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MicroRNAs are dysregulated in peripheral blood mononuclear cells in multiple sclerosis and correlate with T cell mediators

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ABSTRACT

T cell mediators and microRNAs are involved in the pathogenesis of multiple sclerosis (MS), but their interaction largely remains undetermined. We investigated by RT-qPCR the dysregulation of microRNAs in peripheral blood mononuclear cells of MS patients versus healthy controls, according to radiological disease activity or treatment. Several microRNAs correlated positively/negatively with *IL21/FOXP3* mRNA expression, but not with serum neurofilament light chain levels. Cytokine expression is conceivably balanced by several regulators, whereas microRNAs possibly target upstream transcription factors rather than directly cytokine mRNAs. Functional studies are needed to investigate their interaction, notably for the predicted targeting of *FOXP3* by miR-34c-5p.

1. Introduction

Multiple sclerosis (MS), a chronic inflammatory disorder of the central nervous system (CNS), is characterised by acute demyelination, and concomitant neurodegeneration. Although the role of the immune system in its pathophysiology is indisputable, the elementary mechanisms and triggers underlying it are only partially elucidated (Dendrou et al., 2015).

Both pro-inflammatory T helper (Th) 1 cells induced by interleukin (IL)12, a heterodimer of IL12p35 and IL12p40, and Th17 cells, induced by IL23, a heterodimer of IL23p19 and IL12p40, play an important role in acute MS-related inflammatory disease activity (Hiltensperger and Korn, 2018). On the other hand, forkhead box protein (FOXP)3-expressing regulatory T cells (Treg) are functionally impaired in MS (Fletcher et al., 2009), leading to an imbalance of the immune system favouring pro-inflammatory processes.

MicroRNAs are small non-coding RNAs of 18 to 22 nucleotides. They are recognised players in immune cell fating by directly or indirectly inhibiting the expression of transcription factors or cytokines, or by being themselves directed by these proteins (Garavelli et al., 2018). Several microRNAs are dysregulated in MS, whether it be in brain lesions, cerebrospinal fluid (CSF), serum or plasma, peripheral blood mononuclear cells (PBMCs), or in specific cell subtypes. Some microRNAs are proposed as biomarkers predicting or confirming the response to a disease modifying treatment, as for interferon beta (IFNb) or glatiramer acetate (GA) (Huang et al., 2016; Martin and Illes, 2014). The role of microRNAs on the expression of cytokines, that are important in the acute neuroinflammatory events occurring in MS, is, however, largely unknown. We aimed to analyse the expression of microRNAs, of cytokines and transcription factors participating in Th and Treg cell activation and differentiation and of the markers of IFNb response in PBMCs of MS patients either treated (IFNb or GA) or untreated and according to disease activity on brain magnetic resonance imaging (MRI), compared to healthy controls (HC). In parallel, serum neurofilament light chain (sNfL) levels were measured. We then investigated the possible association between the expression of microRNAs and T cell mediators as well as sNfL levels in MS patients and HC.

2. Methods

2.1. Patients

Blood and serum samples of MS patients and HC were collected at the Cliniques universitaires Saint-Luc (Brussels, Belgium). PBMCs were

Abbreviations: AMS, active multiple sclerosis; GA, glatiramer acetate; HC, healthy controls; IMS, inactive multiple sclerosis; IFNb, interferon beta therapy; microRNA, microRNA; mRNA, messenger RNA; MS, multiple sclerosis; PBMCs, peripheral blood mononuclear cells; PCA, principal component analysis; sNfL, serum neurofilament light chain; Th, T helper cells; Treg, regulatory T cells.

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isolated by Ficoll-Paque PLUS (GE Healthcare) density gradient centrifugation within one hour and stored in Qiazol at −80 °C until RNA/microRNA extraction. MS diagnosis was revised with the 2017 McDonald criteria (Thompson et al., 2018).

Patients were enrolled during a regular outpatient visit at the neurology department, after signing informed consent (ethical approval 2007/31AOUT/222). They were classified according to disease activity based on the presence of gadolinium enhancing lesions on brain MRI (Lublin et al., 2014), performed concomitantly to blood sampling. None of the patients were clinically in relapse. Patients were either untreated or treated with IFNb or GA, and had no concomitant infection. The subjects' characteristics are summarised in Fig. 1.

2.2. mRNA and microRNA extraction, reverse transcription, preamplification, and real time quantitative polymerase chain reaction (RT-qPCR)

Messenger RNAs (mRNAs) and microRNAs were isolated separately from 11 to 13.10^6 PBMCs using the miRNeasy Mini Kit, following the manufacturer's protocol (Qiagen). Extracted mRNAs and microRNAs were stored at −80 °C until reverse transcription (RT). Total mRNA was quantified with Qubit and reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen). ABsolute qPCR SYBR Green Mix (ThermoScientific) was used for RT-qPCR. Primers are listed in Supplementary Table 1 (IL12p35, IL12p40, IL17A, IL21, IL22, IL23p19, FOXP3, TGFB1, IL10, IL27p28, MXA, JAK2). MicroRNAs were reverse transcribed using the miScript II RT Kit (Qiagen). For the complementary DNA preamplification and the RT-qPCRs we used the miScript PreAmp PCR Kit and the miScript SYBR® Green PCR Kit (Qiagen) respectively with the miScript Primer Assays (Qiagen, see Supplementary Table 1: miR-15a-3p, miR-20a-5p, miR-21-5p, miR-24-3p, miR-27a-3p, miR-27b-3p, miR-29c-3p, miR-33a-3p, miR-34a-5p, miR-34c-5p, miR-124-5p, miR-125b-5p, miR-126-3p, miR-145-5p, miR-146a-5p, miR-149-3p, miR-150-5p, miR-155-5p, miR-181c-5p, miR-214-3p, miR-297) following the manufacturer's recommendations. The microRNAs were selected based on their differential expression within the CSF, serum or PBMCs of relapsing-remitting MS patients in our previous study (Perdaens et al., 2020). All PCRs were performed on Rotor-Gene Q (Qiagen).

2.3. RT-qPCR analysis

All samples were run in duplicates and the specificity was verified with the melt curves. The mRNA transcripts were normalised to Abelson gene (ABL) and their relative expression was determined by the $2^{-\Delta\Delta Ct}$ method (with $\Delta\Delta Ct = \Delta Ct$ [target mRNA-ABL]$_{sample}$ − mean ΔCt [target mRNA-ABL]$_{HC}$) (Livak and Schmittgen, 2001). Cycle thresholds (Cts) of ABL were within a close range for all samples. The microRNA transcripts were normalised by the global mean method (ΔCt = Ct [target microRNA]$_{sample}$ − mean Cts [all target microRNAs]$_{sample}$) (D'haene et al., 2012). The fold change was calculated as $2^{-\Delta(\Delta)Ct}_{target,sample}$ / median of $2^{-\Delta(\Delta)Ct}_{target,HC}$.

2.4. In silico analysis

We searched in six databases whether the investigated microRNAs

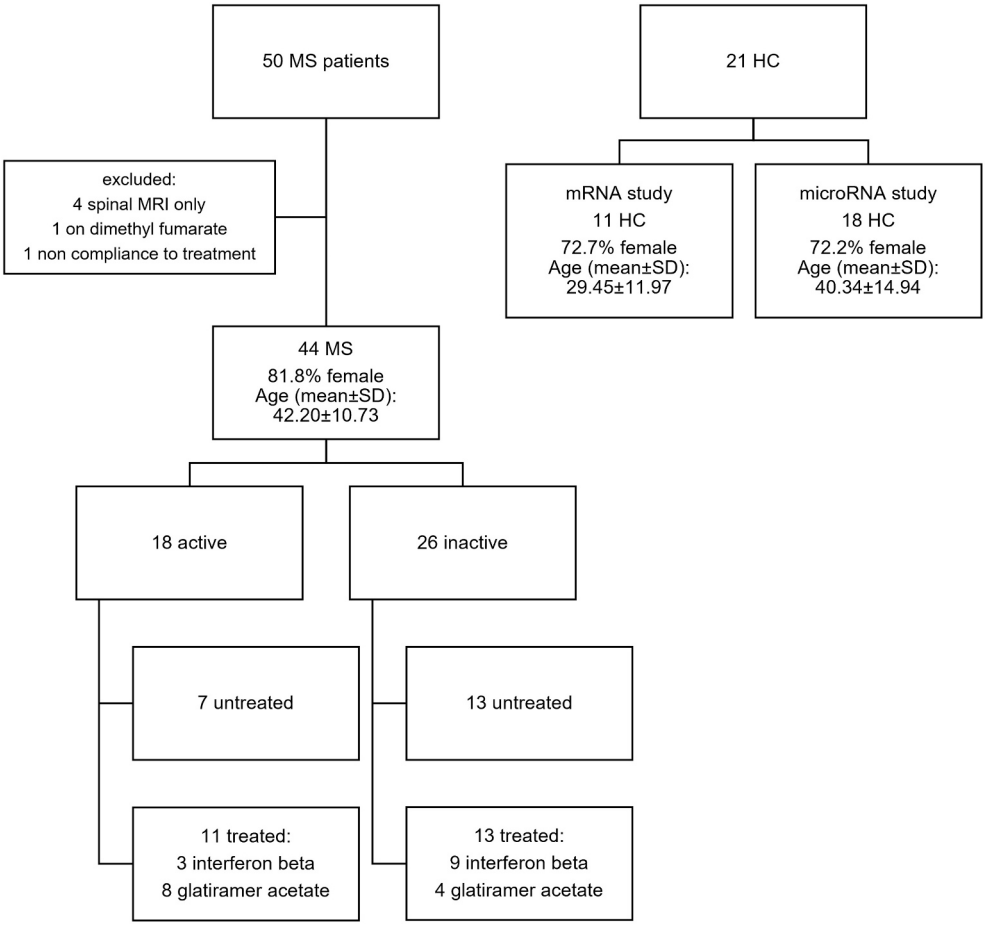

Fig. 1. Study population characteristics. This chart shows the subjects' characteristics (age and sex distribution) as well as the subdivision of MS patients according to disease activity (based on the presence of gadolinium enhancing lesions on brain MRI), and treatment. HC = healthy controls, MS = multiple sclerosis, SD = standard deviation.

were predicted to target the T cell mediator mRNAs of this study: miRNet (https://www.mirnet.ca/), miRDB (http://www.mirdb.org/index.html), RNA22 (https://cm.jefferson.edu/rna22/Precomputed), DIANA-microT-CDS (http://www.microrna.gr/microT-CDS), miRWalk 3 (http://mirwalk.umm.uni-heidelberg.de/), and miRMAP (https://mirmap.ezlab.org/app/) (Chang et al., 2020; Chen and Wang, 2020; Miranda et al., 2006; Paraskevopoulou et al., 2013; Sticht et al., 2018; Vejnar and Zdobnov, 2012).

2.5. Serum neurofilament light chain

sNfL levels were measured in duplicate using the highly sensitive immunoassay Single Molecule Array technology (NF-LIGHT™ (SR-X version), SIMOA®, Quanterix) following the manufacturer's protocol.

2.6. Statistical analysis

We used GraphPad Prism 5.0 for all statistical analyses on the relative mRNA ($2^{-\Delta\Delta Ct}$) and microRNA (ΔCt) expression levels: a nonparametric one-way analysis of variance (ANOVA) Kruskal-Wallis test, followed by a Dunn's multiple comparison post-test, across subgroups; a Mann-Whitney t-test (MW t-test) between MS and HC, as well as a nonparametric Spearman's correlation test. *P* values were corrected by the Benjamini-Hochberg false discovery rate (FDR) method (https://tools.carbocation.com/FDR) and considered significant if the adjusted *p*-value (adj.p) was $\leq$0.05 following correction. Principal component analysis (PCA) on the relative expression levels (-ΔCt) of microRNAs of MS patients and HC as continuous variables was performed in R using the *FactoMineR* package. Results were plotted using the *factoextra* package. Heatmaps, correlogram and linear regression were also built in R using the packages *pheatmap, corrplot, Hmisc* and *ggplot2* (https://cran.r-project.org/).

3. Results

3.1. FOXP3 and JAK2 mRNA are differentially expressed in PBMCs of MS patients

We first aimed to explore the differential mRNA expression of markers of T cell activity (cytokines and transcription factors) and of IFNb response in PBMCs of MS patients and HC. *FOXP3* was significantly upregulated in PBMCs of MS patients compared to HC, in particular in inactive MS (IMS) (Fig. 2A, Supplementary Fig. 1A, Supplementary Table 2), but independently of treatment status (data not shown). *JAK2* was significantly upregulated in MS patients, to the same extent in both active (AMS) and IMS.

The mRNAs of the interferon-induced antiviral protein, human myxovirus resistance protein 1 (*MXA),* as well as of *IL10* and *IL27p28* were significantly upregulated in IFNb-treated patients compared to untreated patients and/or HC (Fig. 2B, Supplementary Fig. 1B, Supplementary Table 2). *JAK2* was the only marker increased in both IFNb- and GA-treated patients as compared to HC. Conversely *IL12p40* was significantly upregulated in untreated patients versus (vs) HC (Fig. 2B). These markers were, however, not affected by disease activity (data not shown).

3.2. MicroRNAs are differentially expressed in PBMCs of MS patients

We quantified 21 microRNAs, previously found dysregulated in the CSF, serum or PBMCs of relapsing and/or remitting MS patients (Perdaens et al., 2020). Of these, miR-15a-3p/20a-5p/27a-3p/34c-5p/125b-5p/181c-5p/297 were significantly downregulated in MS vs HC, and miR-21-5p/29c-3p/34a-5p/146a-5p/150-5p/155-5p were upregulated (Fig. 3A). This differential expression was independent of disease activity for miR-21-5p/34c-5p/155-5p/181c-5p, while it was more strongly directed by IMS than AMS for the other microRNAs, except for miR-27a-3p (Supplementary Fig. 2A, Supplementary Table 3). Interestingly, up- and downregulated microRNAs consistently correlated with each other (Supplementary Fig. 2C). Treatment status did not affect microRNA expression, except for miR-29c-3p that was upregulated in untreated MS patients compared to treated MS patients and HC (Fig. 3B). Interestingly, when analysing the data according to treatment, miR-27a-3p was significantly downregulated for GA-treated patients vs HC, miR-34a-5p was significantly upregulated for IFNb-treated vs GA-treated patients and HC. Moreover, miR-150-5p and miR-297 were respectively up- and downregulated in untreated MS patients vs HC and tended to be similarly dysregulated in GA- and IFNb-treated patients respectively (Supplementary Fig. 2B, Supplementary Table 3).

In PCA on all significantly dysregulated microRNAs, the first two principal components explained 57.40% of the sample population's variance, while seperately, on the significantly up- and downregulated microRNAs, they represented 68.61% and 62.17% respectively (data not shown). Interestingly, for miR-21-5p, miR-155-5p and miR-181c-5p, the three most significantly dysregulated microRNAs, they accounted for 93.26% of the population's variance (Fig. 4A). However, they segregated only partially MS patients from HC (Fig. 4B).

3.3. MicroRNAs mainly correlated with IL21 and FOXP3

In a nonparametric correlation analysis between the mRNA ($2^{-\Delta\Delta Ct}$) and microRNA (-ΔCt) levels of expression in this study, significantly down- and upregulated microRNAs correlated, respectively, negatively and positively with the T cell mediators, independently of their pro- or anti-inflammatory character, while the opposite was noticed with *JAK2* (with regard to the significant correlations only) (Fig. 5A, Supplementary Fig. 3A). Furthermore, microRNAs with similar dysregulation and T cell mediators of similar nature were not strictly grouped together by the unsupervised hierarchical cluster analysis (Fig. 5A). We identified most correlations with *IL21* and *FOXP3* (Figs. 5A-B, Supplementary Fig. 3A-B-D). miR-21-5p and miR-155-5p corelated positively with both *IL21* and *FOXP3*; miR-146a-5p and miR-150-5p with *IL21* and miR-29c-3p and miR-34a-5p with *FOXP3*. On the contrary, miR-20a-3p, miR-34c-5p and miR-297 correlated negatively with *IL21* and *FOXP3*, and miR-24-3p and miR-181c-5p with *FOXP3* only. Interestingly, miR-150-5p/29c-3p and miR-20a-5p/24-3p were also negatively and positively correlated with *JAK2* respectively. Furthermore, miR-125b-5p correlated negatively with *IL10* and *IL27p28*. Of note, miR-27b-3p/33a-3p/126-3p/145-5p and miR-149-3p/297 were respectively positively and negatively correlated with *TGFB1*, but these microRNAs, except miR-297, were not significantly dysregulated in our MS population (Fig. 5A, Supplementary Fig. 3A-B-D).

All investigated microRNAs were predicted to target several T cell mediators of this study, although these predictions were mostly limited to one or two out of the six explored databases, except for miR-21-5p/*IL12p35*, miR-27a-3p/*IL12p40*, miR-27a-3p/*IL10*, miR-34a/c-5p/*FOXP3* (Supplementary Fig. 3C), However, this in silico analysis was overall variably consistent with the mRNA expression profile and the correlation analysis, except for miR-34c-5p/*FOXP3*. Moreover, it did not identify any pattern between the up- and downregulated microRNAs and the pro- and anti-inflammatory T cell mediators (Supplementary Fig. 3A-B-C).

3.4. Serum neurofilament light chain levels increase with radiological disease activity

AMS patients had significantly higher sNfL levels than MS patients with inactive disease or HC, independently of treatment status (Fig. 6). sNfL levels did not, however, correlate with any of the microRNAs and mRNAs of interest of this study.

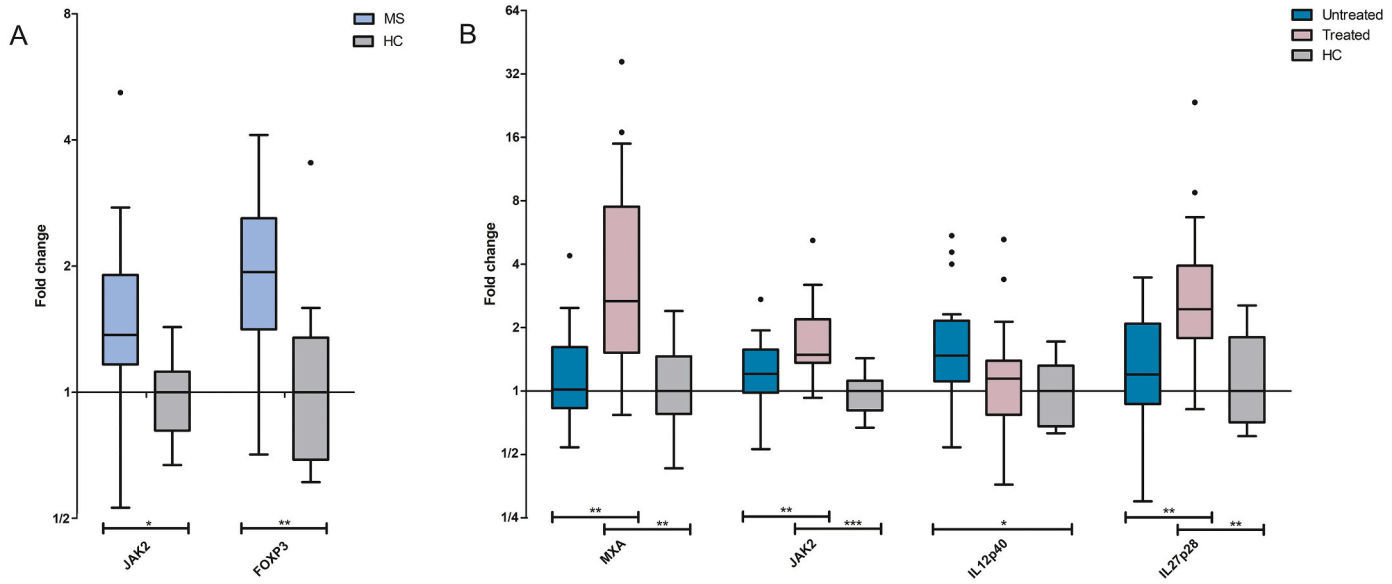

Fig. 2. Relative mRNA expression of T cell mediators and IFNb markers in PBMCs of MS patients and HC. The box plots, following the Tukey method, show the fold change ($2^{-\Delta\Delta Ct}_{target,sample}$ / median of $2^{-\Delta\Delta Ct}_{target,HC}$) between the relative expression of the significantly dysregulated mRNAs of interest of this study and their median relative expression in HC for (A) MS patients vs HC and (B) untreated and treated MS patients vs HC. The y-axis is log2-scaled. Statistical significance was determined by (A) a Mann-Whitney *t*-test and (B) a one-way ANOVA Kruskal-Wallis test followed by a Dunn's post-test in between the subgroups. The *p*-values of the t-test and the one-way ANOVA have been corrected by the Benjamini-Hochberg method. $p \leq 0.05$: *; $p \leq 0.01$: **; $p \leq 0.001$: ***. HC = healthy controls, MS = multiple sclerosis.

4. Discussion

We investigated the expression of microRNAs and of markers of T cell activation (cytokines and transcription factors), and of IFNb response in PBMCs of treated (IFNb or GA) and untreated MS patients, according to their disease activity, based on the presence/absence of gadolinium enhancing lesions on brain MRI, compared to HC.

We confirmed the upregulation of interferon-induced proteins *MXA* and *JAK2* in MS patients treated with IFNb independently of disease activity (Goertsches et al., 2008; Hecker et al., 2013a), while only *JAK2* was upregulated with GA. MXA strongly monitors IFNb response, but its potential as a biomarker of relapse risk is disputed (Libertinova et al., 2017; Matas et al., 2014, 2016; Serana et al., 2014; van der Voort et al., 2010). We did not detect differences in *MXA* expression levels between AMS and IMS, but only 25% of IFNb-treated patients had AMS vs 67% of GA-treated patients. Moreover, anti-IFNb neutralizing antibodies, which can affect levels of *MXA* expression, were not measured in IFNb-treated patients (Bertolotto, 2004). Cytokine expression and cytokine-induced signalling occur through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signalling pathway, and JAK2/STAT3 signalling is especially involved in Th17 differentiation (Benveniste et al., 2014). Interestingly, JAK2 stabilizes IFNg receptor 2 on Th17 cells and IFNb therapy induces an antiproliferative response through IFNg signalling (Conti et al., 2012). The effect of GA on JAK2 is unknown, however GA was proven to inhibit JAK2 and STAT3 phosphorylation in experimental autoimmune encephalomyelitis, possibly by means of suppressor of cytokine signalling (SOCS) 7 as it was found upregulated in PBMCs of GA-treated MS patients (Chen et al., 2009; Rojas-Morales et al., 2019). Nevertheless, we did not measure the level of phosphorylated JAK2 in this study.

We further highlighted that *IL27p28* (a subunit of IL27) was upregulated in PBMCs of treated MS patients, in particular with IFNb. Its expression was found to be induced by both GA and IFNb (Mindur et al., 2017; Sweeney et al., 2011). IFNb inhibits the proliferation and differentiation of Th17 cells, directly, or indirectly through its effect on dendritic cells, and in correlation with *IL27p28* overexpression (Sweeney et al., 2011).

In our cohort *FOXP3* was overexpressed in MS vs HC, independently of treatment status as seen by others (Namdar et al., 2010; Sellebjerg et al., 2012). *FOXP3* was previously found upregulated in relapsing vs remitting MS (Dalla Libera et al., 2011). Conversely, unstimulated PBMCs of newly diagnosed MS patients expressed in vitro lower levels of *FOXP3* compared to HC (Etesam et al., 2016).

Regarding the microRNAs, we highlight the dysregulation of several microRNA species in PBMCs of MS patients, independently of radiological disease activity for miR-21-5p/34c-5p/155-5p/181c-5p and more pronounced in AMS for miR-27a-3p only. Moreover, the three most significantly dysregulated microRNAs, miR-21-5p/155-5p/181c-5p, described 93.26% of the population's variance as shown by PCA. miR-21-5p/146a-5p/155-5p were previously found upregulated in PBMCs of RRMS, while miR-34a was found downregulated in CD4+ T cells and miR-297a in B cells of RRMS patients, and miR-150/181c in PBMCs of MS (Huang et al., 2016; Ma et al., 2014; Martin and Illes, 2014) (Table 1). Conversely, miR-27a-3p was previously found upregulated in CD4+ T cells of relapsing MS patients and was thus postulated to target negative Th17 regulators (Ahmadian-Elmi et al., 2016). Yet on the contrary miR-27a-3p modulates dendritic cell maturation resulting in its mediated accumulation of Tregs instead of the mediated Th1/Th17 differentiation (Dong et al., 2020; Min et al., 2012). These apparently contradictory pro- and anti-inflammatory effects of miR-27a-3p were further observed in our in silico analysis. Herein, miR-27a-3p was predicted to target both *IL12p40* and *IL10*. Hence, the discrepancy between total PBMCs and CD4+ T cell microRNA expression for miR-34a-5p and miR-27a-3p highlights the difficulty in interpreting the pathophysiological significance of these changes, and should therefore be further functionally explored.

In our study treatment status did not significantly affect microRNA dysregulation, except for miR-29c-3p that was upregulated in untreated MS patients. Similarly, miR-29c-3p expression decreased after one month of IFNb therapy as compared to baseline (Hecker et al., 2013b). Furthermore, in GA-treated patients, miR-27a-3p was significantly downregulated and miR-150-5p tended to be upregulated, while in IFNb-treated patients, miR-34a-5p was significantly upregulated and miR-297 tended to be downregulated. Conversely, others highlighted the upregulation of miR-146a-5p and the downregulation of miR-20a-5p in untreated RRMS patients, but their expression level was comparable

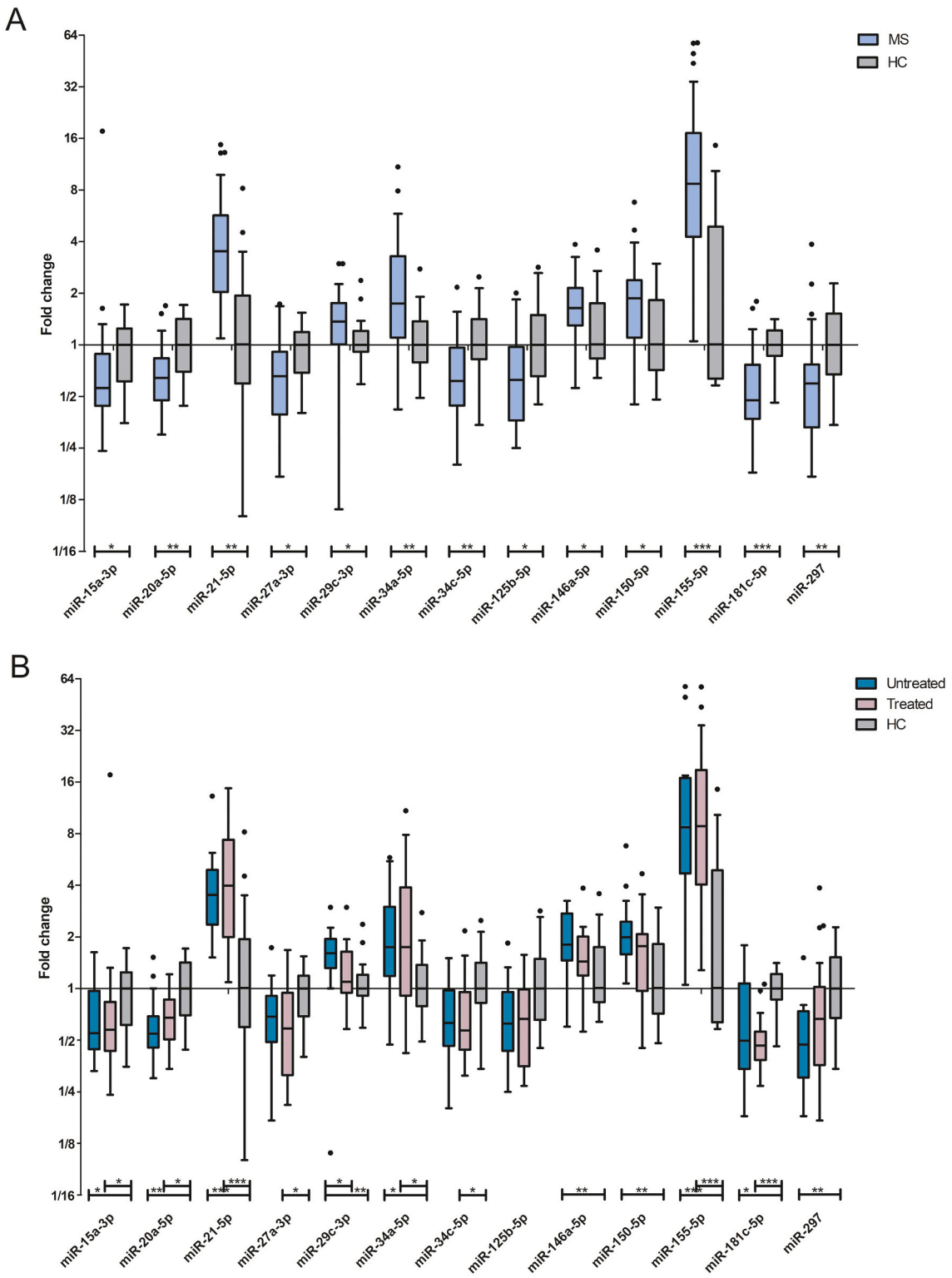

Fig3. Relative microRNA expression in PBMCs of MS patients and HC. The boxplots following the Tukey methods show the fold change ($2^{-\Delta Ct}_{target,sample}$ / median $2^{-\Delta Ct}_{target,HC}$) between the relative expression of the significantly dysregulated microRNAs of interest of this study and their median relative expression in HC for (A) MS patients vs HC and (B) untreated and treated MS patients vs HC. The y-axis is log2-scaled. Statistical significance was determined by (A) a Mann-Whitney t-test and (B) a one-way ANOVA Kruskal-Wallis test followed by a Dunn's post-test in between the subgroups. The p-values of the t-test and the one-way ANOVA have been corrected by the Benjamini-Hochberg method. $p \leq 0.05$: *; $p \leq 0.01$: **; $p \leq 0.001$: ***. HC = healthy controls, MS = multiple sclerosis.

to HC in GA- and in IFNb-treated patients respectively (Ehtesham et al., 2017; Waschbisch et al., 2011).

Several microRNAs of this study correlated with the mRNA levels of *IL21* and/or *FOXP3* or with *TGFB1*. We could however not identify a general and consistent pattern between the microRNAs and mRNAs of this study by in silico analysis, except for miR-34c-5p/*FOXP3*. The direct interaction of miR-34c-5p and *FOXP3* remains so far unexplored but has been evidenced for miR-34a-5p, although our correlation analysis was

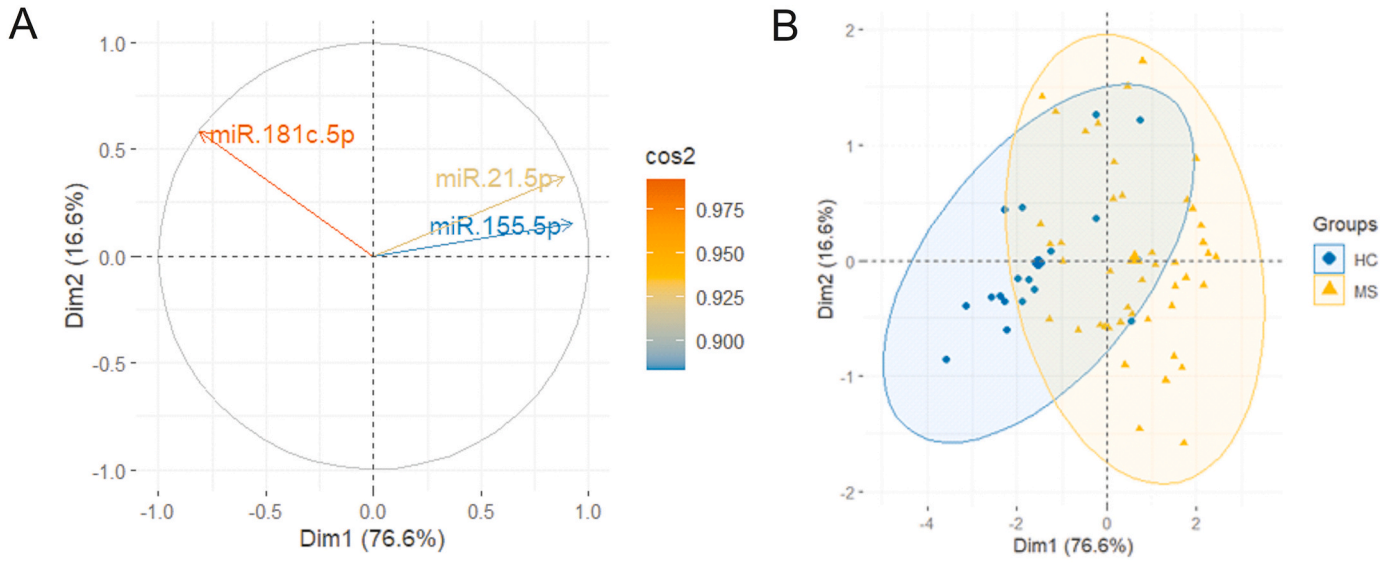

Fig. 4. Principal component analysis of the relative expression of microRNAs. The relative expression (-ΔCt) of the three most significantly dysregulated microRNAs (miR-21-5p/155-5p/181c-5p) are reduced to the first two principal components of the PCA to explain the population's variance (%) in these two dimensions (Dim1/Dim2). The loadings plot (A) represents the correlation of each variable (-ΔCt of the microRNA) to each component and is colour-scaled according to its cos^2 value. The score plot (B) helps to cluster both populations, HC (yellow) and MS (blue), as illustrated by the ellipses. HC = healthy controls, MS = multiple sclerosis.

contrary to this (Xie et al., 2019). An integrated view on cytokine regulation by microRNAs is missing as many studies have been investigating their interaction in a single setting. Cytokine expression conceivably results from a balanced effect of several microRNAs and other regulators. Moreover, chemokines and cytokines are rarely predicted as targets, but might be indirectly, positively or negatively, affected by microRNA regulation dependent whether the upstream target is a promoter or a repressor (Asirvatham et al., 2009; Salvi et al., 2019), which supports the positive and negative correlations found in our study as well as the difficulties identifying a causal interaction. The expression level of upregulated microRNAs of this study correlated only positively with the T cell mediators, independently of their pro−/anti-inflammatory nature. Given the mainly inhibitory effect of a microRNA on its direct target, we could hypothesise that these microRNAs rather target an upstream negative regulator. On the contrary, the expression level of downregulated microRNAs of this study correlated negatively with the T cell mediators and they might thus rather target them directly or an upstream positive regulator. Consistently with our correlation results, miR-125b-5p inhibition in macrophages increases the expression of *IL10,* and miR-24 and miR-149-3p target *FOXP3* (Fayyad-Kazan et al., 2012; Jiang et al., 2021; Liu et al., 2020). The latter could support our negative correlation between *TGFB1* and miR-149-3p. miR-27b-3p and miR-126-3p were both positively correlated with *TGFB1*. Accordingly miR-126-3p preferentially induced Treg differentiation over Th17 in a sepsis model (Zou et al., 2020). In contrast, miR-27b inhibited inducible Treg development in vitro by targeting TGFb receptor 1 and *SMAD4* (Severin et al., 2016). In accordance with the positive correlations we observed with *IL17A*, *IL21* and *FOXP3*, miR-155-5p promotes Th17 (IL6/STAT3/IL21/IL17) and Treg differentiation (IL2/STAT5/FOXP3), mainly through the JAK/STAT signalling pathway (Yao et al., 2012), as reported as well for miR-21-5p although with some discrepancies probably due to the experimental setting (Dong et al., 2014; Ji et al., 2021; Murugaiyan et al., 2015). Our in silico analysis further predicted miR-21-5p to target *IL12p35*, of which the interaction has already been proven by luciferase assay, but it was not significant in our correlation analysis (Cui et al., 2017; Lu et al., 2009). On the contrary, miR-146a-5p induces Treg differentiation at the expense of Th17 cells, which was also not reflected by our correlation analysis (Li et al., 2017).

We acknowledge the limitations of this study in investigating the dysregulation of microRNAs in PBMCs of MS patients as well as their interplay with pathogenic cytokines and their related transcription factors. Firstly, the microRNA dysregulation was overall independent of radiological disease activity and treatment status, with a few exceptions. Although the size of our entire MS cohort was reasonable, the number of patients per subgroup when considering both parameters together were rather small, limiting the power of further statistic analyses. Furthermore we chose a relatively small amount of microRNAs (21) based on the results of our previous study, that however investigated 64 microRNAs, selected from microarray data on 127 microRNAs (Perdaens et al., 2020). Many other microRNAs have been linked to MS before, and are still being identified by indiscriminate techniques with a higher discovery power such as small RNA sequencing (Cuomo-Haymour et al., 2022; Huang et al., 2016; Martin and Illes, 2014). Therefore, this study offers a partial vision on the mutual involvement of microRNAs and T cell mediators in MS. We are aware that other unexplored microRNAs could be uncovered as stronger functional players in the regulation of T cell mediator/cytokine expression.

It is also challenging to draw an overall conclusion on T cell mediators' expression or a peculiar profile of microRNA dysregulation in MS due to differences between studies regarding the population's characteristics and size, the type of sample analysed (serum, plasma, PBMCs, T cell subtypes) and the methodologies used (micro-array, sequencing or RT-qPCR for microRNA and mRNA levels, ELISA or flow cytometry for protein levels). Moreover, PBMCs might not be the best surrogate for disease activity markers, since the CNS is the site of active inflammation. Interestingly, as compared to our previous study on microRNA expression in CSF of relapsing-remitting MS patients (Table 1), miR-15a-3p/20a-5p/21-5p/29c-3p/34c-5p/146a-5p/150-5p/155-5p/297 were similarly dysregulated, while miR-27a-3p and miR-125b-5p were dysregulated in the opposite direction (Perdaens et al., 2020). CSF-dysregulated miR-24-3p/27b-3p/33a-3p/124-5p/145-5p/149-3p/214-3p were not confirmed in PBMCs of this MS population.

To conclude, in PBMCs of MS patients on platform injectable therapies or untreated, with radiologically active or inactive disease, T cell mediators were not dysregulated at the mRNA level, except for *FOXP3* and *JAK2*. We investigated microRNAs previously linked to MS and confirmed for several their dysregulation in PBMCs, although they could only partially segregate MS patients from HC. Interestingly, the

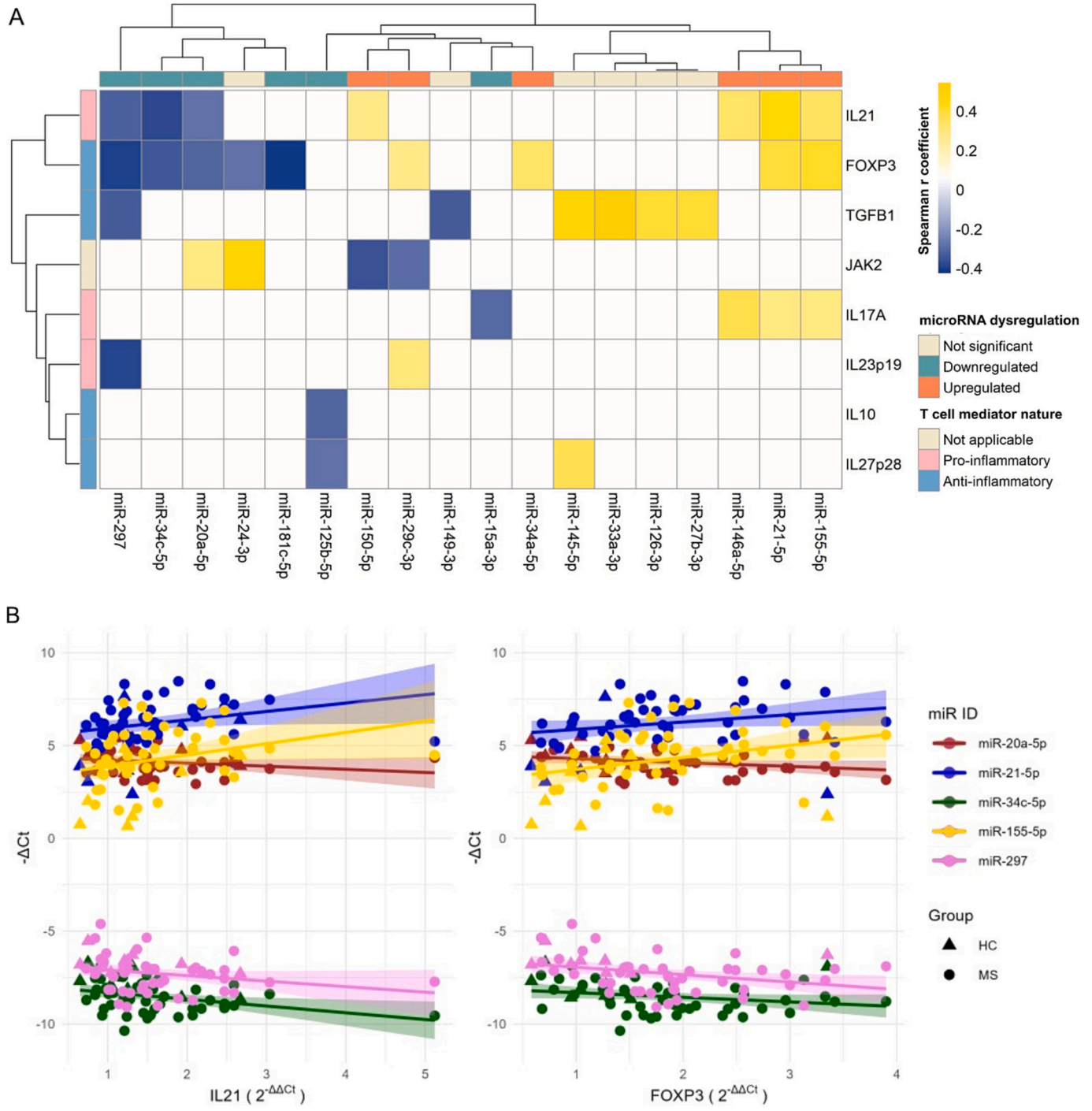

Fig. 5. Nonparametric correlation analysis between the relative expression of the mRNAs ($2^{-\Delta\Delta Ct}$) and the microRNAs (-ΔCt). (A) This heatmap with unsupervised hierarchical clustering, horizontally among the mRNAs, vertically among the microRNAs, displays the significant correlations between both, represented by the Spearman r coefficient in a blue-to-yellow colour scale, blue corresponding to a negative correlation, yellow to a positive. The horizontal colour bar on top of the heatmap indicates the dysregulation of each microRNA (not significant/downregulated/upregulated) and the vertical colour bar on the left side of the heatmap informs about the nature of each T cell mediator (not applicable/pro-inflammatory/anti-inflammatory) correspondingly to the colours in the plot legend. (B) miR-21-5p/155-5p and miR-20a-5p/34c-5p/297 correlate, respectively, positively and negatively with *IL21* (left) and *FOXP3* (right) as further highlighted by this linear regression (line) with confidence interval of 95% (shaded area), wherein each colour corresponds to one of these microRNAs as specified in the plot legend. Moreover, the shape of the dots allows to visualise the distribution of samples of healthy controls (HC, ▲) and multiple sclerosis patients (MS, ●). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression level of some of them correlated positively or negatively with *IL21* and/or *FOXP3* mRNA expression. In particular, the direct interaction of miR-34c-5p and *FOXP3*, supported by our findings on their opposite dysregulation and negative correlation, should be further explored. Cytokine expression conceivably results from the balanced effect of several regulators, of which microRNAs possibly do not target directly the mRNAs of cytokines but rather of their transcription factors. Their dysregulation in MS patients versus HC might not only reflect disease status but possibly contribute substantially to understanding MS pathogenicity, advocating further functional studies.

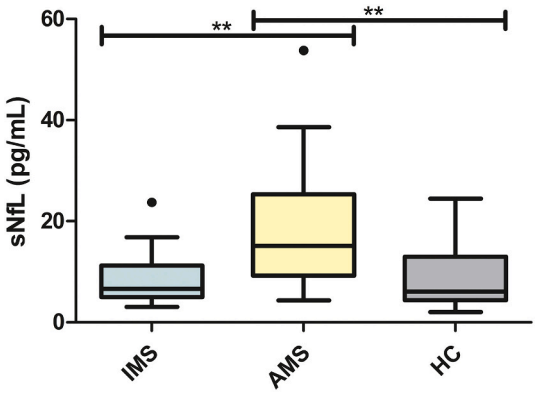

Fig. 6. Serum neurofilament light chain levels of MS and HC. The box plot, following the Tukey method, shows the concentration (pg/mL) of neurofilament light chain in the serum (sNfL) of IMS, AMS patients and HC, measured by single molecule immunoassay (SIMOA, Quanterix). The one-way ANOVA Kruskal-Wallis test results in an unadjusted p-value of 0.0017, the Dunn's post-test shows a significant difference between the subgroups, $p \leq 0.01$: **. HC = healthy controls, AMS = active multiple sclerosis, IMS = inactive multiple sclerosis.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Table 1
Comparison of microRNA dysregulation in MS of this current study on PBMCs with our previous study and with the literature. The significantly dysregulated microRNAs of this study are in bold. Mismatching dysregulations (compared to the current study) are italicised. CSF = cerebrospinal fluid, ns = non significant, NA = not applicable, ND = not done, PBMCs = peripheral blood mononuclear cells, MS = multiple sclerosis, AMS = active MS, IMS = inactive MS, RelMS = relapsing MS, RemMS = remitting MS, RRMS = relapsing-remitting MS, PPMS = primary progressive MS, SPMS = secondary progressive MS.

microRNA ID	**PBMCs study**	CSF study (Perdaens et al., 2020)			Literature	References
		CSF	serum	PBMCs		
miR-15a-3p	**down in MS**	down in RRMS	down in RRMS	ns	NA	
miR-20a-5p	**down in MS**	down in RemMS	ns	ns	down in whole blood of MS (RR/PP/SP) down in gray matter lesions of PP/SPMS	(Cox et al., 2010)) (Tripathi et al., 2021)
miR-21-5p	**up in MS**	up in RelMS	ND	ND	up in active lesions of MS (RR/PP/SP) ns/up in PBMCs of RelMS	(Junker et al., 2009) (Fenoglio et al., 2011; Gandhi et al., 2013)
miR-24-3p	ns	*up in RelMS*	*down in RRMS*	ns	*up in serum of RR/PPMS*	(Ehya et al., 2017; Vistbakka et al., 2018)
miR-27a-3p	**down in (A) MS**	*up in RelMS*	ND	ND	*up in active lesions of MS (RR/PP/SP)*	(Junker et al., 2009)
miR-27b-3p	ns	*up in RelMS*	ND	ND	*up in naïve CD4$^+$ of RemMS*	(Guerau-de-Arellano et al., 2011)
miR-29c-3p	**up in MS**	up in RelMS	ns	ns	*down in gray matter lesions of PP/SPMS*	(Tripathi et al., 2021)
miR-33a-3p	ns	*down in RemMS*	ND	ND	NA	
miR-34a-5p	**up in (I)MS**	ns	ns	*down in RelMS*	up in active lesions of MS (RR/PP/SP) *down in CD4$^+$ of RRMS*	(Junker et al., 2009) (Lindberg et al., 2010)
miR-34c-5p	**down in MS**	down in RRMS	ns	ns	NA	
miR-124-5p	ns	*down in RelMS*	ND	ND	NA	
miR-125b-5p	**down in (I)MS**	*up in RelMS*	ND	ND	down in PBMCs of RRMS	(Hadi et al., 2022)
miR-126-3p	ns	ns	*down in RRMS*	ns	*up in inactive lesions of MS (RR/PP/SP)* *up in CD4$^+$ of RRMS*	(Junker et al., 2009) (Lindberg et al., 2010)
miR-145-5p	ns	*up in RelMS*	ns	ns	*up in PBMCs/serum of RemMS*	(Søndergaard et al., 2013)
miR-146a-5p	**up in (I)MS**	up in RelMS	*down in RRMS*	ns	up in active lesions of MS (RR/PP/SP) up in PBMCs of RRMS	(Junker et al., 2009) (Waschbisch et al., 2011)
miR-149-3p	ns	*up in RemMS*	ND	ND	*up in gray matter lesions of PP/SPMS*	(Tripathi et al., 2021)
miR-150-5p	**up in MS**	up in RRMS	ns	ns	*down in PBMCs of MS (RR/PP/SP)* *ns in PBMCs of RelMS* up in CSF of RRMS	(Martinelli-Boneschi et al., 2012) (Fenoglio et al., 2011) (Bergman et al., 2016)
miR-155-5p	**up in MS**	up in RRMS	ns	ns	up in active lesions of MS (RR/PP/SP) ns/up in PBMCs of RRMS	(Junker et al., 2009) (Fenoglio et al., 2011; Waschbisch et al., 2011)
miR-181c-5p	**down in MS**	ns	down in RRMS	ns	down in active lesions of MS (RR/PP/SP) down in PBMCs of MS (RR/PP/SP) *up in CSF of RRMS*	(Junker et al., 2009) (Martinelli-Boneschi et al., 2012) (Haghikia et al., 2012)
miR-214-3p	ns	*down in RemMS*	*down in RRMS*	ns	*up in active/inactive lesions of MS (RR/PP/SP)*	(Junker et al., 2009)
miR-297	**down in (I)MS**	down in RRMS	ns	ns	down in B cells of RRMS	(Sievers et al., 2012)

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Data availability

Data will be made available on request.

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