


Plasma miR-34a-5p and miR-545-3p as Early Biomarkers of Alzheimer's Disease: Potential and Limitations

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Abstract Plasma microRNAs (miRNAs) have been proposed as potential biomarkers in Alzheimer's disease (AD). Here, we explored their use as early sensors of the preclinical phase of the disease, when brain pathology is being developed and no cognitive loss is detected. For this purpose, we analyzed a set of ten mature plasma miRNAs in symptomatic patients with AD from a cohort that also included healthy controls (HC) and patients with preclinical Alzheimer's disease (PAD) (cohort 1). Plasmas from subjects with Parkinson's disease (PD) were used to control for disease specificity. We found that miR-15b-5p, miR-34a-5p, miR-142-3p, and miR-545-3p levels significantly distinguished AD from PD and HC subjects. We next examined the expression of these four miRNAs in plasma from subjects with PAD. Among these, miR-34a-5p and miR-545-3p presented good diagnostic accuracy to distinguish both AD and PAD from HC subjects,

according to the receiver operating characteristic (ROC) curve analysis. Both miRNAs also demonstrated a significant positive correlation with A β 1–42 levels in cerebrospinal fluid (CSF). Taking into account the clinical potential of these findings, we decided to validate the diagnostic accuracy of miR-34a-5p and miR-545-3p in plasma samples from an independent cohort (cohort 2), in which we did not observe the alterations described by us and others in AD and PAD samples. Although miR-34a-5p and miR-545-3p might be promising early biomarker candidates for AD, our study highlights possible sources of variability in miRNA analysis across hospitals, which currently prevents their use as reliable clinical tools.

Keywords miRNA · Alzheimer's disease · Plasma biomarker · Preclinical · miR-34a-5p · miR-545-3p

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Introduction

Converging evidence from both genetic at-risk cohorts and clinically normal older individuals suggests that the pathophysiological process of Alzheimer's disease (AD) is characterized by a first, preclinical stage of slow and asymptomatic development that can last for decades, during which biological changes associated with the emerging pathology are already present. This stage is followed by clinical symptoms of mild cognitive impairment (MCI) and a final stage of AD dementia. As a result, the pathology is diagnosed too late, when effective therapeutic intervention is difficult [1, 2]. Early detection and early intervention in preclinical AD subjects (PAD, defined as a biomarker positive while asymptomatic) would help provide the basis for preventive approaches involving modifiable and lifestyle risk factors of disease progression [3, 4]. Within this context, there is an increasing interest in secondary prevention trials in persons with preclinical AD (e.g., A4 study, which is a 3-year pharmacological trial for testing an anti-A β antibody [5]).

Recent studies have reported encouraging data on the possibility of diagnosis of AD pathophysiology in MCI stages based on the analysis of proteins, antibodies, or miRNA in human blood [6–8]. However, there are only few biomarker changes currently known to precede the appearance of clinical symptoms that can be used to define preclinical Alzheimer's disease (PAD): (1) brain accumulation of amyloid- β (A β) detectable by the positron emission tomography (PET) imaging technique and (2) low-A β 1–42 levels in cerebrospinal fluid (CSF), elevated CSF total tau (t-tau), and phosphorylated tau (p-tau). Invasiveness, complexity, and/or the economic burden of PET and CSF collection render them unsuitable for routine clinical screening [2, 9–13]. Thus, the finding of biomarkers to be measured by non-invasive and relatively inexpensive techniques remains a pending and extremely necessary task. Quantification of biomarkers in the bloodstream complies with these requirements and can be performed from different biological samples (i.e., whole blood, plasma, and serum). Among them, EDTA-treated plasma samples seem to be more reliable for some analyses since coagulation is not required and less blood cell lysis is observed [14–17].

Recently, microRNAs (miRNAs) have drawn attention as potential peripheral biomarkers of brain and systemic physiological disturbances [18]. These small molecules (approximately 22 nucleotides) bind to their target messenger RNA (mRNA), inhibiting its translation or promoting its degradation; thus, these represent one of the main epigenetic mechanisms for the regulation of gene expression [19]. Since miRNAs play an important role in the developing nervous system and in the physiology of high-order brain functions, their alteration can contribute to the manifestation of neurological disorders [20–26]. Particularly, during the last years, many studies in both AD animal models and patients have

highlighted the impact of miRNA dysregulation on AD pathophysiology [27–31]. miRNAs can reach the blood circulation through different mechanisms, including being exported from the cell through exosomes, through shedding vesicles, or in association with RNA-binding proteins. It is thought that they can mediate paracrine, endocrine, and/or juxtacrine signaling, as exosomes and shedding vesicles can donate their miRNA to recipient cells by the process of endocytosis, phagocytosis, direct fusion with the plasma membrane, or uptake of the RNA-binding protein-miRNA complex through specific cell membrane receptors [32–35]. Circulating miRNA might represent biomarkers of several age-associated diseases [36]. In particular, some circulating miRNAs in CSF, whole blood, plasma, and serum appear to be altered in both AD animal models and patients [6, 7, 27, 37–52]. However, methodological heterogeneity among studies and other uncontrolled factors (e.g., bodily fluid collection and storage, miRNA purification protocols, miRNA normalization strategies) has led to contradictory results in the literature; thus, there is a great need for validation studies and standardization of procedures [27, 53, 54].

Here, we have analyzed whether plasma levels of miRNA characterized as AD candidate biomarkers are already altered in PAD. For this, we examined miRNA expression in plasma from subjects classified as having AD compared with PAD subjects and healthy control (HC) subjects. Moreover, we compared data from two independent cohorts to examine the replicability of the results in samples obtained at different hospitals. Findings and limitations for the use of miRNA as biomarkers of AD are discussed.

Materials and Methods

Subjects

Subjects were recruited at Hospital Clínic de Barcelona (cohort 1 $n = 20$ –21/group; groups HC, AD, PAD, and PD) and at Hospital de Sant Pau de Barcelona (cohort 2 $n = 15$ /group; groups HC, AD, PAD). All participants gave informed written consent. Subjects were selected based on their clinical and neuropsychological characteristics and their levels of AD CSF biomarkers A β 1–42, tau phosphorylated at the threonine 181 position (p-tau), and total tau protein (t-tau). All participants were Caucasians from Barcelona, Spain.

Selection Criteria and Demographics

HC subjects were healthy individuals with cognitive performance within the normal range without biomarker-level abnormalities. PAD subjects were defined according to National Institute on Aging–Alzheimer's Association (NIA-AA) criteria [2], including cognitive performance within the

normal range (performance within 1.5 standard deviations [SD]) in all tests from a specific test series, no significant psychiatric symptoms or previous neurological disease, and decreased CSF A β 1–42.

AD subjects were selected according to International Working Group (IWG) consensus criteria (Dubois B. et al. 2007) and NIA-AA criteria [2]. They presented decreased CSF A β 42 levels (<500 pg/ml for cohort 1, <550 pg/ml for cohort 2) plus high CSF t-tau (>450 pg/ml for cohort 1, >350 pg/ml for cohort 2) and CSF p-tau (>75 pg/ml for cohort 1; >61 pg/ml for cohort 2) [55–57].

Demographic and clinical characteristics of cohorts 1 and 2 are summarized in Table 1.

Apolipoprotein E (APOE) genotyping was performed by PCR-based restriction enzyme digestion and restriction length polymorphism analysis (RLPA) on polyacrylamide gels as described previously [58].

Regarding patients with PD, all individuals were characterized with clinically definite PD [59]. Uncertain PD diagnosis, severe depression, and dementia were exclusion criteria.

Total RNA Isolation from Human Plasma

Blood and CSF samples were obtained from all subjects at the same visit. Blood samples were processed within 2 h after collection with EDTA-containing tubes (BD Vacutainer) and centrifuged at 2000 \times g at 4 °C for 10 min. Plasma was then separated and stored in 1 ml aliquots at –80 °C until further use.

Samples were thawed on ice and total RNA was isolated from 300 μ l of plasma utilizing the mirVana™ PARIS™ Isolation Kit (Applied Biosystems) according to the manufacturer's instructions for liquid samples. To allow for normalization of sample-to-sample variation in RNA isolation, 12.5 fmols of synthetic *Caenorhabditis elegans* miRNA cel-miR-39 (Qiagen) was added to each sample during the first step of the RNA isolation protocol. RNA was then eluted with 100 μ l of H₂O according to the manufacturer's protocol.

Measurement of Plasma miRNA Levels Using TaqMan qRT-PCR Assays

RNA was reverse transcribed using the TaqMan miRNA Reverse Transcription Kit and miRNA-specific stem-loop primers (Applied Biosystems) in a 5- μ l reverse transcription (RT) reaction containing 1.67 μ l of the RNA extract, 0.05 μ l of 100 mM dNTP (with dTTP), 0.33 μ l of MultiScribe™ reverse transcriptase (50 U/ μ l), 0.5 μ l of 10 \times RT buffer, 0.063 μ l of RNase inhibitor (20 U/ μ l), 1.387 μ l of RNase-free water, and 1 μ l of 5 \times miRNA-specific stem-loop RT primer (Applied Biosystems, Foster, CA, USA), as previously described [60, 61] (Online resource 1). RT reactions were performed in triplicate for each specific miRNA and were incubated at 16 °C for 30 min, at 42 °C for 30 min, and at 85 °C for 5 min and then maintained at 4 °C. The complementary DNA (cDNA) products were stored at –20 °C until analysis.

For relative gene expression analysis of both miR-34a-5p and miR-301a-3p, the RT products were preamplified prior to

Table 1 Demographic and clinical characteristics of cohorts 1 and 2

	Cohort 1			Cohort 2		
	HC subjects	AD patients	PAD subjects	HC subjects	AD patients	PAD subjects
Total N	21	21	21	15	15	15
Males	9	9	11	7	6	7
Females	12	12	10	8	9	8
Age (years)	69.55 (67.19–71.91)	71.83 (69.55–74.11)	73.27(70.25–76.29)	62.57 (60.48–64.67)	62.99 (60.75–65.24)	62.69 (58.76–66.63)
MMSE	28.41 (27.86–28.96)	23.20 (20.82–25.58)	27.8 (27.11–28.49)	28.73 (28.12–29.34)	21.93 (19.76–24.10)	28.73 (27.97–29.50)
GDS	47.6 % GDS = 1; 52.4 % GDS = 2	23.8 % GDS = 3; 76.2 % GDS = 4	52.4 % GDS = 1; 47.6 % GDS = 2	100 % GDS = 1	93.3 % GDS = 4; 6.6 % GDS = 5	100 % GDS = 1
CSF A β 1–42 (pg/ml)	772.79 (703.71–841.87)	347.22 (310.15–384.28)	366.5 (327.75–405.26)	859.35 (755.23–963.47)	366.1 (319.87–412.33)	445.67 (400.70–490.64)
CSF total tau (pg/ml)	265.15 (230.71–299.59)	846.49 (699.39–993.59)	440.87 (252.43–629.30)	235.05 (202.48–267.62)	908.9 (673.38–1144.41)	316.99 (13.44–620.56)
CSF p-tau (pg/ml)	54.26 (48.94–59.58)	108.84 (93.77–123.91)	75.63 (56.79–94.47)	47.59 (41.43–53.75)	109.03 (85.92–132.14)	45.71 (20.86–70.56)
% ApoE ϵ 4 carriers (N, %)	3; 14.29 %	14; 66.67 %	7; 33.33 %	4; 26.67 %	10; 66.67 %	9; 60 %

Confidence intervals (CI) of 95 % are indicated between parentheses

real-time quantitative PCR to enhance sensitivity, in that the corresponding fluorescence signal was undetectable without the preamplification step. The preamplification step was carried out in a small-scale (5 μ l) reaction comprised of 2.5 μ l Taqman PreAmp Master Mix (2 \times) (Applied Biosystems), 1.25 μ l Taqman miRNA assay (0.2 \times in TE) (Applied Biosystems), and 1.25 μ l undiluted RT product. The preamplification reactions were incubated at 95 °C for 10 min, followed by 14 cycles of 95 °C for 15 s and at 60 °C for 4 min and then maintained at 4 °C.

For real-time quantitative PCR (qRT-PCR), 1 μ l of the cDNA product was employed as a template in a 10- μ l reaction containing 0.5 μ l of TaqMan miRNA Assay (Applied Biosystem), 3.5 μ l of RNase-free water, and 5 μ l of QuantiMix Easy Probes Kit Master Mix (Biotools, Spain). The qRT-PCR was performed with Bio-Rad CFX96 real-time PCR system (Bio-Rad) at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Duplicates were run for each sample on a 96-well plate. Data were then analyzed with Bio-Rad CFX Manager (Bio-Rad) with the automatic Ct setting for assigning baseline and threshold for Ct determination. The relative expression level of each specific miRNA was calculated using the $2^{-\Delta\Delta C_t}$ method after normalization to the mean of internal housekeeping miRNA (hsa-miR-106a-5p and hsa-miR-17-5p) and to the spiked cel-miR-39.

Statistical Analysis

Comparisons among groups were performed by the Student *t* test or non-parametric statistical tests for independent samples, the Mann–Whitney *U* test, and Kruskal–Wallis test, together with the corresponding post-hoc tests, as indicated. Receiver operating characteristic (ROC) curve analyses were conducted to assess the diagnostic value of the miRNA for distinguishing patients with AD or subjects with PAD from HC. Area under the curve (AUC) values and the associated maximal values of specificity and sensibility are indicated throughout the manuscript. Partial correlations (controlling for group effect), Spearman's bivariate correlations, and multiple linear regression models were utilized to analyze the relationship between miRNA plasma levels and physiological and/or neuropsychological and clinical variables (CSF A β 1–42 levels, p-tau, t-tau, age, gender, the APOE allele variant, the mini mental state examination [MMSE] and the Geriatric Depression Scale [GDS]). Data were normalized (square root or log10) for performing partial correlation analysis. *P* values of <0.05 were considered statistically significant. Statistical outliers (≥ 2 SD from the mean) were removed from the analyses. The statistical analysis was performed using the Statistical Package for Social Sciences (SPSS, ver. 15.0) and MedCalc (ver. 14.12.0) [62].

Results

Expression and Specificity of Plasma miRNA in Alzheimer's Disease

Ten mature microRNAs have been previously reported to be altered in plasma from AD patients compared with HC (i.e., miR-34a-5p, miR-146a-5p, miR-34c-5p, let-7d-5p, let-7g-5p, miR-15b-5p, miR-142-3p, miR-191-5p, miR-301a-3p, and miR-545-3p) [38, 41, 43] (Online resource 2).

Here, we evaluated the levels of these miRNAs in plasma samples from HC and AD and determined the disease specificity of the validated miRNA using plasma samples from sex- and age-matched patients with PD. We did not include in our study miRNAs reported to be altered in biological samples other than plasma. Since autopsy studies demonstrate that a substantial percentage of MCI patients does not develop AD pathology, we did not include miRNAs described to be altered in MCI (see Online resource 2).

We found that only six of the set of ten miRNAs showed alterations that were consistent with previous reports. Plasma miR-15b-5p, miR-34a-5p, miR-142-3p, and miR-545-3p levels were significantly lower in AD samples than in HC ($U(39) = 96.0$, $Z = -2.62$, $P < 0.01$; $U(42) = 100.0$, $Z = -3.03$, $P < 0.01$; $U(40) = 121.0$, $Z = -2.13$, $P < 0.05$; $U(36) = 80.0$, $Z = -2.58$, $P < 0.01$, respectively). AD plasma samples also presented reduced levels of let-7d-5p and miR-146a-5p compared with HC ($U(40) = 128$, $Z = -1.94$, $P = 0.053$; $U(35) = 100.0$, $Z = -1.91$, $P = 0.056$). In contrast to previous studies [41, 43], we did not find alterations in miR-34c-5p, let-7g-5p, miR-191-5p, and miR-301a-3p plasma levels in patients with AD compared with HC. Data are summarized in Table 2 (HC and AD).

The diagnostic accuracy of miRNA differentially expressed between patients with AD and HC subjects was further tested by the ROC-curve analysis (Fig. 1). The AUC for miR-34a-5p was 0.77, indicating good diagnostic value for AD, with 76.19 % sensitivity and 71.43 % specificity. Similarly, the AUC for both miR-545-3p and miR-15b-5p was 0.75, indicating clinical relevance [63], with 94.12 and 72.22 % of sensitivity and 76.01 and 71.43 % of specificity, respectively. On the other hand, the AUC for miR-142-3p, let-7d-5p, and miR-146a-5p were lower than 0.70, indicating poor diagnostic value for AD.

In order to check for disease specificity, we measured those miRNAs found to be altered in AD samples in plasma samples from PD (Table 2, PD).

Kruskal–Wallis test analysis showed that miR-15b-5p, miR-34a-5p, miR-142-3p, and miR-545-3p levels were different among HC, AD, and PD groups ($\chi^2(2) = 22.92$, $P < 0.001$; $\chi^2(2) = 12.86$, $P < 0.01$; $\chi^2(2) = 21.54$, $P < 0.001$; $\chi^2(2) = 13.87$, $P < 0.001$).

Table 2 Expression of plasma miRNA in cohort 1

miRNA	Condition							
	HC		AD		PD		PAD	
	Mean (IC)	<i>n</i>	Mean (IC)	<i>n</i>	Mean (IC)	<i>n</i>	Mean (IC)	<i>n</i>
let-7d-5p	1.000 (0.640–1.360)	21	0.557 (0.381–0.733)	19	0.919 (0.482–1.356)	20	–	–
let-7g-5p	1.000 (0.693–1.307)	21	0.752 (0.427–1.076)	19	–	–	–	–
miR-15b-5p	1.000 (0.690–1.309)	21	0.512 (0.357–0.667)	18	2.469 (1.571–3.367)	20	0.951 (0.730–1.172)	21
miR-34a-5p	1.000 (0.461–1.540)	21	0.216 (0.125–0.307)	21	0.914 (0.502–1.325)	19	0.181 (0.107–0.254)	20
miR-34c-5p	1.000 (0.448–1.552)	17	0.561 (0.253–0.870)	19	–	–	–	–
miR-142-3p	1.000 (0.668–1.332)	21	0.623 (0.366–0.880)	19	2.723 (1.697–3.748)	20	0.700 (0.418–0.983)	21
miR-146a-5p	1.000 (0.682–1.318)	19	0.554 (0.400–0.709)	16	1.046 (0.613–1.480)	20	–	–
miR-191a-5p	1.000 (0.683–1.317)	19	0.850 (0.602–1.098)	19	–	–	–	–
miR-301a-5p	1.000 (0.643–1.357)	21	0.722 (0.455–0.990)	20	–	–	–	–
miR-545-3p	1.000 (0.668–1.333)	17	0.546 (0.307–0.785)	19	1.629 (0.995–2.262)	20	0.423 (0.268–0.578)	19

Confidence intervals (CI) of 95 % are indicated between parentheses

The same statistical analysis showed that let-7d-5p and miR-146a-5p levels were similar among groups ($\chi^2(2) = 3.53$, $P = 0.17$); $\chi^2(2) = 4.10$, $P = 0.13$, thus discarding these two miRNAs as candidates as specific AD biomarkers.

The corresponding post-hoc pairwise comparison tests revealed that miR-15b-5p, miR-34a-5p, miR-142-3p, and

miR-545-3p levels were significantly different between patients with AD and patients with PD ($P < 0.01$). While, miR-34a-5p and miR-545-3p did not show different levels between HC and patients with PD ($P = 0.800$, $P = 0.322$, respectively), miR-15b-5p and miR-142-3p presented higher levels in patients with PD compared with HC ($P < 0.05$, $P < 0.01$, respectively).

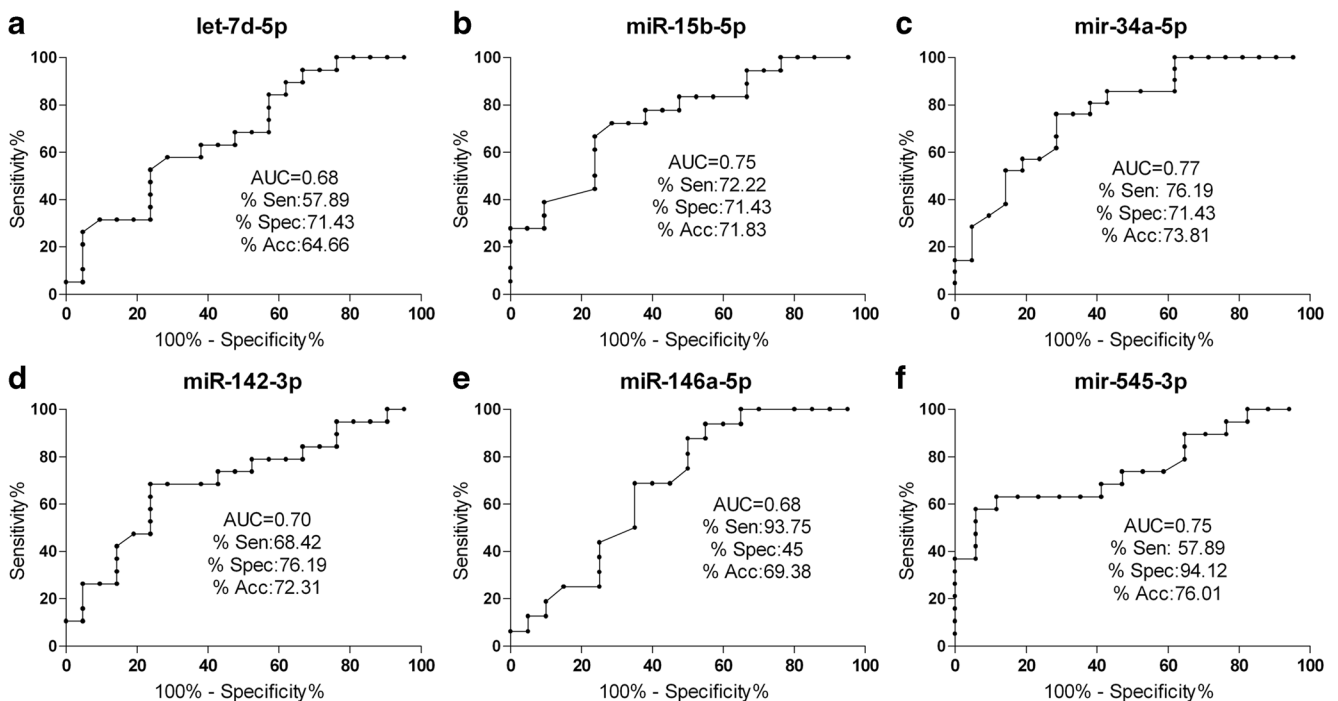


Fig. 1 Receiver operating characteristic (ROC) plots for plasma miRNAs differentially expressed between HC and AD from cohort 1. The true positive rate (sensitivity %) is plotted as a function of the false positive rate (100 %—specificity %) for the six miRNAs individually.

Area under the curve (AUC) values, % sensitivity, % specificity, and % accuracy at cut-off points are indicated in each plot as AUC, %Sens., %Spec., and %Acc., respectively

Expression of miRNA miR-15b-5p, miR-34a-5p, miR-142-3p, and miR-545-3p in Plasma Samples from Subjects in the Preclinical Phase of AD

In order to determine whether the validated miRNA (those differentiating AD from HC and PD) could represent early biomarkers of AD, we measured their plasma levels in 21 sex- and age-matched samples from PAD subjects [2, 64] (Table 2, PAD).

As determined by the Kruskal–Wallis non-parametric test, all four miRNAs showed differences among the HC, AD, PD, and PAD groups (miR-15b-5p $\chi^2(3) = 26.46$, $P < 0.001$; miR-34a-5p $\chi^2(3) = 21.83$, $P < 0.01$; miR-142-3p $\chi^2(3) = 26.56$, $P < 0.001$; miR-545-3p $\chi^2(3) = 21.97$, $P < 0.001$).

Post-hoc pairwise comparison tests showed that miR-34a-5p and miR-545-3p presented reduced levels in PAD subjects compared to both HC and patients with PD (PAD subjects vs. HC $P < 0.001$, $P < 0.01$; PAD subjects vs. PD patients $P < 0.001$, $P < 0.001$, respectively), while their expression levels were similar to those detected in AD samples ($P = 0.785$, $P = 0.870$, respectively). In contrast, miR-15b-5p and miR-142-3p expression levels did not differ between HC and PAD subjects ($P = 0.760$, $P = 0.122$, respectively) (Online resource 3, Fig. 2).

ROC-curve analysis revealed good diagnostic values for both miR-34a-5p and miR-545-3p (AUC values of 0.80 and 0.81, diagnostic accuracy of 78.34 and 76.78 %, respectively). Most importantly, our results indicate that both miR-34a-5p and miR-545-3p present good early diagnostic value for distinguishing HC from subjects presenting decreased CSF A β 1-42 (<500 pg/ml) (including patients with AD and PAD subjects) (Fig. 3).

We assessed the potential effect of gender or the presence of the APOE ϵ 4 allele for both miR-34a-5p and miR-545-3p by two-way analysis of variance (ANOVA) analysis. Our results showed no significant differences between females and males (miR-34a-5p $n = 33$ vs. 29, $F(1, 58) = 1.25$, $P = 0.27$; miR-545-3p $n = 29$ vs. 26, $F(1, 51) = 0.018$, $P = 0.89$), as well as between APOE ϵ 4 allele non-carriers and APOE ϵ 4 allele carriers (miR-34a-5p $n = 39$ vs. 23, $F(1, 58) = 0.349$, $P = 0.56$; miR-545-3p $n = 36$ vs. 19, $F(1, 51) = 1.707$, $P = 0.20$).

Associations Between Validated miRNA and Other Parameters

Interestingly, miR-15b-5p, miR-34a-5p, miR-142-3p, and miR-545-3p plasma levels positively correlate with each other ($P < 0.01$) as well as with A β 1-42 levels in CSF ($\rho = 0.452$, $P < 0.001$; $\rho = 0.384$, $P < 0.01$; $\rho = 0.293$ and $P < 0.05$; $\rho = 0.309$ and $P < 0.05$, respectively). On the other hand, miR-34a-5p was the only miRNA associated with t-tau, exhibiting a negative correlation ($\rho = -0.266$, $P < 0.05$). None of the miRNA was correlated with p-tau.

In order to determine the strongest factors predicting A β 1-42 levels in CSF, we entered the data from the four validated miRNA for AD (miR-15b-5p, miR-34a-5p, miR-142-3p, and miR-545-3p) together with gender, age, and APOE ϵ 4 allele variables into a multiple linear regression model. Our results highlighted miR-34a-5p as the best predicting miRNA for A β 1-42 levels in CSF, also influenced significantly by age and the presence of APOE ϵ 4 allele, $F(3, 49) = 10.488$, $P < 0.001$ (Table 3).

Validation of Plasma miR-34a-5p and miR-545-3p as Biomarkers of AD and PAD Across Hospitals

In order to test the replicability of our results, we measured the two miRNAs identified here as potential early biomarkers of AD in an independent cohort (cohort 2; Hospital de Sant Pau de Barcelona, Spain).

While the majority of sample characteristics were comparable between cohorts 1 and 2, they presented some significant differences. Mean age was significantly lower in all groups from cohort 2 ($t(126) = 6.36$, $P < 0.001$), and mean levels of CSF A β , t-tau, and p-tau in the PAD group were significantly different between the cohorts ($U(36) = 74.5$, $Z = -2.663$, $P < 0.01$; $U(36) = 71.0$, $Z = -2.78$, $P < 0.01$; $U(36) = 57.0$, $Z = -3.23$, $P < 0.01$, respectively) (Table 1).

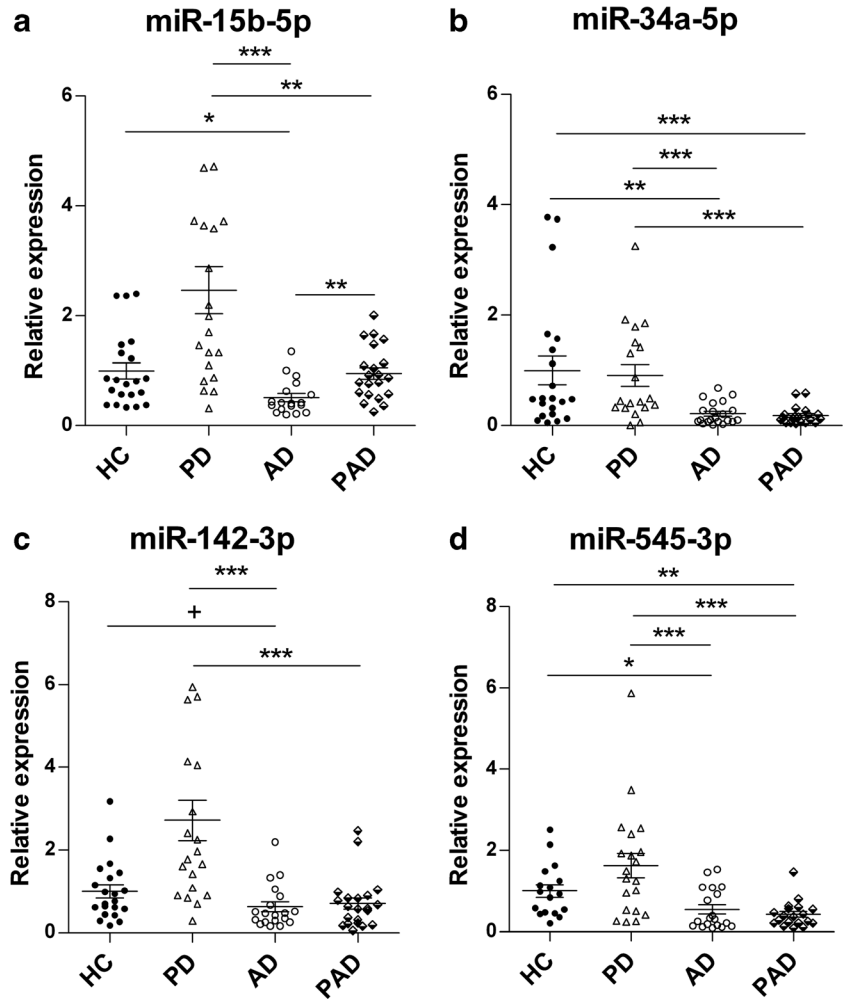
As an internal control, we compared the plasma levels of two housekeeping miRNAs between cohorts. As expected, both miR-106a-5p and miR-17 were similarly expressed between cohorts (coefficient of variation (CV) = 12 and 11 %, respectively) (Online resource 4).

However, in contrast to previous data, including our own results from cohort 1, the miR-34a-5p and miR-545-3p plasma levels from cohort 2 were not diminished in patients with AD as compared with HC ($P = 1.00$). Moreover, PAD subjects demonstrated higher plasma levels of miR-545-3p and a tendency toward higher levels of miR-34a-5p compared with HC ($P < 0.05$, $P = 0.07$, respectively), as well as higher plasma levels of miR-545-3p compared with AD ($P < 0.05$) (Table 4, Online resource 5). As a consequence, in this case, ROC curves did not show clinically relevant diagnostic values for distinguishing HC from subjects presenting decreased CSF A β 1-42 (<500 pg/ml) (both in patients with AD and PAD subjects) (miR-34a-5p AUC = 0.624, 46.67 % sensitivity, 86.67 % specificity; miR-545-3p AUC = 0.648, 55.17 % sensitivity, 80 % specificity).

Discussion

Previous studies have identified a set of ten plasma miRNAs as potentially involved in the pathogenesis of AD. The aims of this study were as follows: (i) to validate the alteration of these miRNAs in plasma from patients with AD, using samples of

Fig. 2 Expression levels of validated miRNAs in cohort 1. Mean \pm standard error (SE) are indicated. Cel-39-mir (spike-in external control) and the means of hsa-miR-106a-5p and hsa-miR-17-5p (endogenous housekeeping) were employed for normalization. Values were normalized relative to the HC. Kruskal–Wallis test and corresponding post-hoc paired-group comparisons were performed. The results are indicated as *** P value <0.001 , ** P value <0.01 , * P value <0.05 , and + P value <0.1 . Healthy control (HC), Alzheimer disease (AD), preclinical Alzheimer disease (PAD), and Parkinson's disease (PD)



PD subjects as controls for disease specificity (cohort 1), (ii) to explore the potential use of the validated miRNA as early biomarkers for the preclinical phase of AD (cohort 1), and (iii) to explore the consistency of our results across hospitals from the same city (cohort 2). Our data showed that only six of the ten previously described miRNAs were validated in

patients with AD, four of whom demonstrated disease specificity utilizing PD samples as control (miR-15b-5p, miR-34a-5p, miR-142-3p, and miR-545-3p). Moreover, two of these miRNAs (miR-34a-5p and miR-545-3p) presented good diagnostic accuracy for distinguishing both PAD subjects and patients with AD from HC and PD controls. Although these

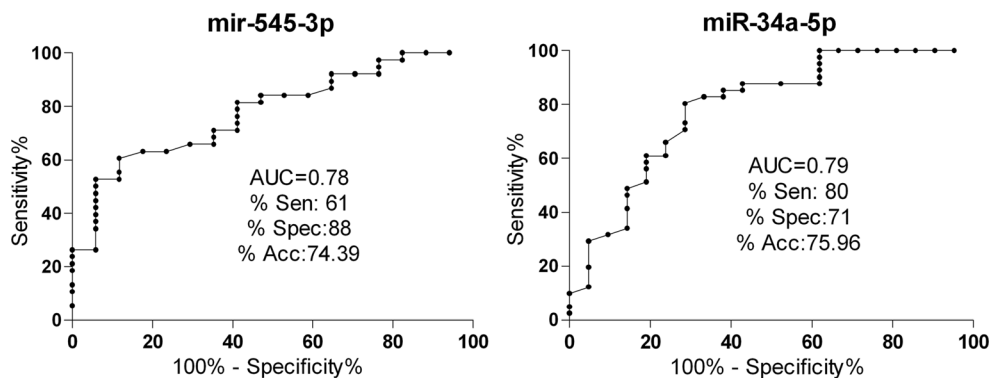


Fig. 3 Receiver operating characteristic (ROC) plots of miR-34a-5p and miR-545-3p for HC vs. individuals with decreased CSF A β 1–42 levels (AD + PAD) from cohort 1. The true positive rate (sensitivity %) is plotted as a function of the false positive rate (100 %—specificity %) for each

miRNA individually. Area under the curve (AUC) values, % sensitivity, % specificity, and % accuracy at cut-off points are indicated in each plot as AUC, %Sens., %Spec., and %Acc., respectively

Table 3 Factors influencing CSF A β 1–42 levels in cohort 1

Variables predicting CSF A β 1–42 levels	Multiple linear regression model			
	<i>B</i>	<i>P</i> value	<i>R</i>	<i>R</i> ²
miR-34a-5p	66.011	<0.001	0.625	0.391
APOE ϵ 4 allele	–117.989	0.018		
Age	–11.208	0.014		
Constant	1401.010	<0.001		

A multiple linear regression model was used in which CSF A β 1–42 level was the dependent variable, while age, gender, the presence of the APOE ϵ 4 allele, and AD-altered plasma miRNA were considered as potential predictor factors

findings might be promising, we did not reproduce these results in samples from cohort 2, highlighting some clinical and methodological issues in the use of plasma miRNA as biomarkers for AD.

In the first cohort, we were able to reproduce previously reported differences between patients with AD and HC in plasma levels of let-7d-5p, miR-15b-5p, miR-34a-5p, miR-142-3p, miR-146a-5p, and miR-545-3p [38, 43]. Thereby, these six miRNAs may represent a reliable miRNA signature of AD because they maintained their diagnostic potential across different studies and countries. In particular, miR-15b-5p, miR-34a-5p, and miR-545-3p presented the best diagnostic value among the six validated miRNA, reaching AUC values with high clinical relevance (>0.75) [63]. In contrast with previous reports [41, 43], we did not find significant changes in patients with AD compared with HC for let-7g-5p, miR-34c-5p, miR-191-5p, and miR-301a-5p plasma levels.

A limitation of the previous studies that have investigated the potential of plasma miRNA as biomarkers for AD is the lack of control of disease specificity in age- and sex-matched subjects with an unrelated neurodegenerative disorder. In our study, we included samples of patients with PD, which showed that some of the miRNAs found to be altered in AD

samples were not specific for AD pathology. Indeed, let-7d-5p and miR-146a-5p showed reduced plasma levels in PD plasmas compared with HC, thus suggesting common mechanisms concerning the deregulation of these specific miRNA in both AD and PD pathologies. Contrariwise, patients with PD presented significantly higher plasma levels of miR-15b-5p, miR-34a-5p, miR-142-3p, and miR-545-3p compared with patients with AD, thus supporting the specificity of the downregulation of these four miRNAs in AD. Moreover, according to our analyses in PAD subjects from cohort 1, our data indicate that miR-34a-5p and miR-545-3p represent potential early plasma biomarkers for AD, which are already altered in PAD when compared with HC. Both miRNA exhibited good diagnostic values for predicting decreased CSF A β 1–42 in AD and PAD subjects. Particularly, the multiple linear regression model revealed that the best miRNA for predicting CSF A β 1–42 levels is miR-34a-5p and that both AD and PAD subjects with lower CSF A β 1–42 levels also display lower miR-34a-5p plasma levels. Our data are consistent with several studies that have also found upregulation of miR-34a-5p in brains from AD mouse models, as well as its downregulation in CSF and plasma samples from patients with AD [38, 65–68].

Table 4 Expression of miRNA in cohort 2

Condition	miR-34a-5p		miR-545-3p	
	Mean (CI)		Mean (CI)	
HC	1.000 (0.664–1.356)		1.000 (0.670–1.330)	
AD	1.062 (0.599–1.524)		1.114 (0.603–1.624)	
PAD	1.615 (1.268–1.962)		1.628 (1.301–1.954)	
	Kruskal–Wallis test (<i>df</i> = 2)		Kruskal–Wallis test (<i>df</i> = 2)	
	χ^2	<i>P</i> value	χ^2	<i>P</i> value
	6.283	<0.05	8.47	<0.05
	Post-hoc test		Post-hoc test	
		<i>P</i> value		<i>P</i> value
HC vs. AD	–0.53	1.000	–0.37	1.000
HC vs. PAD	–10.67	0.078	–12.07	0.030
AD vs. PAD	–10.13	0.104	–11.69	0.043

Group comparisons, assessed by Kruskal–Wallis and the associated post-hoc test, are indicated. The corresponding *P* values are indicated. Confidence intervals (CI) of 95 % are indicated between parentheses; *df* stands for degrees of freedom

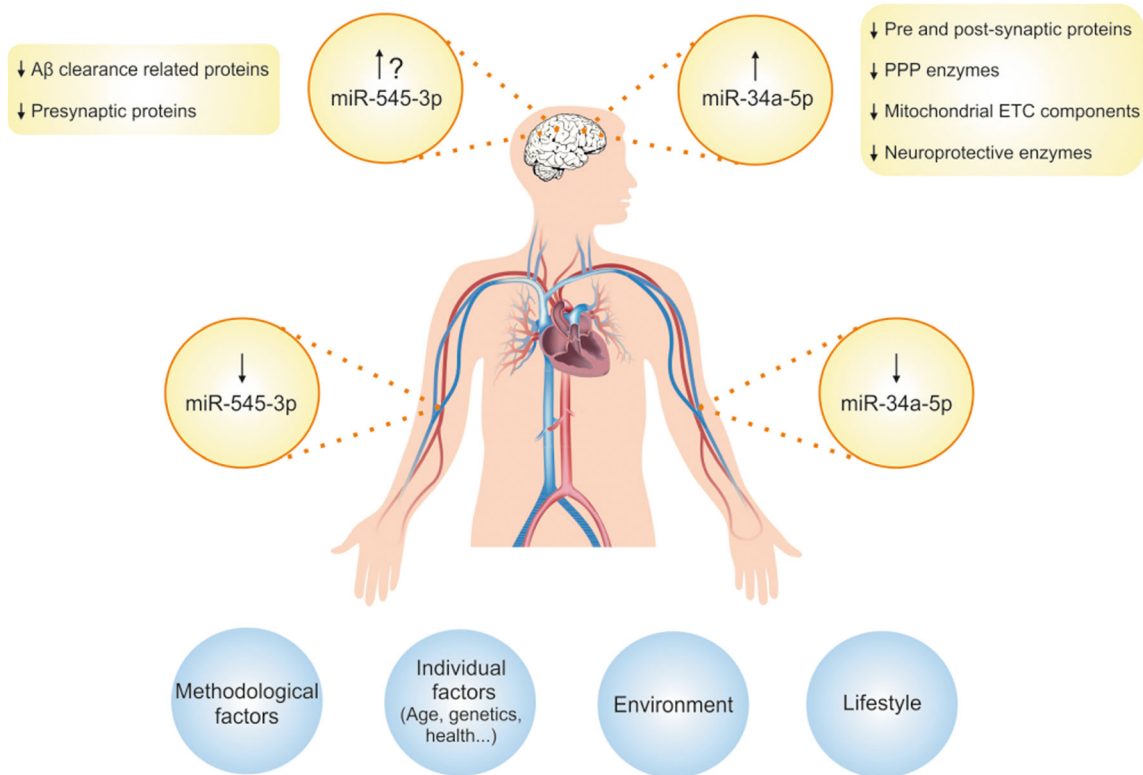


Fig. 4 miR-34a-5p and miR-545-3p as potential biomarkers for AD: a summary of proposed mechanisms and limitations. Both miR-34a-5p and miR-545-3p showed decreased levels in plasma from clinical and preclinical AD subjects in cohort 1, indicating their potential value as early AD biomarkers. Both miRNAs are involved in synaptic and energy metabolism, A β production and clearance, and apoptosis (summary of the corresponding targets indicated in *squares*). A downregulation of both miRNAs in AD plasma samples and in CSF in the case of miR-34a-5p has been reported in different studies, including ours [38, 43, 65, 68]. A downregulation of its targets together with an upregulation of the miRNA itself in the case of miR-34a-5p has been observed in brain from AD

patients. Notably, inversed alterations in biological fluids compared with brain levels have also been reported for other AD and other neurological disorder biomarkers [38, 86, 87]. The lack of reproducibility in cohort 2 indicates that the conditions of sample processing across hospitals as well as the contribution of several individual variables (such as age, genetic, health, and environmental factors) need to be further characterized to determine the accuracy of these miRNAs as early biomarkers for AD. The possibility of a mechanism involving disruption in the secretion or clearance systems for these miRNAs leading to their accumulation in brain and the reduction of their levels in plasma warrants further studies. *PPP*, pentose phosphate pathway; *ETC*, electron transport chain

Contrary to our findings in plasma, miR-34a-5p and miR-545-3p have been not identified by other authors as altered in serum from AD subjects [36, 40, 51]. Indeed, it is frequent that miRNAs detected in plasma are not detected in serum and vice versa. Tiberio et al. [69] reviewed numerous studies comparing different plasma/serum preparation protocols and analyzing differences between the two types of biological fluids in terms of circulating miRNAs. McDonald et al. [70], Mitchell et al. [61], and Wang et al. [71] reported inconsistencies between miRNA levels in plasma and serum. To explain this difference, it has been postulated that the variability may be due, at least in part, to miRNA release from blood cells (such as platelets) during the coagulation and centrifugation processes [71] [60, 72].

From a mechanistic point of view, miR-34a-5p is up-regulated in affected brain regions of AD patients and several AD mouse models [66, 67, 73]. In the brain, the upregulation of miR-34a-5p correlates with

concurrent repression of its target genes involved in synaptic plasticity, such as presynaptic-related proteins VAMP2 and SYT1, the NR2A subunit of the NMDA glutamate receptor, the potassium/sodium channel HCN1, and the glutamate-gated ion channel family member GLUR1 [74]. In addition, miR-34a can inhibit the pentose phosphate pathway (PPP) in neurons, which is essential for its long-term survival (it increases oxidative stress contributing to neurodegeneration), and disrupt mitochondrial oxidative phosphorylation by repressing the expression of electron transport chain components [74]. Other important targets of this miRNA are the antiapoptotic protein BCL-2, which inhibits caspases and promotes neuroprotection [38, 67], and the nicotinamide adenine dinucleotide-dependent deacetylase SIRT1 [75]. The latter is a well characterized neuroprotective factor involved in the regulation of A β production and downstream targets such as ROCK1, AMPK, NF- κ B, or

ADAM10, and it is also downregulated in brains of AD patients [76, 77, 78]. Intriguingly, miR-34a-5p is also altered in age-related macular degeneration (AMD), a late-onset neurodegenerative retinal disease which shares several clinical and pathological features with AD such as detrimental intra- and extracellular iron and lipofuscin deposits, increased oxidative stress, and mitochondrial and lysosomal dysfunction [79, 80]. Overall, miR-34a-5p represses several targets involved in synaptic, energy, and A β metabolism. Regarding miR-545-3p, one of its targets is the mRNA of LDL receptor-related protein 1 (LRP1), a plasma membrane receptor of APOE involved in A β clearance across the blood brain barrier (BBB) and cholesterol importation into neurons [81]. Patients with AD exhibit reduced levels of LRP1 and it has been proposed as a potential target for AD therapy [82, 83]. Another target of this miRNA is the presynaptic vesicle protein synaptophysin [81]. AD patients show reduced levels of synaptophysin in the hippocampus, and this correlates with their cognitive decline [84, 85].

In summary, a downregulation of miR-34a-5p and miR-545-3p in AD plasma samples has been reported in different studies, including ours [38, 43, 65, 68]. Moreover, a downregulation of some of their targets together with an upregulation of miR-34a has been observed in the brain from AD patients. Notably, inversed alterations in biological fluids compared with brain levels have also been reported for other AD and other neurological disorder biomarkers [38, 86, 87]. The possibility of a mechanism involving disruption in the secretion or clearance systems for these miRNAs leading to their accumulation in brain and the reduction of their levels in plasma warrants further studies. This is the first time that two miRNAs are reported to be altered in preclinical AD subjects; nevertheless, these findings need further validation due to the small number of participants included in our study and the fact that all the samples were collected in the Barcelona metropolitan area.

Regarding the second cohort, our results demonstrated no alterations in miR-34a-5p and miR-545-3p expression levels in AD or in PAD plasma samples. These data are especially difficult to explain in AD samples, in that we validated in cohort 1 low levels of these two miRNAs previously reported by other authors [38, 43]. This lack of reproducibility across hospitals evidences the need to identify and standardize the sources of variability that might be causing the inconsistencies between cohorts.

Different factors can account for the variability across miRNA studies including methodological heterogeneity, conditions of sample collection and storage, differences in miRNA purification protocols and miRNA normalization strategies, and environmental influences [27, 53, 54]. In our study, the methodology for collection and sample processing employed by the two centers was similar. The fact that the

expression levels of two housekeeping miRNAs (miR-106a-5p and miR-17) were stable between cohorts suggests that the lack of reproducibility observed for miR-34a-5p and miR-545-3p miRNAs may not be related with technical issues such as sample collection and processing.

One of the main differences between cohorts was their mean age, which in cohort 2 was significantly younger than in all previous studies examining miRNA alterations in AD, including our cohort 1. Several evidences indicate that age is a critical variable to consider when defining thresholds or cut-off values for some biomarkers. Van Harten et al. have recently proposed that differences in age could be underlying non-reproducible results among miRNA biomarker studies in AD [88, 89], and Kester et al. found that the association of A β 42, t-tau, and p-tau CSF levels with APOE genotype is modified by age in both controls and AD patients [90]. Similarly, biomarkers such as t-tau or prostate-specific antigen (PSA) are adjusted for age [91, 92]. On the other hand, as reviewed by many authors [93–98], multiple studies have shown that miRNA expression is altered following exposure to environmental factors. Some of these factors include sensorial elements such as light, noise or odors, physical activity, diet, environmental contaminants such as lead or aluminum, drugs such as alcohol or nicotine, and psychological stressors such as chronic social stress. Figure 4 summarizes findings, proposed mechanisms, and current limitations discussed here.

Secondary prevention is based on low specificity and low cost biomarkers that allow the detection of preclinical subjects [3, 4, 99]. Accordingly, here, we propose plasma levels of miR-34a-5p and miR-545-3p, both involved in AD pathogenesis, as potential early biomarkers of the disease. Further studies are required to better explore the influence of individual and environmental factors on the alterations of these miRNAs. Advances in this field will help develop blood-based biomarker tests for early detection of AD as valuable tools for the delay of the disease progression [3, 4, 99].

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Compliance with Ethical Standards Subjects were recruited at Hospital Clínic de Barcelona (cohort 1 $n = 20$ –21/group; groups HC, AD, PAD, and PD) and at Hospital de Sant Pau de Barcelona (cohort 2 $n = 15$ /group; groups HC, AD, PAD). All participants gave informed written consent.

References

- Morris JC (2005) Early-stage and preclinical Alzheimer disease. *Alzheimer Dis Assoc Disord* 19(3):163–165
- Sperling RA et al. (2011) Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement* 7(3):280–292
- Molinuevo JL et al. (2016) Ethical challenges in preclinical Alzheimer's disease observational studies and trials: results of the Barcelona summit. *Alzheimers Dement* 12(5):614–622
- Dubois B et al. (2016) Preclinical Alzheimer's disease: definition, natural history, and diagnostic criteria. *Alzheimers Dement* 12(3):292–323
- Sperling RA et al. (2014) The A4 study: stopping AD before symptoms begin? *Sci Transl Med* 6(228):228fs13
- Cheng, L., et al. (2014) Prognostic serum miRNA biomarkers associated with Alzheimer's disease shows concordance with neuropsychological and neuroimaging assessment. *Mol Psychiatry* 20(10):1188–1196
- Sheinerman KS et al. (2013) Plasma microRNA biomarkers for detection of mild cognitive impairment: biomarker validation study. *Aging (Albany NY)* 5(12):925–938
- Ray S et al. (2007) Classification and prediction of clinical Alzheimer's diagnosis based on plasma signaling proteins. *Nat Med* 13(11):1359–1362
- Jack CR Jr et al. (2010) Hypothetical model of dynamic biomarkers of the Alzheimer's pathological cascade. *Lancet Neurol* 9(1):119–128
- Jack CR Jr et al. (2013) Tracking pathophysiological processes in Alzheimer's disease: an updated hypothetical model of dynamic biomarkers. *Lancet Neurol* 12(2):207–216
- Hansson O et al. (2006) Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: a follow-up study. *Lancet Neurol* 5(3):228–234
- Villemagne VL et al. (2013) Amyloid beta deposition, neurodegeneration, and cognitive decline in sporadic Alzheimer's disease: a prospective cohort study. *Lancet Neurol* 12(4):357–367
- Mistur R et al. (2009) Current challenges for the early detection of Alzheimer's disease: brain imaging and CSF studies. *J Clin Neurol* 5(4):153–166
- Guder WG, Nayaranan S, Wissner H, Zawta B (2014) Diagnostic samples: from the patient to the laboratory: the impact of preanalytical variables on the quality of laboratory results. Wiley
- Banfi G, Deom A, Fräser CG, Hagemann P, Henny J, Kaliner A, Leppänen EA, Narayanan S, Neumaier M, Gomes MPA, Probst R (2009) Serum, plasma or whole blood? Which anticoagulants to use? *Journal of Laboratory Medicine*:297–312
- Lehmann S et al. (2009) Preanalytical guidelines for clinical proteomics investigation of biological fluids. *Ann Biol Clin (Paris)* 67(6):629–639
- Pritchard CC et al. (2012) Blood cell origin of circulating microRNAs: a cautionary note for cancer biomarker studies. *Cancer Prev Res (Phila)* 5(3):492–497
- Kim YK (2015) Extracellular microRNAs as biomarkers in human disease. *Chonnam Med J* 51(2):51–57
- Bartel DP (2009) MicroRNAs: target recognition and regulatory functions. *Cell* 136(2):215–233
- Wang W, Kwon EJ, Tsai LH (2012) MicroRNAs in learning, memory, and neurological diseases. *Learn Mem* 19(9):359–368
- Mastroeni D et al. (2011) Epigenetic mechanisms in Alzheimer's disease. *Neurobiol Aging* 32(7):1161–1180
- Van den Hove DL et al. (2014) Epigenetically regulated microRNAs in Alzheimer's disease. *Neurobiol Aging* 35(4):731–745
- Xu B et al. (2012) MicroRNA dysregulation in neuropsychiatric disorders and cognitive dysfunction. *Neurobiol Dis* 46(2):291–301
- Eacker SM, Dawson TM, Dawson VL (2009) Understanding microRNAs in neurodegeneration. *Nat Rev Neurosci* 10(12):837–841
- Dwivedi Y (2016) Pathogenetic and therapeutic applications of microRNAs in major depressive disorder. *Prog Neuro-Psychopharmacol Biol Psychiatry* 64:341–348
- Cao DD, Li L, Chan WY (2016) MicroRNAs: key regulators in the central nervous system and their implication in neurological diseases. *Int J Mol Sci* 17(6)
- Femminella GD, Ferrara N, Rengo G (2015) The emerging role of microRNAs in Alzheimer's disease. *Front Physiol* 6:40
- Maes OC et al. (2009) MicroRNA: implications for Alzheimer disease and other human CNS disorders. *Curr Genomics* 10(3):154–168
- Delay C, Mandemakers W, Hebert SS (2012) MicroRNAs in Alzheimer's disease. *Neurobiol Dis* 46(2):285–290
- Cosin-Tomas M et al. (2014) Epigenetic alterations in hippocampus of SAMP8 senescent mice and modulation by voluntary physical exercise. *Front Aging Neurosci* 6:51
- Noh H et al. (2014) Prediction of miRNA-mRNA associations in Alzheimer's disease mice using network topology. *BMC Genomics* 15:644
- Weilner S et al. (2013) Secretion of microvesicular miRNAs in cellular and organismal aging. *Exp Gerontol* 48(7):626–633
- Gibbins DJ et al. (2009) Multivesicular bodies associate with components of miRNA effector complexes and modulate miRNA activity. *Nat Cell Biol* 11(9):1143–1149
- Iguchi H, Kosaka N, Ochiya T (2010) Secretory microRNAs as a versatile communication tool. *Commun Integr Biol* 3(5):478–481
- Chen X et al. (2012) Secreted microRNAs: a new form of intercellular communication. *Trends Cell Biol* 22(3):125–132
- Dorval V, Nelson PT, Hebert SS (2013) Circulating microRNAs in Alzheimer's disease: the search for novel biomarkers. *Front Mol Neurosci* 6:24
- Sheinerman KS et al. (2012) Plasma microRNA biomarkers for detection of mild cognitive impairment. *Aging (Albany NY)* 4(9):590–605
- Kiko T et al. (2014) MicroRNAs in plasma and cerebrospinal fluid as potential markers for Alzheimer's disease. *J Alzheimers Dis* 39(2):253–259
- Tan L et al. (2014) Circulating miR-125b as a biomarker of Alzheimer's disease. *J Neurol Sci* 336(1–2):52–56
- Tan L et al. (2014) Genome-wide serum microRNA expression profiling identifies serum biomarkers for Alzheimer's disease. *J Alzheimers Dis* 40(4):1017–1027
- Bhatnagar S et al. (2014) Increased microRNA-34c abundance in Alzheimer's disease circulating blood plasma. *Front Mol Neurosci* 7:2
- Geekiyana H et al. (2012) Blood serum miRNA: non-invasive biomarkers for Alzheimer's disease. *Exp Neurol* 235(2):491–496
- Kumar P et al. (2013) Circulating miRNA biomarkers for Alzheimer's disease. *PLoS One* 8(7):e69807
- Bekris LM, Leverenz JB (2015) The biomarker and therapeutic potential of miRNA in Alzheimer's disease. *Neurodegener Dis Manag* 5(1):61–74
- Bekris LM et al. (2013) MicroRNA in Alzheimer's disease: an exploratory study in brain, cerebrospinal fluid and plasma. *Biomarkers* 18(5):455–466
- Hu YB et al. (2016) Diagnostic value of microRNA for Alzheimer's disease: a systematic review and meta-analysis. *Front Aging Neurosci* 8:13
- Zi Y et al. (2015) Circulating MicroRNA as potential source for neurodegenerative diseases biomarkers. *Mol Neurobiol* 52(3):1494–1503
- Tan L, Yu JT, Hu N, Non-coding RNA (2013) In Alzheimer's disease. *Mol Neurobiol* 47(1):382–393
- Sorensen SS, Nygaard AB, Christensen T (2016) miRNA expression profiles in cerebrospinal fluid and blood of patients with

- Alzheimer's disease and other types of dementia—an exploratory study. *Transl Neurodegener* 5:6
50. Muller M et al. (2016) Validation of microRNAs in cerebrospinal fluid as biomarkers for different forms of dementia in a multicenter study. *J Alzheimers Dis* 52(4):1321–1333
 51. Danborg PB et al. (2014) The potential of microRNAs as biofluid markers of neurodegenerative diseases—a systematic review. *Biomarkers* 19(4):259–268
 52. Leidinger P et al. (2013) A blood based 12-miRNA signature of Alzheimer disease patients. *Genome Biol* 14(7):R78
 53. Sheinerman KS, Umansky SR (2013) Circulating cell-free microRNA as biomarkers for screening, diagnosis and monitoring of neurodegenerative diseases and other neurologic pathologies. *Front Cell Neurosci* 7:150
 54. Moldovan L et al. (2014) Methodological challenges in utilizing miRNAs as circulating biomarkers. *J Cell Mol Med* 18(3):371–390
 55. Alcolea D et al. (2014) Relationship between beta-secretase, inflammation and core cerebrospinal fluid biomarkers for Alzheimer's disease. *J Alzheimers Dis* 42(1):157–167
 56. Alcolea D et al. (2015) Amyloid precursor protein metabolism and inflammation markers in preclinical Alzheimer disease. *Neurology* 85(7):626–633
 57. Antonell A et al. (2011) Different profiles of Alzheimer's disease cerebrospinal fluid biomarkers in controls and subjects with subjective memory complaints. *J Neural Transm (Vienna)* 118(2):259–262
 58. Zivelin A et al. (1997) Improved method for genotyping apolipoprotein E polymorphisms by a PCR-based assay simultaneously utilizing two distinct restriction enzymes. *Clin Chem* 43(9):1657–1659
 59. Hughes AJ et al. (1992) Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases. *J Neurol Neurosurg Psychiatry* 55(3):181–184
 60. Kroh EM et al. (2010) Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). *Methods* 50(4):298–301
 61. Mitchell PS et al. (2008) Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A* 105(30):10513–10518
 62. Stephan C et al. (2003) Comparison of eight computer programs for receiver-operating characteristic analysis. *Clin Chem* 49(3):433–439
 63. Fan J, Upadhye S, Worster A (2006) Understanding receiver operating characteristic (ROC) curves. *CJEM* 8(1):19–20
 64. Dubois B et al. (2010) Revising the definition of Alzheimer's disease: a new lexicon. *Lancet Neurol* 9(11):1118–1127
 65. Rohn TT et al. (2008) Lack of pathology in a triple transgenic mouse model of Alzheimer's disease after overexpression of the anti-apoptotic protein Bcl-2. *J Neurosci* 28(12):3051–3059
 66. Li X et al. (2011) Circulatory miR34a as an RNAbased, noninvasive biomarker for brain aging. *Aging (Albany NY)* 3(10):985–1002
 67. Wang X et al. (2009) miR-34a, a microRNA up-regulated in a double transgenic mouse model of Alzheimer's disease, inhibits bcl2 translation. *Brain Res Bull* 80(4–5):268–273
 68. Ferreira E et al. (2007) Bcl-2 overexpression protects against amyloid-beta and prion toxicity in GT1-7 neural cells. *J Alzheimers Dis* 12(3):223–228
 69. Tiberio P et al. (2015) Challenges in using circulating miRNAs as cancer biomarkers. *Biomed Res Int* 2015:731479
 70. McDonald JS et al. (2011) Analysis of circulating microRNA: preanalytical and analytical challenges. *Clin Chem* 57(6):833–840
 71. Wang K et al. (2012) Comparing the MicroRNA spectrum between serum and plasma. *PLoS One* 7(7):e41561
 72. Cheng HH et al. (2013) Plasma processing conditions substantially influence circulating microRNA biomarker levels. *PLoS One* 8(6):e64795
 73. Cogswell JP et al. (2008) Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways. *J Alzheimers Dis* 14(1):27–41
 74. Sarkar S et al. (2016) Expression of microRNA-34a in Alzheimer's disease brain targets genes linked to synaptic plasticity, energy metabolism, and resting state network activity. *Brain Res* 1646:139–151
 75. Yamakuchi M, Ferlito M, Lowenstein CJ (2008) miR-34a repression of SIRT1 regulates apoptosis. *Proc Natl Acad Sci U S A* 105(36):13421–13426
 76. Julien C et al. (2009) Sirtuin 1 reduction parallels the accumulation of tau in Alzheimer disease. *J Neuropathol Exp Neurol* 68(1):48–58
 77. Ng F, Wijaya L, Tang BL (2015) SIRT1 in the brain—connections with aging-associated disorders and lifespan. *Front Cell Neurosci* 9:64
 78. Pallas M et al. (2008) Modulation of SIRT1 expression in different neurodegenerative models and human pathologies. *Neuroscience* 154(4):1388–1397
 79. Kaarniranta K et al. (2011) Age-related macular degeneration (AMD): Alzheimer's disease in the eye? *J Alzheimers Dis* 24(4):615–631
 80. Hill JM, Pogue AI, Lukiw WJ (2015) Pathogenic microRNAs common to brain and retinal degeneration; recent observations in Alzheimer's disease and age-related macular degeneration. *Front Neurol* 6:232
 81. Hsu SD et al. (2014) miRTarBase update 2014: an information resource for experimentally validated miRNA-target interactions. *Nucleic Acids Res* 42(Database issue):D78–D85
 82. Martiskainen H et al. (2013) Targeting ApoE4/ApoE receptor LRP1 in Alzheimer's disease. *Expert Opin Ther Targets* 17(7):781–794
 83. Donahue JE et al. (2006) RAGE, LRP-1, and amyloid-beta protein in Alzheimer's disease. *Acta Neuropathol* 112(4):405–415
 84. Sze CI et al. (1997) Loss of the presynaptic vesicle protein synaptophysin in hippocampus correlates with cognitive decline in Alzheimer disease. *J Neuropathol Exp Neurol* 56(8):933–944
 85. Yuki D et al. (2014) DHA-PC and PSD-95 decrease after loss of synaptophysin and before neuronal loss in patients with Alzheimer's disease. *Sci Rep* 4:7130
 86. Glatt SJ et al. (2005) Comparative gene expression analysis of blood and brain provides concurrent validation of SELENBP1 up-regulation in schizophrenia. *Proc Natl Acad Sci U S A* 102(43):15533–15538
 87. Ma X et al. (2014) Expression, regulation and function of microRNAs in multiple sclerosis. *Int J Med Sci* 11(8):810–818
 88. van Harten AC et al. (2013) Cerebrospinal fluid Abeta42 is the best predictor of clinical progression in patients with subjective complaints. *Alzheimers Dement* 9(5):481–487
 89. van Harten AC et al. (2015) Differential expression of microRNA in cerebrospinal fluid as a potential novel biomarker for Alzheimer's disease. *J Alzheimers Dis* 47(1):243–252
 90. Kester MI et al. (2009) CSF biomarkers in Alzheimer's disease and controls: associations with APOE genotype are modified by age. *J Alzheimers Dis* 16(3):601–607
 91. Humpel C (2011) Identifying and validating biomarkers for Alzheimer's disease. *Trends Biotechnol* 29(1):26–32
 92. Moul JW et al. (2007) Age adjusted prostate specific antigen and prostate specific antigen velocity cut points in prostate cancer screening. *J Urol* 177(2):499–503 discussion 503–4
 93. Hollins SL, Cairns MJ (2016) MicroRNA: small RNA mediators of the brains genomic response to environmental stress. *Prog Neurobiol* 143:61–81
 94. Codocedo JF, Inestrosa NC (2016) Environmental control of microRNAs in the nervous system: implications in plasticity and behavior. *Neurosci Biobehav Rev* 60:121–138
 95. Flowers E, Won GY, Fukuoka Y (2015) MicroRNAs associated with exercise and diet: a systematic review. *Physiol Genomics* 47(1):1–11
 96. Xu T et al. (2015) Circulating microRNAs in response to exercise. *Scand J Med Sci Sports* 25(2):e149–e154

97. Zacharewicz E, Lamon S, Russell AP (2013) MicroRNAs in skeletal muscle and their regulation with exercise, ageing, and disease. *Front Physiol* 4:266
98. Palmer JD et al. (2014) MicroRNA expression altered by diet: can food be medicinal? *Ageing Res Rev* 17:16–24
99. Vanderstichele H et al. (2012) Standardization of preanalytical aspects of cerebrospinal fluid biomarker testing for Alzheimer's disease diagnosis: a consensus paper from the Alzheimer's biomarkers standardization initiative. *Alzheimers Dement* 8(1):65–73