

# CSF microRNA Profiling in Alzheimer's Disease: a Screening and Validation Study

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**Abstract** MicroRNAs (miRNAs) are short non-coding RNA molecules that regulate gene expression through post-transcriptional repression of target genes. They have been shown to be implicated in the pathophysiology of Alzheimer's disease (AD) and proposed as disease biomarkers. In the present work, we have studied the expression levels of 754 miRNAs in cerebrospinal fluid (CSF) from AD patients and control subjects. We have explored a first screening cohort ( $N = 20$ ) and selected 12 miRNAs to be further tested in a second independent validation cohort ( $N = 69$ ).

We have found a significant upregulation of miR-222 and miR-125b in AD CSF. Of these, the association of miR-222 with AD is novel and reported here for the first time whereas upregulation of miR-125b has been previously reported in AD brain. Yet we do not find association with other miRNAs which were previously linked to AD. Our results shed light on potential underlying pathophysiological processes of AD and also point out the need for consensus procedures in CSF miRNA detection and data analysis.

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**Keywords** Alzheimer's disease · microRNAs · Biomarkers · Cerebrospinal fluid · miR-125b · miR-222

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## Introduction

Alzheimer's disease (AD) is the most common cause of dementia accounting for 60–70 % of cases [1], an estimated 46.8 million people worldwide which are predicted to double every 20 years [2]. Available cholinesterase inhibitors and memantine treatments are symptomatic but do not tackle disease progression [3]. In the last years, several novel therapies targeting the AD pathological hallmarks beta-amyloid and phosphorylated tau deposits have been tested in clinical trials, but no new drug has shown significant improvement of symptoms. Only recently, it has been recognized that new disease modifying therapies should ideally focus on preclinical stages of disease when neurodegenerative damage is still not widespread [4]. Currently available cerebrospinal fluid (CSF) biomarkers such as beta-amyloid peptide 1-42 ( $A\beta_{42}$ ) and tau protein species—total tau (t-tau) and tau phosphorylated at threonine 181 (p-tau)—combined with clinical and neuropathological diagnostic criteria have good sensitivities and specificities even at early stages of AD [5] but do not efficiently predict disease onset or follow progression. Thus, additional

biomarkers for AD prognosis and monitoring are needed, potentially suitable to be used as new therapeutic targets.

MicroRNAs (miRNAs) are endogenous ~23-nucleotide non-coding RNA molecules highly conserved in eukaryotes that regulate gene expression through post-transcriptional repression [6]. MiRNAs were discovered in *C. elegans* [7] and subsequently identified as a molecular entity of major relevance in regulating gene expression in animals including humans [8–10]. A single gene can be targeted by multiple miRNAs, while each miRNA may target up to hundreds of genes. Globally miRNAs are predicted to bind up to more than half of all human protein-coding genes [11], ultimately controlling relevant physiological processes such as development, neurogenesis and neural differentiation [12, 13]. MiRNA dysregulation has been found in human diseases including neurodegenerative diseases [14, 15], even at early presymptomatic stages [15].

After first pioneering work identifying differential miRNA expression in AD hippocampus [16], other brain studies (reviewed in [17]) have identified AD-associated miRNAs involved in regulation of pathophysiological events of the disease such as amyloid precursor protein (APP) expression and splicing [18] and Tau phosphorylation [19]. Recently, major interest has been dedicated to circulating miRNAs as potential biomarkers for AD [20]. Blood and plasma have been explored in search for AD-related miRNA alterations and, although several signatures have been proposed [21–23], results from these studies have been heterogeneous. To date, only few studies have focused on miRNAs from CSF, which is the biological fluid most directly related to the central nervous system. Although some CSF miRNAs have been pointed out as possible diagnostic biomarkers including miR-27a-3p [24], miR-29a [25, 26], and miR-125b [25], reproducibility in the results has been limited, highlighting the need of additional miRNA studies in AD using larger cohorts and standardized protocols [17, 27].

The aim of the current study is threefold: first, to describe a CSF miRNA expression profile; second, to investigate the differential expression of CSF miRNAs in AD patients; and third, to explore reproducibility of several miRNAs previously associated with AD in CSF samples. To this end, we have performed a CSF miRNA profiling study in screening and validation cohorts of phenotypically well-characterized AD patients and healthy controls.

## Subjects and Methods

### Subjects

Both AD patients and control subjects were recruited by the Alzheimer's disease and other cognitive disorders unit, at the Hospital Clínic de Barcelona, contributing to the screening

cohort and the independent validation cohort, and by the Memory unit, at the Hospital de la Santa Creu i Sant Pau, contributing to the validation cohort. Extensive clinical, neuropsychological and MRI examinations were performed in all the subjects, and CSF biomarkers A $\beta$ 42, t-tau, and p-tau of all the samples were determined by ELISA (Innogenetics, Ghent, Belgium).

We studied a total of 20 individuals in the screening cohort and 69 additional individuals in the validation cohort (Fig. 1). All AD patients fulfilled clinical criteria for probable AD according to the revised NIA-AA criteria [28] and had a CSF biomarker profile consisting of decreased A $\beta$ 42 levels plus high t-tau and p-tau levels, indicating high likelihood of being due to AD. The cut-off values we used to define our AD cohort in this study were 550 pg/mL for A $\beta$ 42, 350 pg/mL for t-tau, and 70 pg/mL for p-tau. The control group (CTR) was defined according to the following criteria: objective cognitive performance within the normal range (performance within 1.5 SD) in all tests from a specific test battery, clinical dementia rating scale score of 0, no significant psychiatric symptoms or previous neurological disease, and a non-pathological CSF biomarker profile. AD and CTR groups were age-matched at CSF collection. Demographic information of our cohorts is presented in Table 1.

### CSF Processing

CSF samples were collected by lumbar puncture between 9 am and noon. There was no report of traumatic lumbar puncture. Centrifugation at 4 °C for 10 min at 2000 $\times$ g and storage of 500  $\mu$ L aliquots in polypropylene tubes at –80 °C were accomplished within 1 h after collection.

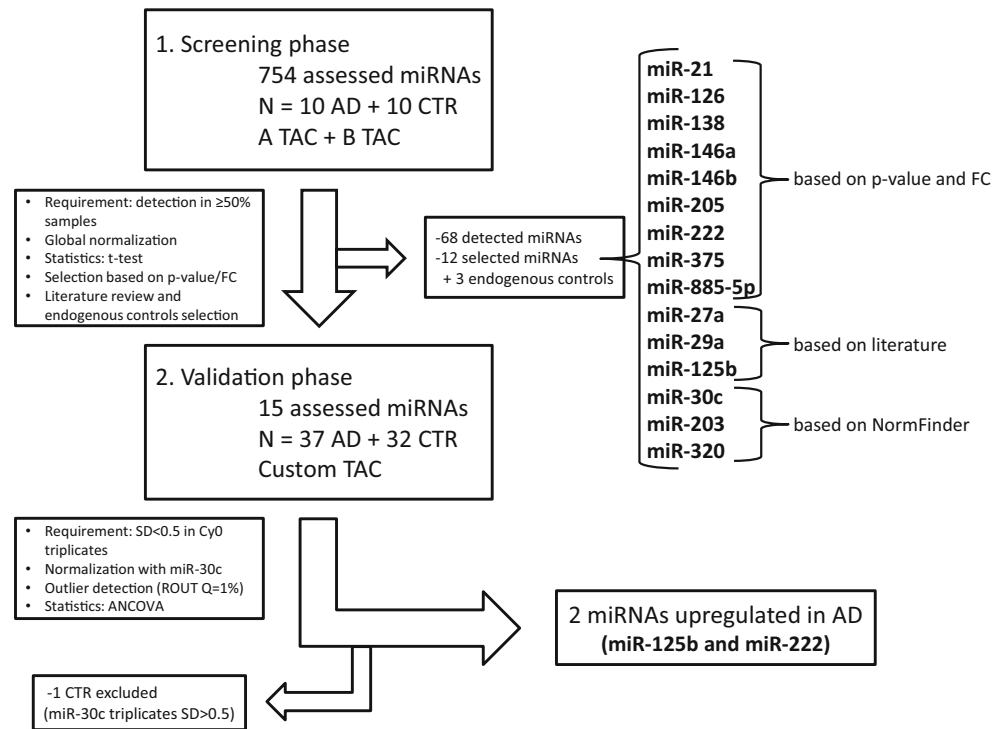
### MiRNA Extraction and Concentration

One 500  $\mu$ L aliquot of each sample was used for miRNA extraction with *mirVana* PARIS RNA and Native Protein Purification Kit (Life Technologies, Carlsbad, USA). Subsequently, extraction product underwent concentration into a final elution volume of 15  $\mu$ L using RNA Clean & Concentrator Kit (Zymo Research, Irvine, USA).

### Retro-Transcription (RT), Pre-amplification (Preamp), and Quantitative PCR (qPCR)

TaqMan Array Human MicroRNA A/B Cards (A/B TAC) and Custom TaqMan Array MicroRNA Cards (Custom TAC) were used as the qPCR platform (Life Technologies). Two different sets of 377 human miRNAs were assessed with the A/B TAC in the cohort 1 (screening phase) and a selection of 12 miRNAs was further explored with the Custom TAC in the cohort 2 (validation phase) (Fig. 1). A unique qPCR reaction for each miRNA was measured in the A/B TAC, whereas

**Fig. 1** Workflow diagram showing miRNAs assessed, samples used, data analysis specifications and main results in each phase of the study. *AD* Alzheimer's disease patients, *CTR* control subjects, *TAC* TaqMan Array Human MicroRNA Cards



triplicate measurements were performed for the miRNAs in the Custom TAC.

TaqMan MicroRNA Reverse Transcription Kit and Human Pool A/B Megaplex RT Primers (for A/B TAC) or a Custom RT Primer Pool (for Custom TAC) were used for RT. A Preamp step prior to qPCR was performed using TaqMan PreAmp Master Mix Kit and Human Pool A/B Megaplex PreAmp Primers (for A/B TAC) or a Custom PreAmp Primer Pool (for Custom TAC). Both RT and Preamp were executed according to Life Technologies technical note for *qPCR microRNA profiling* “Optimized protocols for human or rodent microRNA profiling with precious samples”: 3  $\mu$ L of the concentrated extraction product were used in a RT with a final volume of 10  $\mu$ L (for A/B TAC) or 15  $\mu$ L (for Custom

TAC), and 10  $\mu$ L of the RT product were used in a 14 cycles Preamp step, with a final volume of 50  $\mu$ L. A final dilution ratio of 1:100 of the Preamp product was used in the A/B and Custom TAC. ViiA7 Real-Time PCR System was used to run the TAC qPCR.

### Data Analysis

Cy0 algorithm [29], implemented in QPCR software [30], was used to quantify qPCR amplifications. This algorithm has the advantage of requiring neither the choice of threshold value nor the assumption of similar amplification efficiency among samples and standard curve, being completely objective and assumption-free. A simultaneous exploration of the data using

**Table 1** Demographic values of cohorts 1 (screening phase) and 2 (validation phase)

Variable	Cohort 1			Cohort 2		
	CTR (N = 10)	AD (N = 10)	p value	CTR (N = 31)	AD (N = 37)	p value
Age (years)	65.23 $\pm$ 1.16	66.01 $\pm$ 1.1	0.139	70.28 $\pm$ 3.87	72.97 $\pm$ 4.87	0.014
Sex (male:female)	4:6	2:8		15:16	14:23	
APOE4 (carriers:non carriers)	1:9	7:3		6:25	10:27	
MMSE (score)	27.89 $\pm$ 2.15	19.22 $\pm$ 5.7	0.002	28.58 $\pm$ 1.12	23.52 $\pm$ 3.11	<0.001
A $\beta$ 42 (pg/mL)	768.64 $\pm$ 114.51	369.18 $\pm$ 34.75	<0.001	804.7 $\pm$ 189.56	342.01 $\pm$ 103.5	<0.001
t-tau (pg/mL)	257.11 $\pm$ 76.98	1110.32 $\pm$ 491.65	<0.001	247.41 $\pm$ 63.06	801.47 $\pm$ 337.66	<0.001
p-tau (pg/mL)	51.68 $\pm$ 10.59	127.2 $\pm$ 45.81	<0.001	50.47 $\pm$ 11.13	109.33 $\pm$ 37.65	<0.001

All values are expressed as mean  $\pm$  SD unless otherwise stated. *P* values are the result of a *t* test between biological groups in each cohort  
*AD* Alzheimer's disease patients, *CTR* control subjects

the Ct values obtained through ExpressionSuite Software (Life Technologies) was performed and no major differences were observed comparing to Cy0 results (data not shown).

When no amplification was observed, the maximum Cy0 value (Cy0 = 40) was given to the amplification of a given sample. However, in order to be considered in the analysis, detection of amplification (Cy0 < 40) had to be reached in at least 50 % of the samples for a certain miRNA.

Fold change values (FC), in order to obtain a symmetric scale from  $-\infty$  to  $-1$  and from  $+1$  to  $+\infty$ , were calculated as:

$$\text{AD-upregulated miRNA} : \frac{2^{-\Delta\text{Cy}0}\text{AD\_mean}}{2^{-\Delta\text{Cy}0}\text{CTR\_mean}}$$

$$\text{AD-downregulated miRNA} : -\frac{2^{-\Delta\text{Cy}0}\text{CTR\_mean}}{2^{-\Delta\text{Cy}0}\text{AD\_mean}}$$

Being  $2^{-\Delta\text{Cy}0}\text{AD\_mean}$  and  $2^{-\Delta\text{Cy}0}\text{CTR\_mean}$  the averages of normalized individual  $2^{-\Delta\text{Cy}0}$  values for AD and CTR groups.

### Screening Phase

For data normalization of A/B TAC Cy0 values, the mean expression value of all expressed miRNAs in each sample was used (global normalization) [31]. Statistical analysis in the miRNA expression profiles was performed by a t-test using  $2^{-\Delta\text{Cy}0}$  values, being  $p < 0.05$  considered as significant (SPSS Statistics software, IBM, New York, USA).

Selection of 9 miRNAs (miR-21, miR-126, miR-138, miR-146a, miR-146b, miR-205, miR-222, miR-375, and miR-885-5p) from the screening phase for the validation phase was based on  $p$ -value significance and/or FC magnitude (greater than 2 or lower than  $-2$ ). Three other miRNAs were selected from the literature, where reported as differentially expressed in AD CSF: miR-27a [24], miR-29a [25, 26], and miR-125b [25]. Three more miRNAs (miR-30c, miR-203, and miR-320) were chosen as potential endogenous controls for data normalization in the validation phase based on NormFinder algorithm identification [32] with similar levels of amplification between all the samples tested in the screening phase (Fig. 1).

### Validation Phase

For Custom TAC, miR-30c was used as an endogenous control in order to normalize miRNA expression values. The two other miRNAs selected initially for normalization (miR-203 and miR-320) were discarded due to lack of robustness as they showed no amplification in several samples of the validation phase. Triplicate Cy0 values from the Custom TAC were averaged when the SD between them was lower than 0.5. When higher, the discordant Cy0 value was discarded, and if the SD remained higher than 0.5 the sample was not considered in the analysis for a particular miRNA. One CTR sample was finally

excluded from all analysis due to exceeding the maximum SD in the amplification of the endogenous control miR-30c (Fig. 1). Subsequently, an outlier analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, USA) ROUT algorithm [33] with a Q value of 1 %. In order to find differentially expressed miRNAs, an ANCOVA test was performed with SPSS. In the ANCOVA model, type II sum of squares was used. Initially the variables included in the model were age as a covariate, and gender, APOE E4 allele carrying, center of extraction and batch of RNA extraction as fixed factors. However, as it was confirmed that the center of extraction and the batch of RNA extraction did not have a significant effect on the model, these variables were excluded in order to reduce noise.  $P$  values smaller than 0.05 were considered significant.

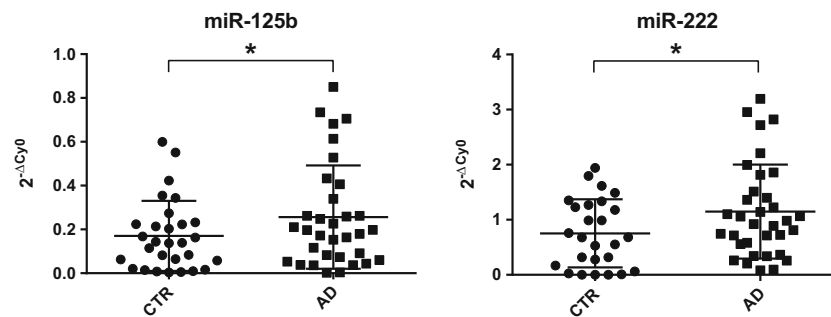
## Results

### Screening Phase

In the screening phase, we measured the expression of a total of 754 human miRNAs in CSF. We found 68 miRNAs with detectable expression levels (57 from A TAC and 11 from B TAC) showing amplification in at least 50 % of the samples (Online Resource 1). Of these, we found nine candidate miRNAs which were dysregulated ( $p < 0.05$  and/or  $|\text{FC}| > 2$ ) in AD with respect to controls: miR-21, miR-126, miR-138, miR-146a, miR-146b, miR-205, miR-222, miR-375, and miR-885-5p. These miRNAs were therefore selected to be further explored in the validation phase.

### Validation Phase

In the validation phase, we quantified the expression levels of the 9 candidate miRNAs mentioned in “Screening phase” section and 3 additional miRNAs previously associated with AD in the literature which showed detectable amplification in the screening phase: miR-27a [24], miR-29a [25, 26], and miR-125b [25]. We found that miR-125b and miR-222 were significantly upregulated in the AD group (FC of 1.51 and 1.52 and  $p$  values of 0.040 and 0.006, respectively, Fig. 2). We also observed an upregulation trend for miR-146b in the AD group when adjusting only by gender and age in the ANCOVA test, but this association did not hold significance when also adjusting by APOE E4 allele carrying. The other assessed miRNAs did not display significant differences between groups (miR-21, miR-27a, miR-29a, miR-146a, and miR-885-5p, Online Resource 2) or their detection levels were insufficient in more than 50 % of the samples (miR-126, miR-138, miR-205, and miR-375).



**Fig. 2** Expression levels in CSF of miR-125 and miR-222 in the validation phase showing an upregulation of both miRNAs in AD patients with respect to control subjects (miR-125b: FC = 1.51 and

$p = 0.040$ ; miR-222: FC = 1.52 and  $p = 0.006$ ). Individual values of control subjects (CTR) are represented with *circles*, Alzheimer's disease patients (AD) with *squares*, and *horizontal lines* show mean  $\pm$  SD

## Discussion

In this study, we have described a CSF miRNA expression profile and further investigated a differential expression of several expressed miRNAs in AD patients with respect to controls, validating data in a larger cohort. We have shown miR-125b and miR-222 to be significantly upregulated in AD.

Our study is exploratory and systematic analyzing all miRNAs described in humans available in the used qPCR platform at the time of the study design. Out of the 754 assessed miRNAs, we were able to satisfactorily detect only a relatively small fraction of 68 miRNAs, which is consistent with the observation that CSF is one of the body fluids with less detectable miRNAs [34]. In the first miRNA profiling study in AD CSF [35], in which post-mortem samples were used, a total of 201 out of 242 miRNAs were detected, but it has been reported that miRNA abundance in post-mortem CSF is much higher than in CSF from living patients [24]. In contrast, CSF miRNA expression detectability from our study is largely in line with other reports using also CSF from living patients and a similar qPCR platform [24, 36].

After analyzing the expression of the 12 candidate miRNAs in the validation cohort, we have identified miR-222 and miR-125b significantly upregulated in the CSF of AD patients. We report for the first time the upregulation of miR-222 in AD CSF. Regarding miR-125b, although it was initially reported as downregulated in AD CSF in one study [25], this miRNA has been shown to be upregulated in AD brain [16, 35, 37] and in post-mortem CSF [38]. Our findings are in line with these studies reporting increased miR-125b expression in AD. However, it is worth mentioning that, although a tendency of upregulation of miR-125b in AD CSF ( $p = 0.058$ ) was also reported by another group [26], they have very recently tried to validate this result in a multicenter study involving 57 AD patients and 40 control subjects and the previous result has not been replicated [27]. Only when

comparing a group of mild cognitive impairment due to AD patients (MCI-AD,  $N = 37$ ) with the control group they found increased levels of miR-125b in MCI-AD, but differences were lost after correcting for confounding factors in the analysis.

Although our results show an overlap between groups in the expression levels of miR-125b and miR-222, it is interesting to link the higher levels in AD CSF that we report with their possible implication in the pathophysiology of the disease. MiR-125b upregulation in AD has been shown to induce tau phosphorylation by increasing the expression of several tau kinases and inhibiting the expression of phosphatases [39]. Additionally, miR-125b has been associated with astrogliosis in neurodegeneration [40]. Regarding miR-222, it has been suggested that its expression is related to increased expression of matrix metalloproteinases (MMPs) through repression of the MMP inhibitor TIMP3, which may lead to increased neuronal apoptosis and inflammatory processes contributing to neurodegeneration [41].

Other CSF miRNAs have been previously linked to AD. Reported miR-27a-3p downregulation [24] has not been replicated in our study nor in two other validation studies [26, 27]. Increased levels of miR-29a were reported in two studies [25, 26] and, although we also detect a marginal upregulation, it is not statistically significant. Similarly, previous differential expression of miR-29a [26] has not been replicated in the recent multicenter validation study [27]. MiR-146a was reported downregulated in two studies [25, 42] and upregulated in one study [43]. When trying to validate previous findings in the multicenter validation study, decreased levels of miR-146a have been found only in one center, but not in the cohort where samples from the three participating centers were combined [27]. In line with these recent results, we observe a downregulation of this miRNA which does not reach statistical significance. Finally, a recent study using the same screening miRNA array platform as in our study (A/B TAC) reported different main findings [36] which might possibly be due to

differences in data normalization, statistical analysis, and validation qPCR platform.

Collectively, these studies indicate that reproducibility of miRNA association findings in AD CSF is currently limited [24–27, 36, 42, 43]. We propose several factors that should be addressed to improve comparability and consistency across studies. First, as a pre-analytical limiting factor, the low amount of miRNAs in the CSF has been reported to be a major issue [44] that compromises detection due to limited sensitivity of current technologies, which perhaps need to be refined if miRNAs are to be studied in CSF. Second, at the analytical level, different miRNA detection platforms might perform differently for each given miRNA and thus give rise to different findings. For instance, Taqman (Life Technologies) and miRCURY (Exiqon) miRNA assays have been shown to display different performances that impact on miRNA measurements [45, 46] and hence results from the two different platforms may not be directly comparable. Even when using Taqman technology, different performances of A/B TAC, Custom TAC, and individual assays for certain miRNAs have been observed [47, 48]. Therefore, ideally a standardization of the different procedures should be achieved. Third, the normalization of miRNA expression is a limiting factor in data analysis. In a screening study, a global normalization with the expression values of all detected miRNAs can be performed, but in candidate or validation studies typically a small set of miRNAs (normally between 1 and 3) are used as endogenous controls depending on their expression levels stability across samples and thus differing between studies. A consensus about the normalization strategy and the endogenous controls would be helpful to achieve comparability and reproducibility.

A limitation of the current study is the relatively reduced sample size of the cohorts. Although they are slightly larger than in other AD CSF miRNA profiling studies, the fact that miRNAs are most likely to be contributors to a complex and multifactorial process calls for the need of larger cohorts in order to have sufficient statistical power to see their potential contributory effect.

## Conclusions

In summary, we have reported for the first time the upregulation in CSF of miR-222 in our AD cohort, as well as an upregulation of miR-125b which was previously reported to be increased in the AD brain. Yet we report a lack of reproducibility between exploratory biomarker studies of miRNAs in CSF which may be related to the absence of a consensus in data normalization

and the variability of analytical conditions across studies together with the technical difficulty in detecting the low amounts of miRNAs available in CSF. In light of these results, an effort in harmonizing detection and analysis of CSF miRNA expression in further studies is needed in order to improve their value as AD biomarkers and to bring new insights into the pathophysiology of the disease.

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

**Informed Consent** Written informed consent was obtained for all subjects.

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