Tampere University

JULIA VISTBACKA

Circulating microRNAs as Biomarkers for Multiple Sclerosis

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ACADEMIC DISSERTATION
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of Tampere University,
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ACADEMIC DISSERTATION
Tampere University, Faculty of Medicine and Health Technology
Finland

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PunaMusta Oy – Yliopistopaino
Joensuu 2022

To my family

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ABSTRACT

Background: Multiple sclerosis (MS) is an immune-mediated demyelinating and degenerative inflammatory disease of the central nervous system that causes severe neurological dysfunction and leads to disabilities in patients. Despite a large number of molecular biomarkers proposed for MS, only a few of them have been rigorously validated and used clinically. MicroRNAs (miRNAs) are small non-coding RNAs, shown to associate with pathogenesis and development of various diseases, including MS. miRNAs regulate gene expression at the post-transcriptional level through translational inhibition or degradation of target mRNA. Single miRNA can regulate expression of multiple target genes, and one mRNA can be targeted by multiple miRNAs, creating a complex regulatory network. Considering their stability and relative ease of detection, circulating miRNAs are promising new biomarker candidates for MS and its clinical subtypes that may provide significant additive value for disease diagnostics, prediction of disease course, and monitoring therapeutic responses.

Aims: This doctoral thesis focused on exploring the role of human circulating miRNAs as biomarkers of MS disease and its clinical subtypes and association with the clinical activity and severity of the disease.

Subjects and Methods: The expressions of circulating miRNAs were studied in serum of a total of 290 subjects: 81 relapsing-remitting (RRMS), 66 primary progressive (PPMS), 45 secondary progressive (SPMS), 18 clinically isolated syndrome (CIS), and 80 healthy controls (HC) (Studies I-III). The expression of miR-128-3p, miR-191-5p, miR-24-3p, and miR-223-3p was evaluated over the four-year follow-up in 57 MS cases, 18 CIS patients, and 32 age- and sex-matched HCs (Study III). MiScript serum miRNA RT-PCR assay techniques were used to measure relative expression levels of selected miRNAs. Statistical analyses were performed using SPSS version 22.0 (IBM corporation, Armonk, NY, USA) in Studies I-III.

Results: From 84 miRNAs studied, miR-128-3p expressed the most potential as a diagnostic biomarker for progressive MS, as it was overexpressed in PPMS, as

compared to SPMS (Study I) and HCs (Study I, III). In turn, miR-191-5p was overexpressed in all the MS subtypes in comparison to HCs, but not to CIS or each other (Study I-III). In addition, miR-24-3p was overexpressed in PPMS to SPMS (Study I), PPMS to HCs (Study I-II), and RRMS to HCs (Study II) comparisons. As for miR-376c-3p, its overexpression was observed in PPMS to HCs comparison (Study I). While miR-223-3p, included in study III, showed no statistically significant differences between MS subtypes and HCs or CIS. The expression of miR-128-3p and miR-24-3p was stable over the four-year follow-up period, while temporal changes of miR-191-5p and miR-223-3p were observed in MS but not in CIS. When the clinical activity was taken into the consideration, temporal changes in miR-191-5p were observed among the patients with an increase in expanded disability status scale (EDSS) or magnetic resonance imaging T1 or FLAIR lesion volumes, while miR-223-3p fluctuated in relapse active RRMS.

Conclusions: The results of this doctoral thesis suggested the potential of circulating miRNAs, especially miR-191-5p and miR-24-3p, as diagnostic biomarkers for MS and miR-128-3p for PPMS. In addition, miR-191-5p and miR-223-3p can potentially reflect temporal changes related to MS pathology.

TIIVISTELMÄ

Tausta: Multippeliskleroosi (MS) on immuunivälitteinen keskushermoston demyelinisoiva ja rappeuttava tulehduksellinen sairaus, joka aiheuttaa vakavia neurologisia toimintahäiriöitä ja johtaa potilaiden vammautumiseen. Huolimatta MS-tautiin ehdotettujen molekyylibiomarkkereiden suuresta määrästä vain muutamia niistä on tiukasti validoitu ja käytetty kliinisesti. MikroRNA:t (miRNA:t) ovat pieniä ei-koodaavia RNA:ita ja niiden on osoitettu toimivan avainmolekyyleinä useiden sairauksien patogeneesissä ja kehityksessä. MiRNA:t säätelevät geenin ilmentymistä transkription jälkeisellä tasolla joko estämällä mRNA:n translaatiota tai edesauttamalla kohde-mRNA:sa hajoamista. MiRNA-järjestelmä on uusi genominen säätelykerros, joka yhdistää kymmeniä tai satoja kohdegeenejä tai niiden muodostamia aineenvaihduntareittejä yhden miRNA:n säätelyn alaisuuteen. Huomioiden miRNA:iden hyvä stabiilius ja niiden suhteellisen helppo analysointi, verenkierron miRNA:t ovat lupaavia uusia biomarkkeriehdokkaita MS-tautiin ja sen kliinisten alatyyppien diagnostiikkaan ja voivat tarjota merkittävää lisäarvoa myös taudin kulun ennustamisessa ja sen terapeuttisten vasteiden optimoinnissa.

Tavoitteet: Tämä väitöskirjatyö keskittyi tutkimaan ihmisen verenkierrossa olevien miRNA:iden osuutta MS-taudin ja sen kliinisten alatyyppien biomarkkereina sekä yhteyttä taudin kliinisen aktiivisuuden ja vaikeusasteen kanssa.

Aineistot ja menetelmät: Verenkierrossa olevien miRNA:iden (n=84) ilmentymistä tutkittiin yhteensä 290 henkilön seerumista: 81 aaltomaista MS-tautia (RRMS), 66 ensisijaisesti etenevää MS-tautia (PPMS), 45 toissijaisesti etenevää MS-tautia (SPMS) ja 18 kliinisesti eriytynyttä oireyhtymää (CIS) sairastavilla sekä 80 terveellä kontrollihenkilöllä (osatyöt I-III). MiR-128-3p:n, miR-191-5p:n, miR-24-3p:n ja miR-223-3p:n ilmentymistä tutkittiin myös neljän vuoden seurannan aikana 57 MS-potilaalla, 18 CIS-potilaalla ja 32 ikä ja sukupuoli kaltaistetussa kontrollilla (osatyö III). MiScript seerumin miRNA RT-PCR-määritystekniikoita käytettiin valittujen

miRNA:iden suhteellisten ilmentymistasojen mittaamiseen. Tilastolliset analyysit suoritettiin käyttämällä SPSS-versiota 22.0 (IBM Corporation, Armonk, NY, USA) tutkimuksissa I-III.

Tulokset: Tutkituista miRNA:ista (n=84) miR-128-3p ilmensi eniten potentiaalia etenevän MS-taudin diagnostisena biomarkkerina, koska se oli yli-ilmentynyt PPMS:ssä verrattuna SPMS:ään (osatyö I) ja terveisiin kontrolleihin (osatyöt I-III). MiR-191-5p yli-ilmentyi RRMS:ssä (osatyöt II-III), PPMS:ssä (osatyöt I-II) ja SPMS:ssä (osatyö I) verrattuna terveisiin kontrolleihin. Kun taas miR-24-3p yli-ilmentyi PPMS:ssä verrattuna SPMS:ään (osatyö I) ja terveisiin kontrolleihin (I-II) sekä RRMS:n verrattuna terveisiin kontrolleihin (II). Mitä tulee miR-376c-3p:hen sen yli-ilmentymistä havaittiin PPMS:ssä verrattuna terveisiin kontrollihenkilöihin (osatyö I). Tutkimukseen III sisältyvä miR-223-3p ei osoittanut tilastollisesti merkitseviä eroja minkään MS-alatyypin ja terveiden kontrollihenkilöiden tai CIS:n välillä. MiR-128-3p:n ja miR-24-3p:n ilmentyminen oli vakaa neljän vuoden seurantajakson ajan, kun taas miR-191-5p:n ja miR-223-3p:n ajallisia muutoksia havaittiin MS:ssa mutta ei CIS:ssä. Kun taudin kliininen aktiivisuus otettiin huomioon miR-191-5p:ssä havaittiin ajallisia muutoksia potilailla, joilla oli lisääntyvä disabiliteetti EDSS-pisteillä luokiteltuna tai aivojen magneettikuvauksessa havaitut lisääntyvät T1- tai FLAIR-leesiovolyymit kun taas miR-223-3p vaihteli relapsiaktiivisessa RRMS:ssä**.**

Johtopäätökset: Yhdistettynä tämän väitöskirjatyön tulokset heijastelevat verenkierron miRNA:iden, erityisesti miR-191-5p:n ja miR-24-3p:n merkitystä MS-taudin diagnostisina biomarkkereina ja miR-128-3p:n tärkeyttä PPMS:n diagnostisena biomarkkerina. Lisäksi miR-191-5p ja miR-223-3p voivat mahdollisesti heijastaa MS-taudin patologiaan liittyviviä ajallisia muutoksia.

TABLE OF CONTENTS

List of Figures

List of Tables

LIST OF THE ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals I – III. The original publications have been reproduced with the permission of the copyright holders.

Study I Vistbakka J, Elovaara I, Lehtimäki T, Hagman S Circulating miRNAs as biomarkers in progressive multiple sclerosis. Multiple Sclerosis Journal. 2017; 23(3):403-412

Study II Vistbakka J, Sumelahti ML, Lehtimäki T, Elovaara I, Hagman S. Evaluation of serum miR-191-5p, miR-24-3p, miR-128-3p and miR-376c-3p in multiple sclerosis patients. Acta Neurol Scand. 2018; 138(2):130-136

Study III Vistbakka J, Sumelahti ML, Lehtimäki T, Hagman S. Temporal changes of serum miR-191, miR-223, miR-128 and miR-24 in multiple sclerosis. (Submitted, under revision)

ABBREVIATIONS

APC	Antigen-presenting cells
BBB	Blood-brain barrier
BMI	Body mass index
cDNA	Complementary deoxyribonucleic acid
CIS	Clinically isolated syndrome
CNS	Central nervous system
CSF	Cerebrospinal fluid
DC	Dendritic cell
DIS	Dissemination in space
DIT	Dissemination in time
DMT	Disease-modifying therapy
EBV	Epstein-Barr virus
EDSS	Expanded disability status scale
EV	Extracellular vesicles
FC	Fold change
FLAIR	Fluid attenuated inversion recovery
Gd	Gadolinium
GM	Grey matter
HLA	Human leukocyte antigen
MHC	Major histocompatibility complex
miRNA	MicroRNA
MRI	Magnetic resonance imaging

MS	Multiple sclerosis
NAWM	Normal-appearing white matter
OCBs	Oligoclonal bands
OR	Odds ratio
PBMC	Peripheral blood mononuclear cell
PMS	Progressive MS
PPMS	Primary progressive multiple sclerosis
pre-miRNA	Precursor miRNA
pri-miRNA	Primary miRNA
RRMS	Relapsing-remitting multiple sclerosis
RT-PCR	Real-time polymerase chain reaction
SPMS	Secondary progressive multiple sclerosis
WM	White matter

AUTHOR'S CONTRIBUTION

The author of this thesis was the sole main author of all the publications (Studies I – III). This thesis work was supervised by docent Sanna Hagman, Professor Irina Elovaara, and Professor Terho Lehtimäki. The author designed the studies I-III. In addition, the author

Study I: performed laboratory works, analysed data, and wrote the paper

Study II: conducted the laboratory works and data analysis, wrote the paper, and led the submission as the corresponding author

Study III: executed laboratory works, performed statistical analysis, and wrote the paper

1 INTRODUCTION

Multiple sclerosis (MS) is a chronic inflammatory autoimmune disease of the central nervous system (CNS). Being one of the most common causes of non-traumatic neurological disabilities in young adults, it afflicts more than 2.8 million people worldwide [1].

The exact cause of MS is not known, but a complex interaction between genetic susceptibility and environmental factors seems to contribute. Different theories are proposed on how MS is triggered. One view suggests that MS is a primary inflammatory disease, where demyelination is driven by immune-mediated mechanisms. While according to the other view, MS is a primary neurodegenerative disease, with inflammation occurring as a secondary response [2]–[4]. However, it is generally accepted that immunopathogenesis of MS is mediated by a breakdown of immunological tolerance to CNS myelin or myelin-like antigens. The pathogenesis of MS is complex, with main pathological characteristics as demyelination, inflammation, and formation of lesions in CNS. Considering the complex pathology, the clinical course and presentation of MS are highly heterogeneous between the patients and can vary over time within one patient [5].

The diagnosis of MS is mainly based on clinical evaluation, although it is supported by laboratory and radiological investigations. Magnetic resonance imaging (MRI) is used to observe MS-specific changes in the CNS, and cerebrospinal fluid (CSF) analysis is used to detect intrathecal production of IgG to indicate immune responses in the CNS [6], [7].

No MS-specific laboratory tests are available for the diagnosis and monitoring of the disease [7]. Thus, there is a need for biomarkers that would enable early diagnosis and respectively early treatment, so that accumulation of disability can be slowed or stopped as early as possible. Also, biomarkers that would recognize high-risk patients for rapid disability accumulation and evaluate the treatment responses to therapies are needed.

The biomarker research field is very active in MS. Yet, regardless of the multitude of candidate molecular biomarkers proposed, very few have been validated and implemented in clinical practice.

Circulating microRNAs (miRNAs) showed notable potential as a possible diagnostic biomarker for MS. miRNAs are small non-coding regulatory RNAs. They are powerful post-transcriptional regulators, that can inhibit translation or degrade their target messenger RNAs (mRNA) to modulate and fine-tune gene expression levels. Regulatory networks formed by miRNAs combine tens or hundreds of target genes of a single miRNA, but also multiple miRNAs that regulate the same gene. Notably, the expression levels of miRNAs can be influenced both by genetic and environmental factors [8], [9].

Deregulation of miRNA expression is associated with numerous human diseases, including MS. Up- and down-regulation of miRNAs upon the abnormal conditions create a signature pattern that can be considered as a biomarker or molecular therapeutic target for corresponding diseases [10].

Different cells have different miRNA expression profiles, but mature miRNAs have been detected also in extracellular fluids [11]. These so-called circulating miRNAs are exceptionally stable and can survive unfavourable physiological conditions, including extended storage, multiple freeze-thaw cycles, and variations in pH [11]–[14], making them a promising biomarker candidate. Therefore, the present doctoral thesis focused on exploring the role of human circulating miRNAs as biomarkers of MS disease and its clinical subtypes and association with the clinical activity and severity of the disease.

2 REVIEW OF THE LITERATURE

2.1 Clinical subtypes and disease course of multiple sclerosis

MS is a chronic neurodegenerative autoimmune disease with a complex clinical course characterized by inflammation, demyelination, and axonal degeneration. MS is highly heterogenic in terms of pathology, clinical features, and response to treatment. Thus, several disease subtypes are recognized (Figure 1) that are classified according to initial and current clinical disease course. In approximately 85% of cases, the disease starts with a relapsing-remitting clinical course (RRMS) when the patients are in their third decade of life. RRMS is characterized by interchanging periods of relapsing neurologic symptoms and complete or partial clinical recovery. Relapses concur with focal CNS inflammation and demyelination. Relapses develop over hours to days, reach a plateau lasting several weeks, followed by a recovery period. Over time recovery becomes less and less effective, leading to the disability accumulation (discussed more precisely in Chapter 2.3 Neuropathology of MS) [5], [15], [16]. In 10 to 15 years 65-80% of RRMS patients will convert into a secondary progressive MS (SPMS) phase, characterized by a gradual, irreversible decrease in neurological function, that occurs independently of the relapses [2], [15]. There is no distinct transition period from RRMS to SPMS, and relapses occur on a background of subtle progression until progression is dominant [16]. In SPMS inflammatory lesions are no longer a central event in the pathogenesis, and progressive neurological decline is instead accompanied by axonal loss and reduction in brain volume [5]. About 10 to 20% of patients will develop primary progressive disease course (PPMS) from the onset, characterized by progressive disability development without relapses [15]. Similar to SPMS, the mean age at PPMS onset is the fifth decade of life [2], [5]. Overall, progressive MS (PMS) is defined as a gradual increase of neurological disability independently of relapses and occurs in different MS courses, such as PPMS and SPMS [2], [17], [18].

Clinically isolated syndrome (CIS) is a pre-clinical stage of MS (Figure 1). It represents the first episode of neurologic symptoms suggestive of MS, caused by

demyelination or inflammation in the CNS. To be classified as CIS, the episode should last for at least 24 h and occur in the absence of fever or infection [19]. A longitudinal study showed that 63% of CIS patients develop MS in 20 years of follow-up [20].

Depending on their anatomical location, lesions can cause various symptoms, such as optic neuritis, caused by the lesions of the optic nerve, motor weakness, as a result of lesions of the cortical tracts, abnormal somatic sensations, due to lesions of somatic sensory pathways, or double vision, caused by lesions of medial longitudinal fasciculus [2]. Lesions can also appear in areas of the brain that do not produce any symptoms (Figure 1).

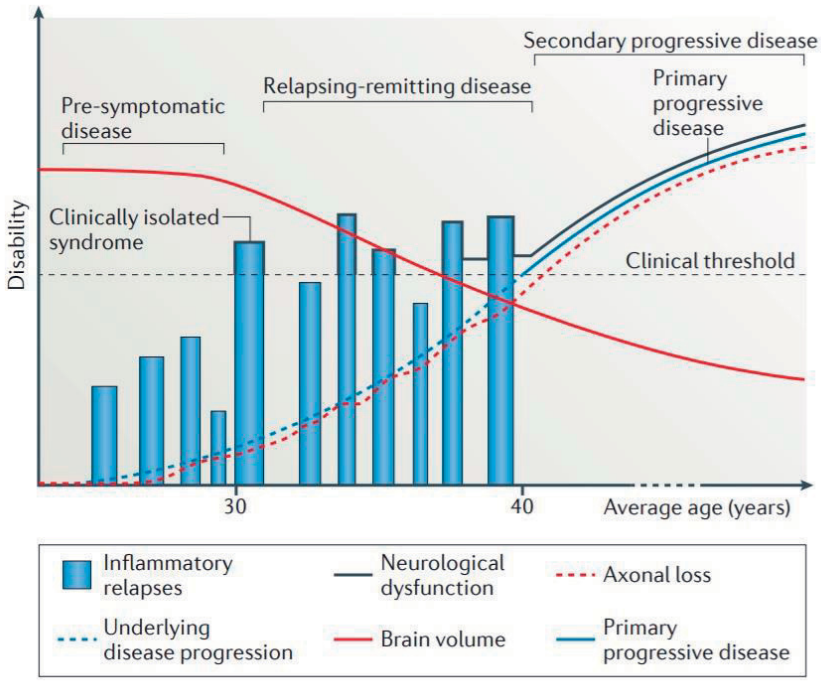

Figure 1. Schematic representation of MS disease courses. MS begins before clinical symptoms are evident. Inflammatory relapse column above the clinical threshold line, peaks represent lesions that cause visible neurological dysfunction. Those columns that remain below the threshold line represent clinically silent lesions. The first episode of neurologic symptoms suggestive of MS manifests clinically isolated syndrome. It progresses into the relapsing-remitting form, eventually evolving to secondary progressive disease. Some patients have progressive disease course from onset (primary progressive MS). Picture from Dendrou et.al 2015, reproduced with permission from the publisher [4].

2.2 Epidemiology and aetiology of MS

With over 2.8 million people affected worldwide, MS is considered one of the most common causes of non-traumatic neurological disability among young adults.
MS prevalence is highly heterogenic worldwide depending on the continent and geographical latitude. Its prevalence is highest in North America and northern Europe countries (more than 200 cases per 100 000) to much lower in East Asia, Latin America, and sub-Saharan Africa (0-25/100 000 cases) [1], [21].

Finland belongs to a high-risk MS region, with an estimated prevalence between 180-200 cases per 100 000 [1], [22]. The heterogeneity in MS prevalence is also seen in Finland, with high-risk areas located in south-western regions (251/100 000 in 2016) [23]. The prevalence of MS has increased by 13% since 2013, which can be partly explained by progress in diagnostics and by the global decrease in disease-related mortality [1].

2.2.1 Gender

Epidemiological studies have shown that women are more likely to develop MS. Over the past decades, the female-to-male ratio has increased, from 1 to 1 reported in the 1940s to 1.5-2.8 to 1 in the 2000s [24]–[26]). This trend is associated primarily with RRMS, with the reported ratio of 3:1. In turn, among the PPMS patients from 20 to 59 years of age and the RRMS patients older than 59, the ratio was 1 to 1 [27], [28]. The decline in female prevalence was observed with increasing age at diagnosis and was no longer present among the RRMS patients older than 58 [27].
Gender seems to influence MS susceptibility. Females tend to have an earlier disease onset, less progression, and disability accumulation, but higher inflammatory activity and higher relapse rate throughout the disease than males [29], [30]. In addition, the disease-modifying effect of pregnancy is well established, with a reduction in relapse rate especially during the third trimester, possibly driven by the immunomodulatory properties of the oestrogens and the progesterone. In contrast, the postpartum period holds increased relapse risk, which may be due to the abrupt removal of protective pregnancy factors [29]–[32]).
In turn, men, in general, are less likely to develop MS, but they are more prone to progressive forms and earlier conversion from RRMS to SPMS [27], [30]. Men tend

to display more progressive and severe disease course with significant disability accumulation over time, in contrast to women [30]. Notably, men tend to worsen more in relapse-free study intervals than women, with no observed effect of relapses on the expanded disability status scale (EDSS) change [33]. In addition, men tend to develop less inflammatory lesions but a higher number of degenerative lesions with extensive axonal loss [30]. Inconsistent evidences suggest an association of male sex with higher incidence and severity of cognitive impairment [34]–[36].

2.2.2 Environmental factors

MS is a multifactorial disease, which results from a complex interaction between genetic susceptibility and environmental agents. However, the exact cause is still unknown. Twin studies have highlighted the contribution of environmental factors at 29-74% for shared and 11-23% for unique variants [37], [38]. The lifestyle and environmental factors that contribute to MS susceptibility include exposure to viral infections, low levels of D vitamin, smoking, and adolescent obesity. In contrast, oral tobacco, alcohol use, and high coffee consumption are potentially associated with reduced risks [39]. Environmental factors impact the disease risk and progression, possibly through epigenetic changes, which, in turn, regulate the responses of immune and neural cells. Yet, the evidence is insufficient to establish a causative role.

Viral infections: The infectious origin of MS was proposed at the end of 19th century. Since then various viruses, including measles, cytomegalovirus, varicella zoster, human endogenous retroviruses, human herpesvirus 6 (HHV-6), and Epstein-Barr virus (EBV) were considered as risk factors for MS [40]. Currently, among the infectious agents highest risk factors for MS susceptibility are the herpes viruses the Human Herpesvirus 6 (HHV-6) (OR=4.2 in developed countries, OR=3.4 in developing countries) [41] and the Epstein-Barr virus (EBV, also called HHV-4)(OR=2.3) [42]. More than 90% of the adult population is estimated to be infected by the HHV-6 [41] and EBV [43]. Nevertheless, most infected individuals do not develop MS, suggesting that infection is a prerequisite for developing MS, but is not sufficient by itself. The infections occurred during adolescence or later in life seem to increase the risk of MS. Particularly, after symptomatic EBV infection (infectious mononucleosis), the risk to develop MS is two to three-fold higher than

among those who had an asymptomatic infection [25], [44]. In turn, the latest study reported a 32-fold increase in risk to develop MS after EBV infections [45]. The link between EBV and MS is also supported by the elevated antibody levels in MS patients, as compared to healthy individuals (OR=3.6) [46], [47]. Studies examining virus particles from the brain tissues are controversial, as several studies reported EBV to be rare or even absent [48]–[51], while others recorded the presence of EBV-infected cells in the brain tissue of MS patients [52]–[55]. As for the HHV-6, several studies describe a possible correlation with risks to develop MS, with the odd range varying between 2.5 and 6 depending on the choice of the specimen, such as saliva, tissues, cerebral spinal fluid (CSF), peripheral blood mononuclear cells (PBMC) or serum [41].

In the light of the recent COVID-19 pandemic, where severely ill patients develop neurological symptoms, SARS-CoV-2 can in the future develop into a new risk factor for MS. Remarkably, the high frequency of anti-neuronal autoantibodies was reported in serum and CSF of critically ill COVID-19 patients [56].

Vitamin D: Increasing evidence indicates that higher levels of serum calcidiol (25(OH)D), a pre-active form of vitamin D, are associated with a lower risk to develop MS [57], [58] and reduced relapse rate [59]–[62]. Increased levels of vitamin D before the age of 20 were found to contribute to a decreased risk of MS later in life [63]. While, insufficient vitamin D intake during pregnancy seems to increase the risk of developing MS in the offspring [57]. Vitamin D levels have also impact on disease course since lower vitamin D levels in plasma or serum are strongly associated with the development of new T2 [64], [65] and gadolinium (Gd)-enhancing MRI brain lesions [65], greater disease severity [62], [64]–[66] and conversion from CIS to MS [64], [67].

It is still unknown what levels of serum calcitriol are protective for MS in a large majority of individuals. However, according to the Endocrine Society daily vitamin D intake should be 10-25 μg (400-1000 IU) a day for infants, 15-25 μg (600-1000 IU) for children, and 37-50 μg (1500-2000 IU) for adults [68].

The contribution of vitamin D as a risk factor is possibly based on its essential role in immunological processes. It has anti-inflammatory properties, through suppressing the adaptive and innate immune systems. It influences the CD4 + T cells differentiation in favour of anti-inflammatory T helper 2 (Th2), as well as enhances the differentiation of hematopoietic stem cells into natural killer cells (NK)

and impedes the function of the dendritic cells (DC). Further, vitamin D was also proposed to have a role in the demyelination and remyelination processes [69].

Smoking: Several studies reported smoking to be associated with an increased risk of developing MS [70]–[72] and faster clinical progression [73]. In addition, smoking is associated with the risk of developing neutralizing antibodies against natalizumab and interferon-β (IFN-β) treatments [39]. Notably, passive smoking also seems to contribute to MS susceptibility [72], [74]. In contrast, nicotine on its own may have a protective effect [75], [76].

Obesity: Several studies report obesity (BMI>30), especially in childhood and adolescence, to increase the risk of MS (OR=1.26) [77], [78]. The adipose tissue is involved in metabolic, endocrine, and immune processes. Adipocytes secreted adipokines, such as adiponectin, leptin, and resistin, influence the immune system by promoting a pro-inflammatory state ([78]). In addition, obesity leads to decreased bioavailability of vitamin D [79].

2.2.3 Genetic risk factors and epigenetics

Twin studies have shown a genetic predisposition to MS with a concordance rate of 20-30% in monozygotic twins, 2-14% in dizygotic twins, and 3% for non-twin siblings of affected individuals [37], [38], [80]. An estimated MS heritability is reported to range between 15 and 50% [37], [38], [81], emphasizing the contribution of a genetic component. Nevertheless, the inheritance model of MS pathology is complex, and MS-prone genotype results from multiple, interacting, or independent genes, each representing moderate to low risk effects [82]. Although MS is not a hereditary disease, susceptibility to genetic variations increases the risk.

In total 32 single nucleotide polymorphisms (SNPs) contained in the major histocompatibility complex (MHC), one variant in X chromosome and 200 in the non-MHC region of the genome, were reported to associate with MS susceptibility [83]. MHC, called HLA (human leukocyte antigen) in humans, is the first described and still the major MS susceptibility locus. Located on chromosome 6p21.3, it contains many genes that have pivotal roles in the immune system. Its association with MS risk has been observed across all populations studied, both in PPMS and RRMS patients [82]. In particular, the HLA-DRB1 gene and especially the HLA-DRB1*15:01 allele has been associated with age at disease onset, response to

immunomodulators, and brain MRI outcomes [84]. In addition, the HLA-DRB1*09 allele was reported to be significantly associated with a high risk of PPMS (OR=8.13), while HLADRB1*03 and *15 were associated with increased risk of RRMS (OR=1.83 and 2.76) [85]. Some polymorphisms seem to interact with environmental risk factors. For example, the HLA-DRB1*15:01 allele, confers higher risks in smokers (OR 13.5), in individuals with previous EBV infections (OR 16.0), and those with adolescent obesity (OR 16.2) [5].

Emerging data show that also epigenetic mechanisms, such as histone modification, DNA methylation, and mRNA-associated post-transcriptional regulation of gene expression via the miRNAs, contribute to the pathophysiology, susceptibility, and progression of MS [86]. In general, epigenetics refers to control of gene expression without direct altering of the DNA sequence and is believed to be involved in the interaction between environmental and genetic factors. The contribution of miRNAs to MS will be discussed further in detail (Chapter 2.9 Circulating miRNAs in MS).

2.3 Diagnosis of MS and therapy

The diagnostic criteria for MS, called McDonald criteria, were first developed by the International Panel on Diagnosis of Multiple Sclerosis in 2001. Since that time, they were revised in 2005, 2010, and 2017 [6], [87]–[89]. Currently, the diagnosis of MS is based on the 2017 criteria [6] and is primarily based on clinical evaluation, facilitated by supportive laboratory and radiological investigations, such as MRI and CSF investigation. The cornerstone of MS diagnosis is clinical and MRI demonstration of disease dissemination in time (DIT) and space (DIS). According to the 2017 McDonald criteria revision, DIS can be demonstrated by a new clinical attack, or by MRI with at least one T2-hyperintense lesions that are characteristic of MS in at least two of the areas: infratentorial, cortical/juxtacortical, and periventricular brain regions, and the spinal cord. DIT, in turn, can be demonstrated by a new clinical attack, the presence of oligoclonal bands (OCB) in CSF or by MRI with the simultaneous presence of Gd-enhancing and non-enhancing lesions at any time or by a new T2-hyperintense or Gd-enhancing lesion on follow-up, irrespective of the timing of the baseline scan [6]. The MRI and OCB are further discussed in Chapter 2.6 Biomarkers.

Table 1. Disease-modifying therapies in MS.

Agent	Trade names (manufacturer in Europe; FDA approval)	Mechanism of action	Subtype
Interferon beta-1a	Avonex (Biogen; 1996), Rebif (Merck; 2002)	Enhances activity of T reg cells, reduces pro-inflammatory cytokine production and antigen presentation, prevents leucocytes trafficking into the CNS.	CIS, RRMS, active SPMS
Interferon beta-1b	Betaferon (Bayer AG; 1993), Extavia (Novartis; 1993)		
Peginterferon beta-1a	Plegridy (Biogen; 2014)		
Glatiramer acetate	Copaxone (Teva; 1996, 2014), Glatopa (Novartis; 2015, 2018)	Shifts immune responses from Th1 to Th2.	CIS, RRMS, active SPMS
Ocrelizumab	Ocrevus (Roche; 2017)	Anti-CD20 mAb, B-cell depletion.	CIS, RRMS, active SPMS, PPMS
Alemtuzumab	Lemtrada[b] (Sanofi Belgium; 2014)	Anti-CD52 mAb, T- and B-cell depletion.	RRMS, active SPMS
Natalizumab	Tysabri[b] (Biogen; 2004)	Anti-VLA4 mAb, prevents leucocytes trafficking into the CNS.	CIS, RRMS, active SPMS
Daclizumab	Zinbryta[a,b](2016)	Anti-CD25 mAb, T-cell depletion.	RRMS
Mitoxantrone	Novantrone (MEDA; 2000)	Impairs DNA synthesis, inhibits lymphocyte proliferation.	RRMS, SPMS
Ponesimod	Ponvory (Janssen-Cilag International; 2021)	Through S1P receptors downregulates the egress of lymphocytes from lymph nodes, reducing their number in peripheral blood.	CIS, RRMS, active SPMS
Siponimod	Mayzent (Novartis; 2019)		CIS, RRMS, active SPMS
Fingolimod	Gilenya[b] (Novartis; 2010)		CIS, RRMS, active SPMS
Cladribine	Mavenclad[b] (Merck; 2019)	Impairs DNA synthesis. T and B cell depletion.	RRMS, active SPMS
Dimethyl fumarate	Tecfidera (Biogen; 2013)	Anti-inflammatory, antioxidant, and neuroprotective action, mediated by the activation of Nrf2 pathway.	CIS, RRMS, active SPMS
Diroximel fumarate	Vumerity (Biogen; 2013)		CIS, RRMS, active SPMS
Teriflunomide	Aubagio (Sanofi-Aventis; 2012)	Inhibition of lymphocyte proliferation.	CIS, RRMS, active SPMS

mAb: monoclonal antibody, CIS: clinically isolated syndrome, RRMS: relapsing-remitting MS, SPMS: secondary progressive MS, PPMS: primary progressive MS, S1P: sphingosine 1-phosphate
[a]no longer authorised in the EU, [b]second-line medication
Based on European Medicines Agency, US Food and Drug Administration, and Biotti et.al. 2018 [90].

Several immunomodulatory therapies are available for treating MS. These therapies are targeted against peripheral inflammation inhibiting the pro-inflammatory responses or preventing the transmigration of immune cells in the CNS.

IFN-β, the first MS treatment, was approved in 1993 by FDA [91]. Since then, multiple disease-modifying therapies (DMT) were established (Table 1). However, the cure for MS is not yet found. The treatment of MS is primarily based on the use of immunomodulators. Current treatment strategies focus on recovery from attacks, slowing the disease progression, and managing MS symptoms, mainly in CIS, RRMS, and active SPMS. So far, only ocrelizumab is approved for the treatment of PPMS [90]. The therapies are divided into moderate efficacy DMTs (IFN-β preparations, dimethyl fumarate, teriflunomide, fingolimod, and glatiramer acetate), with a well-defined safety profile, and high efficacy DMTs (alemtuzumab, natalizumab, ocrelizumab), which are more effective but carries a higher risk of serious side effects [92], [93]. The current European and American treatment guidelines recommend the prescription of high efficacy drugs to patients with highly active disease course. Treatments that have the best ratio between safety and effectiveness are considered as first-line treatments.

In recent decades experimental stem cell therapies, such as autologous hematopoietic stem cell (AHSC) and mesenchymal stem cell (MSC) transplantation were considered as a treatment for aggressive MS, including active PMS, that can slow down the progression of the disease [94], [95].

2.4 Neuropathology of MS

The pathological hallmarks of MS are demyelination, oligodendrocyte loss, axonal degeneration, astrogliosis, and inflammation. The areas of demyelination, so-called lesions, or plaques are the central pathological hallmark of all the MS phenotypes, but they vary over time quantitatively and qualitatively. Lesions typically occur in white matter (WM) but can be also detected in grey matter (GM) of CNS. Typical locations include the periventricular, and juxtacortical WM, the corpus callosum, infratentorial areas (particularly pons and the cerebellum), and GM of the spinal cord, cerebral cortex, brainstem, and optic nerve [2], [5], [96]. Based on the degree and nature of the immune reactions WM demyelinating lesions are characterized into

several subtypes: highly inflammatory active demyelinating, chronic active or slowly expanding, and inactive lesions [2], [5].

Active demyelinating lesions are more typical for CIS and RRMS and become rare in patients with progressive forms of MS, due to reduced frequency of inflammatory events in these patients [2], [5]. These lesions are highly infiltrated by activated microglia, macrophages, and lymphocytes, mainly CD8+ T cells and CD20+ B cells and the lesser number of CD4+ T cells and plasma cells [2], [5], [97].

Slowly expanding or chronic active lesions are more prominent in progressive MS (PMS) patients, including PPMS and SPMS [2], [5]. They are slowly expansive due to active demyelination at the lesion edge. Chronic active plaques are distinguished by a rim of activated microglia and deposits of complement components at the lesion edge, surrounding a hypocellular and gliotic core. Remarkably in a recent study, chronic lesions were reported to associate with aggressive disease course and poor clinical outcome [98].

Inactive lesions are also more common to progressive forms of MS and are the dominating lesion type in patients with a disease duration of more than 15 years and SPMS without disease activity. They are characterized by a prominent loss of oligodendrocytes and axons, pronounced astrogliosis and reduced density of microglia. While the inflammation, less number of T cells, macrophages and activated microglia are no more predominant[5], [99].

NAWM and NAGM. Outside of the lesions, there are signs of inflammation and neuro-axonal damage in the macroscopically normal white matter, or so-called normal-appearing white matter (NAWM) and normal-appearing grey matter (NAGM) [2], [96]. Immunopathological changes in the NAWM are pervasive in PMS but have also been observed in RRMS. These changes comprise diffuse axonal injury and microglial activation, as well as scattered lymphocytes [5].

Remyelination. Demyelination can be compensated by remyelination, a process of generation of new myelin sheaths around demyelinated axons. In MS, remyelination is insufficient and its degree is highly variable between the patients and even between the lesions in the same individual, which could in part be due to oligodendrocyte heterogeneity and amount of oligodendrocytes and macrophages in lesions [2], [5]. Over factor that seems to affect the degree of remyelination, are lesion location, the presence of oligodendrocyte progenitor cells, axonal integrity and the occurrence of repeated demyelination of remyelinated areas as well as disease

duration and patients' age [2], [5]. Remyelination is frequently observed in the earliest phases of MS, and it is rarer or absent in PMS [5].

Brain atrophy is the gradual loss of brain volume, which occurs as the result of extensive axonal transection and demyelination [100]. At the rate of nearly 0.5–1.35% per year it is almost ten times faster in MS than observed in normal aging [101]. It appears early in the course of the disease, is associated with disease progression, but can be reduced by DMTs [100].

2.5 Pathogenesis of MS

Immunopathogenesis of MS is a complex multicellular process, where both innate and adaptive immune responses play important roles. Th17, CD4+ T helper 1 (Th1), CD8+, B cell, NK, and phagocytic cells, have a role in the initiation and progression of MS [102]. Inflammation is most pronounced in RRMS, while PMS is considered less inflammation dependent, with compartmentalized inflammation of the CNS remaining a key characteristic [2].

It is still unclear why immune responses are initiated. According to the CNS extrinsic model, the initial event occurs outside the CNS where activated autoreactive T cells migrate to the CNS [102] (Figure 2). Alternatively, the CNS-intrinsic model proposes a primary abnormality in the CNS that triggers disease development, with the transmigration of autoreactive lymphocytes occurring as a secondary event [4], [102]. Regardless of the disease initiation place, events described in both of the theories flow into a determined circle: tissue damage leads to the release of the antigens to the periphery, which in turn initiates new immune responses, followed by the transmigration of activated immune cells to the CNS, driving the inflammation and tissue damage [102]. Immunopathogenesis can be divided into the following subprocesses immune cell activation, dysfunction of the blood-brain barrier (BBB), and tissue damage in the CNS (Figure 2).

2.5.1 Activation and transmigration of immune cells to the central nervous system

One of the main components of immune cell activation is the presentation of antigens bound by MHC on the surface of antigen-presenting cells (APC), such as B

cells and DCs to naïve T lymphocytes in the peripheral lymphoid organs [103]. The nature of such antigens is not thoroughly understood, but it could be a viral (for example, EBV) protein mimicking CNS proteins, bystander activation, or DC transferred CNS proteins [5], [103]. Notably, MS-derived T cells display spontaneous proliferation in the absence of exogenous stimuli or antigen, with memory B cells suggested playing a key role through HLA-dependent mechanisms [104].

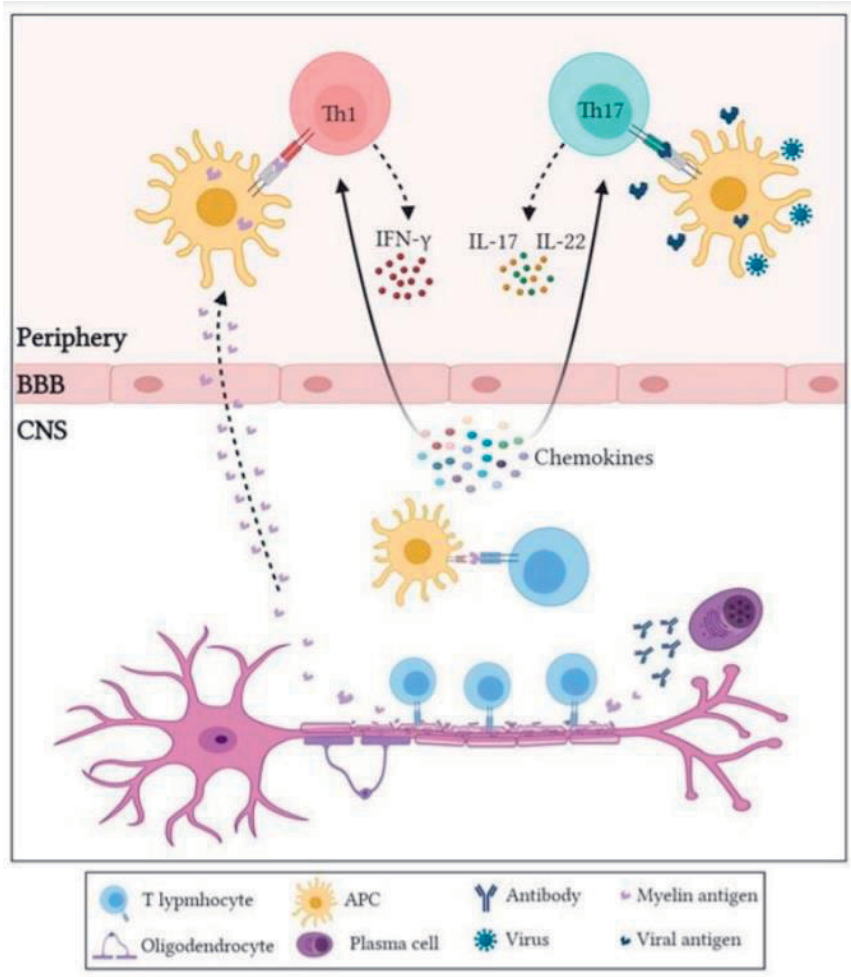

Figure 2. Immune responses in MS. At the periphery activated immune cells traffic through the BBB leading to inflammation and tissue damage in the CNS. Picture from Dhaiban et.al. 2021 [103], reproduced with permission from the publisher.

Once activated, CD8+ and differentiated CD4+ T cells, B cells, macrophages, and DCs migrate into the CNS through the disturbed BBB in the CNS parenchyma, blood-CSF barrier (BCB) within choroid plexus and the subarachnoid space (SAS) [5], [15], [103]. Normally tight junctions between endothelial cells of the BBB and between the epithelial cell of the BCB hinder the access to the brain parenchyma and CSF, respectively. Loss of BBB integrity is an early hallmark of RRMS. However, BBB and BCB are also affected by aging [105]. In addition, immune cells express pro-inflammatory cytokines (IFN-γ, IL-17A, etc.), reactive oxygen species (ROS), matrix metalloproteinases (MMPs), and enzymes that can promote their migration to the CNS by influencing BBB function, either directly or indirectly [106]–[109]. Upon neuroinflammation, the BBB endothelium endures alterations such as increased expression of pro-inflammatory cytokines, adhesion molecules, and chemokines, as well as reduced expression of junctional molecules, promoting increased recruitment of circulating leukocytes across the BBB [109], [110].

2.5.2 Mechanisms of neuronal tissue damage

Infiltrating immune cells, resident-activated microglia, and astrocytes, contribute to demyelination and neuronal loss through cell contact-dependent mechanisms and the secretion of soluble factors. Matrix metalloproteinases, pro-inflammatory cytokines, and nitric oxide, secreted by infiltrating immune cells, contribute to the destruction of the myelin sheath and neurons, but also activate other cell types. Demyelination, in turn, increases the inflammatory activation processes inducing BBB damage and stimulating macrophage activation and oxidative stress pathways [4], [5], [103].

T cells are central players in lesion formation. Upon entry into the CNS, CD4+ effector and CD8+ cytotoxic T cells establish and maintain an inflammatory environment, contributing to oligodendrocyte apoptosis, demyelination, and ultimately neuronal loss [111]. APCs mediate activation and proliferation of transmigrated T cells into different committed helper cell subsets characterized by distinct cytokine profiles (Th1, Th17, Th2, Treg). Activated T cells also can transmigrate from the periphery to CNS. The main subsets of CD4+ T cells associated with MS are Th1 and Th17.

CD4+ Th1 cells play a pathogenic role in MS by driving the recruitment and activation of immune cells or by activation of CNS-resident microglia and trigger their differentiation into the inflammatory and neurotoxic M1-like phenotype [112]. Th1 cells secrete the pro-inflammatory cytokines tumour necrosis factor (TNF) and interferon-gamma (IFN-γ), important contributors in MS pathology [113], [114]. In turn, **Th17** cells contribute to BBB disruption, inhibit maturation, survival, and apoptosis of oligodendrocytes and regulate functions of astrocytes [112], [115]. They secrete pro-inflammatory cytokines IL-17, IL-22, and IFN-γ [112]. IL-17 and IFN-γ-secreting T cells activate local glia and APC, allowing them to restimulate myelin-reactive effector T cells. IFN-γ not only can activate microglia to become phagocytic and present antigens but also directly kill oligodendrocytes [111]. In turn, Th2 cells may have a protective role in MS. **Th2 cells** produce a pro-inflammatory IL-4, which suppresses Th1 cells, even in an environment rich in IFN-γ. Besides, IL-4 inhibits Th1-activated macrophages and represses the secretion of pro-inflammatory mediators such as TNF, IL-1, and ROS or reactive nitrogen species (RNS) [116]. Th2 cells also secrete cytokines (IL-13) that activate B cells to produce antibodies and thus promote demyelination through anti-myelin antibody production [116].

CD8+ T cells are more prevalent in MS lesions than the CD4+ T cells. CD8+ cells can also produce IL-17. Particularly IL-17-secreting CD4+ and CD8+ T cells can secrete granzyme B, killing neurons through the glutamate receptor (GluR3)[103], [111].

As APCs, **B cells** contribute to demyelination and neuronal damage. They produce antibodies that have damaging effects on myelin, oligodendrocytes, and other neuronal structures [5]. In MS, B cells tend to produce pro-inflammatory cytokines, such as IL-6, GM-CSF, TNF-α and lack regulatory cytokines, including IL-10 [5]. Upon the disease progression, B cells may gather in highly organized ectopic lymphoid follicles (eLFs). These B cell populations support the constant antigen-driven expansion of B cells in sites of chronic inflammation, independent of the peripheral B cell population [117], [118].

Mononuclear phagocytes such as macrophages, microglia, and DCs are the main players in innate immunity. In MS, they are abundantly present in lesions [119]–[121]. While microglia are CNS-residential cells, activated macrophages and DCs transmigrate from the periphery in the CNS. Microglia and macrophages, in response to different signals, can acquire classically activated pro-inflammatory M1 or alternatively activated anti-inflammatory M2 phenotypes [122].

M1 macrophages and microglia potentially contribute to the pathological changes through the secretion of pro-inflammatory cytokines, such as IL-1β and TNF-α, ROS, RNS, and multiple chemokines [122]–[124]. In turn, M2 cells of the neuroprotective phenotype contribute to the resolution of inflammation and re-establish homeostasis, through anti-inflammatory cytokines (such as IL-4, IL-10, IL-13, IL-33, and TGFβ), growth factors (fibroblast growth factor, insulin-like growth factor I,), neurotrophic factors (brain-derived neurotrophic factor, glial cell-derived neurotrophic factor, nerve growth factor) and pro-survival factor progranulin [122], [124], [125]. The phagocytic activity of macrophages/microglia also serves as a neuroprotective mechanism [126], as the accumulation of myelin debris inhibits remyelination [127]. M1 type cells are predominant during the early stage or acute phase of MS. In turn, the alternatively activated M2 cells undergo a gradual increase during the process of inflammation until the peak of disease, whereas the amount of M1 cells is decreased, thus M2 become predominant during the later phase of the disease [122]

The pathogenesis of MS is also mediated independently of inflammation. Other mechanisms that seem to contribute to neuro-axonal degeneration are loss of myelin trophic support, acute or chronic oxidative stress promoted by innate and adaptive immune cell activation, hypoxia, altered glutamate homeostasis, and age-dependent extracellular free iron accumulation [5], [102]. Neuronal energy deficit linked to the mitochondrial dysfunction seems to contribute to the neurodegenerative processes, both during the acute and chronic phases [102].

2.6 Biomarkers

Biomarker is "a defined characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention" [128]. Thus, biomarkers can include molecular, histologic, radiologic, and physiologic characteristics [129]. Molecular biomarkers refer to biomarkers that can be measured in biological samples (biofluids, biopsy samples, etc.) and include, among other nuclei acids-based biomarkers (DNA, mRNA, miRNA), peptides, proteins, and lipid metabolites.

Characteristics of a biomarker that could be effectively translated into clinical use should include ease of detection and measure, cost-effectiveness, high sensitivity,

specificity, and reproducibility [130], [131]. Specificity and sensitivity are an ability of a biomarker to avoid false positive or negative results, in other words, to classify a person as one with the condition or without.

According to the FDA-NIH Biomarker Working Group, biomarkers can be classified onto diagnostic, monitoring, pharmacodynamic/response, predictive, prognostic, susceptibility/risk, and safety biomarkers [129].

Diagnostic biomarkers can detect or confirm the presence of a disease or condition of interest [129]. Thus, they should discriminate patients with MS from other diseases and HCs and distinguish between MS subtypes, including CIS, to confirm the diagnosis.

OCBs are clonally restricted immunoglobulins (Ig) that can be found in CSF upon chronic or acute immune activation in the CNS. They are not unique to MS and can be found in other chronic inflammatory diseases, such as systemic lupus erythematosus, HIV infection, and aseptic meningitis, among others [132]. Thought, if other diagnoses are excluded, OCBs support MS diagnosis. OCBs are created by immunoglobulin G (IgG) and M (IgM) and can be detected by agarose gel electrophoresis with isoelectric focusing and immunoblotting or immunofixation [6]. Among the CIS patients, the presence of IgG-OCBs indicates an increased risk for developing MS independently of other risk factors [6]. In addition, IgG-OCB-positive patients are shown to have a worse disease prognosis_than IgG-OCB-negative patients [133].

A CSF IgG index is also used as a diagnostic biomarker for MS. It is measured as a quantitative relationship between CSF IgG and serum IgG, divided by the same relationship for albumin [134], [135]. Index value higher than 0.7 indicates an increased intrathecal B cell response and thus suggests the presence of MS. Its sensitivity is lower than the OCBs, as estimated 60-70% of MS patients have an increased IgG index [135]. However, the IgG index higher than 0.7 has a positive predictive value of 99% for OCBs and the test is less time-consuming than OCB [134].

Prognostic biomarkers are used to identify the probability of an occurrence of a clinical event, disease exacerbations, or progression in patients with a certain disease. In terms of MS, they can provide information on the disease activity, progression and indicate conversion from CIS to RRMS or from RRMS to SPMS.

To some extent, IgG-OCBs can be considered as a prognostic biomarker, as IgG-OCB positive patients are shown to have a worse disease prognosis, in comparison

to IgG-OCB negative patients [133]. IgG-OCB is a prognostic marker for CIS to RRMS conversion [135]. The presence of CSF-restricted IgM OCB (but not of IgG OCB) was reported to be associated with an active inflammatory disease phenotype in PPMS patients [136]. It should be taken into consideration, that nearly 95% of MS patients and 85% of CIS are IgG-OCB-positive [132].

Neurofilaments (NF) CSF and serum levels are also promising prognostic biomarkers. NFs are neuronal cytoskeletal proteins that determine the diameter of axons, are involved in axonal transport, and are important for electrical-impulse transmission. Neurofilaments consist of light (NFL), intermediate (NFM), and heavy (NFH) chains [137]. Upon the neuronal damage, NFs are released from neurons and thus can be detected in the CNS and blood [135], [137]. The development of ultra-sensitive techniques, such as the single-molecule arrays (SIMOA), allows the reliable measurement of NFL from serum, which previously was challenging due to low NFL concentrations (Hendricks, Baker et al. 2019). In MS, NFL serum levels correlate with clinical and MRI disease activity, degree of disability, and brain atrophy rate [135], [138], [139]. Withal, NFLs are not MS-specific, as their increased levels were documented in other neurodegenerative conditions, including Alzheimer's and Parkinson's diseases, amyotrophic lateral sclerosis, and traumatic brain injury [137].

Susceptibility/risk biomarkers indicate the increased or decreased chances of developing a disease or a medical condition. This type of biomarkers can be detected many years before the appearance of clinical signs or symptoms [129]. Numerous genetic variations, especially in the HLA cluster, are proposed as risk biomarkers of MS (discussed in Chapter 2.2.3 Genetic risk factors and epigenetics) [85].

Monitoring biomarker is a biomarker assessed serially to reflect disease progression, including the incidence of new disease effects and changes in disease severity, as well as to monitor treatment or an environmental agent response, either favourable or unfavourable [129]. Especially disease activity biomarkers that can reflect high disease activity and rapid worsening in the early phase of MS are important for the choice of therapeutic strategy. For instance, previously discussed serum NFL is proposed as a monitoring biomarker that can reflect disease activity [138], [140], [141], and disability accumulation [142], by several longitudinal studies. Among other possible disease activity biomarkers are CXCL13 [143], NFL [143], [144], GFAP [145], and BDNF [146].

A pharmacodynamic/response biomarker is a biomarker whose level changes in response to exposure to a treatment or an environmental agent [129]. For example,

NFL can be considered a pharmacodynamic/response biomarker. Its expression levels were found to be influenced by IFN-β [138], fingolimod [147], alemtuzumab [148], and natalizumab treatments [149].

Neutralizing antibodies against IFN-β is another example. They are produced in 40% of patients, usually during the first two years of treatment. They reduce the positive effect of treatment on annual relapse rate, disability progression, and MRI activity. Thus a change of therapy is recommended [135].

Neutralizing antibodies against natalizumab are produced in 6% of patients, mainly during the first three months of treatment. They lower natalizumab serum levels and, with continuous presence, are associated with a reduced therapy efficacy [135].

Predictive biomarkers are used to identify individuals who will likely experience a favourable or unfavourable effect from exposure to a treatment or an environmental agent [129]. IgM bands can be considered as a predictive biomarker for progressive multifocal leukoencephalopathy (PML) risk, as their presence was found to be associated with decreased PML risk (OR=45.9) in natalizumab-treated patients [136].

Safety biomarkers can detect or predict the likelihood, presence, or extent of adverse drug or environmental agent exposure effects. Periodic monitoring of such biomarkers is required for many drugs to ensure that their potential toxicity is detected and managed. Ideally, a safety biomarker would signal developing toxicity before clinical signs and irreversible damage will occur [129]. For example, the presence of antibodies against the John Cunningham virus (JCV) was correlates with an increased risk of developing PML in long-term natalizumab-treated patients [135].

2.7 Magnetic resonance imagining

MRI is a sensitive non-invasive tool that complements clinical and laboratory evaluation. It is critical for MS diagnostics, as it can show DIS and DIT. MRI is used for the assessment of the disease diagnosis, monitoring disease activity, and progression, and evaluating the treatment efficacy [5], [150]. Different types of MRI images, like non-contrast T1-weighted MRI and T2-weighted MRI, provide different information on disease pathology. In addition, Gd, a contrast agent, can be injected to help detect areas of new inflammations. Normally, it cannot pass through the

BBB, due to its large molecule size. Thought, upon active inflammation and BBB disruption, Gd can enter and highlight the inflamed areas.

Non-contrast T1-weighted MRI image demonstrates WM lesion hypointensity associated with axonal loss in chronic plaques, as well as helps to evaluate brain atrophy [151].

T1-weighted Gd-enhancing MRI, focusing on newer active lesions, helps estimate current inflammatory activity, indicating a breakdown of the BBB and reflecting focal and transient inflammation of the WM [152], [153].

T2-weighted MRI shows the total amount of lesion area old and new. It is used for the illustration of DIS. T2-weighted Gd-enhancing images can also illustrate DIT with a reference to a baseline scan [6]. Thus, enlarging or newly formed T2 lesions indicate new areas of MS-related tissue damage. However, all T2 hyperintensity is nonspecific to the actual pathological changes within lesions [153].

2.8 MicroRNAs

MicroRNA (miRNA) was originally discovered in 1993 in *Caenorhabditis elegans* nematode [154]. Thereafter microRNAs were described in other organisms and characterized as a class of biological regulators in the early 2000s [11]. Since then, they have been widely studied in association with various conditions, including MS. They are small, about 22 nucleotides in length, single-stranded, non-coding regulatory RNA molecules [155] that play a critical role in different biological processes by modulating gene expression at the post-transcriptional level through mRNA degradation or translation inhibition, eventually resulting in down-regulation of protein expression [156].

The 22nd release of the miRNA database (miRbase) contains 38 589hairpin precursor microRNAs, from 271 species and predicts that the *Homo sapiens* genome contains 1917 annotated hairpin precursors, and 2654 mature miRNA sequences [157].

Binding to mRNA happens by perfect or partial complementarity between the mRNA's 3'UTR and a 6–8 nucleotides long "seed" sequence at the 5' end of the microRNA. It has been predicted that miRNAs modulate the expression of 60% of human protein-coding genes [158]. A single miRNA can modify the expression of hundreds of target genes, while a single gene may be regulated by multiple miRNAs,

creating a complicated regulatory network [10], [159]. This network, in turn, is involved in various biological processes: developmental and post-developmental. miRNAs have a role in cell homeostasis as they are key players in cell differentiation and proliferation [160], stress responses [161], intracellular signalling [11], cellular metabolism [162], and immunity [163]. Thus, abnormal miRNA expression is associated with many human conditions, including various types of cancer [164], autoimmune [165], [166], cardiovascular [167], and neurodegenerative diseases [168]–[170]. Up- and down-regulation of miRNAs upon the abnormal conditions create a signature pattern that can be considered a biomarker or molecular therapeutic target for corresponding diseases [10].

2.8.1 Biogenesis of microRNAs

Transcribed from the genome microRNAs are generally classified as "intragenic" or "intergenic" based on their location. Intragenic can be found in intronic or exonic regions and co-transcribed with their host by RNA Polymerase II (Pol II). Intergenic miRNAs are situated in non-coding regions of the genome and are transcribed independently, with the use of their own promoter by either Pol II or Pol III [11], [156]. In addition, miRNAs are either encoded in the genome as individual genes or as clusters of several hundred different miRNAs. Clustered miRNAs are transcribed together as polycistronic transcripts, which will be eventually processed to the individual mature miRNAs [156].

Biogenesis involves several steps, including the maturation of miRNA precursors, assembly of mature miRNAs into the microprocessor complexes, and the regulation of gene expression (Figure 3). It begins in the nucleus, where miRNA genes are transcribed into primary miRNA transcripts (pri-miRNA) mainly by RNA polymerase II (Pol II) or, in the case of intergenic miRNAs RNA polymerase III (Pol III). pri-miRNAs are usually several thousand nucleotides long, and each harbours one or more RNA hairpin structures and is capped and polyadenylated [171], [172]. At this stage, miRNA expression can be negatively or positively regulated by transcription factors, such as p53, MYC, ZEB1, ZEB2, etc., or through DNA methylation and histone modifications [172], [173]. Next, the pri-miRNA is cleaved by the nuclear microprocessor complex into one or more, approximately 70-100 bp long, precursor miRNA (pre-miRNA). The complex is formed by the RNase

III enzyme Drosha, the Di George Syndrome critical region 8 (DGCR8) protein, and in some cases by other proteins such as RNA helicases, double-stranded RNA binding proteins, heterogeneous nuclear ribonucleoproteins, etc. [156]. At this step, miRNA processing can be modified among others by c-myc, p53, MeCP2 [172], [174], [175].

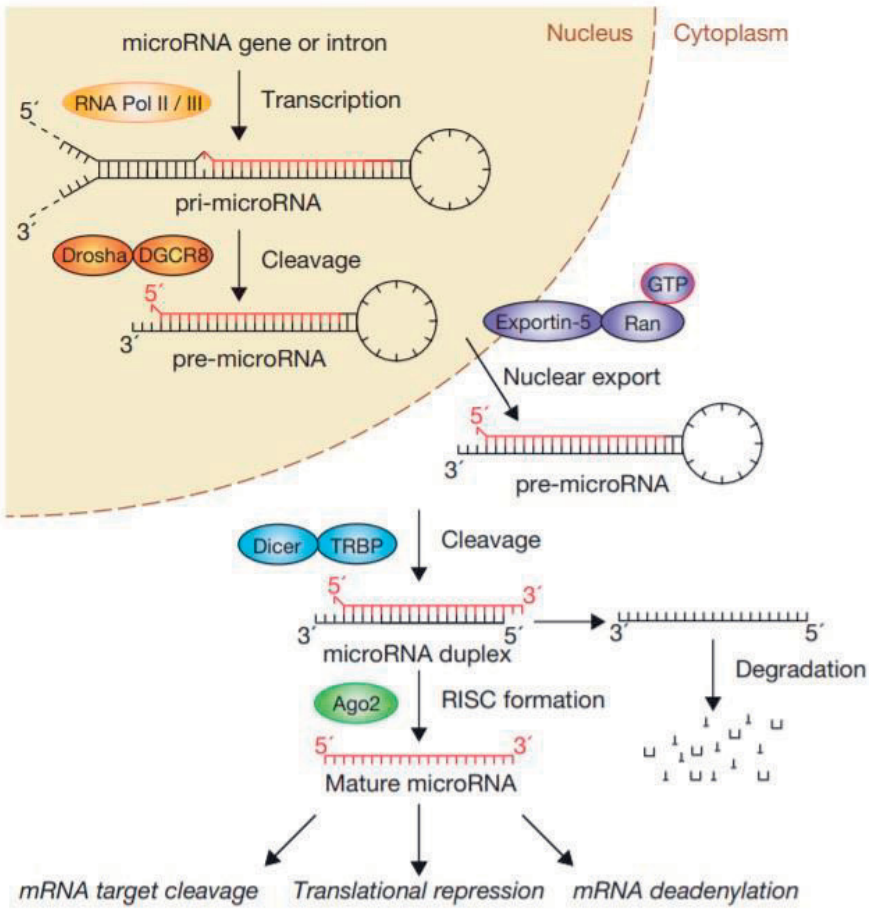

Figure 3. The canonical miRNA biogenesis pathway. In the nucleus, the miRNA gene is transcribed to primary miRNA (pri-microRNA) and cleaved to miRNA precursors (pre-microRNA). Next, it is exported to the cytoplasm and processed to mature miRNA through several steps. Picture from Winter et.al 2009 [176], reproduced with permission from the publisher.

The pre-miRNA is exported out of the nucleus to the cytoplasm through the nuclear pore complex by direct interaction with the export receptor exportin 5

(Exp5), a Ran-GTP dependent Nucleo/cytoplasmic cargo transporter, and further processed by another RNase III endonuclease Dicer, in complex with the dsRBP trans-activation-responsive RNA-binding protein (TRPB, also known as TARBP2), resulting in a mature miRNA–miRNA* (sense-antisense* strand) duplex [11], [156]. The duplex is further loaded onto an Argonaute (AGO) protein to form an effector RNA-induced silencing complex (RISC). Often, one strand (guide strand) of the miRNA duplex is selectively anchored into the AGO protein and becomes part of the miRISC, and the other strand (passenger) is degraded [177]. Both arms (5' and 3') of the precursor have the potential to produce functional mature miRNAs and the dominant may change between developmental stages and from tissue to tissue.

The described mechanism of miRNA biogenesis is the canonical pathway, but also other non-canonical were described, including Dicer-independent and Drosha-independent pathways [178].

2.8.2 mRNA translational regulation

The miRISC complex is guided by miRNA to mRNA complementary target sequence, commonly located within the 3' untranslated region (UTR). Other miRNA binding sites were reported in 5' UTR and within promoter regions and coding sequences [11]. Silencing occurs through a combination of translational repression, deadenylation, decapping, and 5′-to-3′ mRNA degradation [179], [180]. The degree of miRNA–mRNA sequence homology dictates the process of miRNA target repression. In the case of complete homology, post-transcriptional repression is mediated by endonucleolytic cleavage (AGO2-dependent slicing). This is rare in mammals, as mammalian miRNAs bind their targets with partial complementarity, preventing AGO2 endonuclease activity and generally leading to translational inhibition or mRNA decay [11], [180].

Other factors including the number of target sites for the same miRNA, their relative position and accessibility, structure of miRNA effector complex, and RNA secondary structure can influence the outcome of miRNA-mRNA interaction [180].

Some miRNAs may also work as translational activators or regulators [181]. For example, miR-373 was reported to activate the expression of E-cadherin and cold shock domain-containing protein c2 (CSDC2) by binding to complementary sequences in the promoter region [182]. In turn, let-7i specifically binds to the

TATA-box motif of the IL-2 gene and up-regulates IL-2 mRNA and protein production in CD4(+) T-lymphocytes [183].

2.8.3 miRNA nomenclature

The two mature miRNAs that originate from opposite arms of the same pre-miRNA are denoted with a -3p (3' side of the pre-miRNA) or -5p (5' side of the pre-miRNA) suffix. Previously the miRNA strand detected at lower levels was marked with an aster, for example, miR-20b*. The miR/miR* nomenclature was updated in favour of the -5p/-3p in 2011, acknowledging the functional potential of each miRNA strand (miRbase 17th release).

Several mature miRNAs can be produced from the same precursor, and the same mature miRNA can be produced from a different precursor. Dash-number suffix marks pre-miRNAs, located at different regions of the genome, but that lead to identical mature miRNAs. For example, the pre-miRNAs has-miR-128-1, located in chromosome 2, and hsa-miR-128-2, located in chromosome 3, lead to an identical mature hsa-miR-128 (miRbase).

2.9 Circulating miRNAs in MS

Alongside tissues, mature miRNAs have been detected in plasma, serum, CSF, and other biological fluids [11], [184]. Circulating miRNAs are miRNAs found in the extracellular environment. Contrary to cellular RNA species, are exceptionally stable, resisting unfavourable physiological conditions such as variations in pH, boiling, multiple freeze-thaw cycles, and extended storage [11]–[14]. This stability is explained by the association of circulating miRNAs with various carriers, such as extracellular vesicles (EV) or RNA-binding proteins.

Circulating miRNAs are actively or passively released to extracellular space and can be transported between cells and tissues. Actively released miRNAs are encapsulated in exosomes and microvesicles (MV). They enter the extracellular space upon the fusion of endosomes with the plasma membrane, and in the case of MV, by the outward budding of the plasma membrane [11], [185]. Consequent to the passive release, miRNAs are incorporated into apoptotic bodies upon cell death

[186]. In addition to EVs, miRNAs are found in the form of free-floating complexes with high-density lipoproteins (HDLs), low-density lipoproteins (LDLs) [187], AGO 2 [188], and nucleophosmin 1 [189]. EV-associated miRNAs may enter cells by endocytosis, phagocytosis, or direct fusion with the plasma membranes. Vesicle-free miRNAs, in turn, may be taken up by specific receptors on the cell surface [11].

Through the last decade circulating miRNAs were extensively studied in MS as potential diagnostic, prognostic, monitoring, treatment response, and safety biomarkers. Next, we will go through the findings on circulating miRNA in CSF, plasma, serum, and exosomes extracted from plasma or serum.

2.9.1 Cerebrospinal fluid

CSF is a valuable source of biomarkers as it represents the proximity to MS pathology. However, the CSF collection method is relatively invasive, requires a neurologist, and rarely can cause adverse effects, such as spinal headaches.

Most of the miRNAs studied in CSF are associated with the RRMS subtype or predict conversion from CIS to RRMS. The first study on circulating miRNAs in CSF of MS patients was published in 2012 by Haghikia et.al. They profiled 760 miRNAs and showed down-regulation of miR-922 and up-regulation of miR-181c and miR-633 in the combined group of RRMS, SPMS, and PPMS patients as compared to patients with other neurologic diseases. In the subgroup comparison, miR-181c and miR-633 were upregulated in RRMS as compared to SPMS [190]. Another study with the same miRNAs showed higher expression levels of miR-922 in CSF and serum and miR-181c only in serum of CIS patients who converted to RRMS (CIS-RRMS) as compared to non-converted CIS patients. Notably, expression levels of miR-181c were significantly higher in serum in comparison to CSF. In CSF, miR-181c was significantly associated with conversion from CIS to RRMS [191].

Another miRNA, miR-150, was reported to associate with MS when 754 most common human miRNAs were analysed in CSF [192]. Its overexpression was detected in MS and CIS compared to controls, as well as in patients converted from CIS to RRMS in comparison with non-converted. In addition, positive correlations between miR-150 expression levels and immunologic parameters including CSF cell count, immunoglobulin G index, presence of oligoclonal bands, and candidate

protein biomarkers C-X-C motif chemokine 13 (CXCL13), matrix metallopeptidase 9 (MMP9), and osteopontin (OPN) were also reported [192]. Another screening study (754 miRNAs) reported miR-150 to associate with MS, as its expression levels were up-regulated in CSF obtained from MS patients in comparison to HCs, and in patients positive for lipid-specific oligoclonal IgM bands (LS_OCMB+) when compared to OND controls [193]. In the same screening study, up-regulation of miR-328, miR-30a-5p, and miR-645 and down-regulation of miR-21, miR-199a-3p, miR-191, miR-365, miR-106a, and miR-146a were reported in MS in comparison to OND patients. Considering only MS patients with LS_OCMB+, miR-30a-5p and miR-645 were up-regulated and miR-191 down-regulated as compared to OND controls [193].

Some CSF miRNAs have been associated with disease activity. A significant increase of miR-448 [194] and miR-590 [195] expression levels was reported in CSF of RRMS patients during relapse compared with RRMS patients at remission and healthy individuals. In addition, both miRNAs miR-448 and miR-590 positively correlated with the CSF levels of NF-L, CXCL13, and CHI3L1. Moreover, miR-142-3p [196] and miR-125a-3p [197] are associated with the active disease course, as they are reported to be up-regulated among patients with active inflammatory lesions as compared to non-active and healthy individuals. Overexpression of miR-125a-3p was also observed in comparison between MS andAlzheimer's disease [197]. Also, a recent study showed increased levels of miR-21 and miR-146a/b in MS patients with Gd+ enhancing lesions MS. All three miRNAs positively correlated with a number of Gd+ enhancing lesions, miR-146b correlated also with neurofilament light chain (NF-L) levels, and miR-21 with EDSS [198].

Significantly decreased levels of let-7b-5p in PMS in comparison to RRMS. Its levels negatively correlated with cytokines (IL-2, IL-6, IL-10, IL-12p70, IL-15, IL-17, GM-CSF), chemokines (Eotaxin, MIP-1β), and growth factors (bFGF, PDGF-BB), while positively correlated with cytokines (IFNy, IL1RA, IL-5), chemokines (IL-8, IP-10, RANTES,) and growth factor G-CSF in the combined group of RRMS, PMS and CIS/RIS patients as well as among non-progressive (RRMS, CIS/RIS) patients. In PMS patients solely, let-7b-5p positively correlated with IL-5, RANTES, G-CSF, and age, also negatively with EDSS [199].

2.9.2 Plasma and serum

Regarding the minimal invasiveness of the method, extraction of circulating miRNAs from serum or plasma can be considered advantageous to CSF. However, serum and plasma can exhibit variations in miRNA levels [200], [201]. The main difference between serum and plasma collection methodology is clotting. In plasma, clotting is prevented and platelets, cellular elements that contaminate plasma, as well as anticoagulants such as heparin could alter the results [202]. On the other hand, the coagulation process in serum can stimulate the release of miRNAs from blood cells and platelets [200]. Upon the comparison, some studies report higher miRNA concentrations in serum [200], while others in plasma [203]. The discrepancy could result from other factors, such as differences in sample preparation and miRNA extraction methods, the use of different quantification platforms, and normalization strategies [13], [202], [204].

The current search on the PubMed database revealed 26 articles focused on the expression of circulating miRNAs in serum or plasma of patients with MS, excluding studies done for this thesis. Only in four studies plasma samples were used, sixteen were serum-based and five analysed serum or plasma-derived exosomes.

Plasma. The first study on circulating miRNAs extracted from the plasma of MS patients was published in 2012 by Siegel et.al. The focus of this small patient cohort (4 MS, 3 HCs) study was on profiling over 900 plasma miRNAs, up-regulation of miR-648, miR-614, miR-22, miR-572, miR-422a, and miR-1826, as well as down-regulation of miR-1979, were observed in MS to HC comparison [205]. In another profiling study (368 miRNAs), aberrant expression levels of miR-500, miR-574, miR-92a-1*, miR-135, and miR-145 were detected in RRMS compared with SPMS and HC. While let-7a could discriminate SPMS from HC and RRMS, and miR-886-5p discriminated HC from RRMS and SPMS. In addition, miR-22, mir-30e, miR-140-3p, miR-210, and miR-221 were aberrantly expressed in RRMS to HC comparison and miR-454 and let-7d in comparison to SPMS. Furthermore, miR-454positively correlated with disease severity (EDSS) and miR-92a-1* with disease duration and severity [206]. Out of 38 miRNAs studied, up-regulation of miR-145 and down-regulation of miR-660 and miR-939 were reported in treatment-naïve RRMS patients compared to HCs [207]. In addition, let-7a and miR-648a were down-regulated in MS patients in remission as compared to HC, and miR-92a negatively correlated with EDSS in the relapse group [208].

Serum. A wide profiling study (652 serum miRNAs), with a cohort of 296 participants, including patients with MS, other neurological (OND) and inflammatory (OID) diseases, such as Alzheimer´s disease, amyotrophic lateral sclerosis, asthma, and rheumatoid arthritis, showed miR-486-5p and miR-25-3p to discriminate patients with MS (RRMS, SPMS, PPMS) from HCs, OND, and OID. While miR-320a and miR-320b discriminated MS from HC and OND and miR-140-3p, let-7c-5p MS from HC and miR-27a-3p RRMS from PPMS and SPMS. Correlations with EDSS were observed only for miR-199a-5p and miR-142-5p [209]. In their later study, miR-484 was found to discriminate all MS, RRMS, and SPMS from HC. In addition, miR-140-5p, miR-142-5p, miR-320a, miR-320b, miR-320c were able to significantly discriminate SPMS from HC. Correlations with EDSS were observed for miR-320a, miR-337-3p, miR-199a-5p, miR-142-5p and miR-941 [210].

In another profiling study (88 serum miRNAs), down-regulation of miR-15b, miR-23a, and miR-223 was reported in the serum of MS patients (RRMS, PPMS), and correlated with EDSS in PPMS [211]. The same miRNAs were addressed in a follow-up study, where their expression levels were analysed in the serum of RRMS patients at baseline and after 3, 6, 9, and 12 months after starting the fingolimod treatment. At the baseline miR-15b, miR-23a and miR-223 were down-regulated in RRMS compared to controls. Upon the fingolimod treatment, their expression levels increased 6 months after the start of treatment and were stable until the end of the study (12 months) [212].

Up-regulation of miR-223 in the combined group of RRMS and SPMS was later reported in a study focused on serum expression of selected miR-223, miR-145, and miR-326 [213]. In the same study, overexpression of miR-145 was reported in the combined group of RRMS and SPMS patients when compared to HCs. Notably in the earlier mentioned study, overexpression of plasma miR-145 was reported in RRMS to HCs comparison [214].

In a study focused solely on miR-572, its down-regulation was reported in the combined group of patients with RRMS, SPMS, and PPMS. While in the subtype comparison it was down-regulated in PPMS as compared to HC and SPMS and among the patients in remission as compared to those in relapse and HC. It also significantly positively correlated with EDSS independently from the clinical phenotype [215].

Other findings included overexpression of miR-16, miR-24, miR-137, and decreased levels of miR-181 in the combined group of MS, which consisted of

patients with RRMS, SPMS, and PPMS and overexpression of miR-196 in RRMS and down-regulation of miR-9 in PPMS as compared to HCs [216]. In addition, miR-18b was up-regulated in relapse RRMS patients as compared to remission and HC [217], while miR-96 was up-regulated in remission as compared to relapse and HC and down-regulated in relapse as compared to HC [218]. Higher levels of serum miR-128-3p were reported in PMS as compared with RRMS, and in patients without relapses compared to those with relapses. In addition, inverse correlation between miR-128-3p and relapse rate was reported [219]. In turn, expression levels of miR-137 [220], miR-300 and miR-450b-5p were decreased [221] and miR-155 and miR-146a increased in MS to HC comparison [222].

Exosomes participate in cell-to-cell communication by transporting cargo, including miRNAs, to target cells. Possibly, exosome release is a cellular adaptation mechanism and its biogenesis, structure, and secretion are affected by the microenvironment of cells [223]. Thus, exosomal miRNAs can reflect pathological, disease-related changes and can be suggested as possible diagnostic and prognostic biomarkers for various diseases, including MS. Exosomal miRNAs are a relatively new research area in MS, with the first articles published in 2017.

One of the first serum exosomal miRNA profiling studies reported a decrease of 4 miRNAs (miR-15b-5p, -451a, -30b-5p, -342-3p) in RRMS and 6 miRNAs (miR-370-3p, -409-3p, -432-5p, -15b-5p, -23a-3p, -223-3p) in PMS, as compared to HCs. In addition, 6 miRNAs (miR-374a-5p, -30b-5p, -433-3p, -485-3p, -342-3p, -432-5p) could distinguish PMS from RRMS [224]. In a recent study by the same group, a large profiling of 1924 miRNAs was performed on 29 RRMS patients before the start of fingolimod treatment and in 6 months after. On the baseline, miR-194-5p and miR-374a-5p could discriminate active RRMS patients from stable. In six months after the fingolimod treatment, 15 miRNAs (miR-1246, -122-5p, -127-3p, -19b-3p, -134-5p, -323b-3p, -370-3p, -375, -379-5p, -382-5p, -411-5p, -432-5p, -485-5p, -493-3p and -889-3p) could discriminate active RRMS patients from stable. In addition, among the active patients who positively responded to the treatment, decrease of miR-150-5p and miR-548e-3p as well as increase of miR-130b-3p, miR-654-5p and miR-487b-3p were observed in response to treatment. Consecutively, among the stable patients, in response to fingolimod treatment increase in expression levels of 11 miRNAs (miR-203a, -193a-5p, -379-5p, -370-3p, -382-5p, -493-3p, -432-5p, -485-5p, -2110, -1307-3p and -1908-5p) was reported [225]. Another serum exosome miRNA profiling study reported decrease in expression levels of miR-122-

5p, miR-196b-5p, miR-532-5p and miR-301a-3p in RRMS, especially during relapse as compared to HC. These miRNAs were also decreased in patients with a Gd enhancement on brain magnetic resonance imaging. In addition, miR-122-3p, miR-196b-5p and miR-532-5p were down-regulated in relapse when compared to remission [226]. In a small cohort (11 RRMS) profiling study (179 miRNAs) down-regulation of 14 miRNAs (miR-486-5p, -451a, -320b, -122-5p, -215-5p, -320d, -19-3p, -26a-5p, -142-3p, -146a-5p, -15b-3p, -23a-3p, -223-3p and let-7b-5p) as well as up-regulation of miR-22-3p and miR-660-5p were observed in serum exosomes of IFN -β–responsive RRMS patients as compared to those without response and to treatment-naive patients [227].

One study used plasma as a source of exosomes and reported an up-regulation of let-7i, miR-19b, miR-25, and miR-92a in MS to HCs comparison. Particularly, let-7i was reported to suppress induction of Treg cells by targeting insulin-like growth factor 1 receptor and transforming growth factor beta receptor 1 [228].

To conclude, 36 previously published MS-focused studies reported 33 same miRNAs at least in two studies (Table 2). The most frequent associations to MS were found with the miRNAs miR-122-5p, miR-145, miR-15b, miR-223-3p, miR-23a-3p and miR-320b in serum or plasma, but not in CSF. Among them, decreased levels of miR-23a-3p, miR-223-3p and miR-15b were observed in MS [211], [212], PMS [224] and RRMS [229] as compared to HCs in serum and serum extracted exosomes. In contrast, increased levels of miR-223-3p were detected in serum of MS as compared to HC [213], while other studies reported no statistically significant differences in expression levels of miR-223-3p and miR-23a-3p in MS to HC comparison [219], [230]. In turn, increased levels of miR-145 were reported in serum of MS patients [213] and serum and plasma of patients with RRMS [214] as compared to HC. It also could discriminate RRMS from SPMS and HC [206]. Inconsistency and discrepancy of the miRNA findings can be explained by several analytical and pre-analytical aspects, including various patient-derived factors and a lack of standardized methodology and normalization strategy. These will be further addressed in the discussion (6.4 Reproducibility and 6.5 Methodological considerations and limitations of the study). In addition, most of the reported miRNAs are not specific to MS and found in association with other conditions, including cancer, emphasizing the low potential of these miRNAs as independent disease-specific biomarkers.

Table 2. Variations in circulating miRNA expression levels reported in at least two publications.

miRNA	Sample type	Changes	Comparison	Number of patients	Ref.
let-7a	plasma	decreased	remission to HC	20 relapse, 17 remission, 30 HC	[208]
		discriminate	SPMS from HC and RRMS	60 RRMS, 60 SPMS, 41 HC	[206]
let-7b-5p	serum exosomes	decreased	IFN-β–responsive RRMS to non-responsive and to treatment-naive	11 RRMS	[227]
	CSF	increased	RRMS to PMS	117 RRMS, 24 PMS, 25 CIS/RIS	[199]
miR-122-5p	serum exosomes	decreased	RRMS to HC, relapse to remission	82 RRMS, 42 HC	[226]
		decreased	IFN-β–responsive RRMS to non-responsive and to treatment-naive	11 RRMS	[227]
		discriminate	active non-treated RRMS to stable in 6 months after fingolimod treatment	29 RRMS	[225]
miR-137	serum	decreased	MS to HC	108 MS, 104 HC	[220]
		increased	MS (RRMS, SPMS, PPMS) to HC	16 RRMS, 11 SPMS, 6 PPMS, 30 HC	[216]
miR-140-3p	plasma	discriminate	RRMS from HC	60 RRMS, 60 SPMS, 41 HC	[206]
	serum	discriminate	MS (RRMS, PPMS, SPMS) from HC	84 MS, 50 HC, 74 ODC	[209]
miR-142-5p	serum	no	MS to HC and OD	84 MS, 50 HC, 74 OD	[209]
		discriminate	SPMS from HC	95 RRMS, 51 SPMS, 88 HC	[210]
miR-145	serum	increased	RRMS to HC	40 RRMS, 40 HC	[214]
			MS (RRMS, SPMS) to HC	18 RRMS, 19 SPMS, 23 HC	[213]
	plasma	increased	RRMS to HC	22 RRMS, 15 HC	[214]
		discriminate	RRMS from HC and SPMS	60 RRMS, 60 SPMS, 41 HC	[206]
miR-146a	CSF	decreased	RRMS to OND	86 RRMS, 55 OND	[193]
		increased	Gd-positive (Gd+)	46 MS	[198]
	serum exosomes	decreased	IFN-β–responsive RRMS to non-responsive and to treatment-naïve	11 RRMS	[227]

miR-150	CSF	increased	MS to HC, CIS-RRMS to CIS-CIS	145 CIS, 178 MS, 296 HC	[192]
			RRMS to OND, LS_OCMB+ to OND	86 RRMS, 55 OND	[193]
	serum exosomes	decreased	active RRMS positively responded to fingolimod, upon the treatment	29 RRMS	[225]
miR-155	serum	increased	RRMS and SPMS to HC	15 RRMS, 11 SPMS, 4 PPMS, 30 HC	[222]
		no	MS to HC	37 MS, 25 HC	[230]
		decreased	RRMS to HC, stable to post-acute	36 RRMS, 10 HC	[231]
miR-15b	serum	decreased	MS (RRMS, PPMS) to HC	19 RRMS, 16 PPMS, 33 HC	[211]
		decreased	MS to HC	30 MS, 11 HC	[212]
		decreased	RRMS to HC	11 RRMS, 12 HC	[229]
	serum exosomes	decreased	RRMS to HC, PMS to HC	14 RRMS, 22 PMS, 11 HC	[224]
miR-181c	CSF, serum	increased (CSF)	CIS-RRMS to CIS-CIS	30 CIS-RRMS, 28 CIS-CIS	[191]
	CSF	increased	MS to OND, RRMS to SPMS	53MS, 39OND	[190]
miR-194-5p	serum exosomes	discriminate	active non-treated RRMS to stable	29 RRMS	[225]
	serum	no	all MS, RRMS, SPMS, HC	95 RRMS, 51 SPMS, 88 HC	[210]
miR-19b-3p	plasma exosomes	increased	MS to HC	25 RRMS, 6 SPMS, 18 HC	[228]
	serum exosomes	discriminate	active non-treated RRMS to stable in 6 months after fingolimod treatment	29 RRMS	[225]
miR-21	CSF	decreased	RRMS to OND	86 RRMS, 55 OND	[193]
		increased	Gd-positive (Gd+)	46 MS	[198]
miR-22	plasma	increased	MS to HC	4 MS, 4 HC	[205]
		discriminate	RRMS from HC	60 RRMS, 60 SPMS, 41 HC	[206]
miR-223-3p	serum	decreased	MS (RRMS, PPMS) to HC	19 RRMS, 16 PPMS, 33 HC	[211]
		decreased	MS to HC	30 MS, 11 HC	[212]
		decreased	RRMS to HC	11 RRMS, 12 HC	[229]
		increased	MS (RRMS, PPMS) to HC	18 RRMS, 19 SPMS, 23 HC	[213]
		no	MS to HC	74 MS, 17 HC	[219]
	serum exosomes	decreased	PMS to HC	14 RRMS, 22 PMS, 11 HC	[224]

	serum exosomes	decreased	IFN-*β*–responsive RRMS to non-responsive and to treatment-naive	11 RRMS	[227]
miR-23a-3p	serum	decreased	MS (RRMS, PPMS) to HC	19 RRMS, 16 PPMS, 33 HC	[211]
		decreased	MS to HC	30 MS, 11 HC	[212]
		decreased	RRMS to HC	11 RRMS, 12 HC	[229]
	serum exosomes	decreased	PMS to HC	14 RRMS, 22 PMS, 11 HC	[224]
	serum exosomes	decreased	IFN-*β*–responsive RRMS to non-responsive and to treatment-naive	11 RRMS	[227]
	serum	no	MS to HC	37 MS, 25 HC	[230]
miR-25-3p	plasma exosomes	increased	MS to HC	25 RRMS, 6 SPMS, 18 HC	[228]
	serum	discriminate	MS (RRMS, SPMS, PPMS) from HC and OD	84 MS, 50 HC, 74 OD	[209]
miR-301a-3p	serum	decreased	RRMS to HC, stable to post-acute	36 RRMS, 10 HC	[231]
	serum exosomes	decreased	RRMS to HC	82 RRMS, 42 HC	[226]
miR-320a	serum	discriminate	MS (RRMS, SPMS, PPMS) from HC and OND	84 MS, 50 HC, 74 OD	[209]
		discriminate	SPMS from HC	95 RRMS, 51 SPMS, 88 HC	[210]
miR-320b	serum	discriminate	MS (RRMS, SPMS, PPMS) from HC and OND	84 MS, 50 HC, 74 OD	[209]
		discriminate	SPMS from HC	95 RRMS, 51 SPMS, 88 HC	[210]
	serum exosomes	decreased	IFN-*β*–responsive RRMS to non-responsive and to treatment-naive	11 RRMS	[227]
miR-365a-3p	CSF	decreased	RRMS to OND	86 RRMS, 55 OND	[193]
	serum	discriminate	MS (RRMS, PPMS, SPMS) from HC	84 MS, 50 HC, 74 OD	[209]
miR-370-3p	serum exosomes	increased	stable RRMS in responce to figolimod treatment	29 RRMS	[225]
		discriminate	active non-treated RRMS to stable in 6 months after fingolimod treatment	29 RRMS	[225]
		decreased	PMS to HC	14 RRMS, 22 PMS, 11 HC	[224]
miR-432-5p	serum exosomes	increased	stable RRMS in responce to figolimod treatment	29 RRMS	[225]
		discriminate	active non-treated RRMS to stable in 6 months	29 RRMS	[225]

			after fingolimod treatment		
		decreased	PMS to HC, PMS to RRMS	14 RRMS, 22 PMS, 11 HC	[224]
miR-451a	serum exosomes	decreased	IFN-*β*–responsive RRMS to non-responsive and to treatment-naive	11 RRMS	[227]
		decreased	RRMS to HC	14 RRMS, 22 PMS, 11 HC	[224]
miR-486-5p	serum exosomes	decreased	IFN-*β*–responsive RRMS to non-responsive and to treatment-naive	11 RRMS	[227]
	serum	discriminate	MS from HC and OD	84 MS, 50 HC, 74 OD	[209]
miR-572	plasma	increased	MS to HC	4 MS, 4 HC	[205]
	serum	increased	MS to HC	31 RRMS, 16 PPMS, 15 SPMS, 15 HC	[215]
		decreased	PPMS to HC, PPMS to SPMS		
		no	MS to HC	37 MS, 25 HC	[230]
miR-648	plasma	increased	MS to HC	4 MS, 4 HC	[205]
		decreased	remission to HC	20 relapse, 17 remission, 30 HC	[208]
miR-660-5p	plasma	decreased	RRMS to HC	22 RRMS, 15 HC	[214]
	serum exosomes	increased	IFN-*β*–responsive RRMS to non-responsive and to treatment-naive	11 RRMS	[227]
miR-922	CSF	decreased	MS to OND	53 MS, 39 OND	[190]
	CSF, serum	increased	CIS-RRMS to CIS-CIS	30 CIS-RRMS, 28 CIS-CIS	[191]
miR-92a	plasma exosomes	increased	MS to HC	25 RRMS, 6 SPMS, 18 HC	[228]
	plasma	no	remission, relapse, HC	20 relapse, 17 remission, 30 HC	[208]

RRMS: relapsing-remitting MS, PMS: progressive MS (SPMS and PPMS), SPMS: secondary progressive MS, PPMS: primary progressive MS, HC: healthy controls, OD: other diseases, OND: other neurological diseases

3 AIMS OF THE STUDY

This thesis is based on the hypothesis that circulating miRNAs have strong biomarker potential, and their aberrant expression levels are detected in MS. This thesis project is focused on exploring the potential role of human circulating miRNAs as biomarkers of MS disease concomitant with its clinical subtypes and association with the clinical activity and disability of the MS disease.

Therefore, the specific aims of this study were to:

1. Characterize the circulating miRNAs profiles in progressive MS, including SPMS and PPMS (Study I).
2. Utilize selected circulating miRNAs as diagnostic biomarkers to distinguish between MS subtypes (Study II-III).
3. Explore the association of circulating miRNAs with disability progression and disease activity in MS patients (Study I-III).
4. Assess the stability of selected miRNA expression in a longitudinal study to highlight its promising biomarker potential (Study III).

4 PATIENTS AND METHODS

4.1 Study population (Study I-III)

The studies I-III in total had 290 subjects, of whom 192 were patients with clinically defined MS (81 RRMS, 66 PPMS, and 45 SPMS), 18 with clinically isolated syndrome (CIS), and 80 were healthy individuals (HC) (Table 3). Study I was performed on two cohorts of participants: screening and validation cohorts The screening cohort included 18 patients with PPMS and 10 HCs, and the validation cohort included 31 PPMS, 31 SPMS, and 21 HCs. In total 18 MS patients and 10 HCs were included in both screening and validation phases. The study II cohort consisted of 73 patients with MS (53 RRMS, 20 PPMS) and 27 HCs, and study III included 57 patients with MS (28 RRMS, 15 PPMS, 14 SPMS), 18 CIS, and 32 HCs. Study I shared 14 MS patients and 5 HCs with study II, while study II and III shared 3 HCs. Active DMT treatment was ongoing in 37/53 (71%) patients with RRMS in study II and in 16/28 (57%) patients with RRMS and 1/14 (7%) with SPMS in study III.

The diagnosis of MS was based on the revised McDonald criteria and all the diagnoses were definite [89]. CIS patients were defined as patients who had their first acute demyelinating event suggestive of MS [232]. All the patients were studied clinically and neurologically, including the assessment of neurological disability expressed by EDSS score [233]. In the follow-up study III, patients were examined on the baseline (BL) and in 2- and 4-years after the enrolment in the study. MRI was performed on BL and year-2. For all the studies the blood was drawn on the same day as the neurological examination. The progression index (PI) was calculated by dividing the EDSS score by disease duration. An annualized relapse rate (ARR) was calculated in the RRMS group starting from the time of diagnosis (Study II-III).

The control group consisted of age- and sex-matched healthy subjects with no history of any autoimmune disease or use of any immunomodulatory therapy. In the study III, HCs were studied only on the BL.

Table 3. Demographic and clinical characteristics of the MS patients and healthy controls(Studies I-III).

Study I: Circulating microRNAs as biomarkers in progressive multiple sclerosis

	Screening cohort		Validation cohort			
	PPMS	**HC**	**All MS**	**PPMS**	**SPMS**	**HC**
Number of patients	18	10	62	31	31	21
Gender (F/M)[a]	10/8	6/4	42/20	18/13	24/7	12/9
Age[b]	53.5±8.5 (39-68)	51.8±8.2 (40-65)	53±10.3 (28-67)	56.3±9.7 (39-75)	48.8±9.5 (28-67)	52.7±8.1 (38-65)
Time since first symptoms (y)[b]	15.6±10.0 (3.1-43)	-	20.2±10.2 (3.1-49.3)	16.8±10.9 (3.1-49.3)	23.4±8.3 (9.0-36.4)	-
Disease duration from diagnosis (y)[b]	11.0±7.7 (0.1-25.8)	-	13.8±8.8 (0-35.4)	10.9±8.4 (0-29.3)	16.6±8.3 (3.3-35.4)	-
EDSS[a]	5.9±1.3 (3.0-8.0)	-	5.5±1.6 (1.5-8.0)	5.3±1.8 (1.5-8.0)	5.7±1.4 (2.5-8.0)	-
Progression index[b,c]	0.8±1.0 (0.2-3.9)	-	0.6±0.6 (0.1-3.9)	0.7±0.8 (0.1-3.9)	0.4±0.3 (0.1-2.0)	-

Study II: Evaluation of serum miR-191-5p, miR-24-3p, miR-128-3p, miR-376c-3p in MS patients

	All MS	**RRMS**	**SPMS**	**PPMS**	**CIS**	**HC**
Number of patients	73	53	-	20	-	27
Gender (F/M)[a]	51/22	41/12	-	10/10	-	18/9
Age[b]	40.0±10.7 (22-65)	35.3±7.1 (22-50)	-	52.7±8.0 (40-65)	-	38.2±11.8 (22-65)
Time since first symptoms (y)[b]	8.3±6.2 (0.4-30.8)	7.0±5.1 (0.4-20.6)	-	12.3±7.3 (3.1-30.8)	-	-
Disease duration from diagnosis (y)[b]	6.0±5.3 (0-25.8)	5.4±5.4 (0.0-18.1)	-	7.6±6.5 (0.1-25.8)	-	-
EDSS[b]	2.6±2.4 (0.0-8.0)	1.6±1.7 (0.0-6.5)	-	5.4±1.8 (1.5-8.0)	-	-
Progression index[b,c]	2.5±6.0 (0-28.7)	2.1±5.0 (0.0-27.5)	-	3.8±8.2 (0.3-28.7)	-	-

Study III: Temporal variability of serum miR-191, miR-223, miR-128, and miR-24 in multiple sclerosis: A 4-year follow-up study

	All MS	RRMS	SPMS	PPMS	CIS	HC
Number of patients	57	28	14	15	18	32
Gender (F/M)[a]	37/20	19/9	9/5	9/6	16/2	20/12
Age[b]	45.9±12.0 (18-69)	38.1±9.0 (18-50)	49.0±8.8 (35-61)	57.5±8.5 (39-69)	36.1±7.4- (23-50)	42.2±1.2 (21-65)
Time since first symptoms (y)[b]	13.07±9.5 (0.7-42.1)	8.5±7.1 (0.7-28.3)	19.0±8.3 (5.0-32.0)	18.4±9.7 (1.2-42.1)	2.5±2.6 (0.5-8.9)	-
Disease duration from diagnosis (y)[b]	8.4±7.9 (0.1-31.0)	4.3±4.0 (0.1-12.3)	11.3±9.6 (1.9-31.0)	12.8±8.2 (0.3-26.3)	-	-
EDSS[b]	3.1±2.3 (0-7.0)	1.5±1.3 (0-6.0)	4.7±1.7 (2.0-7.0)	4.70±1.9 (1-7.0)	0.1±0.3 (0-1.0)	-
Progression index[b,c]	0.9±1.3 (0-7.2)	0.8±1.1 (0-3.6)	0.9±1.0 (0.2-3.0)	1.0±1.8 (0.1-7.2)	na	

RRMS: relapsing-remitting MS, SPMS: secondary progressive MS, PPMS: primary progressive MS, CIS: clinically isolated syndrome, BL: baseline, EDSS: expanded disability status scale, IFN: interferon, y: year,
[a] number, [b] mean ± SD (range), [c] from diagnosis

In study III, MS cases were categorized into active or stable groups based on disease activity over the follow-up period, defined by disability accumulation in EDSS (EDSS active vs stable), relapses (relapse active vs stable), or activity observed in MRI (MRI active vs stable). Patients were categorized into the EDSS worsening group by EDSS progression: if EDSS increased by 1.0 or more, for BL EDSS of less than 6.0, or by the increase of 0.5, for EDSS of 6.0 or more [234]. The rest of the patients were included in the not worsening group. The relapse-active group consisted of patients who experienced at least one relapse during the follow-up. The MRI active group includes changes in T1 and FLAIR volumes over the first two years of the study (BL to year-2). The cut-off values (T1 > 0.5 cm3 and FLAIR> 6 cm3) were chosen based on the distribution of changes in lesion volumes among the MS patients.

4.2 Ethic statement

The study was approved by the Ethics committee of Pirkanmaa Hospital District (R05157) and a clinical investigation followed according to the principles of the Helsinki Declaration. Written informed consent was given by all the participants.

4.3 Collection of serum and miRNA extraction (Study I-III)

Venous blood was collected in BD Vacutainer SST II advance tubes (Becton Dickinson, US) and allowed to clot for 30 minutes before the centrifugation at 1500xg for 15 minutes at room temperature. Separated serum was stored at -80°C until further use.

Circulating miRNAs were isolated from 200 µl serum using a Qiagen miRNeasy Serum/Plasma kit (Qiagen Inc, Valencia, CA) according to the manufacturer's protocol. Cel-miR-39 (Qiagen Inc) was used as a spike-in control to monitor RNA recovery and reverse transcription efficiency. Extracted and purified miRNA was eluted with RNase-free water and stored at -80°C until further use.

4.4 Reverse transcription (Study I-III)

Isolated miRNAs were converted to complementary DNA (cDNA) using a miScript reverse transcription kit (Qiagen Inc) following the standard protocol. The reaction mixture consisted of extracted miRNA (4.5 µL), 5× miScript Hi-Spec-buffer (4 µL), 10× miScript Nucleic mix (2 µL), RNase-free water (7.5 µL), and miScript Reverse Transcriptase (RT) mix (2 µL). The RT mix included reverse transcriptase and poly(A) polymerase, 10x miScript Nucleic mix contains oligo-dT primers with a universal tag. The reaction was performed at 37°C for 60 minutes, followed by the incubation at 95°C for 5 minutes to inactivate the RT mix and then held at 4°C until the RT-PCR or storage at −20°C.

4.5 MicroRNA expression analysis (Study I-III)

Circulating miRNA expression levels were analysed using miScript SYBR Green PCR kit (Qiagen Inc), by the SYBR-green-based real time polymerase chain reaction method (RT-PCR), on the ABI 7900HT PCR machine (Applied Biosystems, Foster City, USA). Prior to the RT-PCR, all the cDNA samples were diluted by 200 µl of RNase-free water.

During the screening phase of the study I, the relative expression levels of 84 different miRNAs, as well as of 6 endogenous controls (SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, and RNU6-2), 2 quality controls (miRTC for RT and PPC as positive PCR control) and cel-miR-39-3p were measured using commercial MIHS-106ZE Human Serum & Plasma miScript miRNA PCR arrays (Qiagen; Table 4). The 10-µL reactions included 5 µL 2× QuantiTect SYBR Green PCR Master Mix, 1 µL 10× miScript Universal Primer, 0.9 µL diluted cDNA, and 3.1 µL RNase-free water that were incubated in 384-well plate.

In the validation phase of the study I, as well as in the studies II and III the relative expression levels of circulating miRNAs were measured using miScript Human Serum & Plasma miRNA PCR assays (Qiagen), with cel-miR-39-3p, SNORD68, and RNU6-2 used as controls. The 10-µL reaction mixture included 5 µL 2× QuantiTect SYBR Green PCR Master Mix, 1 µL 10× miScript universal primer, 1 µL 10× miScript primer assay, 1 µL diluted cDNA, and 2 µL RNase-free water. To control the intra-assay variation all samples were run as triplicates. In addition, to control the inter-assay variation control sample was included to each plate and run against miR-21 and miR-39-3p (study I) and miR-191-5p and miR-39-3p (Study II and III).

RT-PCR conditions were following in all the studies: enzyme activation at 95°C for 15 minutes, followed by 40 amplification cycles comprised of denaturation at 94°C for 15 seconds, annealing at 55°C for 30 seconds, and extension at 70°C for 30 seconds, followed by pre-set dissociation stage of 15 seconds at 95°C and 15 seconds at 60°C.

The relative expression levels were analysed using the comparative Ct method (ΔΔCt). In this method the Ct values obtained from two different experimental RNA samples are directly normalized to a housekeeping gene and then compared.

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Table 4. Screening phase Human Serum & Plasma miScript miRNA PCR array layout. Includes 84 different miRNA, endogenous contros (SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, and RNU6-2), quality controls (miRTC for RT and PPC as positive PCR control) and exogenous control cel-miR-39-3p. All genes were analyzed in the duplicate wells.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	hsa-let-7a-5p		hsa-miR-1		hsa-miR-100-5p		hsa-miR-106b-5p		hsa-miR-10b-5p		hsa-miR-122-5p		hsa-miR-124-3p		hsa-miR-125b-5p		hsa-miR-126-3p		hsa-miR-133a		hsa-miR-133b		hsa-miR-134	
B																								
C	hsa-miR-141-3p		hsa-miR-143-3p		hsa-miR-146a-5p		hsa-miR-150-5p		hsa-miR-155-5p		hsa-miR-17-5p		hsa-miR-17-3p		hsa-miR-18a-5p		hsa-miR-192-5p		hsa-miR-195-5p		hsa-miR-196a-5p		hsa-miR-19a-3p	
D																								
E	hsa-miR-19b-3p		hsa-miR-200a-3p		hsa-miR-200b-3p		hsa-miR-200c-3p		hsa-miR-203a		hsa-miR-205-5p		hsa-miR-208a		hsa-miR-20a-5p		hsa-miR-21-5p		hsa-miR-210		hsa-miR-214-3p		hsa-miR-215	
F																								
G	hsa-miR-221-3p		hsa-miR-222-3p		hsa-miR-223-3p		hsa-miR-224-5p		hsa-miR-23a-3p		hsa-miR-25-3p		hsa-miR-27a-3p		hsa-miR-296-5p		hsa-miR-29a-3p		hsa-miR-30d-5p		hsa-miR-34a-5p		hsa-miR-375	
H																								
I	hsa-miR-423-5p		hsa-miR-499a-5p		hsa-miR-574-3p		hsa-miR-885-5p		hsa-miR-9-5p		hsa-miR-92a-3p		hsa-miR-93-5p		hsa-let-7c		hsa-miR-107		hsa-miR-10a-5p		hsa-miR-128		hsa-miR-130b-3p	
J																								
K	hsa-miR-145-5p		hsa-miR-148a-3p		hsa-miR-15a-5p		hsa-miR-184		hsa-miR-193a-5p		hsa-miR-204-5p		hsa-miR-206		hsa-miR-211-5p		hsa-miR-26b-5p		hsa-miR-30e-5p		hsa-miR-372		hsa-miR-373-3p	
L																								
M	hsa-miR-374a-5p		hsa-miR-376c-3p		hsa-miR-7-5p		hsa-miR-96-5p		hsa-miR-103a-3p		hsa-miR-15b-5p		hsa-miR-16-5p		hsa-miR-191-5p		hsa-miR-22-3p		hsa-miR-24-3p		hsa-miR-26a-5p		hsa-miR-31-5p	
N																								
O	cel-miR-39-3p				SNORD61		SNORD68		SNORD72		SNORD95		SNORD96A		RNU6-2		miRTC				PPC			

The selection of an appropriate normalization technique was crucial for the project, because of the low concentration of circulating miRNAs in the samples. For normalization, the mean centre restricted (MCR) method was used in the screening phase of the study I. This method is restricted to miRNAs that are expressed in all samples and uses the mean Ct value of these miRNAs as pseudo-reference gene [235]. In the validation phase of study I, as well as in studies II and III raw Ct values were normalized by the spike-in cel-miR-39 and two endogenous controls SNORD68 and RNU6-2. Endogenous controls were selected from the screening assays (Table 4), based on the variability results and the NormFinder algorithm. This algorithm considers intra- and inter-group variability to identify the best combination of reference genes. Normalized Ct was calculated as mean Ct–assay Ct [235]. While cel-miR-39 was added to reflect technical variability during the miRNA processing steps (Qiagen protocol).

To count the fold changes (FC), mean values of HC were used as a calibrator. Ct values higher than 35 (Study I and II) or 37 (Study III) were considered as undetermined and thus excluded from the analysis

4.6 Magnetic resonance imaging (Study III)

All MRI examinations were performed on a 1.5 Tesla MRI Unit (Siemens Avanto, Erlangen, Germany). The MRI protocol included a T1-weighted header followed by axial T1-weighted magnetization prepared rapid gradient echo (MP-RAGE) and T2-weighted turbo spin-echo (TSE), fluid attenuated inversion recovery (FLAIR), magnetization transfer contrasts (MTC), diffusion weighted imaging (DWI), and Gd-enhanced T1-weighted MP-RAGE sequences.

In study III T1-weighted MP-RAGE and FLAIR were used for volumetric analysis. For MP-RAGE, the imaging parameters were as follows: repetition time (TR)=1160ms; echo time (TE)=4.24ms; inversion time (TI)= 600ms; slice thickness=0.9 mm; in-plane resolution=0.45∗0.45mm. In FLAIR images, the following parameters were used: TR=8500ms; TE=100ms; TI=2500ms; slice thickness=5.0mm; in-plane resolution=0.45∗0.45mm. Volumetric segmentation of plaques in the brain was performed using semiautomatic software Anatomatic operating in a PC/Window 95 environment and the images were analysed blind [236].

4.7 Statistical analysis (Study I-III)

Statistical analyses were performed using SPSS version 22.0 (IBM corporation, Armonk, NY, USA) and the GraphPad Prism 7.03 was used to construct figures in studies I-III.

Differences in the miRNA expression levels between MS subtypes, CIS, and HCs were analysed using non-parametric two-tailed Mann-Whitney U test (Study I and III), multivariate logistic regression model, adjusted for age, sex and batch (Study I) and linear regression model, adjusted for age and sex (Study II). In addition, the Mann-Whitney U test was used to evaluate differences in MRI volumes between the MS subtypes and CIS (Study III). The Bonferroni correction was used to reduce the chances of obtaining false-positive results. To study the associations between miRNA expression levels, clinical parameters (Study I-III) and MRI volumes (Study III) Spearman's correlation analysis (Study I and III), Pearson's correlation analysis (Study II), and linear regression, using age and sex as covariates (Study II), were performed. Friedman test and Wilcoxon signed-rank test were used to assess changes in miRNA expression levels during the four-years follow-up (Study III). Receiver operating characteristic (ROC) curve analysis was used for evaluating the diagnostic power of circulating miRNAs (Study I). P-values smaller than 0.05 were considered statistically significant.

5 RESULTS

5.1 Circulating miRNA profiles in progressive MS (PMS) including SPMS and PPMS (Study I)

Study I was performed to explore miRNA profiles in patients with PMS in comparison to HCs, with the emphasis on associations with PPMS. In the screening phase, the expression of 84 circulating miRNAs was studied in sera samples obtained from 18 PPMS and 10 HCs (Table 3). Four miRNAs (miR-141-3p, miR-124-3p, miR-375 and miR-130b-3p) were significantly down-regulated ($p<0.024$; $FC<0.5$), and four (miR-376c-3p, miR-128-3p, miR-191-5p, miR26a-5p) up-regulated ($p<0.03$; $FC>1.5$) in PPMS as compared to HCs (Table 5).

Thereafter, eight miRNAs that were significantly up-or down-regulated in the screening phase were further validated on a larger cohort of progressive patients (n=62), including PPMS (n=31) and SPMS (n=31) and healthy subjects (n=21). In addition, two other miRNAs were included in the validation phase: miR-24-3p, which was statistically significantly deregulated ($FC=1.33$, $p=0.024$) and miR-211-5p, which was strongly but not statistically significantly down-regulated ($FC=0.43$, $p>0.05$). Multivariate logistic regression analysis, adjusted for age, gender, and the batch, was performed for the expression results (Table 6). Notably miR-141-3p was expressed in less than 60% of the samples and was excluded from the analyses. Five out of ten miRNAs studied appeared to be statistically significantly deregulated. More specifically, miR-128-3p and miR-24-3p were overexpressed in the progressive patients (PPMS, SPMS), as well as separately in PPMS, in comparison to HCs and SPMS. miR-191-5p was up-regulated in PMS, and separately in PPMS and SPMS, as compared to HCs. In turn, miR-376c-3p was up-regulated in PMS and separately in PPMS, both in comparison to HCs. While miR-26a-5p was up-regulated only in PMS, but not separately in PPMS or SPMS (Table 6). None of the miRNAs down-regulated in the screening cohort was statistically significantly expressed in the validation cohort.

Table 5. Differently expressed miRNAs in PPMS to HCs comparison in the screening cohort of study I.

microRNA	PPMS (n=18)[a]	HC (n=10)[a]	p-value	FC[b]
Down-regulated				
miR-141-3p	0.12 (0.03-0.36)	2.46 (0.05-22.46)	**0.024**	0.05
miR-124-3p	1.28 (0.04-10.22)	12.98 (0.25-124.71)	**0.024**	0.10
miR-375	0.48 (0.11-1.09)	1.56 (0.39-7.35)	**0.007**	0.31
miR-130b-3p	0.65 (0.01-1.56)	1.35 (0.32-2.58)	**0.006**	0.48
Up-regulated				
miR-376c-3p	1.53 (0.06-3.95)	0.80 (0.24-2.01)	**0.024**	1.90
miR-128-3p	2.29 (0.63-6.89)	1.23 (0.52-2.41)	**0.021**	1.87
miR-191-5p	1.82 (1.02-5.25)	1.03 (0.62-1.77)	**0.003**	1.76
miR-26a-5p	1.63 (0.78-2.77)	1.01 (0.58-1.66)	**0.003**	1.61
miR-24-3p	1.43 (0.74-2.40)	1.08 (0.50-1.88)	**0.024**	1.33

PPMS: primary progressive MS, HC: healthy controls, FC: fold change

[a]mean (range) of ΔΔCt values

[b]FC was calculated by comparing mean miRNA expression values of PPMS and HCs

Table 6. Multivariate logistic regression models, adjusted for age, gender and batch, for the validation set result (Study I).

microRNA	PMS vs HC		PPMS vs HC		SPMS vs HC		RRMS vs SPMS	
	OR	p	OR	p	OR	p	OR	p
miR-124-3p	0.99	0.691	0.98	0.599	1.01	0.908	0.98	0.729
miR-375	0.92	0.567	0.97	0.843	0.63	0.189	0.72	1.071
miR-130b-3	2.76	0.196	5.46	0.110	3.63	0.469	1.39	0.320
miR-211-5p	0.82	0.373	0.90	0.698	0.60	0.206	1.15	0.641
miR-376c-3p	1.89	**0.008**	2.73	**0.010**	1.25	0.051	2.95	0.998
miR-128-3p	1.91	**0.017**	3.74	**0.005**	1.88	0.631	2.13	**0.011**
miR-191-5p	2.07	**0.002**	2.20	**0.001**	1.85	**0.011**	1.36	0.099
miR-26a-5p	2.07	**0.007**	2.72	0.020	1.71	0.050	1.00	0.098
miR-24-3p	1.41	**0.019**	2.06	**0.004**	1.01	0.207	1.49	**0.018**

PMS: progressive MS (includes PPMS and SPMS), PPMS: primary progressive MS, SPMS: secondary progressive MS, HC: healthy controls, OR: odds ratio. p-values are reported before the Bonferroni correction, thus p-values less than 0.20 were considered as statistically significant (marked bold).

5.2 Potential of the selected circulating miRNAs to discriminate progressive MS subtypes from RRMS and CIS (Study II-III)

To explore the potential of selected circulating miRNAs to discriminate between MS subtypes and HCs, studies II and III were performed. Four miRNAs, miR-376c-3p, miR-128-3p, miR-191-5p, and miR-24-3p, were selected for study II based on the study I result. miR-26a-5p was not included, as it was not statistically significantly deregulated in PPMS or SPMS compared to HCs or each other. Selected miRNAs were analysed from the sera samples obtained from patients with RRMS (n=53) and PPMS (n=20), and HCs (n=27) (Table 3). Data were not normally distributed, but natural logarithm transformation allowed the use of linear regression adjusted for age and sex. As a result, overexpression of miR-191-5p and miR-24-3p was observed in comparison with HCs in the combined group of RRMS and PPMS and separately in both subtypes ($p \leq 0.04$, $FC \geq 1.65$). No statistically significant differences were recorded between RRMS and PPMS (Table 7). In addition, no statistically significant differences in expression levels of miR-128-3p and miR-376 were seen between MS subtypes, nor in comparison to HCs.

Table 7. Circulating miRNA expression levels in RRMS, PPMS and HC. Analysed with linear regression model adjusted for sex and age (Study II).

microRNA	All MS vs HC		RRMS vs HC		PPMS vs HC		RRMS vs PPMS	
	FC	p	FC	p	FC	p	FC	p
miR-128-3p	1.81	**0.02**	1.90	0.07	1.72	**0.03**	1.11	0.67
miR-191-5p	1.76	**0.002**	1.65	**0.01***	1.75	**<0.001**	0.95	0.88
miR-24-3p	2.84	**0.002**	2.10	**0.01***	3.58	**0.01**	0.59	0.79
miR-376c-3p	1.20	0.42	1.35	0.42	1.05	0.58	1.29	0.62

All MS group consists of RRMS and PPMS patients. RRMS: relapsing-remitting MS, PPMS: primary progressive MS; HC: healthy controls; FC: fold change. p-values reported prior to Bonferroni correction, thus p-values less than 0.02 were considered as statistically significant (marked in bold).

In study III, the expression levels of miR-24-3p, miR-191-5p, miR-128-3p and miR-223-3p were measured in sera samples from patients with all MS subtypes, RRMS (n=28), PPMS (n=15) and SPMS (n=14), as well as in CIS (n=18) and HCs (n=32) (Table 5). Based on the results of study II, miR-376c-3p was not included in study III, instead, miR-223-3p was chosen based on the literature findings [211], [213], [224], [229]. Data was not normally distributed, thus Mann–Whitney U-test

was an analysis of choice. Overexpression of miR-191-5p was observed in a combined group of RRMS, SPMS, and PPMS and separately in RRMS compared to HCs, but not in PPMS and SPMS. While miR-128-3p was overexpressed in the combined group and separately in PPMS, both in comparison to HCs. No differences in comparison with CIS or between the MS subtypes were observed (Table 8).

Table 8. Baseline circulating miRNA expression in RRMS, PPMS, SPMS and CIS as compared to HC.

microRNA	All MS		RRMS		SPMS		PPMS		CIS	
	FC	p	FC	p	FC	p	FC	p	FC	p
miR-128-3p	2.57	**0.03**	2.29	0.17	2.70	0.33	2.72	**0.02**	2.00	0.20
miR-191-5p	2.63	**0.02**	3.52	**0.03**	2.43	0.61	1.93	0.06	2.12	0.28
miR-24-3p	2.53	0.16	2.65	0.07	2.91	0.89	2.04	0.35	2.27	0.40
miR-223-3p	2.33	0.24	2.27	0.30	2.60	1.00	2.11	0.20	2.11	0.16

All MS group consists of RRMS, SPMS and PPMS patients. RRMS: relapsing-remitting MS, SPMS: secondary progressive MS, PPMS: primary progressive MS, HC: healthy controls, FC: fold change. p-values are Bonferroni corrected a p-values less than 0.05 were considered as statistically significant (marked in bold).

5.3 Association between relative expression levels of selected circulating miRNAs, clinical (Study I-III) and MRI parameters (Study III)

To explore the association between the selected miRNAs and clinical parameters, such as EDSS, progression index, ARR and disease duration, correlation analyses were performed (Study I-III).

miR-191-5p positively correlated with EDSS (r=0.425, p=0.027) and progression index in RRMS (r=0.507, p=0.010, Study III) and with disease duration in SPMS (r=0.637, p=0.014, Study III). **miR-128-3p** positively correlated with the EDSS in RRMS (p=0.407, p=0.035, Study III), progression index in the combined group of RRMS and PPMS patients (r=0.271, p=0.048, Study III), RRMS (r=0.538, p=0.006, Study III) and SPMS (r=0.40, p=0.03, Study I) and ARR in RRMS (r=0.358, p=0.014, Study II). **miR-223-3p** negatively correlated with the progression index in PPMS (r=-0.655, p=0.029, Study III). Expression levels of **miR-24-3p** positively

correlated with the progression index (r=0.343, p=0.004) in the combined group of RRMS and PPMS (Study II).

Association between the expression levels of miR-128-3p, miR-223-3p, miR-191-5p, and miR-24-3p and MRI parameters, such as T1 and FLAIR volumes were assessed in study III. As a result, **miR-223-3p** was found to negatively correlate with T1 volumes in the combined MS group (r=-0.342, p=0.022), and separately in SPMS (r=-0.569, p=0.034) and in PPMS (r=-0.636, p=0.035), but not in RRMS. No other correlations were observed.

5.4 Individual variability of circulating miRNAs (Study I-III)

To evaluate the contribution of age, sex, and immunomodulatory treatment on serum miRNA levels, correlation analyses, and Mann-Whitney U test were performed.

Correlation with age was observed for miR-128-3p and miR-191-5p in the combined group of all MS patients (r=0.271, p=0,048; r=0.326, p=0.014; study III), and separately in RRMS (r=0.508, p=0.007; r=0.481, p=0.011; Study III), and SPMS (r=−0.42, p=0.01, Study I; r=0.662, p=0.010, Study III).

Circulating miRNA expression levels did not differ in IFN-β-treated and untreated patients (Study III). Likewise, no differences in the levels of miRNA were observed between females and males (p>0.05) (Study II-III).

5.5 Temporal variability in circulating miRNA expression levels in patients with MS and CIS over the 4-year follow-up period (Study III)

To explore the temporal stability of selected miRNAs the expression levels of miR-128-3p, miR-191-5p, miR-223-3p, and miR-24-3p were studied over three time points (BL, year-2, year-4) sampled across the four-year follow-up period Patient cohort included 28 RRMS, 15 PPMS, 14 SPMS, 18 CIS and 32 HCs. The results were analysed by the Friedman test to assess their temporal behaviour over the total follow-up period and further evaluated by the Wilcoxon non-parametric test to observe variations between the visits.

Expression levels of miR-191-5p appeared to vary (p=0.028) throughout the follow-up in the combined group of MS patients, which included patients with RRMS, SPMS, and PPMS. However, no changes were observed in the separate subtypes, nor CIS. While miR-223-3p expression levels varied not only in all MS group (p<0.001), but separately in RRMS (p=0.006), PPMS (p=0.010), and SPMS (p=0.013). No temporal changes in expression levels of miR-128-3p and miR-24-3p were observed in any of the MS subtypes studied, nor in CIS (p>0.05) (Table 9). Thus miR-128-3p and miR-24-3p were not included in the Wilcoxon non-parametric test.

Table 9. Circulating miRNA expression levels in MS and CIS during the four-year follow-up.

		Fold change			Friedman test (p-value*)	Wilcoxon signed-rank test (p-values)		
		BL-Year 2	Year 2-Year 4	BL-Year 4		BL-Year 2	Year 2-Yea r4	BL-Year 4
miR-191	All MS	0.68	1.25	0.86	**0.028**	**0.002**	>0.05	>0.05
	RRMS	0.77	1.26	0.97	0.128	**0.023**	>0.05	>0.05
	SPMS	0.61	1.71	1.05	0.236	>0.05	**0.020**	>0.05
	PPMS	0.63	1.03	0.64	0.264	**0.031**	>0.05	>0.05
	CIS	0.94	1.18	1.11	0.819	>0.05	>0.05	>0.05
miR-223	All MS	0.34	3.62	1.22	**<0.001**	**<0.001**	**<0.001**	>0.05
	RRMS	0.49	2.22	1.08	**0.006**	**0.001**	**0.010**	>0.05
	SPMS	0.26	9.68	2.53	**0.013**	**0.040**	**0.003**	>0.05
	PPMS	0.23	3.46	0.8	**0.010**	**0.003**	0.050	>0.05
	CIS	0.82	1.6	1.3	0.199	>0.05	>0.05	>0.05

All MS group consists of RRMS, SPMS and PPMS patients. RRMS: relapsing-remitting MS, SPMS: secondary progressive MS, PPMS: primary progressive MS, CIS: clinically isolated syndrome, FC: fold change. p-values calculated using ΔCt values, values less than 0.05 were considered as statistically significant (marked in bold). FC were included to illustrate the differences in miRNA expression levels between the visits.
* over the course of the follow-up

According to the Wilcoxon non-parametric test, statistically significant decrease in the expression levels of miR-191-5p was detected on year-2 in all MS ($FC_{BL\text{-}year2}$=0.68, p=0.002), RRMS ($FC_{BL\text{-}year2}$=0.77, p=0.023) and PPMS ($FC_{BL\text{-}year2}$=0.63, p=0.031) when compared to BL levels, while in SPMS increase was observed in

year-4 when compared to year-2 ($FC_{year2\text{-}year4}$=1.71, p=0.016). In turn, decrease in the expression levels of miR-223-3p was measured on year-2 as compared to BL and increase on year-4 as compared to year-2 in all MS ($FC_{BL\text{-}year2}$=0.34, p<0.001; $FC_{year2\text{-}year4}$=3.62, p<0.001), RRMS ($FC_{BL\text{-}year2}$=0.49, p=0.001; $FC_{year2\text{-}year4}$=2.22, p=0.011), SPMS ($FC_{BL\text{-}year2}$=0.26, p=0.041; $FC_{year2\text{-}year4}$=9.68, p=0.003) and PPMS ($FC_{BL\text{-}year2}$=0.23, p=0.003; $FC_{year2\text{-}year4}$=3.46, p=0.05).

No statistically significant temporal changes were observed among the CIS patients, and separately in not converted and converted to RRMS during the follow-up.

5.6 The association of temporal miRNA expression changes with clinical activity and MRI changes (III)

To find associations of the temporal changes in expression levels of the selected circulating miRNAs with clinical disease activity and MRI changes, all MS and RRMS patients were categorized into active or stable groups. The categorization was based on the disability accumulation (EDSS worsening or not worsening), relapses (relapse active or stable), or activity observed in MRI (MRI active or stable) (Chapter 4.1 Study population (Study I-III)). Due to the small group size, SPMS and PPMS patients were not included in the analysis. In addition, as MRI was performed on BL and year-2, evaluation of MRI activity was held only over the 2-year follow-up.

Temporal changes of **miR-191-5p** (Table 10) were observed in the EDSS worsening, FLAIR-active, and T1-active groups, but not in the respective stable groups. Specifically, in the EDSS worsening group statistically significant decrease in the expression levels of miR-191-5p was measured at year-2 (all MS: $FC_{BL\text{-}year2}$=0.50, p<0.03; RRMS: $FC_{BL\text{-}year2}$=0.54, p=0.005). However, an increase in expression was observed at year-4 as compared to year-2 in all MS ($FC_{year2\text{-}year4}$=1.93, p<0.03). A decrease in expression levels was detected at year-2 in FLAIR-active in all MS ($FC_{BL\text{-}year2}$=0.52, p<0.001) and RRMS ($FC_{BL\text{-}year2}$=0.50, p=0.012) as well as in T1-active in all MS ($FC_{BL\text{-}year2}$=0.57, p=0.001), but not in the corresponding stable groups. No statistically significant longitudinal changes in miR-191-5p expression levels were observed among the relapse stable and active RRMS patients.

Table 10. Association of circulating miR-191-5p relative expression levels with clinical parameters and MRI measures over the four-year follow-up, among the patients with active and stable disease course.

		Fold change			p-value*		
		BL-Year2	Year2-Year4	BL-Year4	BL-Year2	Year2-year4	BL-Year4
EDSS worsening	All MS (n=24)	0.50	1.93	0.97	**<0.001**	**0.03**	>0.05
	RRMS (n=8)	0.54	1.32	0.72	**0.005**	>0.05	>0.05
EDSS not worsening	All MS (n=33)	0.85	1.03	0.87	>0.05	>0.05	>0.05
	RRMS (n=20)	0.74	0.88	0.84	>0.05	>0.05	>0.05
relapse active	All MS (n=19)	0.57	1.68	0.95	**0.04**	>0.05	>0.05
	RRMS (n=16)	0.68	1.23	0.84	>0.05	>0.05	>0.05
relapse stable	All MS (n=38)	0.75	1.18	0.88	**0.01**	>0.05	>0.05
	RRMS (n=12)	0.70	0.88	0.61	>0.05	>0.05	>0.05
T1 active	All MS (n=37)	0.57	na	na	**<0.001**	na	na
	RRMS (n=16)	0.57	na	na	>0.05	na	na
T1 stable	All MS (n=17)	0.83	na	na	>0.05	na	na
	RRMS (n=10)	0.61	na	na	>0.05	na	na
FLAIR active	All MS (n=36)	0.52	na	na	**<0.001**	na	na
	RRMS (n=17)	0.50	na	na	**0.012**	na	na
FLAIR stable	All MS (n=19)	0.99	na	na	>0.05	na	na
	RRMS (n=9)	0.94	na	na	>0.05	na	na

All MS: group consists of RRMS, PPMS and SPMS patients, RRMS: relapsing-remitting MS, EDSS: expanded disability status scale, FLAIR Fluid attenuated inversion recovery; na: not available; p-values were calculated using ΔCt values. Fold changes (FC) were included for better illustration of the differences in miRNA expression levels between the visits. FC values were calculated by comparing ΔΔCt values between specific timepoints (2^-(ΔΔCtY2-ΔΔCtBL)). p-values of 0.05 or less, considered as statistically significant. (marked bold) * Wilcoxon signed-rank test

Temporal variations of **miR-223-3p** (Table 11) were observed in all the subgroups in all MS and RRMS, except for the relapse stable group of RRMS patients. Notably, the results represented finding in the undivided all MS and RRMS groups. Specifically, decrease in the expression of miR-223-3p was observed between year-2 and BL and increase between year-4 and year-2, both in the EDSS worsening (all MS: $FC_{BL\text{-}year2}$=0.38, $FC_{year2\text{-}year4}$=4.90, p<0.02) and not worsening groups (all MS: $FC_{BL\text{-}year2}$=0.31, $FC_{year2\text{-}year4}$=3.22, p<0.001). The decrease between year-2 and BL was also seen in T1 active (all MS: $FC_{BL\text{-}year2}$=0.33, p<0.001; RRMS: $FC_{BL\text{-}year2}$=0.32, p=0.01) T1 stable (all MS: $FC_{BL\text{-}year2}$=0.28, p=0.002; RRMS: $FC_{BL\text{-}}$

$_{year2}$=0.50, p=0.02) FLAIR active (all MS: $FC_{BL\text{-}year2}$=0.33, p<0.001; RRMS: $FC_{BL\text{-}year2}$=0.30, p=0.01) and FLAIR stable (all MS: $FC_{BL\text{-}year2}$=0.28, p=0.001; RRMS: $FC_{BL\text{-}year2}$=0.50, p=0.03) groups of all MS and RRMS patients. Considering the relapse-based activity, decrease on year-2 and increase on year-4 was observed both among the active ($FC_{BL\text{-}year2}$=0.35, p=0.04; $FC_{year2\text{-}year4}$=5.48, p=0.01) and stable ($FC_{BL\text{-}year2}$=0.34, p<0.001; $FC_{year2\text{-}year4}$=3.13, p<0.001) all MS patients. While among the RRMS patients, temporal changes were observed in the relapse active ($FC_{BL\text{-}year2}$=0.40, p=0.01; $FC_{year2\text{-}year4}$=2.63, p=0.02), but not in the stable group.

Table 11. Association of circulating miR-223-3p relative expression levels with clinical parameters and MRI measures over the four-year follow-up, among the patients with active and stable disease course.

		Fold change			p-value*		
		BL-Year2	**Year2-Year4**	**BL-Year4**	**BL-Year2**	**Year2-year4**	**BL-Year4**
EDSS worsening	All MS (n=24)	0.38	4.90	1.86	**0.003**	**0.012**	>0.05
	RRMS (n=8)	0.41	na	na	**0.03**	na	na
EDSS not worsening	All MS (n=33)	0.31	3.22	0.98	**<0.001**	**<0.001**	>0.05
	RRMS (n=20)	0.53	1.82	0.96	**0.01**	**0.02**	>0.05
relapse active	All MS (n=19)	0.35	5.48	1.93	**0.04**	**0.01**	>0.05
	RRMS (n=16)	0.40	2.63	1.05	**0.01**	**0.02**	>0.05
relapse stable	All MS (n=38)	0.34	3.13	1.08	**<0.001**	**<0.001**	>0.05
	RRMS (n=12)	0.61	1.85	1.11	>0.05	>0.05	>0.05
T1 active	All MS (n=37)	0.33	na	na	**<0.001**	na	na
	RRMS (n=16)	0.32	na	na	**0.01**	na	na
T1 stable	All MS (n=17)	0.28	na	na	**0.002**	na	na
	RRMS (n=10)	0.50	na	na	**0.02**	na	na
FLAIR active	All MS (n=36)	0.33	na	na	**<0.001**	na	na
	RRMS (n=17)	0.30	na	na	**0.01**	na	na
FLAIR stable	All MS (n=19)	0.28	na	na	**0.001**	na	na
	RRMS (n=9)	0.50	na	na	**0.03**	na	na

All MS: group consists of RRMS, PPMS and SPMS patients, RRMS: relapsing-remitting MS, EDSS: expanded disability status scale, FLAIR Fluid attenuated inversion recovery; na: not available; p-values were calculated using ΔCt values. Fold changes (FC) were included for better illustration of the differences in miRNA expression levels between the visits. FC values were calculated by comparing ΔΔCt values between specific timepoints (2^-(ΔΔCtY2-ΔΔCtBL)). p-values of 0.05 or less, considered as statistically significant. (marked bold) * Wilcoxon signed-rank test

6 DISCUSSION

Characteristics of a biomarker include ease of detection and measure, ability to reflect the targeted process, pre-analytic stability, high sensitivity, specificity, and reproducibility. According to multiple studies circulating miRNAs are resistant to RNase degradation also their levels remain stable after exposure to such conditions as multiple freeze-thaw cycles, boiling, extreme pH changes, and extended storage [13], [14], [237], [238]. This, together with the low invasiveness of blood sampling methods and convenient, well-established miRNA extraction methodology, make circulating miRNAs encouraging biomarker candidates. Despite the extensive studies, none of the circulating miRNAs has yet been applied to clinical practice.
The aim of this thesis was to explore miRNA profiles in MS subtypes with an emphasis on PMS and the associations of selected miRNAs with disease progression, clinical activity, and disability accumulation. Here, we will discuss our findings and address limitations and considerations at the current state of miRNA research.

6.1 Circulating miRNA expression profile in MS (Study I-III)

As discussed in chapter 2.3, the diagnosis of MS is currently based on clinical evaluation supported by MRI and CSF investigation. It also tends to be retrospective, based on the patient's history. Behind relapsing and progressive subtypes lie distinct pathogenic mechanisms. In contrast to RRMS, inflammatory events are less prominent in PMS, and neurodegeneration independent of inflammatory responses is the main mechanism of disease progression [239]. In addition, despite wide range of effective DMTs for RRMS and active SPMS, only ocrelizumab was approved for PPMS (Chapter 2.3 Diagnosis of MS and therapy). Therefore, diagnostic biomarkers are highly needed to support diagnosis and reliably distinguish between MS subtypes.

Therefore, the expression of circulating miRNA was profiled in MS subtypes to assess their potential as diagnostic biomarkers. To explore PPMS associated miRNA expression profile, we screened 84 circulating miRNAs in 18 PPMS patients and 10

HC (Study I, screening). Ten significantly deregulated miRNAs were further validated on a wider cohort of PPMS and HCs. Patients with SPMS were included to assess the specificity of deregulated miRNAs to PPMS (Study I, validation). Four miRNAs (miR-191-5p, miR-128-3p, miR-24-3p, miR-376c-3p) were overexpressed in PPMS when compared to HC. Among them, miR-128-3p and miR-24-3p could discriminate between the PPMS and SPMS subtypes. These findings were further addressed in studies II and III.

In study II, the expression levels of miR-191-5p, miR-128-3p, miR-24-3p, and miR-376c-3p were evaluated in RRMS, PPMS, and HCs to understand whether these miRNAs could be linked to inflammatory driven immune responses that are seen in RRMS more prominently than in PPMS patients. The main aim of study III was to explore the temporal variability of selected miRNAs (will be discussed in chapters 6.2 and 6.3), expression levels of miR-191-5p, miR-128-3p, miR-24-3p, and miR-223-3p were also assessed in all MS subtypes, CIS, and HCs. MiR-376c-3p was not included in study III due to the insignificant results in study II. Whereas, miR-223-3p was selected for the analysis based on its links to MS, reported by several studies [211], [213], [224], [227].

Out of all the miRNAs studied, **miR-128-3p** showed the most potential as a diagnostic biomarker for PPMS, as it could discriminate PPMS from SPMS (Study I) and HCs (Study I-III). Thought, no differences were observed between PPMS and RRMS (Study II-III), nor between PPMS and CIS (Study III) (Table 11). A recent report by Zanoni et.al. reported increased levels of circulating miR-128-3p in PMS to RRMS comparison [219]. These results, however, further emphasize its potential as a diagnostic biomarker. At the cellular level, overexpression of miR-128-3p have been reported in PBMC of RRMS [214], [240], in naïve CD4+ T cells of RRMS, PPMS, and SPMS [241], and in CD4+ T cells of RRMS [240], all compared with HCs. The exact role of miR-128 is not known, but it is a brain-enriched miRNA [242], [243] expressed predominantly in neurons [244]. Through its target genes, it plays an important role in neuronal differentiation and regulates the proliferative and apoptotic events [242]. Previous studies have suggested that it would also indicate ongoing brain damage. A study reported higher miR-128 in CSF of stroke patients as compared to controls [245]. Moreover, increased levels of miR-128 were reported in the serum of patients with traumatic brain injury (TBI) (collected at emergency department admission) as compared to healthy controls [243]. In addition, miR-128 may have a role in MS immunopathology. It seems to contribute to the inhibition of

CD4+ Th2 differentiation through the direct suppression of B lymphoma Mo-MLV insertion region 1 homolog (BMI1) and therefore favour pro-inflammatory CD4+ Th1 responses [241]. Nevertheless, miR-128 is not specific to MS, as its deregulation was reported in other neurologic and autoimmune diseases, such as Alzheimer's disease [246], Parkinson's disease [247], and diabetes type 2 [248].

The role of **miR-24-3p** in neurodegenerative and autoimmune diseases, especially in MS, is currently poorly understood. Earlier studies showed that miR-24 targets IFN-γ [249] and IL-4 [250] and thus, can mediate the CD4+ Th1/Th2 homeostasis in both directions. IL-4 mediates the differentiation of naïve helper CD4+ T cells to Th2, while IFN-γ controls the differentiation into pro-inflammatory Th1 cell type. In addition, its other target, platelet-derived growth factor (PDGF) [216], was found to be associated with a modest disease course with a prolonged relapse-free period in the RRMS [251]. Our results showed potential as a diagnostic biomarker for PPMS, as it could discriminate PPMS from SPMS and HCs (Study I) (Table 11). However, in study II, its overexpression was observed in RRMS and PPMS compared to HC, showing no differences between the subtypes. These findings were not replicated in study III. Previously miR-24-3p was proposed as a candidate biomarker for MS, and its upregulation was reported in the combined group of RRMS, PPMS, and SPMS patients as compared to HCs, but not in the separate subtypes [216]. Notably, miR-24 is associated with other diseases such as multiple system atrophy, Parkinson's disease [252], rheumatoid arthritis [253], diabetes [254], and different types of cancer [255], [256]. Thus, its ability to discriminate MS from other diseases should be further evaluated.

Based on the results, we can propose **miR-191-5p** as a potential diagnostic biomarker for MS. Its increased levels could discriminate RRMS (Study II-III), PPMS (Study I-II), and SPMS (Study I) from HCs, but nor CIS (Study III) and could not distinguish between the subtypes (Study I-III) (Table 11). Circulating miR-191-5p has not been previously reported in serum or plasma-focused MS studies. Though, its decreased levels were described in CSF of MS patients compared to HCs [193]. A similar discrepancy was reported in Alzheimer's disease study, where several miRNAs were significantly downregulated in the CSF, but upregulated in serum compared to neurologically healthy subjects [257]. Decreased levels of cellular miR-191 were reported in NAWM of patients with MS as compared to healthy individuals, and its expression negatively correlated with mRNA levels of transcription factor SRY-box 4 (SOX4) [258], a critical factor for T and B cells

development [259] and oligodendrocyte differentiation [260]. Moreover, its decreased levels were described in B cells of untreated patients with RRMS in comparison to natalizumab-treated patients and healthy controls, addressing its role in B cell-mediated immune responses [261]. Through its targets, miR-191 can modulate B cell development [262] as well as the development and survival of T cells [263]–[265] and thereby maintain immune homeostasis. Deregulation of miR-191 was observed in Alzheimer's disease [266], mild cognitive impairment [267], and type 2 diabetes [268]. Thus, additional studies with larger cohorts of MS patients and patients with other neurological and autoimmune diseases are needed to evaluate its potential as a diagnostic biomarker.

miR-223-3p is one of the most reported miRNAs in MS-related studies [211], [213], [229], [258], [269], [270]. We showed no statistically significant differences between any of the MS subtypes and HCs or CIS (Table 11), which is in concordance with a recent study [219], but discordant with several previous reports [211], [213], [229]. Remarkably, there is an inconsistency between the studies, Fenoglio et.al. reported decreased circulating levels of miR-223-3p in the combined group of RRMS and PPMS patients [211] and separately in RRMS [229] as compared to HCs, while according to Sharaf-Eldin et.al. an increase in its levels was detected in the combined group of RRMS and SPMS patients as compared to HCs [213]. Another study reported downregulation of exosomal miR-223 in sera of patients with PMS compared to RRMS and HCs [224]. In addition to serum and plasma, aberrant expression levels of miR-223-3p were reported in the whole blood [271], PBMCs [229], CD4+ T cells and monocytes of MS patients [272], [273]. Specifically, miR-223 was significantly upregulated in CD4+ T-cells during the relapsing phase compared to the remitting phase of RRMS and healthy individuals [273] While in PBMC the upregulation was reported in remission compared to relapse and HCs [274]. Upregulation of miR-223 was reported in active MS lesions compared to normal white matter areas in control subjects [269] and non-MS tissues [275]. Also, significant upregulation of miR-223-3p was described in white matter lesions [270] and NAWM [258]. In addition, its upregulation has been reported in neurons in the experimental autoimmune encephalomyelitis, a mouse model of MS, and in grey matter-containing MS lesions [275]. Increasing evidence suggests that miR-223-3p is actively involved in immune responses and has anti-inflammatory properties. It plays a role in the monocytes and their polarization of anti-inflammatory macrophage M2phenotype [276], [277]. In MS, increased levels of miR-223 have been observed

in monocytes of patients with RRMS and PPMS [278]. It also modulates the activity of NLRP3 inflammasome that is known to be a key function in the deleterious inflammatory responses in MS [279]. It was also demonstrated on the EAE model that miR-223 protects neurons from degeneration [275] by contributing activation of myeloid cells, CNS remyelination, and debris clearance via phagocytosis [276]. In context to other diseases, an increase in miR-223 expression levels was observed in neural-derived small EVs in plasma of patients with Alzheimer's disease, suggesting an ability of miR-223 to reflect pathogenic alterations in brain [280]. miR-223 is not specific to MS, as its aberrant expression was reported in other autoimmune and neurodegenerative conditions, such as rheumatoid arthritis [253], [281], Alzheimer's disease [280], [282], diabetes type 2 [283], [284] and lupus [285].

Table 12. Deregulation of circulating miR-128-3p, miR-24-3p and miR-191-5p in Studies I-III

miRNA	Subtype	Compared to	up↑/down↓	Reference
miR-128-3p	PPMS	HCs	↑	Study I, III
	PPMS	SPMS	↑	Study I
miR-24-3p	PPMS	HCs	↑	Study I-II
	PPMS	SPMS	↑	Study I
	RRMS	HCs	↑	Study II
miR-191-5p	RRMS	HCs	↑	Study II-III
	PPMS	HCs	↑	Study I-II
	SPMS	HCs	↑	Study I

RRMS: relapsing-remitting MS, SPMS: secondary progressive MS, PPMS: primary progressive MS, HCs: healthy controls

6.2 Temporal stability (Study III)

The longitudinal stability is an important factor for the assessment of biomarker reliability. Understanding the normal temporal variations create the basis for identifying diagnostic and prognostic biomarkers. Still, this aspect is poorly studied, and existing studies report controversial results. In one of the few longitudinal studies, temporal stability of 742 circulating miRNAs was assessed during the follow-up ranging from 2 to 17 months in sera of healthy individuals [286]. No statistically

significant variability was observed for all the miRNAs studied, including miR-128-3p, miR-223-3p, and miR-191-5p. While another study focused on post-menopausal healthy women, named only 18 out of 684 serum miRNAs longitudinally stable (hsa-miR-4326, hsa-miR-4433b-3p, hsa-miR-412-5p, hsa-miR-1255a, hsa-miR-218-5p, hsa-miR-7854-3p, hsa-miR-370-3p, hsa-miR-5189-5p, hsa-miR-493-5p, hsa-miR-503-5p, hsa-miR-100-5p, hsa-miR-382-3p, hsa-miR-369-3p, hsa-miR-381-3p, hsa-miR-410-3p, hsa-miR-134-5p, hsa-miR-494-3p, hsa-miR-495-3p), over the 2-5 years follow-up period [287]. Regarding CSF analysis of healthy individuals, the expression levels of 12 out of 217 miRNAs (miR-19a-3p, miR-19b-3p, miR-23a-3p, miR-25a-3p, miR-99a-5p, miR-101-3p, miR-125b-5p, miR-130a-3p, miR-194-5p, miR-195-5p, miR-223-3p, and miR-451a) were significantly altered during the 48 hours interval [288]. Unfortunately, there is a lack of such studies in MS.

In study III, the temporal behaviour of four circulating miRNAs miR-128-3p, miR-24-3p, miR-191-5p, and miR-223-3p was analysed over the four-year follow-up miR-128-3p and miR-24-3p appeared stable, while temporal variability was noticed in expression levels of miR-191-5p and miR-223-3p in all MS subtypes, but not in CIS. Factors that can be associated with these changes are reviewed in Chapter 6.3. The temporal stability of miR-128-3p and miR-24-3p suggests that they cannot associate with disease progression or disease activity in MS.

6.3 Associations of miRNAs expression levels with clinical and MRI parameters (Study I-III)

It is vital to explore the potential of selected miRNAs as prognostic biomarkers reflecting disease activity and progression. Such information is highly needed in personalized medicine predicting which patients would benefit from a specific treatment. Therefore, correlations between miRNAs clinical and MRI parameters were assessed. In addition, factors that may contribute to temporal changes in the expression levels of selected miRNAs were analyzed in study III.

miR-128-3p. Among our findings, we reported the positive correlation between the expression levels of circulating miR-128-3p and disability accumulation, described by EDSS, in RRMS (Study III), disease progression, described by progression index, in RRMS (Study III) and SPMS (Study I) and disease activity, described by ARR, in RRMS (Study II). In contrast to our study, increased levels of

circulating miR-128-3p were reported among patients with no relapses in comparison to those with at least one new relapse during the 12 months follow-up period [219]. In the same study, a negative correlation with ARR was reported among the patients with relapsing disease course, including CIS [219]. While we used the ARR calculated over two years before the study entry, Zanoni et.al. adopted relapse rate counted over 12 months. Notably, in our four-year follow-up study, we observed no correlations between miR-128-3p and the number of relapses or ARR. Importantly, in the same study, we reported temporal stability of miR-128-3p, emphasizing the importance of additional studies on its association with disease progression and activity.

miR-24-3p. In the three performed studies, only in study II miR-24-3p associated with disease progression, described by progression index, in the combined group of RRMS and PPMS patients. In contrast, Ehya et. al. reported a negative correlation between miR-24-3p and disability accumulation represented by EDSS in the combined group of RRMS and PPMS [216]. In all the three studies conducted by our group, no correlations between miR-24-3p and EDSS were observed. In addition, we reported miR-24-3p stable in the four-year follow-up study. Therefore, it has weak potential as a prognostic biomarker.

Out of all the miRNAs explored in studies I-III, **miR-191-5p** has the most potential as a prognostic biomarker that could be linked to disease progression. First, miR-191-5p correlated positively with disability accumulation and disease progression in RRMS, as well as with disease duration in SPMS (Study III). Secondly, its temporal variation observed during the four-year follow-up (Study III) can be associated with the increase in EDSS score and T1 and FLAIR lesion volumes, suggesting the role in the neurodegenerative processes of MS. These observations are supported by the finding of a stable expression pattern of miR-191-5p in CIS patients with no disease progression. In addition, an increase in expression levels of miR-191 was found in serum of patients with traumatic brain injury, thus proposing it as a possible indicator of ongoing brain damage [289]. Therefore, increased levels of miR-191 in the blood of patients with MS may reflect the release of the miRNAs due to the apoptosis of neuronal and immune cells. This is in concordance with our follow-up findings, as degeneration of neurons is one of the main pathological events in MS and is tightly associated with disability accumulation and disease progression.

miR-223-3p. We reported a negative correlation between expression levels of miR-223-3p and disease progression, described by progression index in PPMS and

T1 volumes in the combined group of MS patients, which consisted of RRMS, PPMS, and SPMS patients, and separately in PMS cohort but not in RRMS (Study III). These observations suggest that a higher level of this miRNA is associated with a milder disease course with less disability accumulation or lesional changes observed with MRI. However, temporal variations observed over the four-year follow-up in the expression levels of miR-223-3p seem not to be associated with clinical disability accumulation indicated by EDSS, nor with changes in MRI volumes, as the fluctuations were observed in stable and active groups. Interestingly, disease activity defined by relapses over the follow-up were contributing to these longitudinal changes in miRNA levels, while non-active patients had stable gene expression pattern.

6.4 Reproducibility

Reproducibility reflects the ability to replicate the study results in similar conditions. In 26 studies focused on the expression of circulating miRNAs in serum or plasma (excluding the studies performed for this thesis), 26 miRNAs were reported in at least two studies (Table 2), among these findings, only eight miRNAs (miR-122-5p, miR-145, miR-15b-5p, miR-155, miR-223-3p, miR-23a-3p and miR-320b, miR-572) were observed in three or more studies. Regarding our findings, circulating miR-128-3p, miR-191-5p and miR-24-3p were reported in one study each [193], [216], [219], while miR-223-3p in four studies [211], [213], [224], [229], and miR-376c-3p was not previously associated with MS. miR-223-3p showed the most discrepancy in findings, as it was found to be down-regulated in serum of patients with RRMS [229] and PPMS [211], but up-regulated in the combined group of RRMS and SPMS patients [213], while in some studies [219], including ours (Study III) no statistically significant differences were observed.

The discrepancy between the results may be explained by the pre-analytical and analytical factors. The pre-analytical factors can generally be divided into patient-derived factors and sample-related sources [13]. The selection of the patient cohort has a significant impact on the results, as age, gender, BMI, ethnic origin, physical activity, and underlying diseases have been shown to contribute to variation in expression of some of the miRNAs [155], [290]–[293]. At the same time, no effect of smoking, fasting, diurnal variation, or menstrual cycle phase was detected on levels

of circulating miRNAs among the healthy individuals [286], [294], [295]. In our studies, we have observed correlations between miR-128-3p and miR-191-5p expression levels and age in RRMS and SPMS. No associations were observed with medication (IFN- β) and gender. In contrast, others have reported downregulation of miR-223 in IFN-β treated RRMS patients compared to naive patients [227]. Regarding the association with age, in a recent study, miR-191 expression levels were reported not to vary between children (3-7.4 years), adults (36-48 years), and elders (75-94 years) age groups. miR-128 reached the peak of expression in adulthood but decreased in old age [296]. A negative correlation between miR-191-5p expression levels and BMI was reported [297].

Sample-related factors are also crucial as multiple factors may influence the miRNA levels. Sample type (serum or plasma or CSF) and sample handling affect the quantitative values of individual circulating miRNAs. For example, the choice of anticoagulants and centrifugation speed, contamination with blood cells or platelets, hemolysis, and delayed specimen processing can alter plasma/serum miRNA concentrations [155], [200], [204], [286], [295]. Nonetheless, according to multiple studies, circulating miRNAs are highly stable and resistant to RNase degradation, thus their expression levels remain unchanged through multiple freeze-thaw cycles, extended storage, and extreme pH changes [13], [14], [237], [238], [298].

Among the analytical factors, significant variations were observed between different miRNA extraction methods and between different quantification platforms. Also, the choice of the normalization method is critical for miRNA analysis, and it contributes to inconsistent miRNA results [13], [204], [235], [295]. Importantly most published works on circulating miRNA biomarkers use a case-control study design, often with a single timepoint, small sample sizes, and varying levels of case-control matching [287].

6.5 Methodological considerations and limitations of the study

6.5.1 Limitations in the study populations

The patients' cohorts used in all studies included more than 55 MS patients. That number can be considered acceptable when compared to the other MS-related miRNA studies, where cohort sizes start from several dozens and rarely reach a hundred patients [213], [219], [225], [299].

The limiting factors are the unavailability of MS patient samples, especially with progressive disease form of MS, as well as the high expenses of miRNA technology. Additional samples would have enabled studies in the different sub-populations and could improve results' reliability. Naturally, 65% of patients were treated with immunomodulatory treatment, known to modulate miRNA expression levels [300]. However, we observed no effect of medication on the expression levels of selected miRNAs. The follow-up study could provide more information on how immunomodulatory therapies regulate the miRNA levels. Notably, the cohorts used in this study consist of a homogenous Finnish (Caucasians) population, which should be taken into consideration when compared to other studies.

6.5.2 Limitations in the laboratory methodologies

The main limitation and challenge regarding laboratory methodologies is a low concentration of circulating miRNAs in body fluids, in our case in sera, making it difficult to precisely measure the quantity and quality of isolated miRNAs. Due to this limitation, miRNAs are mostly quantified using amplification-based methods. For the first study, the miScript miRNA PCR arrays for the screening and miScript miRNA PCR assays for the validation (Qiagen) were chosen as most suitable at that time. The method was further used in studies II and III, to make the comparison between the results reliable.

Regarding the low concentration of circulating miRNAs and thus the usage of the amplification-based methods, the choice of correct normalization strategy is essential. The normalization strategy that was used through all the studies was

established in the first study. Due to the large number of miRNAs studied, the MCR method was chosen in the screening phase [235]. Based on the results, two endogenous controls SNORD68 and RNU6-2 were selected for the validation and were used in further studies. As endogenous controls cannot reflect the effects of the miRNA processing, cel-miR-39 was added as the additional control.

6.6 Future prospects

miR-191-5p can be considered for future studies as a possible diagnostic and prognostic MS biomarker, which can reflect neurodegenerative events. While miR-128-3p expressed a potential as a diagnostic biomarker for PPMS. In addition, due to its longitudinal stability, miR-128-3p can be considered for treatment-related studies and evaluated as a possible treatment-response monitoring biomarker, especially in PPMS. In turn, miR-223-3p can be addressed as a prognostic biomarker, depicting disease activity. Yet, we faced several limitations that should be further addressed. Considering the low specificity of selected miRNAs to MS, studies with wider cohorts of patients, possibly with the inclusion of patients with other neurological or autoimmune diseases, are highly needed. Also, evaluation of different miRNA combinations and development of specific panels for different subtypes of MS is one possible direction. In addition, the creation of standardized miRNA extraction and analysis protocols could improve reproducibility and make data more comparable. Longitudinal studies in healthy individuals would give valuable information on the temporal stability of circulating miRNAs. Also, the influence of patient factors such as age, sex, physical activity, BMI, etc. should be taken into consideration and optimized. This indicates that a lot of work is yet required before moving to clinical application.

7 SUMMARY AND CONCLUSIONS

Based on the results of this thesis the following conclusions may be drawn:

1. The circulating miRNA profiles differ in progressive MS patients (PPMS, SPMS) and HCs. Three microRNAs miR-128-3p, miR-191-5p, and miR-24-3p could be associated with PMS.

2. Out of all the miRNAs studied miR-191-5p could discriminate between MS subtyped and HCs and miR-128-3p appeared to be associated with PPMS, suggesting their potential as a diagnostic biomarker

3. The expression levels of miR-191-5p and miR-128-3p are associated with disability accumulation and disease progression in RRMS. In addition, associations with disease progression expressed miR-128-3p in PMS and miR-223-3p in PPMS.

4. The expression levels of miR-128-3p and miR-24-3p were stable in all MS subtypes and CIS during the four-year follow-up. Emphasizing their potential as diagnostic biomarkers. Temporal changes were observed in expression levels of miR-191-5p and miR-223-3p in all MS subtypes but not in CIS. In the case of miR-191-5p, these changes appeared to be associated with disability accumulation and disease activity, while miR-223-3p solely with disease activity.

5. Based on the results, miR-191-5p expressed the strongest potential as a diagnostic biomarker for MS and miR-128-3p for PPMS. In addition, miR-191-5p can be considered as a prognostic biomarker that can reflect disease progression.

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9 PUBLICATIONS

PUBLICATION I

Circulating miRNAs as biomarkers in progressive multiple sclerosis

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Original Research Paper

Circulating microRNAs as biomarkers in progressive multiple sclerosis

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Abstract

Background: In multiple sclerosis (MS), microRNA (miRNA) dysregulation is mostly reported in different immune cells, but less information is available on circulating miRNAs that exert strong biomarker potential due to their exceptional stability in body fluids.

Objective: The aim of this study was to profile expression of circulating miRNAs in primary progressive multiple sclerosis (PPMS) and secondary progressive multiple sclerosis (SPMS) and assess their association with neurological worsening.

Methods: The expressions of 84 different miRNAs were profiled in serum of 83 subjects (62 MS and 21 controls) using miScript miRNA techniques. First, they were screened on 18 PPMS and 10 controls; thereafter, 10 most aberrantly expressed miRNAs were validated on a larger cohort.

Results: In comparison with controls, upregulation of miR-191-5p was found in both progressive MS subtypes, while miR-376c-3p was overexpressed only in PPMS. Additionally, upregulation of miR-128-3p and miR-24-3p was detected in PPMS when compared to controls and SPMS. Progression index correlated with miR-128-3p in PPMS and miR-375 in SPMS.

Conclusion: We detected overexpression of four miRNAs that have not been previously associated with progressive forms of MS. The increased expression of circulating miR-191-5p seems to be associated with progressive forms of MS, while miR-128-3p seems to be associated mostly with PPMS.

Keywords: Multiple sclerosis, PPMS, SPMS, biomarkers, circulating microRNA, miR-128-3p, miR-376c-3p, miR-191-5p, miR-24-3p

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Introduction

MicroRNAs (miRNAs) play a critical role in a variety of biological processes by regulating gene expression at the post-transcriptional level through mRNA degradation or inhibition.[1] In addition to their direct functions within the cells, miRNAs are released into the extracellular space, where they are involved in cell-to-cell communication.[2] Aberrant miRNA profiles are detected in different autoimmune diseases, including multiple sclerosis (MS).[3] Most MS-related miRNA studies are focused on miRNA expressions in blood mononuclear cells and various populations of T (CD4+ and CD8+) and B cells,[4] but less information is available on circulating miRNAs.[5–10] Until now, in MS, only four circulating miRNA profiling studies are available.[5–8] In the first study, over 900 miRNAs were profiled from plasma obtained from four MS patients and three healthy controls (HC). It appeared that in plasma from patients with MS, six miRNAs were upregulated (miR-22, miR-422a, miR-572, miR-614, miR-648, and miR-1826) and one (miR-1979) was downregulated in comparison with controls.[7] Another study, based on treatment-naive relapsing-remitting multiple sclerosis (RRMS) patients and their controls, screened 38 selected circulating miRNAs and reported an upregulation of miR-660 and miR-939, as well as downregulation of miR-145 in plasma of patients with RRMS.[8] Two other studies explored the association of circulating miRNAs not only with RRMS but also with progressive subtypes of MS.[5,6] Gandhi et al.[6] showed that 8 (let-7c, let-7d, miR-92a-1*, miR-135a, miR-145, miR-454, miR-500, and miR-574-3p) of 368 miRNAs were differentially expressed in plasma

Table 1. The clinical characteristics of the multiple sclerosis (MS) patients and healthy controls in screening and validation cohorts.

	Screening cohort		Validation cohort			
	PPMS ($n=18$)	HC ($n=10$)	All MS ($n=62$)	PPMS ($n=31$)	SPMS ($n=31$)	HC ($n=21$)
Gender (female)[a]	10 (56%)	6 (60%)	42 (68%)	18 (58%)	24 (77%)	12 (57%)
Age,[b] years	53.5±8.5 (39–68)	51.8±8.2 (40–65)	53±10.3 (28–67)	56.3±9.7 (39–75)	48.8±9.5 (28–67)	52.7±8.1 (38–65)
Disease duration from first symptoms, years[b]	15.6±10.0 (3.1–43)	–	20.2±10.2 (3.1–49.3)	16.8±10.9 (3.1–49.3)	23.4±8.3 (9.0–36.4)	–
Disease duration from diagnosis, years[b]	11.0±7.7 (0.1–25.8)	–	13.8±8.8 (0–35.4)	10.9±8.4 (0–29.3)	16.6±8.3 (3.3–35.4)	–
EDSS[b]	5.9±1.3 (3.0–8.0)	–	5.5±1.6 (1.5–8.0)	5.3±1.8 (1.5–8.0)	5.7±1.4 (2.5–8.0)	–
Progression index[b,c]	0.8±1.0 (0.2–3.9)	–	0.57±0.6 (0.11–3.90)	0.7±0.8 (0.11–3.90)	0.45±0.3 (0.14–2.00)	–

PPMS: primary progressive multiple sclerosis; HC: healthy controls; SPMS: secondary progressive multiple sclerosis; EDSS: Expanded Disability Status Scale.
[a]Number of patients (%).
[b]Mean ± standard deviation (SD) (minimum to maximum).
[c]Progression index (EDSS/disease duration from diagnosis) calculated only for patients with disease duration more than 1 year (PPMS $n=27$, SPMS $n=31$).

of patients with secondary progressive multiple sclerosis (SPMS) in comparison with RRMS, while study by Fenoglio et al.[5] showed a downregulation of three miRNAs (miR-15b, miR-23a, and miR-223) in serum of both RRMS and primary progressive multiple sclerosis (PPMS) patients, with no detected differences between the MS subtypes.

Relapsing and progressive subtypes of MS have distinct underlying pathogenic mechanisms that drive the disease processes. In RRMS, the inflammation together with blood–brain barrier (BBB) damage is the key mechanism in the initiation of disease process. It is known that in the progressive subtypes, inflammation-promoting effects become less prominent over time while the neurodegenerative events become amplified by various mechanisms including oxidative stress and mitochondrial damage followed by intracellular influx of Na^+ and Ca^{2+} leading to cell death.[11] Pathology of progressive subtypes is characterized by widespread diffuse inflammation with slowly expanding lesions and abundant cortical lesions, as well as lymphocyte infiltration and microglia activation in the normal-appearing white matter. Although in PPMS and SPMS the extent of inflammation in brain tissue is less prominent than in RRMS, some studies have shown that the ongoing peripheral immune activation mediates the neuroinflammation also in these subtypes.[12] Indeed, a recent study showed that the natalizumab therapy decreased the levels of inflammatory and neurodegenerative biomarkers in cerebrospinal fluid in progressive MS indicating that the peripheral immune activation contributes to the disease processes.[13]

In recent years, increased understanding of pathogenesis of progressive MS subtypes facilitated the development of therapeutic strategies for these subtypes. In fact, the results from phase III clinical studies on the efficacy of the ocrelizumab, a monoclonal antibody against CD20 molecule expressed on B-cells, have been recently reported in both RRMS and PPMS.[14,15] Thus, it would be extremely important to identify such biomarkers that could reliably distinguish PPMS from SPMS and progressive MS subtypes from RRMS, as well as to predict response to such treatments. Therefore, the aim of this study was to explore the association of circulating miRNAs with progressive MS and their ability to capture pathological events related to disability progression.

Materials and methods

Study design

miRNA expression analyses from sera were performed on two cohorts (Table 1). First, the expressions of 84 different miRNAs were analyzed (screening phase) from the samples of 18 PPMS patients and 10 HC. Second, 10 most promising miRNAs were studied (validation phase) on a larger group of 31 PPMS, 31 SPMS, and 21 HC.

Study population

The study population comprises a total of 62 MS patients (31 PPMS and 31 SPMS) and 21 controls (Table 1). Screening and validation cohorts shared 18 MS patients and 9 controls. The diagnosis of MS was based on the revised McDonald[16] criteria, and all diagnoses were definite. All patients were studied clinically and neurologically including the assessment of disability expressed by Expanded Disability Status Scale (EDSS) score.[17] None of the patients received immunomodulatory therapy within 6 months prior to blood sampling. Control group consisted of age- and sex-matched healthy subjects. Study was approved by the Ethics committee of Pirkanmaa Hospital District, and clinical investigation followed the principles of Helsinki Declaration. The patients gave their informed consent.

Collection of serum and miRNA extraction

Blood was drawn into BD Vacutainer SST II advance tubes (Becton Dickinson, USA) and allowed to clot for 30 minutes before centrifugation at 1500×*g* for 15 minutes at room temperature. Thereafter, serum was separated and stored at −80°C until use. Circulating miRNA was extracted from 200 µL serum using Qiagen miRNeasy Serum/Plasma kit (Qiagen Inc, Valencia, CA) according to the manufacturer's protocol. Spike-In control cel-miR-39 (Qiagen Inc) was used to monitor RNA recovery and reverse transcription efficiency. Extracted and purified miRNA was eluted into 14 µL of RNase-free water and stored at −80°C until use.

Reverse transcription

Isolated miRNAs were reverse transcribed to complementary DNA (cDNA) using a miScript reverse transcription kit (Qiagen Inc) with the standard protocol. Reaction mix included 4.5 µL of extracted miRNA solution, 4 µL of 5× miScript Hi-Spec-buffer, 2 µL of 10× miScript Nucleic mix, 7.5 µL of RNase-free water, and 2 µL of miScript Reverse Transcriptase (RT) mix that was incubated for 60 minutes at 37°C, 5 minutes at 95°C, and then held at 4°C. The cDNA samples were stored at −20°C until use.

miRNA expression analysis

Expression of circulating miRNAs was analyzed with miScript Human Serum & Plasma miRNA polymerase chain reaction (PCR) arrays/assays (Qiagen Inc), by the SYBR-green-based real-time PCR (RT-PCR) method, on the ABI 7900HT PCR machine (Applied Biosystems, Foster City, CA, USA). Prior to the RT-PCR, all the cDNA samples were diluted by adding 200 µL of RNase-free water. In the screening phase, Human Serum & Plasma miRNA PCR arrays (Qiagen Inc) were used to determine the expression of 84 different miRNAs and 9 controls, including cel-miR-39-3p, 6 housekeeping genes (SNORD61, SNORD68, SNORD72, SNORD95, SNORD96A, and RNU6-2), and 2 quality controls (reverse transcription (miRTC) and positive PCR (PPC) controls). The 10-µL reactions included 5 µL 2× QuantiTect SYBR Green PCR Master Mix, 1 µL 10× miScript Universal Primer, 0.9 µL diluted cDNA, and 3.1 µL RNase-free water that were incubated in 384-well plate. In the validation phase, 10 potential miRNAs from the screening phase (miR-130b, miR-376c, miR-141, miR-211, miR-24-1, miR-128-1, miR-375, miR-124-1, miR-191, and miR-26a-1) were further studied using miScript Human Serum & Plasma miRNA PCR assays (Qiagen) with cel-miR-39-3p, SNORD68, and RNU6-2 as controls. The 10-µL reaction mixture included 5 µL 2× QuantiTect SYBR Green PCR Master Mix, 1 µL 10× miScript universal primer, 1 µL 10× miScript primer assay, 1 µL diluted cDNA, and 2 µL RNase-free water. All samples were run as triplicates in order to control for the intra-assay variation (average coefficient of variation (CV) = 2.0%). RT-PCR conditions in screening and validation phases were 95°C for 15 minutes, followed by 40 amplification cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 70°C for 30 seconds, followed in turn by pre-set dissociation stage of 15 seconds at 95°C and 15 seconds at 60°C. To control for the inter-assay variation, we used an additional control sample which was included to each plate and run against miR-21 (CV 3.0%) and miR-39-3p (CV 2.5%), during the validation phase.

The expression data were analyzed with miScript miRNA PCR Array Data Analysis software (Qiagen) using comparative Ct method (ΔΔCt) using mean values of HC as a calibrator. In the screening phase, results were normalized by the mean center restricted (MCR) method.[18] Validation data were normalized using miR-39-3p, SNORD68, and RNU6-2 as endogenous controls.

Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (IBM corporation, Armonk, NY, USA). As data were not normally distributed, nonparametric statistical tests were used. In the screening set, differences of the miRNA expressions between PPMS and HC were analyzed using Mann–Whitney *U*-test. In the validation set, differences of the miRNA

Table 2. Differently expressed miRNAs in PPMS and healthy controls in the screening cohort (mean (minimum–maximum)).

miRNA	PPMS[a] ($n=18$)	HC[a] ($n=10$)	p value[a]	Fold change[b]
miR-141-3p	0.12 (0.03–0.36)	2.46 (0.05–22.46)	**0.024**	0.05
miR-124-3p	1.28 (0.04–10.22)	12.98 (0.25–124.71)	**0.024**	0.10
miR-375	0.48 (0.11–1.09)	1.56 (0.39–7.35)	**0.007**	0.31
miR-211-5p	0.43 (0.07–0.94)	1.00 (0.13–3.70)	0.314	0.43
miR-130b-3p	0.65 (0.01–1.56)	1.35 (0.32–2.58)	**0.006**	0.48
miR-376c-3p	1.53 (0.06–3.95)	0.80 (0.24–2.01)	**0.024**	1.90
miR-128-3p	2.29 (0.63–6.89)	1.23 (0.52–2.41)	**0.021**	1.87
miR-191-5p	1.82 (1.02–5.25)	1.03 (0.62–1.77)	**0.003**	1.76
miR-26a-5p	1.63 (0.78–2.77)	1.01 (0.58–1.66)	**0.003**	1.61
miR-24-3p	1.43 (0.74–2.40)	1.08 (0.50–1.88)	**0.024**	1.33

miRNAs: microRNAs; PPMS: primary progressive multiple sclerosis; HC: healthy controls.
[a]Comparison between PPMS and controls using Mann–Whitney *U*-test. Values less than 0.05 (bold) were considered to be statistically significant.
[b]Fold changes were calculated by comparing mean miRNA expression values between the PPMS patients and controls.

expressions between the groups were studied using a multivariate logistic regression model adjusted for age, sex, and batch. Bonferroni's correction was used to reduce the chances of obtaining false-positive results. Spearman's correlation analysis was used to study association between miRNA expressions and clinical parameters (EDSS, progression index, disease duration, and age). Receiver operating characteristic (ROC) curve analysis was used for evaluating the diagnostic power of circulating miRNAs. Values of p smaller than 0.05 were considered to be statistically significant.

Results

Circulating miRNA profile in sera of PPMS patients and HC (screening set)

We profiled the expression of circulating miRNAs in serum obtained from 18 PPMS patients and 10 HCs (Table 1). Of the 84 miRNAs analyzed, 4 were significantly downregulated (miR-375, miR-130b-3p, miR-141-3p, and miR-124-3p) and 5 were upregulated (miR-128-3p, miR-376c-3p, miR-191-5p, miR-26a-5p, and miR-24-3p) in PPMS when compared to controls (Table 2). The expression of miR-211-5p was strongly downregulated in comparison with controls, but statistical significance was not reached (Fold change (FC) = 0.43, $p=0.314$).

The expression of selected circulating miRNAs in SPMS, PPMS, and HC (validation set)

To validate the preliminary findings, the gene expressions of 10 most aberrantly expressed miRNAs (Table 2) were analyzed on a larger cohort of 83 subjects including 31 patients with PPMS, 31 with SPMS, and 21 HC (Table 1). Of the 10 miRNAs studied, only one (miR-141) could not be amplified in 60% of the samples and thus was excluded from the analyses.

Data were analyzed using a logistic regression model and adjusted for sex, age, and batch (Table 3).

When the group including all MS patients was compared to controls, five miRNAs (miR-128-3p, miR-376c-3p, miR-26a-5p, miR-191-5p, and miR-24-3p) were upregulated (FC > 1.5; $p<0.05$), but after the Bonferroni correction only the differences for four miRNAs (miR-128-3p, miR-376c-3p, miR-26a-5p, and miR-191-5p) reached statistical significance (Table 3). Of these, miR-191-5p showed the strongest upregulation in progressive MS (adjusted $p=0.01$, odds ratio (OR) = 2.76, FC = 1.9).

Analysis of the miRNA expressions between the groups showed significant upregulation of five miRNAs (miR-128-3p, miR-376c-3p, miR-26a-5p, miR-191-5p, and miR-24-3p) in PPMS ($p<0.05$, FC > 1.5) in comparison with controls (Figure 1). The differences for four of them (miR-128-3p, miR-376c-3p, miR-191-5p, and miR-24-3p) appeared to be significant after the Bonferroni correction. Notably, in SPMS, three miRNAs (miR-376c-3p, miR-26a-5p, and miR-191-5p) were upregulated ($p<0.05$, FC > 1.5), but only miR-191-5p remained statistically significant after the Bonferroni correction (adjusted $p=0.03$, OR = 3.63, FC = 1.6). miR-191-5p also showed the strongest upregulation in PPMS (adjusted $p=0.003$, OR = 5.46, FC = 2.3).

Table 3. Multivariate logistic regression models, adjusted for age, gender, and batch, for the validation set result.

MicroRNA	All MS vs HC		PPMS vs HC		SPMS vs HC		PPMS vs SPMS	
	OR	*p* value[a]	OR	*p* value[a]	OR	*p* value[a]	OR	*p* value[a]
miR-124-3p	0.99	0.691	0.98	0.599	1.01	0.908	0.98	0.729
miR-375	0.92	0.567	0.97	0.843	0.63	0.189	0.72	1.071
miR-211-5p	0.82	0.373	0.90	0.698	0.60	0.206	1.15	0.641
miR-130b-3p	2.76	0.196	5.46	0.110	3.63	0.469	1.39	0.320
miR-376c-3p	1.89	**0.008***	2.73	**0.010***	1.25	**0.051**	2.95	0.998
miR-128-3p	1.91	**0.017***	3.74	**0.005***	1.88	0.631	2.13	**0.011***
miR-191-5p	2.07	**0.002***	2.20	**0.001***	1.85	**0.011***	1.36	0.099
miR-26a-5p	2.07	**0.007***	2.72	**0.020**	1.71	**0.050**	1.00	0.098
miR-24-3p	1.41	**0.019**	2.06	**0.004***	1.01	0.207	1.49	**0.018***

MS: multiple sclerosis; PPMS: primary progressive multiple sclerosis; HC: healthy controls; SPMS: secondary progressive multiple sclerosis; OR: odds ratio.
[a]*p* values before Bonferroni correction. Values less than 0.05 were considered to be statistically significant.
**p* values that remained statistically significant after the Bonferroni correction.

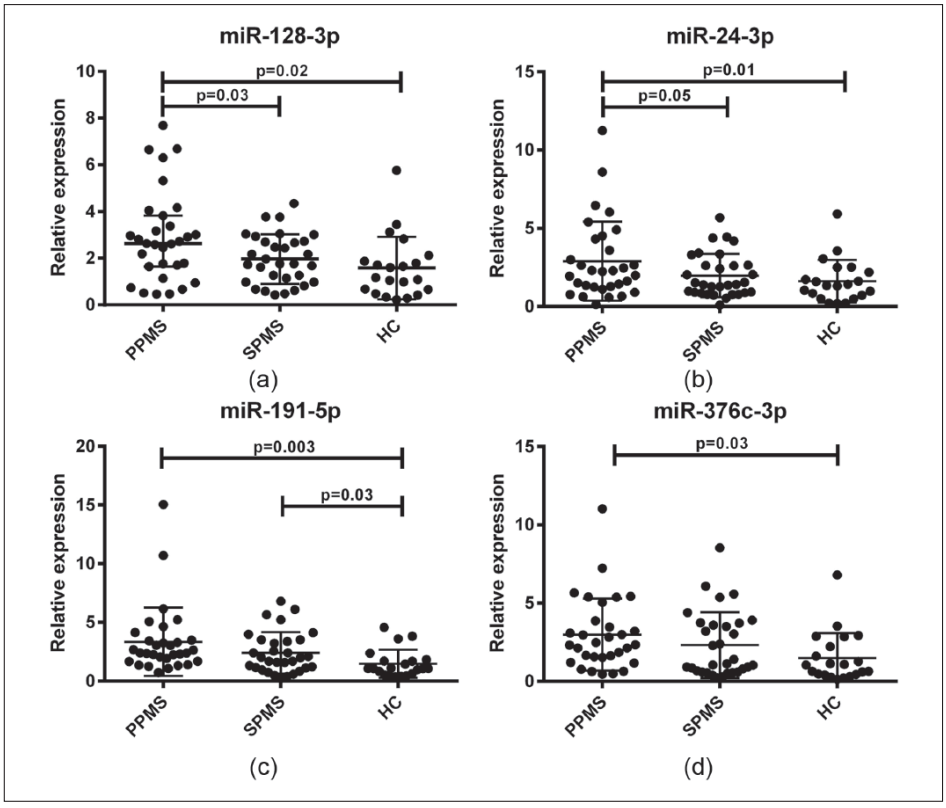

Figure 1. Scatter plot of the distribution of (a) miR-128-3p, (b) miR-24-3p, (c) miR-191-5p, and (d) miR-376c-3p in serum obtained from primary progressive multiple sclerosis (PPMS), secondary progressive multiple sclerosis (SPMS) patients, and healthy controls (HC) (validation cohort). Bars indicate median with interquartile range, and *p* values shown are after Bonferroni correction.

Comparison between the progressive subtypes showed that two miRNAs (miR-128-3p and miR-24-3p) were significantly upregulated in PPMS after the Bonferroni correction (miR-128-3p adjusted $p=0.03$; OR = 2.95; FC = 1.5; miR-24-3p adjusted $p=0.05$; OR = 2.13; FC = 1.5).

In the validation set, we aimed to maximize the number of MS cases and controls for the miRNA analyses; therefore, in the screening and validation cohorts, 18 MS and 9 control samples were the same. In order to confirm that our results could be replicated in the independent cohort, additional logistic regression model analyses were performed excluding all the overlapping samples (Table 4). In the analyses, age and sex were used as covariates.

Analysis showed that three miRNAs (miR-128-3p, miR-26a-5p, and miR-191-5p) were significantly expressed in progressive MS patients (PPMS and SPMS) when compared to controls as it was detected in the whole validation cohort. In turn, the expression of miR-376c-3p did not differ statistically between MS and controls. Comparison between the groups showed that three miRNAs (miR-128-3p, miR-26a-5p, and miR-191-5p) were significantly expressed in PPMS and two miRNAs (miR-26a-5p and miR-191-5p) in SPMS when compared to controls ($p<0.05$). No differences in the expression levels were detected between the progressive subtypes.

Association of miRNAs with clinical parameters

To explore the miRNAs association with clinical parameters, correlation analyses were performed between the expressions of the miRNAs and EDSS, progression index, disease duration, and age.

In the whole MS group, the expression of miR-124-3p correlated positively with the time from first symptoms ($r=0.37$, $p=0.003$), but other statistically significant correlations were not found. Based on the subtype analysis, the SPMS group showed a positive correlation between the expression of miR-128-3p and the progression index ($r=0.40$, $p=0.03$), but negative association with age ($r=-0.42$, $p=0.01$).

In the PPMS group, miR-124-3p correlated with the time from first symptoms ($r=0.48$, $p=0.01$) and miR-375 with age ($r=0.39$, $p=0.03$). When patients with the EDSS scores higher than six were excluded from the analysis, a negative correlation was found between miR-375 and the progression index in PPMS ($r=-0.41$, $p=0.04$, $n=28$). Due to the skewed

Table 4. Multivariate logistic regression models for the microRNA (miRNA) gene expressions in the validation cohort (validation cohort) and in the cohort excluding samples used in the screening phase (independent cohort).

miRNA	MS vs HC[a]				PPMS vs HC[a]				SPMS vs HC[a]				PPMS vs SPMS			
	Whole cohort ($n=62$)		Independent cohort ($n=44$)		Whole cohort ($n=31$)		Independent cohort ($n=13$)		Whole cohort ($n=31$)		Independent cohort ($n=31$)		Whole cohort ($n=31$)		Independent cohort ($n=13$)	
	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p	OR	p
miR-124-3p	0.99	0.78	0.97	0.36	0.99	0.63	1.01	0.89	1.01	0.90	4.14	0.49	0.99	0.70	1.09	0.11
miR-375	0.93	0.46	1.02	0.91	1.00	0.97	1.01	0.97	0.84	0.15	0.92	0.69	1.14	0.36	1.05	0.77
miR-128-3p	1.55	**0.05**	3.17	**0.02**	1.73	**0.02**	2.28	**0.04**	1.13	0.66	2.77	0.06	2.02	**0.01**	1.89	0.07
miR-130b-3p	1.20	0.24	1.09	0.58	1.32	0.11	1.36	0.17	1.03	0.70	1.02	0.74	1.00	0.89	1.01	0.67
miR-211-5p	0.94	0.65	0.85	0.29	1.03	0.86	1.04	0.83	0.79	0.22	0.69	0.07	1.22	0.23	1.34	0.13
miR-376c-3p	1.43	**0.04**	1.37	0.16	1.64	**0.03**	1.76	0.07	1.15	0.41	1.20	0.38	1.14	0.36	1.24	0.26
miR-191-5p	1.95	**0.008**	7.37	**0.009**	2.37	**0.007**	20.43	**0.03**	1.57	0.06	6.00	**0.02**	1.19	0.19	1.16	0.41
miR-24-3p	1.34	0.13	1.82	0.15	1.52	**0.05**	2.30	0.29	1.07	0.77	1.69	0.20	1.46	**0.04**	1.19	0.37
miR-26a-5p	1.58	**0.026**	3.97	**0.006**	1.55	**0.05**	10.36	**0.04**	1.40	0.10	3.62	**0.01**	1.15	0.20	1.28	0.16

PPMS: primary progressive multiple sclerosis; HC: healthy controls; SPMS: secondary progressive multiple sclerosis; OR: odds ratio. p-values less than 0.05 (bold) were considered to be statistically significant.
[a]In the screening and validation cohort, 9 of 21 controls were the overlapping samples.

Table 5. The correlations (*r*) between significantly expressed miRNAs in the validation cohort.

Comparison	PPMS	SPMS	HC
miR-24-3p vs miR-191-5p	0.62**	0.50**	0.56**
miR-24-3p vs miR-128-3p	0.64**	0.60**	0.69**
miR-24-3p vs miR-376c-3p	0.43*	0.44**	0.54**
miR-128-3p vs miR-376c-3p	0.47**	0.37*	0.37
miR-128-3p vs miR-191-5p	0.61**	0.34	0.74**
miR-191-5p vs miR-376c-3p	0.46**	0.26	0.28

miRNA: microRNAs; PPMS: primary progressive multiple sclerosis; SPMS: secondary progressive multiple sclerosis; HC: healthy controls. *$p<0.05$; **$p<0.01$.

distribution of the miRNA data, regression analysis adjusted for age could not be performed.

Correlation analyses between the validated miRNAs

Correlation analyses were performed between the miR-191-5p, miR-128-3p, miR-376c-3p, and miR-24-3p that were seen to be aberrantly expressed in progressive MS (Table 5). Correlations were most notable in the PPMS where the correlations were detected between all four miRNAs. However, in SPMS and HC, miR-24-3p was the only miRNA that correlated with three other miRNAs (miR-191-5p, miR-128-3p, and miR-376c-3p), while MiR-128-3p correlated with miR-376c-3p in SPMS and with miR-191-5p in HC.

Circulating miRNAs' diagnostic values

An ROC curve analysis was performed for most significantly expressed circulating miRNAs in order to test their biomarker potential for progressive MS (Figure S1). High values for sensitivity, specificity, and the area under the curve (AUC) were observed for miR-128-3p ($p=0.006$; 95% confidence interval (CI): 0.59−0.87) (Figure S1A) and miR-191-5p ($p<0.001$; 95% CI: 0.68−0.94) (Figure S1C), suggesting them as potential predictors of PPMS.

Discussion

Due to exceptional stability and relative ease of detection of circulating miRNAs, they are suggested as promising biomarkers for various autoimmune diseases. However, despite growing interest for circulating miRNAs, they are less studied in MS. Until now, an aberrant expression of circulating miRNAs,[5,6,8–10,19] as well as an association of some of them with EDSS score, has been reported in MS.[5,6,10] In our study, four miRNAs (miR-128-3p, miR-376c-3p, miR-191-5p, and miR-24-3p) were upregulated in PPMS, and two of them were expressed differentially when compared to SPMS (miR-128-3p and miR-24-3p), suggesting an important role of these miRNAs in pathophysiology of PPMS.

One of the main findings of this study was the predominant expression of miR-128-3p and miR-24-3p in PPMS. In line with this observation, miR-128 has been previously found to be upregulated in naïve CD4+ T cells obtained from patients with RRMS, SPMS, and PPMS.[20] In addition, in the same study, the role of miR-128 in the suppression of Th2 cell differentiation and promotion of proinflammatory Th1 responses was demonstrated.[20] Based on these data, miR-128 seems to promote proinflammatory immune responses, but it has also been shown to have a role in the neuronal differentiation, proliferation, and apoptosis.[21,22] In fact, miR-128 is one of the most abundant brain-enriched miRNAs that is detected especially in the neurons.[21] Notably, it is shown to be deregulated in various other neurological conditions like Alzheimer's disease,[23] prion-induced neurodegeneration,[24] and autism.[25] Therefore, our observation on miR-128 correlation with the progression index in SPMS patients supports the earlier findings, indicating its role in neurodegenerative processes. In turn, miR-24-3p has not been previously identified in MS, and overall there is little information on its relations to any autoimmune diseases. A recent experimental study described that miR-24-3p inhibits interferon-γ (IFN-γ) expression through direct binding to its mRNA target sites.[26] Since IFN-γ is primary secreted from Th1 cells, upregulation of miR-24-3p in PPMS may indicate presence of regulatory responses in this subtype. Moreover, miR-24-3p had similar correlation patterns between the studied miRNAs in MS patients and controls suggesting its role to maintain immune system homeostasis. Interestingly, according to DianamiRPath database,[27] both miR-24-3p and miR-128-3p are involved in the regulation of p53 signaling pathway, ErbB signaling pathway, ubiquitin-mediated proteolysis, and T-cell receptor signaling pathway, that all contribute to MS pathology.[28–31]

Of all the detected miRNAs, miR-191-5p was the only one upregulated both in PPMS and in SPMS, suggesting its associations with both progressive forms of MS. The exact function of miR-191 is not known, but it was shown to play a role in cellular differentiation and development, as well as in innate immune responses.[32] In fact, in accordance with our finding, it has been proposed that the progressive phase of MS is mainly mediated by the innate rather

than adaptive immune responses.[33] MiR-191 is also reported to be downregulated in blood B lymphocytes of untreated RRMS patients when compared to both controls and RRMS patients treated with the natalizumab, suggesting its role in the B-cell-mediated immune responses.[19] Involvement of the humoral immune responses in progressive MS is demonstrated by the presence of B-cell follicle-like structures in meninges of progressive MS patients and their association with cortical neuronal loss and demyelination. In addition, miR-191-5p has been considered as a biomarker candidate for predicting the development of Alzheimer's disease.[34]

Another interesting finding was the upregulation of miR-376c-3p (previous ID miR-368) in PPMS in comparison with controls, with no differences between PPMS and SPMS. This miRNA has not been previously associated with MS or any other autoimmune and neurodegenerative disorders. Moreover, there is no information available on its involvement in the immune responses. Nevertheless, miR-376c is highly expressed in neurons and is important for the neuronal differentiation.[35] It has been shown to suppress transforming growth factor-β (TGF-β) signaling,[36] which in turn plays a critical role in the pathogenesis of MS by regulating Th17 and Treg responses.[37,38] In fact, in concordance with our finding, reduced levels of TGF-β were observed in sera of patients with MS.[39]

We performed a comprehensive study of circulating miRNAs in progressive forms of MS and found that their aberrant expressions were associated mostly with PPMS subtype, suggesting their involvement in immunopathogenesis of PPMS. In addition, results from the correlation analyses among the validated miRNAs further support the important role of these miRNAs in PPMS, since the correlations were most notable in the PPMS subtype. However, this PPMS emphasis may be explained by the fact that the screening phase included only PPMS patients. Moreover, in order to maximize the validation cohort size, 29% samples overlapped between screening and validation cohorts that might have impact on the validated results. Therefore, additional analyses were performed excluding all the overlapping samples. Analysis confirmed the upregulation of miR-191-5p in SPMS and PPMS as well as upregulation of miR-128-3p in PPMS. However, the expression pattern of miR-24-3p and miR-376c-3p was not replicated, which may be explained by smaller number of patients in the independent cohort. Notably, in contrast with previous findings, miR-26a-5p turned to be significantly upregulated in both PPMS and SPMS, suggesting its potential as predictor of progressive forms of MS, although this needs to be confirmed in the additional studies.

Most of the detected miRNAs were not previously associated with progressive forms of MS and, in some cases, with other MS subtypes. This was expected, considering the low number of available publication. In addition, there is a variability between circulating miRNAs reported, due to differences in patient cohorts, as well as in miRNA study methodologies and statistical approaches, emphasizing an importance of developing consensus protocols for miRNA research as well as a high need for additional studies in all MS subtypes.

In summary, aim of this study was to identify the circulating miRNAs specific to progressive forms of MS and explore their association to neurological worsening. We were able to show the specific circulating miRNA profiles in progressive MS that were not previously linked to MS. The increased expression of circulating miR-191-5p seems to be associated with progressive forms of MS, while miR-128-3p seems to be associated mostly with PPMS. It seems that these miRNAs are involved in the immunopathogenesis of progressive forms of MS and represent interesting and promising candidate biomarkers. In order to validate their specificity for progressive forms of MS, additional studies on large MS cohort and other neurodegenerative disorders are needed.

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Declaration of Conflicting Interests

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PUBLICATION II

Evaluation of serum miR-191-5p, miR-24-3p, miR-128-3p, and miR-376c-3p in multiple sclerosis patients

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ORIGINAL ARTICLE

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Evaluation of serum miR-191-5p, miR-24-3p, miR-128-3p, and miR-376c-3 in multiple sclerosis patients

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Background: Biomarkers that could be used in early diagnosis of multiple sclerosis (MS), segregation of disease subtypes, and discrimination of the aggressive disease course from the benign one are urgently needed.

Objective: The aim of this study was to investigate the specificity of circulating microRNAs: miR-191-5p, miR-128-3p, miR-24-3p, and miR-376c-3p in MS and evaluate their association with disease activity and disability progression.

Methods: The expressions of circulating miRNAs were studied in serum of 100 subjects (53 relapsing-remitting (RRMS), 20 primary progressive (PPMS), and 27 controls), using miScript serum miRNA RT-PCR assay techniques.

Results: In comparison with controls, miR-191-5p and miR-24-3p were overexpressed in RRMS and PPMS, with no differences between the subtypes. miR-24-3p correlated positively with the disability progression index in the combined group of all patients with MS. miR-128-3p showed tendency toward the predominant expression in PPMS and correlated positively with the annual relapse rate in RRMS. miR-376c-3p expression levels did not differ between the groups, and no associations were found to clinical parameters.

Conclusion: This study highlighted the connection of circulating miRNAs to MS. miR-24-3p and miR-128-3p showed a tendency of association with disability accumulation and disease activity, respectively. Further studies should evaluate their suitability for clinical use.

KEYWORDS
biomarkers, circulating microRNA, miR-128-3p, miR-191-5p, miR-24-3p, multiple sclerosis, primary progressive multiple sclerosis, relapsing-remitting multiple sclerosis

1 | INTRODUCTION

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system (CNS) where inflammatory and neurodegenerative events are the key characteristics, although progressive and relapsing subtypes show some differences in underlying immunological mechanisms.[1] Inflammation and focal disruption of the blood-brain barrier (BBB) lie behind the demyelination and neuronal loss in relapsing-remitting MS (RRMS), while in the progressive forms, neurodegeneration is mediated most likely without marked peripheral inflammation, and the role of CNS inflammation has been recognized also in PPMS. Disease-modifying treatments (DMT) decrease inflammation in active RRMS,[2] and this effect has now been observed in ocrelizumab-treated active RRMS and PPMS patients resulting in decrease in disability progression in both subtypes.[3,4]

Blood-derived biomarkers that are able to detect disease activity in MS and segregate the disease subtypes may prove useful in personalized MS medicine, as blood collections are less invasive than collection of cerebrospinal fluid (CSF). Complexity in treatment decision making, due to heterogeneous pathology and clinical course of disease, creates the ultimate need for biomarkers that could enable early diagnosis and discriminate the aggressive disease course from

the benign one. However, despite the large numbers of candidate molecular biomarkers proposed in MS, very few have been successfully validated and used in clinical practice.[5]

Circulating microRNAs (miRNA) have great biomarker potential, due to their exceptional stability in body fluids and relative ease of collection and quantification. miRNAs play critical role in the variety of biological processes by regulating gene expression at the post-transcriptional level through mRNA degradation or inhibition. Because of their key role in the immune system regulation, such as development and differentiation of T and B cells, proliferation of monocytes and neutrophils, antibody switching, and the expression of cytokines and chemokines, miRNAs are associated with several autoimmune diseases including MS.[6,7] It can be hypothesized that in clinical perspective, circulating miRNAs can help in early disease detection and disease activity monitoring, even before the severe clinical symptoms.

Although miRNAs are marginally new era in MS research, there are some significant discoveries published.[7-11] Active and chronic lesions seen in MRI were shown to have distinct miRNA profiles in MS.[7] Moreover, aberrant miRNA expression levels were detected in peripheral blood mononuclear cells, as well as in T and B lymphocytes.[7] Furthermore, deviant expression levels of circulating miRNAs associated with the MS subtypes were reported in CSF[7,8] and in blood.[9-11] For example, miR-660, miR-939, and miR-145 were found to be aberrantly expressed in plasma of RRMS patients,[11] while let-7c, let-7d, miR-92a-1*, miR-135a, miR-145, miR-454, miR-500, and miR-574-3p were differentially expressed in plasma of patients with secondary progressive MS (SPMS) in comparison with RRMS.[9] In addition, miR-15b, miR-23a, and miR-223 were downregulated in serum of both RRMS and primary progressive MS (PPMS) patients, with no prominent differences between the MS subtypes.[10]

This study is based on our previous study on circulating miRNAs in progressive subtypes of MS,[12] where overexpression of miR-128-3p, miR-24-3p, miR-191-5p, and miR-376c-3p was detected. Thus, the aim of this study was to evaluate the specificity of these miRNAs to RRMS and PPMS subtypes and explore their association with disease progression.

2 | MATERIALS AND METHODS

2.1 | Ethics statement

Study was approved by the Ethics committee of Pirkanmaa Hospital District and clinical investigation followed the principles of Helsinki Declaration. Written informed consent was given by all the participants.

2.2 | Patients and sample collection

Study included 100 subjects of whom 73 were patients with MS (53 RRMS and 20 PPMS) and 27 were healthy controls (HC). Demographic and clinical characteristics of the patients with different MS subtypes and HC are shown in Table 1. Diagnosis of MS was based on the revised McDonald criteria,[13] and all the diagnoses were definite. Active DMT treatment was ongoing in 37 (71%) patients with RRMS.

All the patients underwent clinical and neurological examination including the assessment of neurological disability expressed by expanded disability status scale (EDSS) score.[14] Progression index was calculated by dividing EDSS score by disease duration. An average annual relapse rate (ARR) was calculated in RRMS group starting from the time of diagnosis. In addition, the number of relapses during the last 2 years prior to the study entry was assessed. Prior to sampling, none of the patients had any relapses for at least 8 weeks. The mean ARR was 1.7 ± 3.4 (range: 0-20), and mean number of relapses during the last 2 years was 0.9 ± 1.1 (range: 0-4).

Healthy controls had no history of any autoimmune diseases or use of any immunomodulatory therapy. Patients and controls were age-matched and sex-matched.

Venous blood was collected in Becton Dickinson (BD) Vacutainer SST II advance tubes (Becton Dickinson, US). Sera were separated by centrifugation at 1600 × *g* for 15 minutes at room temperature and stored at −80°C until further use.

2.3 | microRNA extraction and reverse transcription

Circulating miRNA was isolated from 200 μL serum using a Qiagen miRNeasy Serum/Plasma kit (Qiagen Inc., Valencia, CA) following

TABLE 1 Demographic and clinical characteristics of patients with different MS subtypes and healthy controls

	All MS[a] (n = 73)	RRMS (n = 53)	PPMS (n = 20)	HC (n = 27)
Gender (Females)	51 (70%)	41 (77%)	10 (50%)	18 (67%)
Age[b]	40.0 ± 10.7 (22.4-65.0)	35.3 ± 7.1 (22.4-49.8)	52.7 ± 8.0 (40.0-65.0)	38.2 ± 11.8 (22.0-65.0)
Time since first symptoms; y[b]	8.3 ± 6.2 (0.4-30.8)	7.0 ± 5.1 (0.4-20.6)	12.3 ± 7.3 (3.1-30.8)	–
Disease duration from diagnosis; y[b]	6.0 ± 5.3 (0-25.8)	5.4 ± 5.4 (0.0-18.1)	7.6 ± 6.5 (0.1-25.8)	–
EDSS[b]	2.6 ± 2.4 (0.0-8.0)	1.6 ± 1.7 (0.0-6.5)	5.4 ± 1.8 (1.5-8.0)	–
Progression index[b,c]	2.5 ± 6.0 (0-28.7)	2.1 ± 5.0 (0.0-27.5)	3.8 ± 8.2 (0.3-28.7)	–

MS, multiple sclerosis; RRMS, relapsing-remitting MS; PPMS, primary progressive MS; HC, healthy controls; EDSS, expanded disability status scale.
[a]All MS patients (RRMS and PPMS).
[b]Mean ± SD (min- max).
[c]Progression index (EDSS/disease duration from diagnosis (y)).

TABLE 2 Circulating miRNA expression levels in RRMS, PPMS, and HC, analysed with the logistic regression model adjusted for sex and age

	miRNA expression			All MS[a] to HC		RRMS to HC		PPMS to HC		RRMS to PPMS	
MicroRNA	RRMS (n = 53)[b]	PPMS (n = 20)[b]	HC (n = 27)[b]	FC	*P*	FC	*P*	FC	*P*	FC	*P*
miR-128-3p	4.89 (0.25-52.22)	4.42 (0.42-29.11)	2.57 (0.14-23.37)	1.81	.02	1.90	.07	1.72	.03	1.11	.67
miR-191-5p	2.68 (0.19-13.15)	2.83 (0.52-7.21)	1.62 (0.22-8.79)	1.76	**.002**	1.65	**.01**	1.75	**<.001**	0.95	.88
miR-24-3p	7.14 (0.08-72.34)	12.17 (0.21-89.26)	3.40 (0.05-27.60)	2.84	**.002**	2.10	**.01**	3.58	**.01**	0.59	.79
miR-376c-3p	3.56 (0.13-33.90)	2.76 (0.32-10.98)	2.64 (0.17-13.90)	1.20	.42	1.35	.42	1.05	.58	1.29	.62

MS, multiple sclerosis; RRMS, relapsing-remitting MS; PPMS, primary progressive MS; HC, healthy controls; FC, fold change.
P -values of .01 or less, marked in bold, considered as statistically significant (Bonferroni correction, n = 4).
[a]All MS patients (RRMS and PPMS).
[b]Mean (min-max).

the manufacturer's protocol. Spike-In control cel-miR-39 (Qiagen Inc.) was used to monitor RNA recovery and reverse transcription efficiency. Extracted and purified miRNA was eluted into 14 μL of RNase-free water and stored at −80°C until use.

Isolated miRNAs were converted to cDNA using a miScript reverse transcription kit (Qiagen Inc.) according to the manufacturer's protocols. Prepared cDNA was stored at −20°C until use.[12]

2.4 | microRNA expression analysis

Circulating miRNA expression was analysed using miScript Human Serum & Plasma miRNA PCR assays (Qiagen Inc), following the SYBR green-based real-time polymerase chain reaction method (RT-PCR), on the ABI 7900HT PCR machine (Applied Biosystems, Foster City, USA). As primers, we used hs-miR-24-1, hs-miR-191-1, hs-miR-368-1, and hs-miR-128-1 miScript primer assays. Prior to the RT-PCR, all the cDNA samples were diluted by adding 200 μL of RNase-free water. Cel-miR-39-3p, SNORD68, and RNU6-2 were used as controls. All the samples were run as triplicates to control for the intra-assay variation (CV = 1.5%). To control for the inter-assay variation, we used a control sample which was included to each plate and run against miR-191-5p (CV = 1.5%) and miR-39-3p (CV = 1.1%).

The relative expression levels were calculated using the comparative Ct method (ΔΔCt) with mean values of HC set as a calibrator. For normalization, miR-39-3p, SNORD68, and RNU6-2 were used as endogenous controls. Ct values higher than 35 were considered as undetermined and thus excluded from the analysis.

2.5 | Statistical analysis

Analysis was performed using SPSS version 22.0 (IBM Corporation, Armonk, NY, USA). Due to skewed distribution, natural logarithm transformation was performed for the miRNA expression data to obtain normal distribution. Differences in miRNAs expression between groups were analysed using linear regression model using sex and age as covariates. To study association between miRNAs and EDSS, ARR and PI, Pearson's correlation analysis and linear regression using age and sex as covariates were used. Bonferroni correction (four comparisons) was used to reduce the chances of obtaining false-positive results, and thus, *P*-values of .01 or smaller were considered as statistically significant. During correlation tests, *P* -values less than .05 were considered statistically significant.

3 | RESULTS

3.1 | Circulating miRNA expression in RRMS, PPMS, and controls

Circulating miRNA expression levels were determined in sera samples from 73 patients with MS and 27 HC. Two miRNAs: miR-24-3p and miR-191-5p were expressed in all the samples, while miR-128-3p and miR-376c-3p were expressed in 95% and 87% of the samples, respectively.

Comparison between MS and controls revealed overexpression of miR-128-3p, miR-191-5p, and miR-24-3p (fold change, FC > 1.5; $P < .05$) in sera of patients with MS (RRMS and PPMS) (Table 2, Figure 1), although after the Bonferroni correction, only miR-191-5p and miR-24-3p reached statistical significance ($P \leq .01$). miR-376c-3p expression levels did not differ between the groups.

Analysis of the miRNA expressions between the groups showed overexpression of miR-191-5p (RRMS: $P = .01$, FC = 1.65; PPMS: $P < .001$, FC = 1.75) and miR-24-3p (RRMS: $P = .01$, FC = 2.10; PPMS $P = .01$, FC = 3.58) in both subtypes and miR-128-3p in PPMS ($P = .03$, FC = 1.72), in comparison with HC (Table 2, Figure 1). However, after the Bonferroni correction, miR-128-3p did not reach statistical significance. No statistically significant differences were detected between RRMS and PPMS.

In addition, comparison between untreated and treated MS patients was performed, but statistical differences between miRNA expression levels were not detected.

The expression of miRNAs was further analysed based on the sexes. In the male population, miR-128-3p and miR-191-5p were overexpressed in RRMS ($P = .007$, FC = 11.33 and $P = .009$, FC = 4.68), PPMS ($P = .023$, FC = 3.56 and $P = .022$, FC = 4.01), and among all the patients with MS ($P = .003$, FC = 7.49 and $P = .004$, FC = 4.40; Table 3) in comparison with HC. In addition, miR-128-3p was overexpressed in

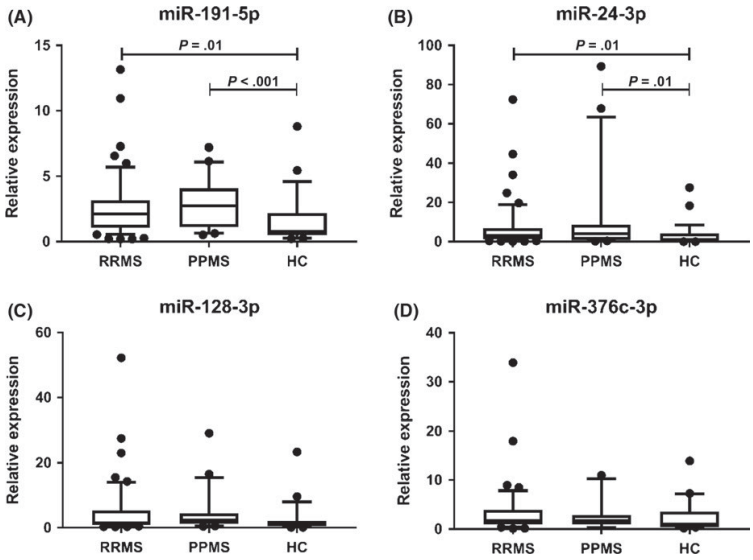

FIGURE 1 Box and whiskers plot of the distribution of miR-191-5p (A), miR-24-3p (B), miR-128-3p (C), and miR-376c-3p (D) in serum obtained from relapsing-remitting (RRMS) and primary progressive (PPMS) patients and healthy controls (HC). Box indicates median with interquartile range (25-75 percentiles) and whiskers indicate the 10-90 percentile range. Dots represent independent values that are of the 10-90 percentile range. *P* -values of .01 (Bonferroni correction, n = 4) or lower were considered significant and are shown here

TABLE 3 Circulating miRNAs expression levels in female and male patients

		All MS[a] to HC		RRMS to HC		PPMS to HC		RRMS to PPMS	
miRNA	**Sex**	**FC**	***P***	**FC**	***P***	**FC**	***P***	**FC**	***P***
miR-128-3p	F	1.21	.63	1.09	.93	1.69	.34	0.64	.21
	M	7.48	**.003**	11.39	**.01**	3.58	.02	3.18	.04
miR-191-5p	F	1.24	.09	1.21	.25	1.38	.07	0.88	.16
	M	4.40	**.004**	4.70	**.01**	4.03	.02	1.16	.13
miR-24-3p	F	2.62	**.01**	1.78	.02	6.07	**.01**	0.29	.20
	M	2.19	.15	3.27	.18	0.89	.41	3.67	.10

MS, multiple sclerosis; RRMS, relapsing-remitting MS; PPMS, primary progressive MS; HC, healthy controls; FC, fold change.
P -values of .01 or less, marked in bold, considered as statistically significant (Bonferroni correction, n = 4).
[a]All MS patients (RRMS and PPMS).

RRMS in comparison with PPMS (*P* = .038, FC = 3.18). However, the results of PPMS to HC and PPMD to RRMS comparisons did not pass the Bonferroni correction. In turn, in female population, comparison between MS and HC showed miR-24-3p overexpression in RRMS (*P* = .020, FC = 1.78), PPMS (*P* = .011, FC = 6.06), and in group of all patients with MS (*P* = .007, FC = 2.62) (Table 3). Out of these, result for RRMS to HC comparison did not pass the Bonferroni correction.

The overexpression of miR-24-3p was detected (*P* = .038, FC = 8.17) in PPMS cohort, in male to female comparison, but it did not pass the Bonferroni correction.

3.2 | Associations of miRNAs relative expression levels with clinical parameters

To explore associations between circulating miRNAs expression levels and clinical parameters, Pearson's correlation test and linear regression model were used.

In the whole MS group, miR-24-3p showed positive correlation with the progression index (*r* = .264, *P* = .026), and after the adjustment for sex, disease subtype, and age, this correlation preserved (adjusted *r* = .343, adjusted *P* = .004). Respective correlations were not detected separately in RRMS and PPMS.

In the RRMS group, miR-128-3p showed positive correlation with ARR (*r* = .286, *P* = .049), and this result became stronger after the adjustment for sex and age (adjusted *r* = .358, adjusted *P* = .014).

In PPMS group (n = 20), miR-376*c*-3p tended to correlate positively with EDSS (*r* = .454, *P* = .051); however, this correlation did not preserve after the age and sex adjustment.

4 | DISCUSSION

Until date, studies on circulating miR-191 in blood of patients with MS are lacking. To bridge this scientific gap, in the present study,

we were able to show that miR-191 was overexpressed in RRMS and PPMS when compared to controls, without statistically significant differences between these subtypes. Notably, our previous study on circulating miRNAs in progressive subtypes of MS showed similar picture revealing upregulation of miR-191 both in PPMS and in SPMS without differences between them.[12] A recent CSF study has shown decreased levels of soluble miR-191 in treatment-naive MS patients especially in those positive for lipid-specific immunoglobulin M oligoclonal bands (LS-OCMB)[8] as compared to patients with other neurological diseases. The presence of LS-OCMB in CSF was found to be associated with poorer long-term outcome.[15] To our knowledge, no studies are available on comparison of miR-191 expression levels in blood and CSF of patients with MS. Therefore, these dichotomous results need to be clarified in the future studies. However, similar findings have been reported with the miR-184 and mir-127-3p in Alzheimer disease where their levels were downregulated in CSF but upregulated in blood in comparison with neurologically healthy subjects.[16] The exact function of miR-191 in immune systems is not clarified yet, but it has been shown to support cytokine-dependent naïve, memory, and regulatory T-cell survival and thereby to maintain immune homeostasis by controlling the levels of insulin receptor substrate 1 (IRS1).[17] In addition, decreased levels of miR-191 were found in B cells of the untreated RRMS patients when compared to the natalizumab-treated patients and healthy controls proposing its role in the B cell-mediated immune responses.[18] Moreover, miR-191 is found to be abundantly expressed in brain.[19] In MS, miR-191 levels have shown to be decreased in normal appearing white matter (NAWM) and its expression correlated negatively with brain-derived neurotrophic factor (BDNF) in addition to four other CNS-related genes.[20] Furthermore, another study showed that miR-191 directly suppress BDNF expression,[21] which, in turn, is known to promote neuronal regeneration and mediate neuronal plasticity.[22] Previous reports of low BDNF serum levels among RRMS patients compared to healthy subjects[23] are in agreement with our findings of miR-191 overexpression in patients with MS. Moreover, increased levels of miR-191 have been reported in serum of patients with traumatic brain injury.[24] In the same publication, it was hypothesized that this increase could be due to active or passive miRNA release by injured brain tissue. Therefore, increased levels of miR-191 in blood of patients with MS may reflect release of the miRNAs due to apoptosis of neuronal as well as immune cells. Taken together, our and other data suggest that miR-191 seems to be associated with disease processes common for all MS subtypes.

miR-128 is another brain-enriched miRNA that is detected especially in neurons, where it plays a role in the neuronal differentiation and regulates the proliferative and apoptotic events.[25] In our study, no differences were found in miR-128 expression levels between RRMS, PPMS, and HC. In our previous study, we have found it to be predominantly expressed in PPMS in comparison both with SPMS and HC.[12] The existing discrepancy between the results can be partly explained by the relatively small size of PPMS cohort. Therefore, the role of miR-128 needs to be clarified in future studies especially in PPMS. As far as we know, no other publications are available on circulating form of miR-128 in MS. On the cellular level, increased expression of miR-128 in RRMS was found in peripheral blood mononuclear cells[11] and in naïve CD4+ T cells obtained from patients with RRMS, SPMS, and PPMS.[26] In the same study, it was proposed that miR-128 together with miR-27 suppress the differentiation of anti-inflammatory Th2 cells in favor of pro-inflammatory Th1 responses, through direct inhibition of polycomb complex protein BMI1 and interleukin 4 (IL-4).[26] BMI-1, among other roles, regulates mitochondrial functions and production of reactive oxygen species,[27] which in turn seems to be associated with pathogenesis, development, and progression of MS.[28] Furthermore, another study reported that miR-128 exerts pro-apoptotic function via p53-dependent and independent manner by upregulating p53, FOXO3A, and PUMA, and downregulating SIRT1 and IGFBP5.[29] Therefore, detected correlation of miR-128 with ARR in RRMS supports its role in the inflammatory responses.

Another interesting finding in this study was the overexpression of miR-24-3p in both patients with RRMS and PPMS, with no differences between the subtypes. Notably, in our earlier study, we found it to be predominantly expressed in PPMS both in comparison with HC and to SPMS. Very little is currently known on miR-24 association with autoimmune or neurodegenerative events. However, it seems to promote pro-apoptotic effects,[30] also by targeting BIM and PUMA. Importantly, miR-24 inhibits interferon (IFN)-γ[31] and IL-4[32] expressions suggesting its role in the regulation of Th1/Th2 balance, thereby regulating inflammatory responses. In addition, our observation of the positive correlation between miR-24-3p and progression index within the whole MS group supports its connection to the neurodegenerative events and disease progression. Furthermore, miR-24 was also shown to regulate aldosterone and cortisol biosynthesis through repression of *CYP11B1* and *CYP11B2* mRNA functions.[33] Aldosterone plays a role in inflammatory processes, promotes autoimmune damage,[34] and regulates VEGF-A production in human neutrophils,[35] which is a potent inducer of BBB disruptions.[36]

Noteworthy, gender-specific analysis showed an overexpression of miR-128-3p and miR-191-5p in male RRMS patients and overexpression of miR-24-3p in female PPMS patients. Although subgroups consist of marginally low number of patients, our data showed the gender-specific differences in miRNA expression in MS that may be related to disease development and progression.

To conclude, miR-24-3p seems to be associated with disability accumulation, while miR-128-3p were associated with disease activity. Moreover, we have found miR-191-5p and miR-24-3p overexpressed in serum of patients with RRMS and PPMS, when compared to controls, without differences between the subtypes. Considering this overlap, they cannot distinguish MS subtypes from each over. Notably, no differences in miR-128-3p and miR-376c-3p expression levels were detected. However, in our previous study, all four miRNAs were overexpressed in PPMS when compared to controls. The discrepancy between the results can be partly explained by the small

size of PPMS cohort. We also observed significant but marginally weak correlations of miR-24-3p with the disability accumulation and miR-128-3p with the annual relapse rate. These promising findings should be further clarified in future studies.

All four miRNAs are aberrantly expressed in cancer and various other conditions. For example, altered expression levels of miR-24-3p were also found in major depression.[37] miR-128 was shown to be deregulated in Alzheimer's disease[38] and prion-induced neurodegeneration.[39] miR-191 as part of seven miRNAs signature expression profile showed high Alzheimer's disease biomarker potential,[40] while miR-376c-3p was proposed as potential marker for active tuberculosis.[41] Thus, considering the fact that each of these miRNAs can be associated with wide variety of physiological and pathological processes, their potential as independent disease-specific biomarkers is low, and thus, further studies on miRNA combinations and development of specific panels for different subtypes of MS are important.[42] In addition, on individual level, miR-191-5p, miR-24-3p, miR-128-3p, and miR-376c-3p showed high inconsistency, ranging from very low to high expression levels, even among healthy individuals. Unfortunately, the biological variability in miRNA levels is yet poorly studied. There are examples of changes in expression levels of several miRNAs in response to exercise, or during different phases of menstrual cycle and between pre- and post-menopausal status,[43] thus emphasizing a high need of such studies as well as development of standardized collection procedures and validation of methodology.

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CONFLICT OF INTEREST

The authors report no conflict of interests.

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PUBLICATION III

Temporal variability of serum miR-191, miR-223, miR-128, and miR-24 in multiple sclerosis: A 4-year follow-up study

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