs with difference in their expression in CSF cells maintain a similar pattern in PBMC, plasma and cell-free CSF. To consider the routine use of miRNAs as biomarkers, comprehensive studies are needed to validate the accuracy, precision, sensitivity, and specificity of their quantification as well as their usefulness with regards to the sample type analyzed as well as the sampling time point.

**MicroRNAs and disease modifying therapy**

The results of the analysis of microRNA show that they have a potential use as biomarkers before and during the treatment of MS patients. In this review we show and discuss the different results of work on microRNA and drugs that are part of DMT.

**IFN-β-1b**

The first drug approved by the FDA for the treatment of multiple sclerosis was interferon beta 1b (IFN-β-1b) in 1993 and later IFN-β-1a in 1996. Both interferons reduce disease severity in some patients and slow disease progression. Mainly used in RRMS patients, it decreases the presence of relapses and the risk of disability progression from the third month of treatment compared to the placebo effect (Traboulsee et al., 2018). The exact mechanism of action of IFN-β is unknown, different studies have shown that IFN-β allows the increase of Th2 lymphocytes through its action on interleukin 10 (IL-10) levels, thus promoting the remission of the inflammatory process, in addition to decreasing plasma levels of metalloproteinases 8 and 9 (Jakimovski et al., 2018).

The use of IFN-β has been shown to modify the levels of miRNAs. Ehtesham et al. (2016) analyzed the expression of miR-145 and miR-20a in blood from 30 RRMS patients, 15 under IFN-β-1a treatment and 15 untreated, in addition to 15 healthy controls. They employed the real-time PCR technique for the measurement of miRNAs expression and used unSp6 synthetic RNA for normalization. They showed that patients on IFN-β-1b treatment have miR-145 and miR-20a levels in the range of healthy controls, and they also mention that miR-145 could function to monitor the response to treatment. In a study with a larger number of miRNAs analyzed, Manna et al. (2018) analyzed the expression of miRNAs in exosomes obtained from serum of 11 RRMS patients, evaluating a total of 179 miRNAs with the help of microarrays. They found that the expression of 16 miRNAs in serum exosomes was different between a group of IFN-treated RRMS patients and a group of untreated RRMS patients. They also employed real-time PCR to assess the expression of these 16 miRNAs and used miR-39 for normalization. Two of the 16 miRNAs, miR-22-3p and miR-660-5p increased their levels and 14 of the 16 miRNAs, miR-486-5p, miR-451a, miR-let-7b-5p, miR-320b, miR-122-5p, miR-215-5p, miR-320d, miR-19b-3p, miR-26a-5p, miR-142-3p, miR-146a-5p, miR-15b-3p, miR-23a-3p, and miR-223-3p decreased. Within this group of miRNAs, miR-23a is involved in oligodendrocyte differentiation, miR-15b regulates the fibroblast growth factor 2 (FGF-2) gene, which is involved in demyelination and remyelination, miR-451 is a regulator of oxidative stress and is involved in neurodegenerative processes, miR-let-7 regulates stem cell differentiation and T-cell activation, activates TLR 7, and is involved in neurodegeneration, and exosome-associated miR-146 inhibits the expression of proinflammatory genes.

**Glatiramer acetate**

Glatiramer acetate was approved by the FDA in 1996 for the treatment of RRMS. It consists of a heterogeneous polymer in size and composition of four amino acids (L-alanine, L-lysine, L-glutamate, and L-tyrosine) present in myelin basic protein. Glatiramer acetate induces a broad immunomodulatory effect: it presents competitive binding to MHC type II, inhibitory effect on monocyte reactivity, in T lymphocytes, decrease of pro-inflammatory T cells and increase of anti-inflammatory T cells; it increases Th2 differentiation which results in an increase in IL-10 and TGF- secretion and a decrease of IL-12 and TNF. In addition, it exerts negative regulation of Th1 and Th17 lymphocytes and increases the expression of Tregs (Aharoni, 2013). In the GALA (Glatiramer Acetate Low-Frequency Administration) study, it was found that, compared to placebo, glatiramer acetate reduces the annual relapse rate and also reduces the number of new lesions or the increase in lesions present in the brain observed by MRI (Khan et al., 2013) in RRMS patients, the follow-up of the patients lasted for one year. Subsequently, with patients enrolled in GALA, they conducted an open-label study with a 3-year follow-up from which they report that glatiramer acetate reduces the annual relapse rate and slows the progression of brain atrophy as measured by MRI (Khan et al., 2017).

Waschbisch et al. (2011) performed measurements of five miRNAs (miR-326, miR-155, miR-146a, miR-142-3p, miR-20b) considered immunologically relevant because of their presence in PBMC. They used real-time PCR to analyze the expression of miRNAs and RNU6/B for normalization. Measurements were made in PBMC from 32 healthy controls, 36 patients with untreated RRMS, 20 patients with RRMS treated with glatiramer acetate and 18 patients with RRMS treated with IFN-β for at least 3 months. They found overexpression of miR-326, miR-155, miR-146a and miR-142-3p among the untreated group of patients compared to the control group. In patients treated with glatiramer acetate, miR-146a and miR-142-3p are found at levels like the control group. miR-146a regulates T lymphocyte differentiation and overexpression of miR-142-3p prevents proper differentiation of Tregs.

**Natalizumab**

Natalizumab is a humanized monoclonal antibody that selectively binds to the α4 subunit of the α4β1 and α4β7 integrins that are expressed on the surface of human leucocytes (Sellebjerg et al., 2016). It was approved in 2004 by the FDA for the treatment of RRMS as a treatment for patients who have not received first-line treatment. Natalizumab is currently recommended by the American Academy of Neurology as one of the starting drugs in patients with active-phase RRMS (Rae-Grant et al., 2018). Blockade of α4β1 and α4β7 integrins by natalizumab reduces leucocyte migration in the central nervous system leading to remission of disease activity. Treatment with natalizumab has been shown to significantly decrease the annual relapse rate in patients with RRMS for 5 years (Butzkueven et al., 2014). In a study in patients with SPMS, treatment with natalizumab for 2 years delayed the loss of upper extremity function and exploratory analyses suggested that this treatment effect occurred independently of active brain lesions (Kapoor et al., 2018). The use of natalizumab was associated with an increased risk of developing progressive multifocal leukoencephalopathy (PML), there were three factors identified that allow estimating this risk which are: the presence of antibodies against JC virus, the previous use of immunosuppressive drugs, and the use of natalizumab for more than 24 months. When all three factors were present the risk for developing PML increased significantly to 2.3% (Mao-Drayer, 2018).

Several clinical studies have measured miRNAs in patients treated with natalizumab. Mameli et al. (2016) selected 4 miRNAs (miR-155, miR-132, miR-146a and miR-26a) previously analyzed in PBMC. The expression of these miRNAs was analyzed by real-time PCR and nuclear RNAs were used for normalization (RNU6-6P and SNORD68). They found that miR-155 and miR-26a from PBMC of 24 RRMS patients.
treated with natalizumab and observed a decrease in the levels of these miRNAs during and after 6 months of treatment. miR-155 induced CD8+ T-cell activation and increased CD4+ T-cell proliferation (Chen et al., 2020) while miR-26a induced Th-17 differentiation by altering the TGF-b signaling pathway (Honardoost et al., 2014). Meira et al. (2014) analyzed miR-126 expression only in CD4+ T lymphocytes from 24 natalizumab-treated RRMS patients, 12 patients with previously untreated RRMS, and 12 healthy controls. They used real-time PCR for miR-126 expression analysis and used RNU44 for normalization. They found an overexpression of miR-126 in the untreated group compared to healthy controls. Between the natalizumab-treated group of patients and healthy controls there is no difference in miR-126 levels. Previous reports indicate that miR-126 is overexpressed in TCD4+ lymphocytes in RRMS. miR-126 interacts with POU2AF1 which is an important regulator of the transcription factor Spi-B18 that mediates B-cell receptor-dependent humoral immune responses and T-cell-dependent T-cell responses. Bergman et al. (2016) analyzed CSF samples in RRMS patients. They first performed a study with a microarray of 754 miRNAs in 15 patients with MS, 15 with isolated clinical syndrome, 14 with inflammatory diseases and 13 healthy controls. When evaluating the healthy control group against the RRMS group they found only two miRNAs with significant differences miR-145 and miR-150. They subsequently analyzed these miRNAs with real-time PCR using cel-miR-39, cel-miR-54, and cel-miR-238 for normalization. In 430 patients only miR-150 showed significant differences. In this study they report that treatment with natalizumab decreases miR-150 levels in CSF in RRMS patients after 12 months of treatment and the opposite happens in plasma where levels increase. Therefore, the usefulness of miR-150 as a biomarker would be exclusive of its expression in CSF. Functional studies of intracellular miR-150 suggest a role in the regulation of B and T lymphocyte development. Ingwersen et al. (2014) analyzed blood samples from 17 RRMS patients before initiating natalizumab treatment as well as one year later. They used a microarray of 866 miRNAs, found 14 miRNAs with changes, 8 over expressed and 6 under expressed, they subsequently decided to perform real-time PCR analysis of the overexpressed miRNAs, used RNU6B for normalization and confirmed the overexpression in miR-18a, miR-29a, miR-20b, miR-103, and miR-326. They report that natalizumab regularizes the levels of these miRNAs one year after treatment initiation. Among the miRNAs evaluated by Ingwersen et al. (2014) miR-26a and miR-126 are also reported to have lower levels in MS patients than in healthy controls, although this difference was not statistically significant. On the contrary, in the studies by Mameli et al. (2016) and Meira et al. (2014) they report overexpression of these miRNAs, the difference between results may be explained by:

- Sample type. In the study by Ingwersen et al. (2014) they obtained miRNA from whole blood samples, while Mameli et al. (2016) extracted them from PBMC and Meira et al. (2014) used TCD4+ lymphocytes.
- Patient characteristics. Ingwersen et al. (2014) and Meira et al. (2014) compare miRNA levels in MS patients and healthy controls, Mameli et al. (2016) compares levels in serial samples of RRMS patients (before natalizumab and at 6 months of treatment).

**Fingolimod**

Fingolimod is the first drug available in oral presentation for the treatment of MS, it was approved by the FDA in 2010. This drug is mainly used in patients with RRMS, it modulates sphingosine 1-phosphate (S1P) receptors which are expressed in lymphocytes and are important for the release of lymphocytes from lymph nodes, i.e. the principal action mechanism of fingolimod is the blockade of lymphocyte egress from lymph nodes (Chaudhry et al., 2017). In addition, fingolimod promotes neurogenesis and oligodendrogenesis in vitro under basal conditions and in clinical studies, it reduces the lymphocyte efflux from lymph nodes (Cipriani et al., 2017) and in clinical studies, it slows down the progression of both diffuse and focal gray matter damage (Bajrami et al., 2018). A study in RRMS patients treated with fingolimod for 4.5 years showed that early initiation of MS treatment with fingolimod had a stronger overall impact on the control of clinical disease activity in comparison to a group of patients who started treatment with IFN-b-1a and later switched to fingolimod (Cohen et al., 2016). In patients with PPMS, treated with fingolimod or placebo for a time period of 3–5 years, fingolimod did not delay disease progression or reduce brain volume loss (Lublin et al., 2016).

In a study in 78 patients with RRMS, plasma levels of seven miRNAs (miR96-5p, miR-211-5p, miR-15a, miR-34a-5p, miR-204-5p, miR-501-5p, and miR-524-5p) selected from a literature review were analyzed. In this study, real-time PCR was used for expression analysis of miRNAs and RNU6B for normalization. It was reported that patients responding to fingolimod therapy had lower levels of miR-34a-5p and higher levels of miR-204-5p compared to non-responders in miR-204-5p had a decreased expression. It has been reported that miR-34a expression was reduced in PBMCs of RRMS patients, whereas its expression in active brain lesions of MS patients was increased which has been associated with decreased membrane glycoproteins CD47, which alters macrophage inhibition leading to myelin phagocytosis (Eftekharian et al., 2019). Fenoglio et al. (2016) analyzed the expression of three miRNAs (miR-15b, miR23a and miR-223) in serum from 30 RRMS patients. They used real-time PCR for miRNA expression analysis and used cell-miR-39 for normalization. They report that treatment with fingolimod, elevated the levels of miR-15b, miR23a and miR-223 after a sixth month treatment. Both miR-15b and miR-23a upregulated the FGF-2 gene, a member of the fibroblast growth factor family, whose protein levels were elevated in the CSF of MS patients, especially those with active disease. FGF-2 was differentially expressed in active and chronic MS lesions in postmortem tissues, suggesting that FGF-2 is a marker for inflammation in MS lesions.

**Dimethyl fumarate**

Dimethyl fumarate was approved in 2013 by the FDA for the treatment of RRMS, it is an orally administered drug. The exact mechanism of action is still unknown, but it is suggested that dimethyl fumarate causes a decrease in the number of lymphocytes due to apoptosis and activation of the Nrf2-dependent antioxidant pathway in the central nervous system (Yadav et al., 2019). Nrf2 helps maintain cellular homeostasis by regulating a number of genes related to various antioxidant agents such as glutathione, thioredoxin, heme oxygenase and NAD(P)H dehydrogenase (Gopal et al., 2017). Hayashi et al. (2017) performed a study in different cell lines and their results showed that dimethyl fumarate stimulated the proliferation of mitochondria. Dimethyl fumarate achieved a 31% reduction in the annual relapse rate compared to placebo in RRMS patients (Saida et al., 2019). Dimethyl fumarate reduced the annual relapse rate and decreased the likelihood of new brain lesions (Kappos et al., 2015; Miller et al., 2015). In a comparison of effectiveness between glatiramer acetate and dimethyl fumarate, RRMS patients treated with dimethyl fumarate were reported to have a lower annual relapse rate (Chan et al., 2017).

Dimethyl fumarate decreased miR-146a and miR-155 levels in in vitro studies performed in human and mouse astrocytes (Galloway et al., 2017) and also decreased miR-155 levels in monocytes from RRMS patients (Michell-Robinson et al., 2015). Giuliani et al. (2021) analyzed the plasma expression of four miRNAs (miR-34a, miR-125a-5p, miR-146a-5p and miR-155) from 16 RRMS patients. They used real-time PCR for miRNA analysis and used cell-miR-39 for normalization. In this study they report a decrease in miR-125a-5p, miR-146a-5p and miR-155 levels after four months of treatment with dimethyl fumarate. miR-125a-5p reduced neuroinflammation by influencing the integrity of the blood-brain barrier preventing the release of proinflammatory cytokines and chemokines by endothelial cells.
Discussion

miRNAs represent an opportunity to offer individualized therapy to MS patients. As they are involved in different processes of the immune system, miRNAs allow us to study and analyze the mechanisms involved in the pathophysiology of MS. As mentioned in the microRNAs section, there are in vitro studies that show that miRNAs play an important role in the differentiation of CD4+, CD8+ and regulatory T lymphocytes, specifically miR-146a (Li et al., 2017) and miR-155 (Chen et al., 2020). In addition, it has been described that inhibition or overexpression of miR-155 in endothelial cells could decrease or increase vascular endothelial permeability, respectively (Sun et al., 2012; Zheng et al., 2017), which could be a determining factor for lymphocyte migration across the blood-brain barrier.

These two miRNAs are among the most studied in autoimmune diseases: In patients with rheumatoid arthritis the expression of miR-146a and miR-155 is higher in PBMC and whole blood compared with healthy controls (Mooskherjee and El-gabalawy, 2013); in Graves’ disease both miRNAs are decreased in serum (Zheng et al., 2018); in systemic lupus erythematosus, there is an overexpression of both miRNAs in blood (Shummalieva et al., 2018); in Sjögren’s syndrome, overexpression of miR-146a and underexpression of miR-155 has been observed in PBMC (Shi et al., 2014); and in patients with oral lichen planus, the expression of miR-146a and miR-155 is increased compared with normal oral mucosa and blood samples from healthy controls (Cristina et al., 2012).

Variations in miRNA expression levels among autoimmune diseases may be explained in part by the pathophysiology of each disease or the stage of the disease. The expression of some miRNAs may differ between MS stages, for example, miR-155 and miR-310a levels have been reported to increase in MS patients during the relapse period (Niwald et al., 2017; Zhang et al., 2014). These types of results suggest that the levels of miRNAs would allow selecting the best drug for each MS patient and thus improve drug efficacy, allow disease remission for a longer period, decrease the number of relapses, and delay MS progression for as long as possible.

Another reason for variation in the expression of miRNAs could be found in the methodology used for the analysis of expression levels.

Real-time PCR is the most widely used method for the study of miRNAs due to its high sensitivity, specificity, and reproducibility. However, both the collection and processing of samples and the miRNA extraction process can influence the quantification of miRNAs, thereore, the use of reference genes is required to normalize the results and reduce quantification errors. Unfortunately, despite the existence of different studies on miRNA expression, there are still no universal reference genes that allow adequate comparison and reproducibility of experiments with different populations. The simplest way to decide which is the ideal reference gene is to perform a review of studies previously performed in similar populations and choose the reference gene most used in those studies. The main disadvantage of selecting the reference gene in this way is that their expression may change under different experimental conditions. Some tools (Excel add-ins) have been developed that allow the analysis and selection of reference genes such as NormFinder (Andersen et al., 2004), geNorm (Vandesompele et al., 2002) and Bestkeeper (Pfaffl et al., 2004).

Normfinder ranks the set of candidate genes for normalization according to their expression stability in a given sample set and experimental design, in addition it can analyze expression data obtained by any quantitative method, e.g., real-time RT-PCR and microarray-based expression analysis (Andersen et al., 2004).

gNorm is a popular algorithm for determining the most stable reference genes from a set of candidate reference genes tested in a panel of samples. It allows the calculation of a gene expression normalization factor for each sample based on the geometric mean of a user-defined number of reference genes (Vandesompele et al., 2002).

Bestkeeper determines the ‘optimal’ reference genes using pairwise correlation analysis of all candidate gene pairs and calculates the geometric mean of the most suitable ones. The weighted index is correlated with up to ten target genes using the same pairwise correlation analysis (Pfaffl et al., 2004).

Another method employed for normalization is delta Ct in which the relative expression of ‘gene pairs’ within each sample is compared to confidently identify useful reference genes. If the delta Ct value between the two genes remains constant when analyzed in different samples, it means that both genes are stably expressed between those samples, or that they are co-regulated (stability of both genes is assumed) (Silver et al., 2006).

Conclusions

Currently, drug therapy is the mainstay of MS treatment. Monitoring drug activity is of vital importance to determine adequate response to treatment. Due to their widespread presence in fluids such as blood and CSF, miRNAs could be a good biomarker of treatment response in MS patients. However, although there are many studies analyzing the relationship between miRNAs and MS, there are important differences in the design of these studies, which complicates their reproducibility. Among these differences are the types of samples, the extraction methods used to obtain miRNAs, the reference genes used in real-time PCR, the presence or absence of healthy controls within the study group, the characteristics of the patients included such as the type of MS, and the time of evolution of the disease. As mentioned in this review, several studies show that there is a relationship between miRNA levels and some drugs available for MS, although it is important to note that for mitoxantrone, teriflunomide, alemtuzumab, Peg-INF-β, ocrelizumab, siponimod and cladribine, there are still no studies available that analyze this relationship. There is a clear need for further research to define the usefulness of miRNAs as biomarkers in patients with MS.

References

Aharoni, R., 2013. The mechanism of action of glatiramer acetate in multiple sclerosis and beyond. Autoimmun. Rev. 12, 543–553. https://doi.org/10.1016/j.aur.2012.09.005.
Andersen, C.L., Jensen, J.L., Ørntoft, T.F., 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res. 64, 5245–5250.
Baecher-Allan, C., Kaskow, B.J., Weiner, H.L., 2018. Multiple sclerosis: mechanisms and immunotherapy. Neuron 97, 742–768. https://doi.org/10.1016/j.neuron.2018.01.021.
Bajrami, A., Pitteri, M., Castellaro, M., Pizzini, F., Romualdi, C., Montemese, S., Calabrese, M., 2018. The effect of fingolimod on focal and diffuse grey matter damage in active MS patients. J. Neurol. 265, 2154–2161.
Beijer, E., Veltkamp, M., Meek, B., Moller, D.R., 2017. Etiology and Immunopathogenesis of Sarcoidosis: Novel Insights. Semin. Respir. Crit. Care Med 404–416.
Bergman, P., Piket, E., James, T., Olsson, T., Piehl, F., Jagodic, M., 2016. Circulating miR-150 in CSF is a novel candidate biomarker for multiple sclerosis. Neuron. Neuroimmunol. Neuroinflamm. 0, 1–10.
Bjornevik, K., Cortese, M., Healy, B.C., Kuhle, J., Mina, M.J., Leng, Y., Elledge, S.J., Niebuhr, D.W., Scher, A.L., Munger, K.L., Ascherio, A., 2022. Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis. Science 375 (8702), 296–301.
Butzkeven, H., Kappos, L., Pellegrini, F., Trojanow, M., Wiendl, H., Patel, R.N., Zhang, A., Hotermans, C., 2014. Efficacy and safety of natalizumab in multiple sclerosis: interim observational programme results. J. Neurol. Neurosurg. Psychiatry 85, 1190–1197.
Chan, A., Cutter, G., Fox, R.J., Xiao, J., Lewin, J.B., 2017. Comparative effectiveness of delayed-release dimethyl fumarate versus glatiramer acetate in multiple sclerosis patients: results of a matching-adjusted indirect comparison. J. Comp. Eff. Res. 6, 313–323.
Chaudhry, B.Z., Cohen, J.A., Conway, D.S., 2017. Sphingosine 1-phosphate receptor modulators for the treatment of multiple sclerosis. Neurotherapeutics 14, 859–873.
Chen, L., Gao, D., Shao, Z., Zheng, Q., Yu, Q., 2020. miR-155 indicates the fate of CD4 + T cells. ImmunoLett. 124, 40–49.
Chen, Y., Wu, T., Zhu, Z., Huang, H., Zhang, L., Goel, A., Yang, M., Wang, X., 2021. An integrated workflow for biomarker development using microRNAs in extracellular vesicles for cancer precision medicine. Semin. Cancer Biol. 74, 134–155.
dimethyl fumarate in patients with relapsing-remitting multiple sclerosis from East Asia and other countries. BMC Neurol. 19, 1–10.

Sakaguchi, S., Mitkami, N., Wing, J.B., Tanaka, A., Ichiyama, K., Okkura, N., 2020. Regulatory T cells and human disease. Annu. Rev. Immunol. 38, 541–566.

Sand, I.K., 2015. Classification, diagnosis, and differential diagnosis of multiple sclerosis. Curr. Opin. Neurol. 28, 193–205.

Schwartz, D.M., Kitakule, M.M., Luo, Y., Mehta, N.N., 2020. T cells in autoimmunity-associated cardiovascular diseases. Front. Immunol. 11. https://doi.org/10.3389/fimmu.2020.588776.

Schwarzenbach, H., Machado, A., Calin, G., Pantel, K., 2015. Data normalization strategies for MicroRNA quantification. Clin. Chem. 61, 1333–1342.

Sellebjerg, F., Cadavid, D., Steiner, D., Villar, L.M., Reynolds, R., Mikol, D., 2016. Selective microRNA expression studies in human reticulocytes using real-time PCR. BMC Mol. Biol. 7, 1–9. https://doi.org/10.1186/1471-2199-7-9.

Sun, H.-X., Zeng, D.-Y., Li, R.-T., Yang, H., Hu, Y.-L., Zhang, Q., Jiang, Y., Huang, L.-Y., Tang, Y.-B., Yan, G.-J., Zhou, J.-G., 2012. Essential role of MicroRNA-155 in regulating endothelium-dependent vasorelaxation by targeting endothelial nitric oxide synthase. Hypertension 60, 1407–1414.

Taleb, F., Ghorbani, S., Chan, W.F., Boghizian, R., Masoumi, F., Ghasemi, S., Vojgani, M., Power, C., Noorbakhsh, F., 2017. MicroRNA-142 regulates inflammation and T cell differentiation in an animal model of multiple sclerosis. J. Neuroinflamm. 14, 1–14. https://doi.org/10.1186/s12974-017-0832-7.

Thompson, A.J., Baranzini, S.E., Guruts, J., Hemmer, B., Ciccarelli, O., 2018. Multiple sclerosis. Lancet 391, 1622–1636. https://doi.org/10.1016/S0140-6736(18)30461-1.

Traboulsee, A., Li, D.K.B., Cascione, M., Fang, J., Dangond, F., Miller, A., 2018. Effect of interferon beta-1a subcutaneously three times weekly on clinical and radiological measures and no evidence of disease activity status in patients with relapsing-remitting multiple sclerosis at year 1. BMC Neurol. 18, 1–9.

Transl., J., Wang, Y., Xie, C., Song, Y., Xiang, W., Peng, J., Han, L., Ding, J., Guan, Y., 2021. mir-20a suppresses treg differentiation by targeting Map3k9 in experimental autoimmune encephalomyelitis. J. Transl. Med. 19, 223.

Tsitsiou, E., Lindsay, M.A., 2009. microRNAs and the immune response. Curr. Opin. Pharmacol. 9, 514–520. https://doi.org/10.1016/j.coph.2009.05.003.

Tufekci, K.U., Oner, M.G., Genc, S., Genc, K., 2011. MicroRNAs and multiple sclerosis. Autoimmun Rev. 11, 1–27. https://doi.org/10.1002/jcla.22266.

Vandesompele, J., Preter, K., De, Poppe, B., Roy, N., Van, Paepe, A.De, 2002. Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol. 034, 1.

Waschbisch, A., Atiya, M., Linker, R.A., Potapov, S., Schwab, S., Derfus, T., 2011. Glatiramer acetate treatment normalizes deregulated microRNA expression in relapsing remitting multiple sclerosis. PLoS One 6, 1–5. https://doi.org/10.1371/journal.pone.0024604.

Yadav, S.K., Soins, D., Ito, K., Dhib-jalbut, S., 2019. Insight into the mechanism of action of dimethyl fumarate in multiple sclerosis. J. Mol. Med. 97, 463–472.

Yasuda, K., Takeuchi, Y., Hirota, K., 2019. The pathogenicity of Th17 cells in autoimmune diseases. Semin. Immunopathol. 41, 283–297.

Zhang, J., Cheng, Y., Cai, W., Li, M., Li, B., Guo, L., 2014. MicroRNA-155 modulates Th1 and Th17 cell differentiation and is associated with multiple sclerosis and experimental autoimmune encephalomyelitis. J. Neuroimmunol. 266, 56–63.

Zheleznyakova, Y.G., Piket, E., Needhamsen, M., Hagemann-jensen, M., Ekman, D., 2021. Small noncoding RNA profiling across cellular and biofluid compartments and their implications for multiple sclerosis immunopathology. Proc. Natl. Acad. Sci. U.S. A. 118. https://doi.org/10.1073/pnas.2011574118.

Zheng, B., Yin, W., Suzuki, T., Zhang, Y., Song, L., Jin, L., Zhan, H., Zhang, H., Li, J., Wen, J., 2017. Exosome-mediated mir-155 transfer from smooth muscle cells to endothelial cells induces endothelial injury and promotes atherosclerosis. Mol. Ther. 25, 1279–1294.

Zheng, L., Zhuang, C., Wang, X., 2018. Serum miR–146a, miR-155, and miR–210 as potential markers of Graves’ disease. J. Clin. Lab. Anal. 32, e2286. https://doi.org/10.1002/jcla.2286.