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What is This?

Blood miRNA expression pattern is a possible risk marker for natalizumab-associated progressive multifocal leukoencephalopathy in multiple sclerosis patients

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Abstract

Background: Natalizumab has shown its efficacy in reducing multiple sclerosis (MS) relapses and progression of disability; however, it has been associated with an increased risk of developing progressive multifocal leukoencephalopathy (PML). The differential expression of microRNA (miRNA), the small non-coding RNAs that regulate gene expression, in natalizumab-treated patients has been reported and miRNA have also been described as good candidates for disease biomarkers.

Objective: To characterize the effect of natalizumab therapy on the miRNA expression pattern and to search for miRNAs that can predict PML on an individual basis.

Methods: The expression of 754 microRNAs was measured in blood samples from 19 relapsing–remitting MS patients at three time points during natalizumab therapy, using TaqMan OpenArray panels. Two patients included in this study developed PML after more than 2 years of therapy.

Results: We found that the expression level of three miRNAs (let-7c, miR-125a-5p and miR-642) was affected after 6 months of therapy (t6). Furthermore, we observed a differential expression of another three miRNAs (miR-320, miR-320b and miR-629) between the PML and non-PML groups after 12 months of treatment (t12); and a positive correlation was found between therapy time and the expression of miR-320.

Conclusions: Natalizumab modified the expression levels of three miRNAs after a 6-month treatment. We suggest miR-320, miR-320b and miR-629 as possible biomarkers for individual PML risk assessment.

Keywords: Adverse effects, biomarker, microRNA, miRNA expression, multiple sclerosis, natalizumab, progressive multifocal leukoencephaly, risk factors

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Introduction

Natalizumab (marketed as Tysabri®) is a monoclonal antibody that was approved in 2006 as second-line therapy for relapsing–remitting multiple sclerosis (MS).¹ Despite being very effective,¹ natalizumab has been associated with an increased risk of developing progressive multifocal leukoencephalopathy (PML),² a severe demyelinating disease of the central nervous system (CNS) caused by the reactivation of a latent infection of JC virus (JCV).³ The global PML risk can

be stratified and it is known that patients with more than 2 years of treatment, previous immunosuppression and anti-JCV antibody seropositivity have an increased risk of PML, up to 11.1 per 1000 treated patients, whereas the lowest risk is estimated at < 0.09 per 1000 treated patients, for those patients with negative anti-JCV antibody status.⁴

It has been reported that natalizumab alters transcriptional expression profiles of peripheral blood cells.⁵

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MicroRNAs (miRNAs) are small, non-coding RNAs that regulate gene expression⁶ and therefore, any alteration in their expression may cause changes in their target mRNAs. Moreover, miRNAs have been proposed as valuable biomarkers in several diseases.⁶⁻⁸ Recently, miRNA expression was explored in B cells from untreated and natalizumab-treated MS patients: It was found that natalizumab alters miRNA expression⁹; however, characterization of the basal miRNA expression pattern and the changes during the treatment period would be very interesting to know, in order to shed light on the effects of natalizumab. Furthermore, exploring the blood miRNA signature could identify a useful biomarker to stratify the individual PML risk.

To address this, we characterized miRNA expression in MS patients during natalizumab therapy at three timepoints and we evaluated the potential of miRNA expression to predict the development of PML.

Materials and methods

Patients and sample collection

We collected blood samples from 19 natalizumab-treated MS patients monthly, before receiving their drug infusion (intravenous 300 mg natalizumab) at the beginning of the therapy (pre-treatment) and during the first year of treatment. Furthermore, we included 3 healthy control (HC) samples (untreated) as the reference group for relative expression calculations. Two patients developed PML after 28 and 49 natalizumab infusions. Sample collection was performed in the Department of Neurology at the University Hospital Donostia, after obtaining informed consent. The study was approved by the hospital's ethics board.

RNA isolation

Blood was collected in PAXgene tubes (PreAnalytix, Qiagen) and the RNA isolation was carried out with the PAXgene Blood miRNA kit (PreAnalytix, Qiagen). The total RNA concentration was measured with a NanoDrop-1000 spectrophotometer and the RNA integrity was assessed using the Bioanalyzer (Agilent). Samples with a RNA integrity number (RIN) above six were included in subsequent experiments.

Our miRNA expression analysis

The expression profile of 754 miRNAs (based on miRBase v14) was analyzed at three time points during therapy: pre-treatment (t0), 6 months after

treatment (t6) and 1 year after receiving therapy (t12). We measured miRNA expression by quantitative PCR (qPCR), using TaqMan probe technology in the OpenArray platform (Life Technologies). We carried out reverse transcription and pre-amplification (12 cycles) steps in two separate reactions that came from 105 ng of total RNA, using the Megaplex™ Reverse Transcription Primers, Human Pool A v2.1, Human Pool B v3.0 (with the TaqMan MicroRNA Reverse Transcription Kit) and Megaplex™ PreAmp Primers, Human Pool A v2.1 and Human Pool B v3.0, respectively. Finally, the qPCR was run in a QuantStudio 12K System (Life Technologies). Validations were carried out using the individual TaqMan miRNA assays for each target and two endogenous controls (U6 and RNU48). We reverse transcribed 50 ng of total RNA using the TaqMan MicroRNA RT Kit without any pre-amplification step.

Data analysis

For data collection the QuantStudio™ 12K System software was used and quality control, normalization, relative quantification (RQ) and statistical analyses were performed using Excel and R 2.15.0 in RStudio v0.96.330. We analyzed both pools separately and considered as detected only Ct values between 10 and 33 and amplification scores higher than 1.1. The rest of the data were discarded and considered as missing values. For downstream analyses, just the miRNAs present in at least 80% of MS samples and in two out of the three healthy donors were included. As an endogenous control, the average Ct value of RNU48 and U6 was used, given that both of these showed the smallest variance at each time point in their pools. We calculated relative expression, taking the healthy control group as a reference for the three timepoints of MS samples; and finally, we transformed those data to a log₂ scale, in order to obtain a normally distributed dataset.

To explore whether any miRNA was differentially expressed among the timepoints or showed any trend with the treatment time, we carried out a repeated-measures analysis of variance (ANOVA) test. Finally, we added other clinical variables, such as the Expanded Disability Status Scale (EDSS), gender and anti-JCV antibody presence in the serum, as the covariates in the ANOVA analysis. For the PML biomarker search, a Wilcoxon Rank sum test was applied at each time point, after removing those miRNAs having no data for any of the two PML cases. To test the relationship between the treatment time and the candidate PML biomarkers, we applied a Pearson correlation test.

Table 1. Demographic and clinical data of the non-PML patients included in this study and the two PML cases, individually.

	Non-PML patients (<i>N</i> = 17)	PML Case 1	PML Case 2
Age when starting natalizumab therapy (yr) (mean ± SD)	36.59 ± 9.97	42	35
Age at disease onset (yr) ^a (mean ± SD)	29 ± 8.6	16	15
Gender ^a	Male = 7; Female = 10	Female	Male
EDSS when starting natalizumab (median (range))	4.5 (3–6.5)	4.5	5
Anti-JCV Ab	Positive = 11; Negative = 6	Positive	Positive
Number of natalizumab infusions received until PML onset	– ^b	28	49
Previous immunosuppression	Yes = 1; No = 16	No	Yes (Imurel)
IRIS	–	No ^c	Yes
Final outcome (EDSS)	–	Death (10)	Aphasia, right hemiplegia (8.5)

Anti-JCV Ab: anti-JC virus antibody presence in the study subject's serum; EDSS: Expanded Disability Status Scale; HC: healthy control; IRIS: immune reconstitution inflammatory syndrome; PML: progressive multifocal leukoencephalopathy.

^aHC subjects were two males and one female of mean age (SD) = 59.67(± 4.16).

^bNon-PML patients had received a variable number of drug infusions, but no other PML cases other than the two given in the accompanying columns have been detected in our cohort, so far.

^cNo clinical/radiological signs of IRIS, although there was an anatomopathological confirmation of IRIS.

Our miRNA target analysis

For the miRNA target search, we used the miRDIP web tool, which gathers up to 12 miRNA target prediction databases.¹⁰ We performed several filtering steps. For a given interaction, a threshold of three databases and a minimum average standard score of 25 was set. We then applied an additional filter, selecting only the genes expressed in the same tissue of the study (blood, in our case) according to the Gene Enrichment Profiler,¹¹ an online database where the expression profiles of 126 normal tissues are available. From this dataset, blood tissue data were selected and considered as detected, for those with log₂ intensity value above 6, as explained in the original article describing the database.¹¹ Gene ontology (GO) enrichment analysis was carried out using ClueGO v2.0.2.¹² We searched for the over-represented biological terms with a minimum of three genes per term, and used Benjamini-Hochberg corrected *p*-value as a measure of statistical significance.

Results

Longitudinal miRNA expression study

We analyzed the expression of 754 miRNAs in whole blood obtained from 19 natalizumab-treated MS patients at baseline, and at 6 and 12 months after their

treatment initiation. The patients' clinical characteristics are presented in Table 1.

After the quality control and filtering steps, we detected 183 miRNAs in blood that were considered for subsequent analyses. The repeated-measures ANOVA test revealed 53 miRNAs having, on average, different expression among the time points (*p* < 0.05); and three of them passed the false discovery rate correction (FDR) of < 0.05 (they were let-7c, miR-125a-5p and miR-642). As seen in Figure 1, Let-7c and miR-125a-5p showed decreased expression levels after treatment initiation (let-7c RQ: t₀ = 2.9, t₆ = 1.1 and t₁₂ = 1.2; miR-125a-5p RQ: t₀ = 1.9, t₆ = 0.7 and t₁₂ = 1.2), while miR-642 exhibited an increased expression level (RQ: t₀ = – 0.4, t₆ = 0.5, t₁₂ = 0.6). For the three differentially expressed miRNAs found, there was a significant change in expression levels between t₀ and t₆, and t₀ and t₁₂ (paired *t*-test *p* < 0.01), but not between t₆ and t₁₂.

We performed the technical validation of let-7c and miR-125a-5p, given that they showed the biggest difference between baseline and t₆: We confirmed that these two miRNAs exhibit a decrease in their expression levels after 6 months of therapy (let-7c, *p* = 0.024; miR-125a-5p, *p* = 0.002). We hypothesized that the first natalizumab infusion might be enough to trigger the change in miRNA expression. Therefore,

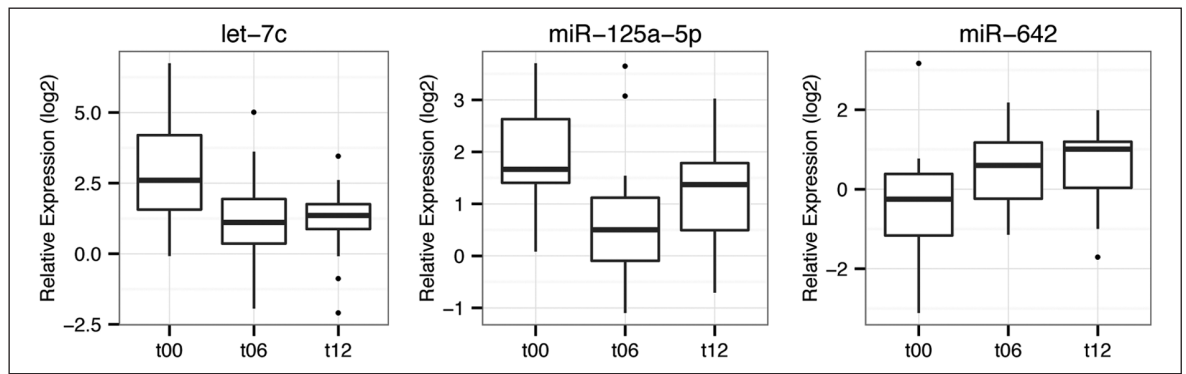


Figure 1. Boxplot of the three miRNAs showing significant differential expression among the study time points (FDR < 0.05). Paired *t*-test *p*-values for let-7c, miR-125a-5p and miR-642 were: t0 versus t6: $p = 2.523 \times 10^{-3}$, $p = 1.496 \times 10^{-4}$ and $p = 6.294 \times 10^{-4}$, respectively; t6 versus t12: $p = 0.911$, $p = 0.077$ and $p = 0.851$, respectively; t0 versus t12: $p = 8.5 \times 10^{-4}$, $p = 3.66 \times 10^{-3}$ and $p = 4.62 \times 10^{-3}$, respectively. Healthy controls' expression level (reference group) is 0. FDR: false discovery rate; miRNA: micro-ribonucleic acid; t: study testing time point, at 0, 6 or 12 months after initiation of therapy with natalizumab.

we analyzed the expression of the two validated miRNAs in the sample after the first treatment infusion (t1), confirming our hypothesis in let-7c (paired *t*-test $p = 0.046$).

The analysis of the influence of other clinical variables, such as gender, anti-JCV antibody status or EDSS on miRNA expression did not give any significant result.

PML biomarker search

We searched the candidate miRNAs for ability to predict PML, by comparing in each time point, the expression in the two patients who developed the disease after more than 2 years of treatment (PML group, $n = 2$), to that in the rest of the natalizumab-treated patients (non-PML group, $n = 17$). After removing those miRNAs with missing values for any of the two PML cases, we had 171, 178 and 162 miRNAs remaining at t0, t6 and t12, respectively. We found that 3, 1 and 15 miRNAs were differentially expressed between the patients with PML and non-PML, at t0, t6 and t12 ($p < 0.05$), respectively. For validations, the three miRNAs (miR-320, miR-320b and miR-629) were selected from those that were significant at t12, as they showed differential expression with no overlap in their data distributions between the PML and the non-PML groups (Figure 2(a)). We also checked these differences, segregating the non-PML group by the status of anti-JCV antibodies in serum (positive/negative), and found that the mean expression level of miR-320 and miR-320b was the highest in the PML patients ($RQ_{\text{miR-320}} = 5.2$; $RQ_{\text{miR-320b}} = 3.8$), followed

by the patients with positive anti-JCV antibody ($RQ_{\text{miR-320}} = 2.7$; $RQ_{\text{miR-320b}} = 1.7$), and finally the negative anti-JCV antibody patients ($RQ_{\text{miR-320}} = 1.8$; $RQ_{\text{miR-320b}} = -0.1$). On the contrary, the expression of miR-629 in the PML group was lower than in both non-PML groups ($RQ_{\text{PML}} = 0$ versus $RQ_{\text{positive}} = 1.4$ and $RQ_{\text{negative}} = 1.6$) (Figure 2(b)).

A technical validation of the miR-320 expression at t12 was carried out, which confirmed its differential expression between the PML and non-PML groups ($p = 0.039$) (data not shown). Moreover, we analyzed the expression of the three selected miRNAs individually at t6, t8, t10 and t12 in a subgroup of five non-PML and the two PML cases, to see whether their expression exhibited any consistent trend through the four study time points. With this analysis, we observed a positive correlation between the months from treatment onset and the expression of miR-320 in the PML group ($r = 0.877$; $p = 0.004$), but not in the non-PML group ($r = 0.253$; $p = 0.281$). As seen in Figure 3, the expression of miR-320b and miR-629 was not correlated with the treatment time, either in the PML or the non-PML groups (miR-320b, PML: $r = 0.482$, $p = 0.227$; miR-320b, non-PML: $r = 0.141$; $p = 0.553$; miR-629, PML: $r = 0.160$, $p = 0.704$; miR-629, non-PML: $r = -0.029$; $p = 0.900$).

Biological role of selected miRNAs

To explore the biological functions of the three miRNAs whose expression changed with natalizumab therapy and for the three candidate miRNAs for PML biomarkers, we searched for their putative targets in

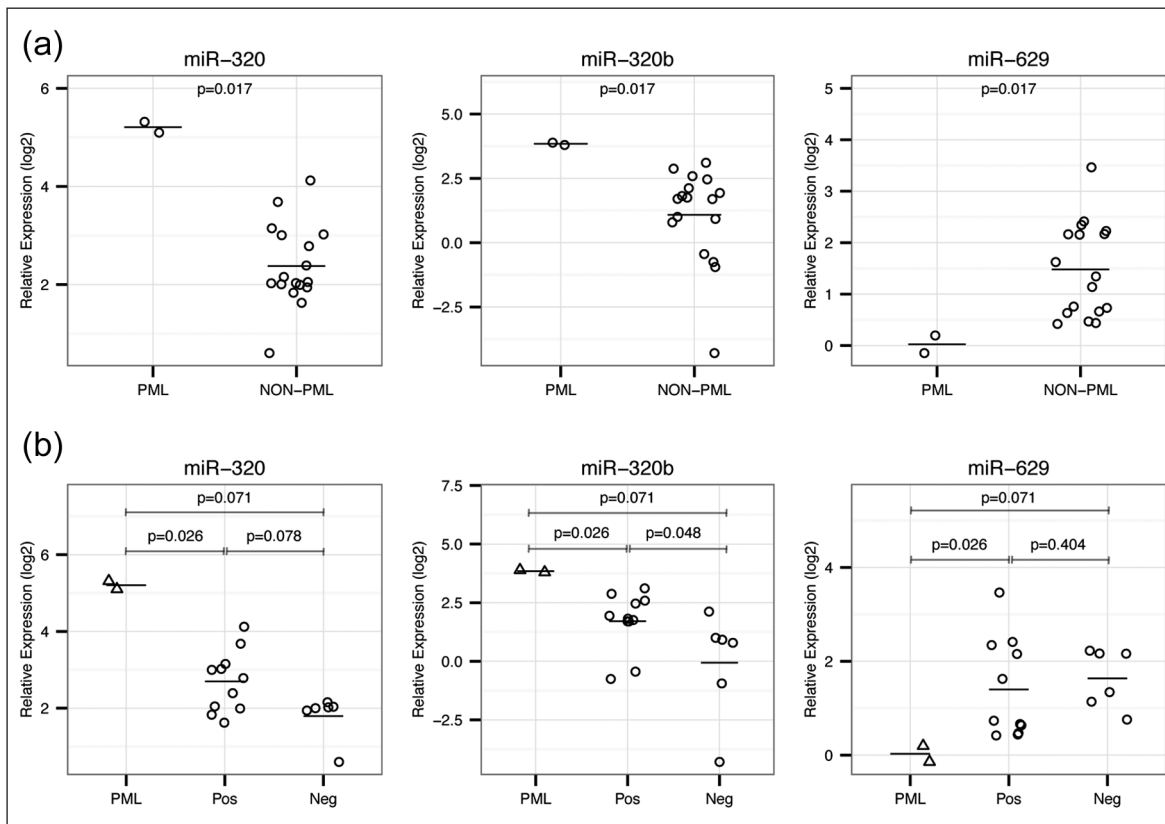


Figure 2. Expression results of the three miRNAs (miR-320, miR-320b and miR-629) selected for validation among the 15 differentially-expressed miRNAs at t12, between the PML and the non-PML groups. (a) Expression values of the three miRNAs in the PML and the non-PML groups. (b) Expression values of the three miRNAs after dividing the non-PML group by the status of JCV antibodies in serum. Triangles: PML cases; circles: non-PML cases.

JCV: JC virus; miRNA: micro-ribonucleic acid; Neg: negative; PML: progressive multifocal leukoencephalopathy; Pos: positive

the miRDIP database applying several filtering steps, as is described in our Materials and methods section. Finally, we obtained a list of 329, 398 and 105 putative target genes for let-7c, miR-125a-5p and miR-642, respectively. GO enrichment analysis resulted in several enriched processes for each miRNA (Supplementary Table 1). Remarkably, among others, GO terms such as filopodium assembly, and terms associated to post-transcriptional gene expression and regulation of channel activity, were enriched among the putative target genes.

Among the miR-320 targets, only four remained after all filtering steps (*FOXPI*, *PAN3*, *STOX2* and *NDRG3*). In addition, the miR-320b and miR-629 target search gave as output a list of 220 and 226 putative targets, whose GO enrichment analysis resulted in 6 and 13 enriched biological processes, respectively (Supplementary Table 2). Interestingly, the putative target genes are involved, among others, in the virus-host interaction process and associated to the mRNA catabolic process.

Discussion

Natalizumab had an effect on miRNA expression

In the present study, we analyzed the effect of natalizumab in global miRNA expression and found that the levels of let-7c and miR-125a-5p miRNA decreased after treatment initiation, while miR-642 expression increased. The effect of the therapy can be seen within the first 6 months, and then the levels of miRNA seem to be stable, during the rest of the period. The change in let-7c expression is detectable even after the first natalizumab infusion.

In other studies analyzing miRNA expression in MS patients, let-7c and miR-125a-5p were found upregulated in blood from MS patients, compared to controls¹³; and let-7c was also found upregulated and miR-642 downregulated in inactive lesions from MS patients, when compared to normal brain.¹⁴ Our study also identified the aberrant expression of those previously-reported miRNAs and showed that their expression might be restored due to natalizumab therapy,

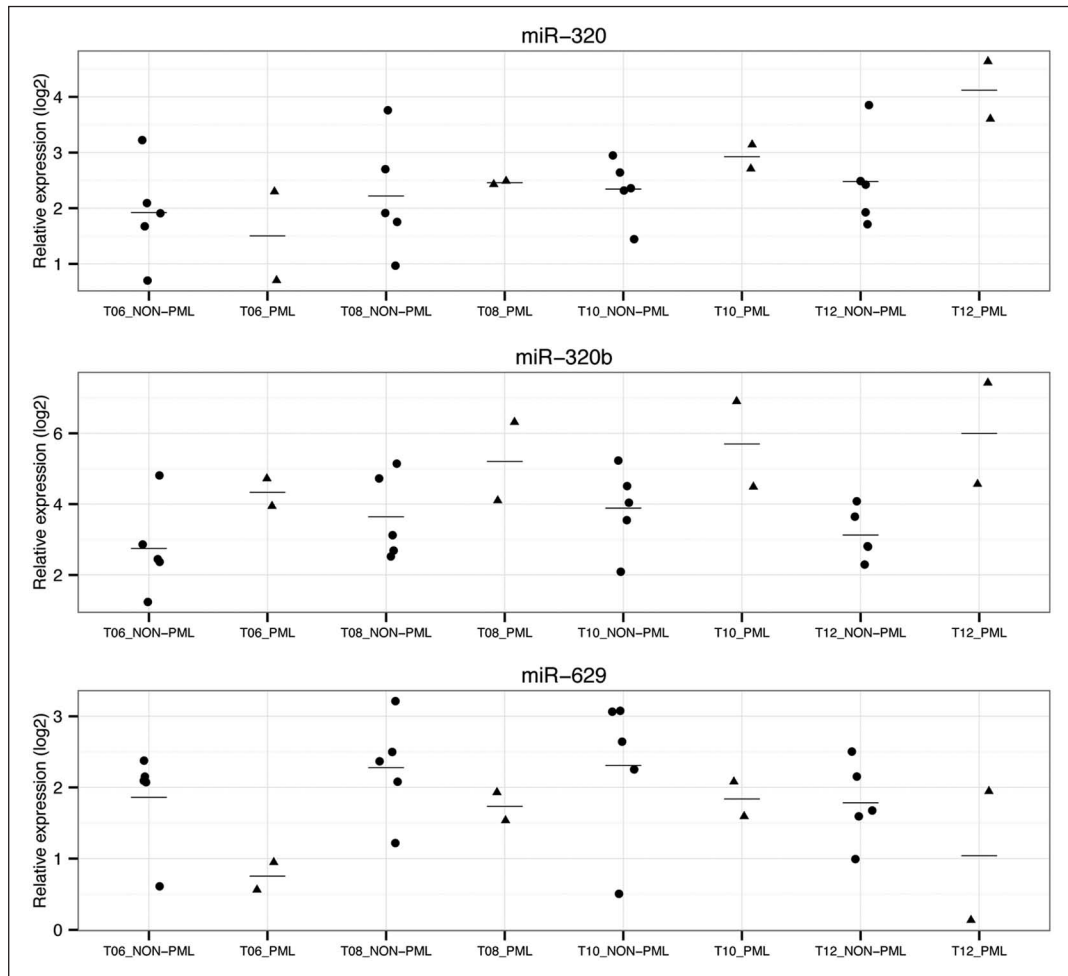


Figure 3. The results of the expression analysis of the three candidate miRNAs at the four study time points (t6, t8, t10 and t12) for predicting PML (Non-PML $n = 5$; PML $n = 2$). The Pearson correlation analysis for miR-320 showed a strong positive correlation for the PML group ($r = 0.877$; $p = 0.004$). The rest of the groups and miRNA tested did not result in any significant correlations (miR-320, non-PML: $r = 0.253$, $p = 0.281$; miR-320b, PML: $r = 0.482$, $p = 0.227$; miR-320b, non-PML: $r = 0.141$, $p = 0.553$; miR-629, PML: $r = 0.160$, $p = 0.704$; miR-629, non-PML: $r = -0.029$, $p = 0.900$). miRNA: Micro-ribonucleic acid; PML: progressive multifocal leukoencephalopathy; t: time in the number of months after initiation of natalizumab therapy

thus suggesting that these miRNAs might be related to the drug's mechanism of action.

In this sense, our *in silico* approach to find miRNA functions revealed that among miR-125a-5p target genes, terms related to *filopodium assembly* are enriched, indicating that miR-125a-5p may regulate the leucocyte migration process, which is one of the pathological steps required for disease development, and it is blocked by natalizumab. On top of that, it has been found that miR-125a-5p is a key regulator of brain endothelial integrity, suggesting the involvement of this miRNA in MS pathogenesis.¹⁵ Reijerkerk et al.¹⁵ reported that the *in vitro* overexpression of miR-125a-5p in brain endothelial cell lines reduces ICAM-1 expression and monocyte transmigration

through the brain endothelial cell barrier. Therefore, it seems that miR-125a-5p has a role in the leucocyte migration process, although it might be different in peripheral blood leucocytes and brain endothelial cells.

Another remarkable result in our miRNA target analysis was finding several genes (*LIN28B*, *SMAD2*, *ZCCHC11*, *DICER1* and *EIF2C2*) involved in the production of miRNAs among the let-7c and miR-125a-5p putative targets. Furthermore, functionally-related terms associated to post-transcriptional regulation were enriched in the miR-125a-5p target gene list. These findings suggested that let-7c and miR-125a-5p might regulate miRNA production by targeting genes from their biogenesis machinery, with

the wide spectra of implications that this regulation could have.

Nonetheless, our target analysis was an *in silico* approach, which gives an idea of the pathways that might be affected by the deregulated miRNAs, but experimental studies must be done in order to validate these results and confirm the implication of these biological processes.

It is reported that T-cells, B-cells and NK cells are increased in the peripheral blood of MS patients after treatment initiation and during a long period of time.¹⁶⁻¹⁹ Therefore, the change in miRNA expression levels might be due to the difference in the percentage of peripheral blood cell subsets. In this sense, miR-642 expression, which is elevated after treatment initiation, could be reflecting the increase in T-, B- and NK cells; however, *let-7c* and miR-125a-5p expression was downregulated after 6 months of therapy, and thus, they could represent the RNA fraction coming from other minor cell types. In any case, whether the miRNA expression pattern change is either a direct consequence of natalizumab therapy or it reflects a change in cell subset populations, it can give valuable information about the mechanism of action of the treatment.

To our knowledge, only one paper has been published to date that studies miRNA expression in natalizumab-treated patients: 10 miRNAs were found to be differentially expressed, compared to untreated MS patients, and only two remained after validation experiments.¹⁹ None of them appeared as being differentially expressed in the present work; however, in the above-mentioned study, the experiment was carried out in the B-cell population; while in the present work, whole blood was analyzed. Moreover, our objective was to evaluate changes in global miRNA expression during therapy; and thus, different time points from the same patients were compared, whereas Sievers and colleagues⁹ evaluated the effect of the treatment by comparing treated versus untreated MS patients. Therefore, differences in the cell type and the design of both studies make these two reports difficult to compare, and may explain the lack of overlapping results.

Putative PML marker miRNAs

We found three PML biomarker candidates showing significant differential expression between the PML and the non-PML groups (Figure 2). In addition, we identified a strong positive correlation between the treatment time and the expression of miR-320 in PML

patients, making this miRNA very interesting for further studies.

It is important to highlight that the two PML patients showed differential expression of these three candidate miRNAs more than 1 year before the onset of PML, which is very valuable when evaluating the individual risk and continuity of the treatment. Interestingly, these three miRNAs show significant differential expression between the PML cases and patients with anti-JCV antibodies in serum, which is the group presenting a higher risk of developing PML. Nonetheless, due to the small sample size of the PML group in this study, our results should be confirmed in a wider sample population.

Remarkably, one of the enriched terms among the putative targets of miR-320b was *virus-host interaction*, as three genes were associated with this term. This finding suggested that miR-320b may interfere between the virus and the host; and as mentioned above, miR-320b shows a higher expression level in patients positive for anti-JCV antibodies, strengthening the idea of its relationship with the virus and PML. In fact, miRNAs are reported to participate in hepatitis B virus chronic infection, thus pointing to the fact that both cellular and viral miRNAs could regulate the host-virus interaction.²⁰ However, the possible role of these miRNA in viral infection is only a hypothesis and it should be addressed with much more detail in future studies.

The *mRNA 3'-end processing* term appears enriched in the miR-320b target lists. One of the associated genes is *PAN3*, which is also targeted by miR-320. Furthermore, among the miR-629 targets, mRNA catabolism-related genes appear enriched as well, suggesting that all these miRNAs may be involved in the regulation of mRNA degradation.

Recently, L-selectin was proposed as a biomarker for individual PML risk in natalizumab-treated MS patients. An unusually low percentage of L-selectin-expressing CD4⁺ T cells have been found in pre-PML samples, compared with non-PML natalizumab-treated patients.²¹ Interestingly, miR-320 and miR-320b have putative binding sites in L-selectin, as predicted by more than one database. Thus, the low expression of L-selectin in CD4⁺ T cells from pre-PML samples reported by Schwab *et al.*²¹ correlates with the overexpression of miR-320 and miR-320b found in our pre-PML samples, provided that L-selectin is a target of those miRNAs.

Natalizumab-associated PML is a life-threatening adverse event with no specific treatment available nowadays. Finding a biomarker that could predict and monitor for PML is crucial, and it could help clinicians to better stratify the risk of PML in MS patients on an individual basis. Our results, although preliminary, are very promising in this sense, given that they offer three candidate miRNAs to predict PML cases. On top of that, the overexpression of miR-320 and miR-320b in our pre-PML samples nicely fits with the downregulation of L-selectin found in another cohort of pre-PML samples, which is, as far as we know, the only biomarker for PML prediction on an individual basis proposed so far.

In summary, we observed that after 6 months of natalizumab therapy there is a change in the expression of three miRNAs, two of which had been previously reported as differentially expressed in MS patients. Furthermore, we propose another three miRNAs as candidate PML biomarkers. Although very promising, these results should be validated in an independent and bigger patient cohort, in order to confirm the usefulness of these three miRNAs as biomarkers for PML risk.

Authorship

MMC: designed the study, performed the experiments, analyzed and interpreted the data and wrote the manuscript. HI and MS: interpreted data and discussed the manuscript. TCT: collected samples, performed patients' clinical characterization and collaborated in writing the manuscript. JO: collected samples, performed patients' clinical characterization and collaborated in writing the manuscript. ALdM: interpreted data and discussed the manuscript. LS: performed the experiments. IL: collected samples and analyzed the data. DO: designed the study, analyzed and interpret the data and wrote the manuscript. SEB: designed the study and analyzed and interpret the data.

Conflict of interest

J Olascoaga received honoraria as a consultant at the advisory board of Biogen-Idec, Genzyme and Novartis; and by participation in meetings as an organizer, speaker or moderator, as well as clinical trials or research projects promoted by: Almirall, Bayer, Biogen Idec, Sanofi-Genzyme, Merck, Novartis and Teva Pharmaceuticals. T Castillo-Triviño has received travel expenses for attending meetings from Biogen-Idec, Merck Serono and Novartis.

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