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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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ABSTRACT

Background: Circulating microRNAs (miRNA) are suggested to be a promising biomarker for multiple sclerosis (MS). Previously, miR-128-3p, miR-24-3p, miR-191-5p and miR-223-3p have been reported to associate with MS pathology. However, their longitudinal changes and association with the disease activity have not been studied.
Objectives: To evaluate the serum temporal variability of miR-128-3p, miR-191-5p, miR-24-3p, and miR-223-3p and their association with disability and disease activity in MS.
Methods: The expression of four miRNAs in serum was studied in 57 MS patients, 18 clinically isolated syndrome patients, and 32 healthy controls over the four-year follow-up.
Results: At the baseline, miR-191-5p was overexpressed in RRMS in comparison to controls, and its levels correlated positively with EDSS and progression index (PI) in RRMS. Increased levels of miR-128-3p were detected in PPMS in comparison to controls, and increased levels correlated with EDSS and PI in RRMS. The expression of miR-24-3p and miR-223-3p did not differ between the subtypes, but miR-223-3p correlated negatively with T1 lesions volumes in SPMS and PPMS. Over the four-years follow-up period, the expression of miR-128-3p and miR-24-3p was stable longitudinally, while temporal changes of miR-191-5p and miR-223-3p were observed in MS. Temporal changes in miR-191-5p were observed to be associated with an increase of EDSS or MRI activity, while the variability of miR-223-3p was associated with relapses.
Conclusion: Temporal variability of miR-191-5p and miR-223-3p are associated with changes in disability accumulation and disease activity. While, miR-128-3p was stably expressed and associated with the PPMS subtype and correlated with disability accumulation.

1. Introduction

Multiple Sclerosis (MS) is an immune-mediated demyelinating disease of the central nervous system, characterized by inflammation and axonal degeneration [1]. Since different immunomodulatory and immunosuppressive treatment options are available for the active disease course, biomarkers that could assess disease course, activity, or progression and evaluate the efficacy of treatment response are urgently needed. There are also no specific biomarkers indicating the ongoing pathogenetic mechanisms in MS. Serum and plasma are easily accessible sample types, while cerebrospinal fluid collection is limited by invasiveness, emphasizing an intensive need for blood-based biomarkers.

MicroRNAs (miRNAs) are a group of small non-coding RNA molecules that regulate gene expression at the post-transcriptional level through mRNA degradation or inhibition of translation into proteins [2]. In addition to intracellular expression, they can be found in extracellular space in most biofluids. These extracellular miRNAs, called circulating miRNAs, are packed in the microparticles, such as exosomes, or bound to RNA-binding proteins or lipoprotein complexes. They are resistant to RNase degradation and are exceptionally stable after exposure to

Abbreviations: MS, Multiple sclerosis; miRNA, MicroRNA; RRMS, Relapsing-remitting multiple sclerosis; SPMS, Secondary progressive multiple sclerosis; PPMS, Primary progressive multiple sclerosis; CIS, Clinically isolated syndrome; HC, Healthy controls; EDSS, Expanded disability status scale; ARR, Annualized relapse rate; BL, Baseline; PI, Progression index; FC, Fold change.

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multiple freeze-thaw cycles, pH changes, and extended storage [3,4,5]. Aberrant expression of circulating miRNAs has been implicated in numerous diseases, including MS [6]. Considering the findings, together with the relative ease of detection and established methodology, circulating miRNAs show strong biomarker potential. However, despite extensive studies on MS, their temporal variability is poorly addressed. Two studies have analysed the longitudinal variability of serum microRNAs with partially controversial results [7,8]. Among them, one study reported stable expression levels of serum miRNAs during over 17 months of follow-up time [7], while another showed fluctuations in 12 of the total 217 miRNAs over 48 h follow-up period [8]. To our knowledge, no data is available on temporal miRNAs expression pattern in MS, nor how it is associated with MS clinical course, activity, or progression.

This study aimed to evaluate the temporal variability of serum circulating miR-128-3p, miR-24-3p, miR-191-5p, and miR-223-3p over the four-year follow-up period and associate their levels with magnetic resonance imagining (MRI) changes and clinical disease activity in MS. These miRNAs have been previously associated with MS. We have heretofore shown overexpression of miR-191-5p in all the MS subtypes. While overexpression of miR-128-3p and miR-24-3p were associated with primary progressive MS (PPMS) and miR-24-3p with relapsing-remitting MS (RRMS) [9,10]. In turn, miR-223-3p was selected for the study as it is one of the most reported miRNAs associated with MS [11,12,13,14,15,16,17].

2. Materials and methods

2.1. Patients, clinical follow-up, and sample collection

The retrospective study was performed with the prospectively collected data. The subjects were recruited and diagnosed in the neurological department at Pirkanmaa Hospital District in Tampere University Hospital, Finland. Inclusion criteria were a definite MS diagnosis based on the revised McDonald criteria [18] or a clinically isolated syndrome (CIS) [19].

A total of 107 subjects were included, consisting of 32 healthy controls (HC), 18 CIS, and 57 MS cases: 28 RRMS, 14 secondary progressive (SPMS), and 15 PPMS (Table 1). 16 RRMS patients and 1 SPMS patient were under IFN-β treatment, no other immunomodulatory therapies were used. HCs were age- and sex-matched and had no history of autoimmune diseases or use of any immunomodulatory therapy.

This four-year follow-up study included three visits: baseline (BL) and visits after two (year-2) and four years (year-4). Each visit included a clinical and neurological examination, blood sample collections, and disability assessment using an expanded disability status scale (EDSS) score [20]. MRI was performed at BL and year-2. HC blood samples were collected only at BL. A total of nine patients (1 RRMS, 2 SPMS, 3 PPMS, and 3CIS) dropped out before the end of the follow-up. The study was performed between 2006 and 2012.

Disease activity at the baseline (Table 1) and at the end of follow-up period (Supplementary Table 1) was defined by the number of clinical relapses during the two and four preceding years, respectively. Also, a baseline annualized relapse rate (ARR) was calculated by dividing the total number of relapses by disease duration in years, starting from the time of diagnosis. The baseline progression index was calculated by EDSS divided by disease duration from diagnosis.

During the follow-up, MS cases were categorized into active or stable groups based on disease activity after enrolment, defined by disability accumulation in EDSS (EDSS worsening vs. not worsening), relapses (relapse active vs. stable), or activity observed in MRI (MRI active vs. stable). Patients were categorized into the EDSS worsening group if EDSS increased by 1.0 or more for baseline EDSS <6.0, or by the increase of 0.5 for EDSS 6.0 or more. The rest of the patients were included in the not worsening group [21] The relapse active group consisted of patients who experienced at least one relapse during the follow-up. The MRI active group included 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 cm^3; FLAIR>6 cm^3) were chosen based on the distribution of changes in lesion volumes among the MS patients. The study was approved by the Ethics committee of Pirkanmaa Hospital District (R05157) and the clinical investigation followed according to the principles of the Helsinki Declaration. Written informed consent was obtained from all the participants.

2.2. Magnetic resonance imaging (MRI) image segmentation and volumetric analysis

MRI was performed using a 1.5 Tesla MRI Unit (Siemens Avanto, Erlangen, Germany). The MRI protocol included a T1-weighted header followed by axial 3D T1-weighted magnetization prepared rapid gradient echo (MP-RAGE) and T2-weighted turbo spin-echo (TSE), fluid-attenuated inversion recovery (FLAIR), T1-weighted spin echo with magnetization transfer contrast, diffusion-weighted imaging (DWI), and

Table 1
Baseline demographic and clinical characteristic of MS patients, clinically isolated syndrome, and healthy controls.

	All MS ($n = 57$)	RRMS ($n = 28$)	SPMS ($n = 14$)	PPMS ($n = 15$)	CIS ($n = 18$)	HC ($n = 32$)
Sex (females)[a]	37 (64.9%)	19 (67.9%)	9 (64.3%)	9 (60.0%)	16 (84.2%)	20 (62.5%)
Age[b]	45.9 ± 12.0 (18.1-68.7)	38.1 ± 9.0 (18.1–49.8)	49.0 ± 8.8 (35.4–61.0)	57.5 ± 8.5 (38.8–68.7)	36.1 ± 7.4 (23.5–50.5)	42.2 ± 12.2 (21.0–65.0)
Timefrom the symptom onset (years)[b]	13.1 ± 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 (years)[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 (0.0-7.0)	1.5 ± 1.3 (0.0–6.0)	4.7 ± 1.7 (2.0–7.0)	4.7 ± 1.9 (1.0–7.0)	0.1 ± 0.3 (0.0–1.0)	–
Progression index[b,c]	0.9 ± 1.3 (0.0-7.2)	0.8 ± 1.1 (0.0–3.6)	0.9 ± 1.0 (0.2–3.0)	1.0 ± 1.8 (0.1–7.2)	–	–
Relapses [a,d]						
0	30 (53%)	5 (18%)	10 (71%)	15 (100%)	4 (22%)	–
1	14 (25%)	11 (39%)	3 (21%)	0 (0%)	11 (61%)	–
2–6	13 (23%)	12 (43%)	1 (7%)	0 (0%)	3 (17%)	–
IFN-β treatment [a]	17 (30%)	16 (57%)	1 (7%)	0 (0%)	0 (0%)	–

RRMS: relapsing-remitting MS, SPMS: secondary progressive MS, PPMS: primary progressive MS, CIS: clinically isolated syndrome, EDSS: expanded disability status scale, IFN: interferon.

[a] Number of patients (percent).

[b] Mean ± SD (range).

[c] From diagnosis.

[d] Number of relapses in 2 years before baseline.

gadolinium enhanced T1-weighted MP-RAGE sequences. T1-weighted MP-RAGE, FLAIR and T2-weighted TSE images were used for volumetric analysis. For MP-RAGE, the imaging parameters were as follows: repetition time (TR) = 1160 ms; echo time (TE) = 4.2 ms; inversion time (TI) = 600 ms; slice thickness = 0.9 mm, interslice gap = 0; in-plane resolution = 0.45*0.45 mm, matrix 256*256. In FLAIR images, the following parameters were used: TR = 8500 ms; TE = 100 ms; TI = 2500 ms; slice thickness = 5.0 mm, interslice gap = 0; in-plane resolution = 0.45*0.45 mm, matrix 256*256. For T2-weighted TSE, the following imaging parameters were used: TR = 750 ms; TE = 115 ms; slice thickness = 3.0 mm; in-plane resolution = 0.90*0.90 mm. Volumetric segmentation of plaques in the brain was performed using semi-automatic software Anatomatic operating in a PC/Window 95 environment and the images were analysed in a blinded fashion [22].

2.3. Blood collection, miRNAs extraction, and reverse transcription

Venous blood was collected in Vacutainer SSTII advance tubes (Becton Dickinson, US). Serum was separated by centrifugation at 1600 ×g for 15 min at room temperature and stored at −80 °C until further use.

Circulating miRNAs were isolated from 200 μl serum using a Qiagen miRNeasy Serum/Plasma kit, eluted into 14 μl of RNase-free water and then 4.5 μl of isolated product was converted to cDNA with a miScript reverse transcription kit (Qiagen Inc., Valencia, CA) as described previously (Vistabakka et al., 2018). Cel-miR-39 (Qiagen Inc) was used as a spike-in control to monitor RNA recovery and reverse transcription efficiency. Extracted and purified miRNAs were stored at −80 °C and cDNA at −20 °C until use [9,10].

2.4. MicroRNA expression analysis

Circulating miRNAs expression was analysed using the miScript SYBR Green PCR kit (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). Prior to the RT-PCR, a 20 μl complementary DNA (cDNA) samples were diluted by adding 200 μl of RNase-free water.

The miScript primer assays for hs-miR-191-1, hs-miR-128-1, hs-miR-24-1 and hs-miR-223-1 were used for target genes, SNORD68, and RNU6–2 were used as endogenous controls and cel-miR-39-3p was used as spike-in exogenous control. The 10-μl reaction mixture included 5 μl 2× QuantiTect SYBR Green PCR Master Mix, 1 μl10× miScript universal primer, 1 μl 10× miScript primer assay, 1 μl diluted cDNA and 2 μl RNase-free water.

All the samples were run as triplicates and intra-assay and inter-assay variability percent (CV %) was calculated. Intra-assay CV% was calculated for each assays (miR-191-5p CV = 1.7%, miR-128-3p CV = 1.9%, miR-24-3p CV = 1.5%, miR-223 CV = 1.6%) with the formula (100xSD/mean) for each triplicate samples and then taking the average of the each values. In turn, to calculate inter-assay CV%, the additional control sample was included in each plates and run against miR-191-5p (CV = 1.2%) and miR-39-3p (CV = 1.4%). In this case the intra-CV% was calculated with formula (100xSD/mean) where SD and mean for miR-191-5p and miR-39-3p was obtained all the analysed plates. Ct values higher than 37 were considered as undetermined and thus excluded from the analysis.

The relative expression levels were analysed using the comparative Ct method (ΔΔCt). In this method, the Ct values are normalized to *endo-* (SNORD68 and RNU6–2) and exogenous controls (cel-miR-39-3p) and then compared to controls as described previously [9]. Cel-miR-39-3p was included in the normalization because it reflects technical variability during the miRNAs processing steps.

Fold changes (FC) were calculated to show differences in miRNAs expression levels between MS subtypes, CIS, and HCs and to visualize differences in miRNAs expression levels between the visits.

2.5. Statistical analysis

Statistical analyses were performed using SPSS version 22.0 (IBM corporation, Armonk, NY, USA). Due to skewed distribution and relatively small group sizes non-parametric statistical tests were used. The Mann-Whitney *U* test was used to evaluate the differences between the groups in miRNAs expression and MRI volumes. To study the association between miRNAs levels and clinical and MRI measures, the Spearman's correlation analysis was used. For the follow-up data, the Friedman test was used to assess changes in miRNAs expression levels from BL to year-4. The Wilcoxon signed-rank test was used to evaluate differences between BL and year-2 as well as between year-2 and year-4. The Bonferroni correction was used to reduce the chances of obtaining false-positive results and p-values <0.05 were considered statistically significant.

3. Results

3.1. Disease activity and disability accumulation during the follow-up

Four years follow-up study included 57 MS cases and 18 CIS patients with the average follow-up time (±SD) 4.4 ± 0.3 years. Clinical disability accumulation was evaluated with the EDSS increase over the four-year follow-up, which was observed in 24/57 (42%) MS patients over the first two years (BL to year-2) and 24/51 (47%) MS patients who participated in all three visits (supplementary table 1). Clinical disease activity was evaluated with number of relapses. Over the follow-up, clinical relapses were observed in 19/57 (33%) MS patients. More specifically, in RRMS 16/28 (57%) and in SPMS 3/14 (21%) patients had at least one new relapse (Supplementary table 1).

Paraclinical disease activity and progression were evaluated by changes in the volumes of T1 and FLAIR lesions over two years (supplementary table 2). From BL to year-2 increase in T1 and FLAIR lesion volumes was observed in all MS patients and CIS (Wilcoxon-signed rank test $p < 0.01$). In RRMS, changes in T1 volumes correlated with FLAIR volume changes ($r = 0.422$, $p = 0.032$) and with EDSS changes ($r = 0.414$, $p = 0.036$).

3.2. CIS: Clinical activity, conversion, and disability accumulation

In CIS, time from the first demyelinating event suggestive of MS to BL ranged from 0.5 to 8.9 years (mean(±SD) 2.5 ± 2.6 years). EDSS at BL ranged from 0 ($n = 16$) to 1 ($n = 2$) (Table 1). Conversion to RRMS during the follow-up was observed in 10/18 cases. Among them, 5 converted during the first two years. EDSS increased in three patients (two converted to RRMS), and four patients experienced a relapse (three converted to RRMS) (Supplementary table 1). No statistically significant changes in T1 and FLAIR lesion volumes were seen among the converted CIS patients (Wilcoxon-signed rank test $p > 0.05$, $n = 10$), while among the non-converted changes in T1 (Wilcoxon-signed rank test $p = 0.015$) and FLAIR (Wilcoxon-signed rank test $p = 0.001$) volumes were observed (Supplementary table 2).

3.3. Baseline expression of circulating miRNAs in MS and CIS

Circulating miRNAs expression levels were determined in serum of 57 MS patients (28 RRMS, 14 SPMS, 15 PPMS), 18 CIS patients, and 32 HCs (Fig. 1). Comparison between all MS, including RRMS, PPMS and SPMS, and HCs showed an overexpression of miR-191-5p (FC = 2.63, corrected $p = 0.02$) and miR-128-3p (FC = 2.57, corrected $p = 0.03$). Separately in MS subtypes, an overexpression of miR-191-5p was observed in RRMS (FC = 3.52, corrected p = 0.03) and miR-128-3p in PPMS (FC = 2.72, corrected p = 0.02) as compared to HCs. No differences were observed between CIS and any of MS subtypes ($p > 0.05$).

Circulating miRNAs expression levels did not differ between IFN-β-treated and untreated patients. Likewise, no differences were seen

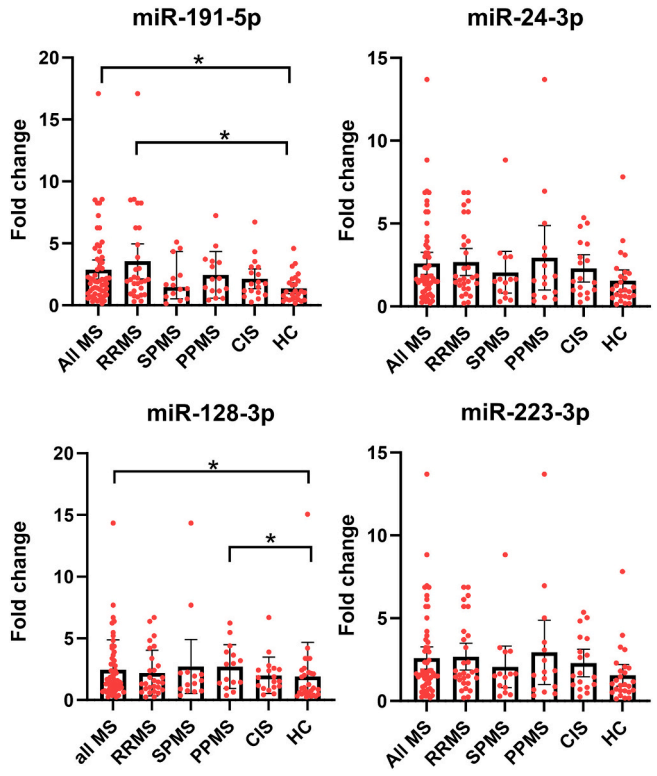

Fig. 1. Baseline levels of A) miR-191-5p B) miR-24-3p C) miR-128-3p and miR-223-3p in All MS, MS subtypes, CIS and HC. Bars indicate median with 95% confidence interval and red dot indicates the individual value of each patient. Black lines indicate the statistical significance of the Mann-Whitney *U* test after Bonferroni correction. *$p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

between females and males (p > 0.05).

3.4. Baseline association of circulating miRNAs relative expression levels with clinical course and MRI parameters

To explore the associations between the BL expression levels of miR-191-5p, miR-128-3p, miR-24-3p and miR-223-3p, and the clinical and MRI parameters, such as EDSS, progression index, ARR, disease duration and volumes of T1 and FLAIR lesions, correlation analyses were performed (Table 2 and Supplementary table 3). Due to the skewed data distribution and relatively small group sizes, non-parametric Spearman's correlation analysis was used, as regression analysis adjusted for age and gender could not be performed.

Expression levels of miR-191-5p positively correlated with EDSS in RRMS ($r = 0.425$, $p = 0.027$, $n = 27$) and progression index in all MS ($r = 0.301$, p = 0.027, $n = 54$) and RRMS ($r = 0.507$, $p = 0.010$, $n = 25$). In turn, miR-128-3p correlated positively with EDSS ($p = 0.407$, $p = 0.035$, $n = 27$) in RRMS and with progression index both in all MS ($r = 0.271$, $p = 0.048$, $n = 54$) and RRMS ($r = 0.538$, $p = 0.006$, $n = 25$). Relapses seem not to have an influence on any of the studied miRNAs expression levels at BL, as no correlation with ARR were seen in RRMS and SPMS groups. No correlations with clinical parameters were detected for miR-24-3p.

As for the MRI, miR-223-3p negatively correlated with T1 volumes in all MS ($r = -0.342$, $p = 0.022$, $n = 45$), SPMS ($r = -0.569$, $p = 0.034$, $n = 14$) and PPMS ($r = -0.636$, p = 0.035, $n = 11$).

Correlation with age were detected for miR-191-5p in all MS ($r = 0.326$, $p = 0.014$, $n = 56$) and separately in RRMS ($r = 0.481$, $p = 0.011$, $n = 28$) and SPMS ($r = 0.662$, $p = 0.010$, n = 14) and for miR-128-3p in all MS ($r = 0.271$, $p = 0,048$, $n = 54$) and RRMS ($r = 0.508$, $p = 0.007$, $n = 27$). In addition, miR-191-5p correlated with disease duration ($r = 0.637$, $p = 0.014$, $n = 13$) in SPMS.

Table 2
Correlations between the clinical and MRI measures and miRNAs expression levels at baseline. Only statistically significant values are shown here.

		r*	p
Clinical measures		**miR-191-5p**	
Progression index	All MS (n = 54)	0.301	0.027
Progression index	RRMS (n = 25)	0.507	0.010
EDSS	RRMS (n = 27)	0.425	0.027
		miR-128-3p	
Progression index	All MS (n = 54)	0.271	0.048
Progression index	RRMS (n = 25)	0.538	0.006
EDSS	RRMS (n = 27)	0.407	0.035
MRI measures		**miR-223-3p**	
T1 volumes	All MS ($n = 45$)	−0.342	0.022
T1 volumes	SPMS (n = 14)	−0.569	0.034
T1 volumes	PPMS (n = 11)	−0.636	0.035

MS: multiple sclerosis, RRMS: relapsing-remitting MS, PPMS: primary-progressive MS, SPMS: secondary progressive MS, EDSS: expanded disability status scale, p-values of 0.05 or less are considered statistically significant. * Spearman's coefficient.

3.5. Correlation analysis between miR-191-5p, miR-24-3p, miR-128-3p, and miR-223-3p

Correlation analysis was performed between the selected circulating miRNAs at BL (Table 3). Interestingly, correlations were observed between all four miRNAs in RRMS and HCs. While, in the SPMS, miR-128-3p and miR-191-5p correlated with miR-24-3p and miR-223-3p, but not with each other. In turn, amon the patients with PPMS, miR-128-3p correlated with miR-24-3p and miR-223-3p, also miR-191-5p correlated with miR-24-3p.

3.6. Temporal changes in circulating miRNAs expression levels over the follow-up period in MS

The levels of circulating microRNAs were also analysed at the year-2 and year-4 in MS patients, but their levels did not differ between the subtypes. Then the temporal variability was analysed by the Friedman test, which showed miR-191-5p (p = 0.028) and miR-223-3p (p < 0.001) expression levels to vary (Fig. 2). In turn, miR-128-3p and miR-24-3p were stable ($p > 0.05$). The subtype analysis showed temporal variability in miR-223-3p in RRMS (p = 0.006), PPMS (p = 0.010), and SPMS ($p = 0.013$), while all other miRNAs were stable. Since miR-128-3p and miR-24-3p showed no change through the follow-up they were excluded from the analysis.

Changes in the expression levels of miR-191-5p and miR-223-3p between the visits were analysed separately using Wilcoxon non-parametric test (Fig. 2). The expression levels of miR-191-5p decreased on year-2 compared to BL ($p = 0.002$), but the levels did not differ between year-2 and year-4 or BL and year-4 ($p > 0.05$) in all MS group. Similar observations were done in RRMS (BL vs. year-2 $p = 0.023$; year-2 vs. year-4 p > 0.05; BL vs. year-4 p > 0.05) and PPMS (BL vs. year-2 $p = 0.031$; year-2 vs. year-4 p > 0.05; BL vs. year-4 p > 0.05). In SPMS, the expression levels of miR-191-5p increased on year-4 compared to year-2 (year-2 vs. year-4 $p = 0.016$), with no differences between other visits (BL vs. year-2 p > 0.05; BL vs. year-4 p > 0.05).

Temporal variation of miR-223-3p was observed on year-2 compared to BL and year-4 compared to year-2 in all MS (BL vs. year-2 $p < 0.001$; year-2 vs. year-4 p < 0.001), RRMS (BL vs. year-2 $p = 0.001$; year-2 vs. year-4 $p = 0.011$), SPMS (BL vs. year-2 $p = 0.041$; year-2 vs. year-4, $p = 0.003$) and PPMS (BL vs. year-2 p = 0.003; year-2 vs. year-4 $p = 0.05$), but no differences were noticed between BL and year-4 in any of the groups p > 0.05).

Table 3
Correlations between the circulating miRNAs at baseline.

	RRMS (n = 27)		SPMS (n = 14)		PPMS (*n* = 15)		CIS (*n* = 18)		HC (*n* = 26)	
	r	p	r	p	r	p	r	p	r	p
miR-191-5p to miR-24-3p	**0.64**	**<0.001**	**0.85**	**<0.001**	**0.74**	**0.002**	0.27	>0.05	**0.68**	**<0.001**
miR-191-5p to miR-128-3p	**0.79**	**<0.001**	**0.81**	**<0.001**	0.45	>0.05	**0.73**	**<0.001**	**0.70**	**<0.001**
miR-191-5p to miR-223-3p	**0.72**	**<0.001**	0.50	>0.05	0.42	>0.05	**0.65**	**0.006**	**0.81**	**<0.001**
miR-128-3p to miR-24-3p	**0.65**	**<0.001**	**0.87**	**<0.001**	**0.53**	**0.04**	0.39	>0.05	**0.54**	**0.004**
miR-128-3p to miR-223-3p	**0.70**	**<0.001**	**0.82**	**<0.001**	**0.82**	**0.002**	0.41	>0.05	**0.61**	**0.002**
miR-24-3p to miR-223-3p	**0.56**	**0.007**	0.61	0.02	0.45	>0.05	0.02	>0.05	**0.66**	**<0.001**

RRMS: relapsing-remitting MS, PPMS: primary-progressive MS, SPMS: secondary progressive MS, CIS: clinically isolated MS, HC: healthy controls, r: Spearman's coefficient, *p*-values of 0.05 or less, marked bold, considered as statistically significant.

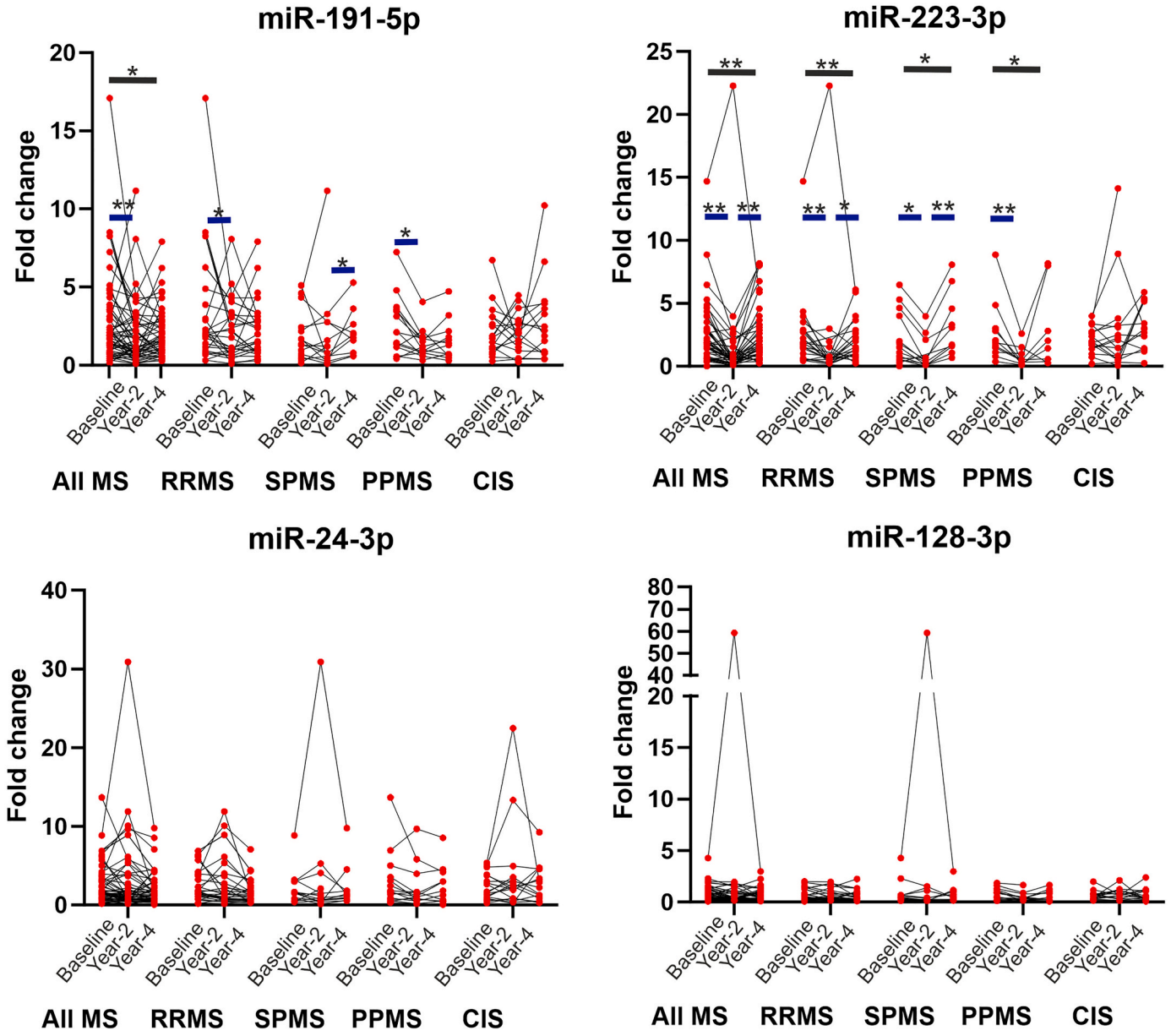

Fig. 2. Circulating miRNAs expression levels in MS and CIS during the 4-year follow-up. Each red dot connected with black lines indicates the individual values of each patient. Black lines with asterisk indicate the statistical significance of the Friedman test over the whole follow-up period and blue lines with asterisk indicate the statistical significance of the Wilcoxon signed-rank test between the two visits.** $p < 0.01$, *$p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.7. Circulating miRNAs expression levels in CIS over the 4-years follow-up

No statistically significant changes were detected in miRNA expression levels in CIS at the baseline and year-4. At year-2, increased levels of miR-223-3p were observed in CIS when compared to RRMS (FC: 3.7 (CIS) vs. 2.91 (RRMS), $p = 0.008$).

Temporal variability was also analysed with Friedman test in the groups of CIS patients, converted, and not converted to RRMS, no statistically significant temporal fluctuations were observed among all CIS patients, as well as in the separate groups.

3.8. Association of circulating miRNAs with clinical and MRI changes over the 4-year follow-up

The aim was to assess the association of temporal miRNAs expression changes with clinical disease activity (relapses and EDSS) and MRI changes (Table 4). Due to a small group size SPMS and PPMS patients were excluded from the analysis.

First patients were categorized into EDSS worsening and not worsening groups according to clinical disability accumulation over the follow-up. Temporal changes in miR-191-5p were observed in the EDSS worsening group ($FC_{BL\text{-}year2} = 0.50$, $p = 0.004$; $FC_{year2\text{-}year4} = 1.93$, $p = 0.028$: $FC_{BL\text{-}year4} = 0.97$, $p > 0.05$), but not the EDSS not worsening group ($FC_{BL\text{-}year2} = 0.85$, $FC_{year2\text{-}year4} = 1.03$, $FC_{BL\text{-}year4} = 0.87$, all $p > 0.05$). Similar observation was detected in RRMS (worsening: $FC_{BL\text{-}year2} = 0.54$, $p = 0.005$; $FC_{year2\text{-}year4} = 1.32$, $p > 0.05$; $FC_{BL\text{-}year4} = 0.72$, $p > 0.05$; not worsening: $FC_{BL\text{-}year2} = 0.74$, $FC_{year2\text{-}year4} = 0.88$, $FC_{BL\text{-}year4} = 0.84$, all $p > 0.05$). The expression levels of miR-223-3p fluctuated in both EDSS worsening ($FC_{BL\text{-}year2} = 0.38$, $p = 0.003$; $FC_{year2\text{-}year4} = 4.90$, $p = 0.012$; $FC_{BL\text{-}year4} = 1.86$, $p > 0.05$) and not worsening ($FC_{BL\text{-}year2} = 0.31$, $p < 0.001$; $FC_{year2\text{-}year4} = 3.22$, $p < 0.001$; $FC_{BL\text{-}year4} = 0.98$, $p > 0.05$) groups of MS patients.

Next RRMS patients were categorized into active and stable groups according to the number of relapses throughout the follow-up. No statistically significant longitudinal changes in miR-191-5p expression levels were observed both among the stable and active RRMS patients. In turn, temporal variations in miR-223-3p expression levels were detected among the active RRMS patients ($FC_{BL\text{-}year2} = 0.40$, $p = 0.008$; $FC_{year2\text{-}year4} = 2.63$, $p = 0.017$; $FC_{BL\text{-}year4} = 1.05$, $p > 0.05$), but not the stable.

Based on changes in T1 or FLAIR volumes over the first two years of the study, MS patients were categorized into MRI-active and stable groups. Statistically significant changes in miR-191-5p were observed among the T1 active ($FC_{BL\text{-}year2} = 0.57$, $p = 0.001$) and FLAIR active ($FC_{BL\text{-}year2} = 0.52$, $p < 0.001$) MS patients, but not the stable (Table 4). Similarly, in the RRMS group, the temporal fluctuation was observed among the active FLAIR patients ($FC_{BL\text{-}year2} = 0.50$, $p = 0.012$), but not the stable. No changes were seen in T1 active nor stable RRMS patients. In turn, miR-223-3p changes were observed both among the active (T1: $FC_{BL\text{-}year2} = 0.33$, $p < 0.001$; FLAIR: $FC_{BL\text{-}year2} = 0.33$, $p < 0.001$) and the stable (T1: $FC_{BL\text{-}year2} = 0.28$, $p = 0.002$; FLAIR: $FC_{BL\text{-}year2} = 0.28$, $p = 0.001$) MS patients, as well as among the active (T1: $FC_{BL\text{-}year2} = 0.32$, $p = 0.01$; FLAIR: $FC_{BL\text{-}year2} = 0.30$, $p = 0.01$;) and the stable (T1: $FC_{BL\text{-}year2} = 0.50$, $p = 0.02$; FLAIR: $FC_{BL\text{-}year2} = 0.50$, $p = 0.03$) RRMS patients.

4. Discussion

Over the last decades, the interest in circulating miRNAs as easily accessible biomarkers for MS has increased, but there are little data available on their temporal behavior. Previously, we have shown an overexpression of circulating miR-128-3p, miR-191-5p, and miR-24-3p in serum of MS patients [9,10], while others have reported decreased levels of miR-223-3p in serum of RRMS [16] and PPMS [11] patients. In this study, the temporal expression of circulating miR-223-3p, miR-128-3p, mi191-5p, and miR-24-3p was assessed in all MS subtypes as well as in CIS over the four-year follow-up period and their levels were associated with temporal variability of clinical and MRI parameters. In addition, correlations were assessed between miRNAs expression levels and the clinical parameters, and MRI findings.

MiR-191-5p, an abundantly expressed miRNA in the brain [23], seems to play an important role in the regulation of T- and B-cell mediated immune responses [24,25]. Its deregulated expression patterns were found in various neurological conditions, such as Alzheimer's disease [26], traumatic brain injury [27], mild cognitive impairment [28], and MS [13,29,25,9,10]. We have previously reported its overexpression in serum of patients with RRMS [10], PPMS [9,10], and SPMS [9] as compared to HCs. In turn, downregulation of cellular miR-191-5p was reported in normal-appearing white matter [13] and B cells [25] of MS patients suggesting its role in the pathogenesis of MS. In the present study, higher expression of miR-191-5p was observed among the MS patients and separately in RRMS, as compared to HCs, with no differences detected in comparison to other disease subtypes, nor CIS. Interestingly, temporal changes in miR-191-5p expression levels over four years were associated with the changes in EDSS score and the growth of T1 and FLAIR lesion volumes in MS and separately in RRMS. Importantly, patients in the EDSS worsening, T1 and FLAIR active groups showed decreased miR-191-5p expression levels in year-2 compared to BL. Also when studing the clinical parameters, most significant changes were seen in the year-2 suggesting miR-191-5p important role in the neurodegenerative processes of MS.

Notably, CIS patients with stable EDSS had a stable temporal expression of miR-191 that further supports its role in the disease progression. Moreover, BL miR-191-5p correlated positively with the EDSS score and progression index. All the findings together suggest miR-191-5p to be associated with disease worsening and progression, possibly through its role in the mediation of immune response. Besides, it can be considered as a possible indicator of ongoing brain damage as it was previously suggested in the traumatic brain injury study [30,31,32].

An immunoregulatory miRNA miR-223 has anti-inflammatory properties in immune cell functions since it seems to reduce inflammatory responses in neutrophils and dendritic cells and promotes the polarization of anti-inflammatory M2 macrophage phenotype [30,31,32]. Notably, among the RRMS patients, it was upregulated in CD14+ monocytes, as compared to HCs, as well as in tissue-regenerating M2-polarized human macrophages and microglia [30]. In addition, as demonstrated on experimental autoimmune encephalomyelitis (EAE) models, miR-223 protects neurons from degeneration [32] and contributes to activation of myeloid cells, CNS remyelination, and debris clearance via phagocytosis [30]. Deregulation of miR-223-3p has been reported in several other MS studies (Keller et al., 2009; De Santis et al., 2010; [11,16]; Hosseini et al., 2016; [33]). Although, it is not specific for MS, as it was found to be aberrantly expressed in osteoarthritis, lupus, hepatitis, tuberculosis, and various types of cancer [3,34]. In the present study, no differences were observed in its expression levels between all MS subtypes, HCs and CIS. In contrast to our findings, decreased serum levels of miR-223-3p in RRMS [16] and PPMS [11] and its positive correlation with EDSS in PPMS [11] were reported. Exosomal miR-223 was found to be downregulated in serum of patients with progressive MS as compared to HCs and was able to discriminate progressive MS from RRMS together with eight other miRNAs [33]. While the expression levels of miR-223-3p did not differ between the subtypes, we found miR-223 to significantly variate over the four-years follow-up period in all the subtypes studied. These variations seem not to be associated with clinical disability accumulation indicated by EDSS, nor with changes in MRI volumes, as the fluctuations were observed in both stable and active groups. The only factor that can possibly contribute to these longitudinal changes is relapse activity measured through the follow-up in RRMS. Though, no correlations were observed between miR-223-3p and ARR on BL. Our current findings of temporal changes in RRMS, but not in CIS, together with a possible contribution of relapse activity, suggest miR-223-3p association with disease pathology, possibly through the regulation of inflammatory responses.

Table 4
Association of circulating miRNAs relative expression levels with clinical parameters and MRI measures over the course of the four-year follow-up, among the patients with active and stable disease course.

		Fold change			p-value*			Fold change			*p*-value*		
		BL-Year2	Year2-Year4	BL-Year4	BL-Year2	Year2-Year4	BL-Year4	BL-Year2	Year2-Year4	BL-Year4	BL-Year2	Year2-Year4	BL-Year4
EDSS worsening (all MS: *n* = 24, RRMS: *n* = 8)								**EDSS not worsening (all MS: *n* = 33; RRMS: *n* = 20)**					
miR-191	All MS	0.50 (0.09–2.74)	1.93 (0.39–8.31)	0.97 (0.13–3.71)	**0.004**	**0.03**	>0.05	0.85 (0.19–8.79)	1.03 (0.28–15.96)	0.87 (0.09–3.66)	>0.05	>0.05	>0.05
	RRMS	0.54 (0.10–2.72)	1.32 (0.39–8.31)	0.72 (0.13–1.61)	**0.005**	>0.05	>0.05	0.74 (0.19–3.74)	0.88 (0.35–4.62)	0.84 (0.09–3.66)	>0.05	>0.05	>0.05
miR-223	All MS	0.38 (0.11–1.52)	4.90 (0.26–12.53)	1.86 (0.34–5.71)	**0.003**	**0.012**	>0.05	0.31 (0.04–1.99)	3.22 (0.16–20.83)	0.98 (0.13–5.71)	**<0.001**	**<0.001**	>0.05
	RRMS	0.41 (0.11–1.52)	na	na	**0.03**	na	na	0.53 (0.07–1.99)	1.82 (0.16–8.41)	0.96 (0.13–3.64)	**0.01**	**0.02**	>0.05
Relapse active (all MS *n* = 19:, RRMS: n = 16)								**Relapse stable (all MS: *n* = 38; RRMS: *n* = 12)**					
miR-191	All MS	0.57 (0.09–2.95)	1.68 (0.70–15.96)	0.95 (0.13–2.17)	**0.04**	>0.05	>0.05	0.75 (0.19–8.79)	1.18 (0.28–5.90)	0.88 (0.09–3.71)	**0.01**	>0.05	>0.05
	RRMS	0.68 (0.10–2.95)	1.23 (0.70–8.31)	0.84 (0.13–1.61)	>0.05	>0.05	>0.05	0.70 (0.19–3.74)	0.88 (0.35–2.92)	0.61 (0.09–3.66)	>0.05	>0.05	>0.05
miR-223	All MS	0.35 (0.07–1.22)	5.48 (1.53–6.19)	1.93 (0.34–2.28)	**0.04**	**0.01**	>0.05	0.34 (0.04–1.99)	3.13 (0.16–20.83)	1.08 (0.13–3.64)	**<0.001**	**<0.001**	>0.05
	RRMS	0.40 (0.07–1.22)	2.63 (1.53–6.19)	1.05 (0.34–2.14)	**0.01**	**0.02**	>0.05	0.61 (0.14–1.99)	1.85 (0.16–8.41)	1.11 (0.13–3.64)	>0.05	>0.05	>0.05
T1 active (all MS: *n* = 37; RRMS: n = 16)								**T1 stable (all MS: *n* = 17; RRMS: n = 10)**					
miR-191	All MS	0.57 (0.09–3.74)	na	na	**0.001**	na	na	0.83 (0.19–8.79)	na	na	>0.05	na	na
	RRMS	0.57 (0.10–3.74)	na	na	>0.05	na	na	0.61 (0.19–2.95)	na	na	>0.05	na	na
miR-223	All MS	0.33 (0.04–1.99)	na	na	**<0.001**	na	na	0.28 (0.07–1.12)	na	na	**0.002**	na	na
	RRMS	0.32 (0.11–1.99)	na	na	**0.01**	na	na	0.50 (0.07–1.12)	na	na	**0.02**	na	na
FLAIR active (all MS: *n* = 36; RRMS: n = 17)								**FLAIR stable (all MS: n = 19; RRMS: *n* = 9)**					
miR-191	All MS	0.52 (0.09–3.74)	na	na	**<0.001**	na	na	0.99 (0.30–8.79)	na	na	>0.05	na	na
	RRMS	0.50 (0.10–3.74)	na	na	**0.012**	na	na	0.94 (0.30–2.37)	na	na	>0.05	na	na
miR-223	All MS	0.33 (0.04–1.99)	na	na	**<0.001**	na	na	0.28 (0.11–1.12)	na	na	**0.001**	na	na
	RRMS	0.30 (0.07–1.99)	na	na	**0.01**	na	na	0.50 (0.14–1.12)	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 to the table for a better illustration of the differences in miRNAs expression levels between the visits. FC values were calculated by comparing ΔΔCt values between specific timepoints: **FC(BL-Year2)** = 2^-(ΔCt(Year2)-ΔCt(BL), **FC (Year2-Year4)** = 2^-(ΔCt(Year4)-ΔCt(Year2), and **FC(BL-Year4)** = 2^-(ΔCt(Year4)-ΔCt(BL). *P*-values of 0.05 or less, marked bold, considered as statistically significant.

* Wilcoxon signed-rank test.

Stable expression profiles over the follow-up period were observed for miR-24-3p and miR-128-3p in all MS subtypes and CIS. Expression levels of miR-24-3p were on the same level in all the studied groups and no associations were found to clinical or MRI parameters. While miR-128-3p was overexpressed in the combined MS group and separately in PPMS as compared to controls and correlated with EDSS and progression index in RRMS, suggesting its association with disease activity and progression of disability. These results are in line with our previous findings [9,10]. The reported findings, including the temporal stability of miR-128-3p, further emphasize its potential as a biomarker that could discriminate progressive MS from other subtypes.

The current study faced limitations, including a small sample size in the subtype analysis, heterogeneous study population and lack of validation cohorts, affecting the generalizability of the results.

The cohort consisted of 57 MS patients, 18 CIS, and 32 HCs that can be considered an acceptable cohort size, but they became marginally small in the subgroups analysis. However, it is noteworthy that this was the first study focused on the temporal nature of miRNAs expression patterns and associations of these changes with clinical parameters. Regarding the heterogenicity, 17 patients were under immunomodulatory treatment. Though, no effect of medication was detected in the current study. Others have earlier reported changed miRNA expression levels in treated patients [35]. Also, disease duration, EDSS score, and disease activity varied between the patients. In future studies, independent study cohorts with more homogenous populations need to be used to reveal the potential of selected miRNAs in clinical practise. Regarding methodology, the main limiting factor is the lack of established standardized protocols, including normalization strategies.

5. Conclusion

To conclude, the follow-up study showed that miR-191-5p and miR-223-3p have potential as biomarkers that can reflect temporal changes related to MS pathology, while miR-128-3p could be considered as a discriminative marker. However, these miRNAs are not specific for MS, as their aberrant expression levels were reported in other autoimmune and neurodegenerative conditions [34,26,36,37]. Thus, further studies, especially in combination with other miRNAs are needed.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declaration of Competing Interest

The authors report no conflict of interests.

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Appendix A. Supplementary data

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