Journal of Alzheimer's Disease 99 (2024) 1317–1331
DOI 10.3233/JAD-230572
IOS Press

Extracellular Vesicle-Enriched miRNA-Biomarkers Show Improved Utility for Detecting Alzheimer's Disease Dementia and Medial Temporal Atrophy

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Accepted 11 April 2024
Pre-press 21 May 2024

Abstract.

Background: Emerging diagnostic modalities suggest that miRNA profiles within extracellular vesicles (EVs) isolated from peripheral blood specimens may provide a non-invasive diagnostic alternative for dementia and neurodegenerative disorders. Given that EVs confer a protective environment against miRNA enzymatic degradation, the miRNAs enriched in the EV fraction of blood samples could serve as more stable and clinically relevant biomarkers compared to those obtained from serum.

Objective: To compare miRNAs isolated from EVs versus serum in blood taken from Alzheimer's disease (AD) dementia patients and control cohorts.

Methods: We compared 25 AD patients to 34 individuals who exhibited no cognitive impairments (NCI). Subjects were Singapore residents with Chinese heritage. miRNAs purified from serum versus blood-derived EVs were analyzed for associations with AD dementia and medial temporal atrophy detected by magnetic resonance imaging.

Results: Compared to serum-miRNAs, we identified almost twice as many EV-miRNAs associated with AD dementia, and they also correlated more significantly with medial temporal atrophy, a neuroimaging marker of AD-brain pathology.

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We further developed combination panels of serum-miRNAs and EV-miRNAs with improved performance in identifying AD dementia. Dominant in both panels was miRNA-1290.

Conclusions: This data indicates that miRNA profiling from EVs offers diagnostic superiority. This underscores the role of EVs as vectors harboring prognostic biomarkers for neurodegenerative disorders and suggests their potential in yielding novel biomarkers for AD diagnosis.

Keywords: Alzheimer's disease, blood biomarker, dementia, diagnosis, extracellular vesicle, medial temporal atrophy, microRNA

INTRODUCTION

Due to shifts in population age distributions to older ages, dementias like Alzheimer's disease (AD) are becoming major health issues. To date, only one drug has been approved, albeit controversially, as a disease-modifying treatment for AD [1]. One of the major challenges in dementia research is the ability to provide accurate diagnoses in premortem settings, especially before clinical symptoms are evident. Advances in positron emission tomography (PET) for amyloid and tau protein, magnetic resonance imaging (MRI) for hippocampal atrophy, and cerebrospinal fluid tests for phospho-tau and amyloid, have made *in vivo* detection of AD brain pathology possible [2–4], but the high costs and invasiveness of these procedures have limited their use in clinical settings. Consequently, biomarkers derived from blood, which offer the advantages of reduced invasiveness and lower costs, have garnered significant interest for their potential roles in understanding the pathogenesis, prognosis, and diagnosis of this disease [5].

MicroRNAs (miRNA), a class of non-coding RNA, have recently been recognized as a novel disease biomarker species due to their characteristics of tissue- and disease-specific expression [6]. Notably, miRNA incorporation into extracellular vesicles (EVs) increases miRNA stability, as the lipid bilayer of EV membranes protects the contents from degradation by nucleases and proteases [7]. Experimental studies have reported that EV-bound extracellular miRNAs are resistant to conditions of severe cellular stress, such as high temperatures, repeated freeze-thaw cycles [8], extreme pH levels [9], boiling and extended storage time [10–12]. The high stability of miRNAs within the EVs means that they have long half-lives in biological fluids [13]. As such, they are attractive as potential diagnostic [14] and prognostic biomarkers [8].

Numerous studies investigating blood expression levels of EV-derived miRNAs in AD patients have been performed in the past decade. Notably, associations of AD with hsa-miR-93-5p, hsa-miR-23a-3p, hsa-miR-3065-5p, hsa-miR-424-5p, hsa-miR-1306-5p, hsa-miR-15b-3p, hsa-miR-342-3p, and hsa-miR-193b have been replicated in independent cohorts [15–24]. Besides being promising in identifying AD, several EV-derived miRNAs have also displayed putative associations with cognitive and functional outcomes [15, 23]. Interestingly, one study reported a significant correlation between EV-derived miR193b and the 42-amino acid beta-amyloid (Aβ42) in cerebrospinal fluid, thus linking EV-derived miRNAs and AD pathology [19].

It remains unknown if EV-derived miRNAs provide diagnostic advantages over serum-miRNAs. We therefore compared EV versus serum miRNAs of AD patients.

MATERIALS AND METHODS

Study cohort

Subjects for this study were selected from a previously described longitudinal case-control study performed on a memory clinic cohort in Singapore, recruited between August 2010 and March 2014. Briefly, all subjects underwent neuroimaging and physical, clinical, and neuropsychological tests at the National University of Singapore. A comprehensive neuropsychological battery of tests was performed to assess seven cognitive domains: Executive Function, Attention, Language, Visuomotor Speed, Visuoconstruction, Verbal Memory, and Visual Memory (see Supplementary Table 1 for details on the component tests for each domain). Diagnoses were made at weekly consensus meetings attended by neuropsychologists and clinicians, at which psychometrics, clinical features, and neuroimaging were evaluated.

AD dementia cases included in this study were recruited from memory clinics from National University Hospital and St Luke's Hospital in Singapore, and diagnosed using the National Institute of Neuro-

logical and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association (NINCDS-ADRDA) clinical criteria [25]. Subjects diagnosed with non-AD etiologies defined by the National Institute of Neurological Disorders and Stroke and Association Internationale pour la Recherche´ et l' Enseignement en Neurosciences (NINDS-AIREN) criteria, such as vascular dementia, were excluded from this study to rule out the possible confounding effects. Concurrently, controls were recruited from both the community and memory clinics and were defined as those who were cognitively normal on an objective neuropsychological assessment—also labelled as having no cognitive impairment (NCI) and being functionally independent. Ethics approval for the current study was obtained from the National Healthcare Group Domain-Specific Review Board (DSRB reference: 2010/00017; study protocol number: DEM4233). Informed consent was obtained from all participants before study recruitment and assessment procedures. The study was conducted in accordance with the Declaration of Helsinki.

Neuroimaging assessment for brain atrophy

MRI scans were performed on a 3-Tesla Siemens Magnetom Trio Tim scanner, using a 32-channel head coil, at the Clinical Imaging Research Centre of the National University of Singapore. Subjects with claustrophobia, contraindications for MRI, or those who were unable to tolerate the procedure were excluded. All MRIs were graded by one radiologist and two clinicians blinded to the neuropsychological and clinical data. Brain atrophy was assessed on the MRI scans, with medial temporal lobe atrophy defined by the widening of the choroid fissure, the widening of temporal horn, and the loss of hippocampal height as seen on coronal sections. The severity of medial temporal atrophy was graded using the 5-point Scheltens' scale (0 – normal, 1 – mild, 2 – mild-moderate, 3 – moderate, 4 – severe) [26]. We used a dichotomized scale in all our analyses, with a score of ≥ 2 defined as significant medial temporal atrophy and a score of < 2 considered to be non-significant, as previously described [27, 28].

Assessments of other risk factors

Risk factors such as hypertension, hyperlipidemia, diabetes, and cardiovascular diseases were ascertained from both clinical interviews and medical records and were classified as present or absent. Hypertension was defined as systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg, or use of antihypertensive medications. Diabetes was defined as glycated hemoglobin $\geq 6.5\%$ or on corresponding medications. Hyperlipidemia was defined as total cholesterol levels ≥ 4.14 mM or on corresponding medications. Cardiovascular disease was classified as a previous history of atrial fibrillation, congestive heart failure, and myocardial infarction. Education status was categorized as low (not exceeding primary school education) or high (beyond primary school education). Apolipoprotein E (*APOE*) genotyping was performed as described previously [29], and *APOE* $\varepsilon 4$ carrier status was defined as having at least one $\varepsilon 4$ allele.

Blood sample preparation

Blood samples collected in ethylenediaminetetraacetic acid (EDTA) and serum-separating tubes (SST) were centrifuged at 2000 g for 10 min at 4°C. The upper layer of plasma (from EDTA tubes) and serum (from SST) were transferred to a new tube, mixed, aliquoted, and stored at –80°C until future use. All samples were subjected to only one freeze-and-thaw cycle.

EV isolation and characterization

We followed the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines of the International Society for Extracellular Vesicles (ISEV) [30] for the isolation, quantification, and characterization of EVs. EVs were isolated from EDTA plasma with 70 nm qEVsingle columns (IZON Science, Christchurch, New Zealand). 200 μl EDTA plasma followed by 0. 2μm-filtered 1x Tris-buffered saline were loaded onto the column. 16 fractions of 250 μl were collected. Fractions 4–8 were combined in a 100k Amicon filter and concentrated to 200 μl by centrifugation (4000 g, 15 min, 4°C). Isolated EVs were stored at –80°C until RNA extraction.

EV quantification and size characterization were performed via nanoparticle trafficking analysis (NTA, Nanosight). RNA and protein concentrations were measured using Nanodrop. To demonstrate the EV identity of our isolated particles, we utilized western blot analysis (30 μg/lane) of the classical EV markers: mouse anti-Alix (1 : 200, sc-53538, Santa Cruz), mouse anti-Hsp70 (1 : 1000, sc-32239,

Santa Cruz), rabbit anti-CD81 (1 : 1000, ExoAB-CD81A-1, Systems Biosciences), rabbit anti-CD9 (1 : 1000, ExoAB-CD9A-1, Systems Biosciences), rabbit anti-GM130 (1 : 1000, ab52649, Abcam), and goat anti-albumin (1 : 1000, NB600-41532, Novus Biologicals). Collected and combined fractions used for EV analysis (F4-F8) contained most of the particles detected by NTA, the bulk of the RNA molecules, and little protein contamination. In accordance with the MISEV guidelines, detailed characterizations of the extracted EVs were performed as shown in Supplementary Figures 1 and 2. We detected classical EV markers in all fractions analyzed with little albumin and Golgi matrix protein (GM130) contamination (Supplementary Figure 1), indicating successful EV separation.

Isolation, augmentation, and quantification of miRNAs

In the present study, "serum-miRNAs" refer to the miRNAs isolated from serum samples, while "EV-miRNAs" refer to the miRNAs isolated from plasma-derived EVs. All reagents and assay kits used for RNA isolation, augmentation, and quantification were from MiRXES, Singapore, unless otherwise stated.

Total RNAs were extracted from patient-derived serum and patient-derived EVs (200 μl each). The miRNeasy Serum/Plasma kit (Qiagen; Hilden, Germany) on a semi-automated QiaCube system was used for the serum, while the Maxwell RSC miRNA Tissue Kit and the Maxwell RSC System (Promega Corp., Wisconsin, USA) were used for the isolated EVs. A combination of synthetic control RNAs with sequences distinct from annotated mature human miRNAs (miRBase versions 21 and 22) and inter-plate calibrators were included in the assay panels to monitor and normalize workflow efficiency.

Isolated total RNA was reverse transcribed on the ID3EAL cDNA Synthesis System using conformational restricted miRNA-specific reverse transcription (RT) primers that were selected based on experimental analysis of more than 1000 high confidence human miRNAs from several hundred serum and plasma specimens. The reaction mixture was incubated at 42°C for 30 min, followed by 90°C for 5 min, to inactivate the reverse transcriptase on a Veriti™ Thermal Cycler (Applied Biosystems; California, United States). cDNAs were further augmented using the MiRXES ID3EAL miRNA Augmentation System.

For serum-miRNA quantifications, qPCR amplification and detection were performed on ViiA7 qPCR system (Applied Biosystems; California, USA) with the following cycling conditions: 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 30 s. In contrast, qPCR amplification and detection were performed on the QuantStudio5-384-well qPCR system (Applied Biosystems, California, USA) for the quantification of EV-miRNAs. Raw Ct values were calculated using the ViiA 7 RUO software and the QuantStudio Design and Analysis software, respectively, with an automatic baseline setting and a threshold of 0.5. Technical variations introduced during RNA isolation and the process of RT-qPCR were normalized using the spike-in control RNAs and inter-plate calibrators. The gene expression levels of each serum and EV-miRNAs were expressed in arbitrary units by subtracting raw Ct numbers from 40.

Statistical analysis of demographic data

Our statistical analysis of demographic information (Table 1) was performed using SPSS software (version 21, IBM Inc.; New York, USA). Kolmogorov-Smirnov tests were used to test for normality. The *t*-test was used for two-group comparisons of means of variables that could be assumed to be approximately normally distributed. For comparisons of mean ranks of distributions not assumed to be normal (Mini-Mental State Exam, MMSE score), the Mann-Whitney U Test was used. Finally, to test for differences in proportions of categorical variables, the Chi-square test was used. p value < 0.05 was considered statistically significant for all analyses.

Statistical analysis of miRNA data

Our statistical analysis for miRNA data was performed as follows. To identify potential miRNA biomarkers, two-tailed t-tests were performed to compare each serum and EV-miRNA gene expression levels (i.e., 40 minus Ct number) between AD and control groups. Mean difference (i.e., average Ct in AD group minus average Ct in control group) and significance (i.e., -Log10 of p-value obtained from corresponding t-test) of each miRNA expression level between AD and control groups were expressed in volcano plots. miRNAs with $p < 0.05$ were selected for further investigation. Logistic regression was used to form odds ratios (OR) and 95% confidence intervals (CI) of each miRNA included in the model; the

Table 1
Baseline demographics of cases and controls ($n = 59$)

	NCI ($n = 34$)	AD ($n = 25$)	p
Age, y, mean (SD)	69.9 (5.2)	73.5 (5.8)	**0.015**[a]
Female, *n* (%)	20 (58.8)	16 (64.0)	0.687[b]
Education, year, mean (SD)	10.1 (4.7)	4.8 (5.1)	**<0.001**[a]
APOE ε4 carrier, *n* (%)	8 (23.5)	12 (48.0)	**0.05**[b]
Hypertension, *n* (%)	15 (44.1)	19 (76.0)	**0.014**[b]
Diabetes, *n* (%)	7 (20.6)	12 (48.0)	**0.026**[b]
Hyperlipidemia, *n* (%)	16 (47.1)	17 (68.0)	0.109[b]
Cardiovascular disease, *n* (%)	1 (2.9)	2 (8.0)	0.382[b]
MMSE, mean (SD)	27.7 (2.1)	18.1 (4.3)	**<0.001**[c]

Pairwise comparison was performed using [a]two-tailed *t*-test, [b]Pearson's Chi-square test, or [c]Mann-Whitney U test. Bold values indicate $p < 0.05$. NCI, non-cognitive impairment; AD, Alzheimer's disease; SD, standard deviation; n, number; *APOE* ε4, apolipoprotein E ε4 allele.

model's output is AD (1) versus control (0). Univariate models without a covariate were assessed. To identify a combination of miRNAs associated with AD, miRNAs that were found to be significantly associated with AD at the univariate level were included in a forward stepwise multiple logistic regression analysis; if adding each new variable to the model lowered the model's Akaike information criterion (AIC) value, a likelihood ratio significance level of 0.10 was used to determine if any variables should be removed from the model. Unless stated otherwise, calculations were performed using R statistical software (version 3.6.3, The R Foundation).

Receiver operating characteristic (ROC) curve analyses of our fitted logistic regression model were performed using the R package *pROC* [31]. ROC area under curves (AUC) and their 95% CIs were computed using DeLong's method [32]. Sensitivity and specificity were calculated via Youden index thresholds using bootstrap procedures with 2,000 iterations [33].

In the final miRNA model, age, education, *APOE4*, hypertension, and diabetes were added as covariates, as these variables were not matched between groups (see Table 1). The same statistical approach (adjusted for age, gender, education, and *APOE4*, but not hypertension or diabetes) was then used to form a model of the occurrence of medial temporal brain atrophy.

miRNA-target enrichment and Gene Ontology (GO) analysis

To predict the potential biological relevance of the miRNAs that are part of the combination panels identified using stepwise multiple regression models shown in Fig. 3, we first confirmed their expression in human brain tissue utilizing a human miRNA tissue atlas published by Ludwig and colleagues in 2016 [34]. We then performed miRNA-target enrichment analyses of each combination panel via miRTarBase 9.0, utilizing only experimentally validated targets [35]. We then performed Gene Ontology analysis [36, 37] of the identified targets via the web-based tool Enrichr [38–40]. The top ten significantly enriched GO annotations with p-value<0.05 were exported from Enrichr and plotted in Prism Graphpad. False Discovery Rates (FDR) were calculated at 5% and 10%.

RESULTS

Baseline demographics of study cohort

Of 59 subjects in our study, 34 (57.6%) had NCI and 25 (42.43%) had AD dementia. AD patients were, on average, three years older, had less education, higher APOE ε4 carrier prevalence, and more hypertension and diabetes. As expected, compared to NCI, AD patients also scored significantly lower in MMSE (Table 1).

EV-miRNAs show stronger associations with AD than serum-miRNAs

Of 291 serum-miRNAs detected, 25 were significantly altered in AD versus NCI patients ($p < 0.05$, two-tailed t-test; Fig. 1B, red dots). Univariate logistic regression analyses demonstrated that 20 serum-miRNAs were significantly associated with AD ($p < 0.05$, univariate logistic regression; Fig. 2A, blue bars). After complete covariate adjustment using multiple regression analyses, none were of statistical significance ($p > 0.05$, multiple logistic regression; Fig. 2A).

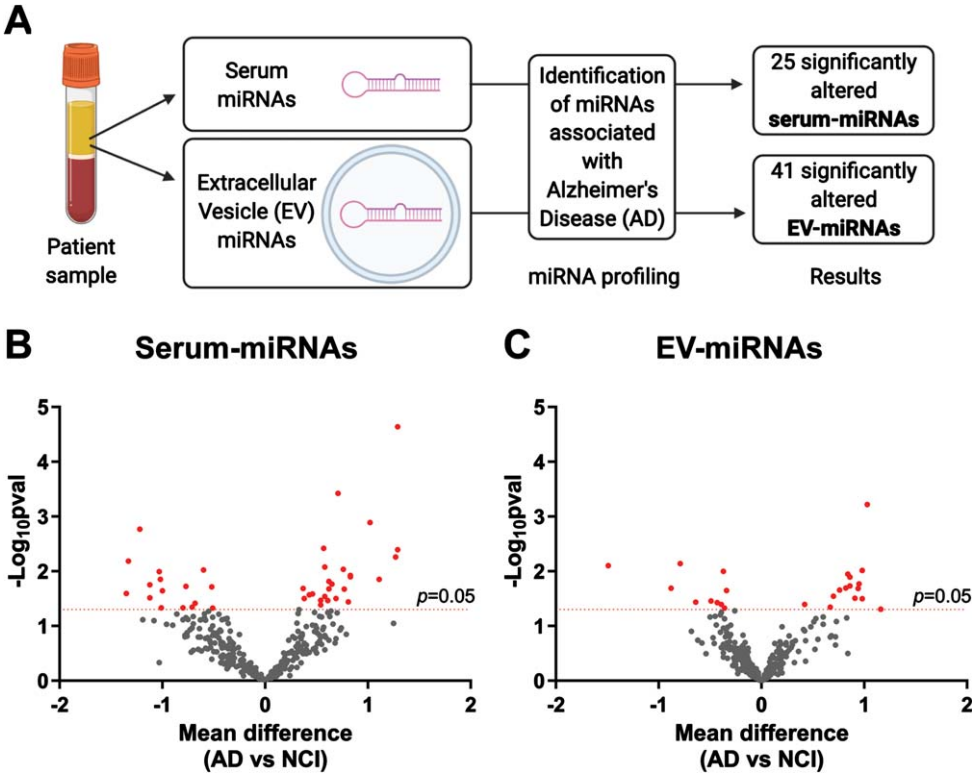


Fig. 1. Profiling of serum-miRNAs and EV-miRNAs in AD. (A) Summary of study design and main results. Volcano plots showing the differential expression of (B) serum-miRNAs and (C) EV-miRNAs in AD patients versus NCI controls. The x-axis shows the mean difference of each miRNA expression level between AD patients and NCI controls and the y-axis shows -log10 of *p*-values obtained from corresponding *t*-tests. Each dot represents miRNAs detectable in the EV ($n = 352$) and serum ($n = 291$) sample pools. Red dots indicate a significant mean difference of $n = 25$ serum-miRNAs and $n = 41$ EV-miRNAs in AD compared to NCI controls ($p < 0.05$, two-tailed *t*-tests).

Of 322 EV-miRNAs detected, 41 were significantly altered in the AD versus NCI patients ($p < 0.05$, two-tailed *t*-test; Fig. 1C, red dots). Using univariate logistic regression analyses we identified 34 EV-miRNAs associated with AD ($p < 0.05$, univariate logistic regression; Fig. 2B, red bars), of which seven miRNAs retained statistical significance after complete covariate adjustment ($p < 0.05$, multiple logistic regression; Fig. 2B, asterisks).

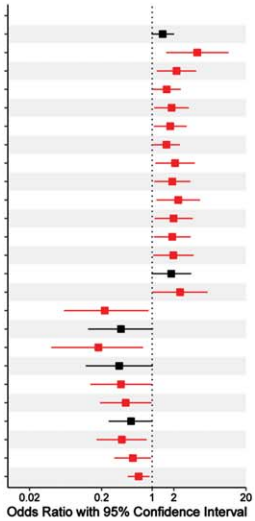
EV-miRNAs outcompete serum-miRNAs utilizing miRNA combination panels

Four serum-miRNAs were identified to be significantly associated with AD in the stepwise multiple regression model, namely hsa-miR-431-3p [Odds Ratio (OR) = 18.4; 95% Confidence Interval (CI)], hsa-miR-1290 (OR = 39.8; 95% CI = 2.6 to 603.4), hsa-miR-330-5p (OR = 0.13; 95% CI = 0.03 to 0.58) and hsa-miR-181a-2-3p (OR = 0.02; 95% CI = 0.0004 to 0.8) (Table 2). The combination panel had better model fitting with a lower AIC value of 30.0 compared to that of the individual miRNAs (individual AIC = 57.8, 64.4, 64.6 and 63.8, respectively; $p < 0.05$, likelihood ratio test). ROC AUCs (Fig. 3A) were 0.79, 0.70, 0.70 and 0.68 for individual miRNAs and 0.97 for all miRNAs combined. Comparing AD to NCI, hsa-miR-431-3p and hsa-miR-1290 were upregulated and hsa-miR-181a-2-3p and hsa-miR-330-5p were downregulated (Fig. 3C).

Two EV-miRNAs were significantly associated with AD in the stepwise multiple regression model: hsa-miR-1290 (OR = 1.17e4; 95% CI = 0.89 to 1.54e8) and hsa-miR-128-3p (OR = 1.77e4; 95% CI = 1.34 to 2.34e6) (Table 2). The combination panel had better model fitting with a lower AIC value of 14.0 compared to those of the individual miRNAs (individual AIC = 24.8 and 39.8, respectively; $p < 0.05$, likelihood ratio test). While the ROC of hsa-miR-1290 alone achieved an AUC of

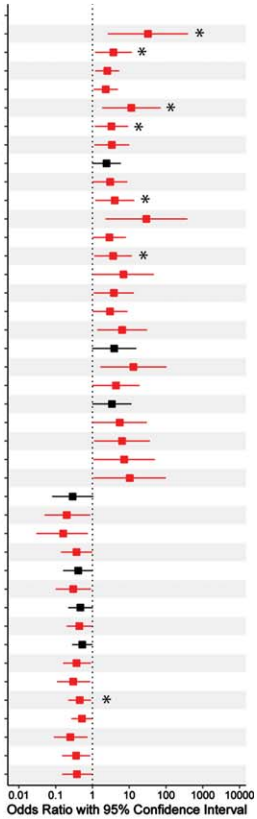
A) Association between serum-miRNAs and AD

miRNA	Mean Difference	AUC (95% CI)	Unadj. OR (95% CI)	Adj. OR (95% CI)
miR−650	1.16	0.65 (0.50 − 0.81)	1.40 (0.98 − 2.01)	1.09 (0.74 − 1.60)
miR−431−3p	1.03	0.79 (0.66 − 0.91)	4.22 (1.57 − 11.4)	2.43 (0.88 − 6.69)
miR−1825	0.98	0.71 (0.56 − 0.86)	2.18 (1.16 − 4.08)	1.38 (0.70 − 2.74)
miR−770−5p	0.98	0.68 (0.53 − 0.83)	1.59 (1.01 − 2.49)	1.19 (0.73 − 1.93)
miR−616−3p	0.95	0.69 (0.54 − 0.84)	1.86 (1.07 − 3.21)	1.20 (0.66 − 2.15)
miR−596	0.94	0.69 (0.54 − 0.84)	1.78 (1.06 − 3.00)	1.29 (0.73 − 2.30)
miR−200c−5p	0.91	0.69 (0.54 − 0.84)	1.58 (1.03 − 2.42)	1.11 (0.68 − 1.83)
miR−1207−5p	0.86	0.70 (0.55 − 0.85)	2.08 (1.11 − 3.89)	1.44 (0.72 − 2.90)
miR−365b−5p	0.86	0.68 (0.53 − 0.83)	1.90 (1.07 − 3.38)	1.40 (0.72 − 2.71)
miR−1290	0.84	0.70 (0.55 − 0.84)	2.30 (1.15 − 4.60)	1.56 (0.70 − 3.48)
miR−34b−3p	0.82	0.69 (0.53 − 0.84)	1.98 (1.08 − 3.63)	1.33 (0.66 − 2.70)
miR−518e−5p	0.76	0.68 (0.53 − 0.83)	1.90 (1.06 − 3.41)	1.87 (0.88 − 3.94)
miR−346	0.7	0.66 (0.51 − 0.82)	1.97 (1.04 − 3.75)	1.30 (0.64 − 2.61)
miR−620	0.67	0.66 (0.51 − 0.82)	1.84 (0.98 − 3.48)	1.23 (0.61 − 2.46)
miR−25−5p	0.42	0.66 (0.50 − 0.82)	2.44 (1.01 − 5.86)	1.84 (0.62 − 5.44)
miR−29c−3p	−0.34	0.72 (0.57 − 0.87)	0.22 (0.06 − 0.89)	0.32 (0.06 − 1.72)
miR−660−5p	−0.36	0.68 (0.52 − 0.83)	0.37 (0.13 − 1.03)	0.58 (0.17 − 1.96)
miR−181a−5p	−0.37	0.72 (0.56 − 0.87)	0.18 (0.04 − 0.75)	0.13 (0.02 − 0.98)
miR−362−3p	−0.39	0.67 (0.52 − 0.83)	0.35 (0.12 − 1.02)	0.80 (0.25 − 2.56)
miR−628−5p	−0.43	0.64 (0.48 − 0.80)	0.37 (0.14 − 0.99)	0.46 (0.14 − 1.50)
miR−29b−3p	−0.49	0.67 (0.52 − 0.83)	0.43 (0.19 − 0.98)	0.33 (0.09 − 1.22)
miR−9−3p	−0.64	0.62 (0.45 − 0.78)	0.51 (0.25 − 1.01)	0.67 (0.30 − 1.49)
miR−181a−2−3p	−0.8	0.68 (0.53 − 0.83)	0.38 (0.17 − 0.84)	0.36 (0.12 − 1.08)
miR−342−5p	−0.89	0.71 (0.56 − 0.86)	0.54 (0.30 − 0.96)	0.48 (0.21 − 1.09)
miR−330−5p	−1.49	0.79 (0.54 − 0.85)	0.65 (0.46 − 0.92)	0.71 (0.46 − 1.11)



B) Association between EV-miRNAs and AD

miRNA	Mean difference	AUC (95% CI)	Unadj. OR (95% CI)	Adj. OR (95% CI)
miR−1290	1.29	0.92 (0.83 − 1)	32.4 (2.63 − 398.6)	6.05 (1.28 − 28.6)
miR−208a−3p	1.29	0.79 (0.63 − 0.96)	3.72 (1.18 − 11.8)	12.9 (1.13 − 147.3)
miR−885−5p	1.27	0.80 (0.63 − 0.97)	2.54 (1.20 − 5.38)	2.14 (0.93 − 4.90)
miR−22−3p	1.11	0.79 (0.59 − 0.98)	2.30 (1.09 − 4.89)	2.15 (0.85 − 5.44)
miR−1246	1.02	0.84 (0.70 − 0.99)	11.4 (1.83 − 71.1)	4.37 (0.98 − 19.4)
miR−193a−5p	0.83	0.79 (0.61 − 0.96)	3.28 (1.16 − 9.31)	5.80 (1.09 − 30.8)
miR−215−5p	0.83	0.75 (0.57 − 0.93)	3.36 (1.13 − 10.0)	6.61 (1.00 − 43.6)
miR−363−3p	0.81	0.71 (0.52 − 0.90)	2.41 (0.96 − 5.90)	2.44 (0.84 − 7.08)
miR−99a−5p	0.77	0.80 (0.61 − 0.98)	3.03 (1.04 − 8.85)	2.28 (0.70 − 7.49)
miR−424−5p	0.76	0.78 (0.61 − 0.96)	4.05 (1.20 − 13.6)	2.97 (0.83 − 10.6)
miR−30a−5p	0.71	0.82 (0.67 − 0.98)	29.3 (2.27 − 376.6)	19.9 (1.16 − 340.4)
miR−92b−3p	0.69	0.72 (0.53 − 0.91)	2.89 (1.03 − 8.12)	4.01 (0.89 − 18.1)
miR−365a−3p	0.65	0.78 (0.60 − 0.96)	3.66 (1.13 − 11.8)	3.06 (0.72 − 13.02)
let−7c−5p	0.62	0.78 (0.61 − 0.95)	6.98 (1.055 − 46.3)	117.6 (1.13 − 1.2e4)
miR−185−5p	0.62	0.71 (0.52 − 0.90)	3.83 (1.09 − 13.4)	8.38 (0.88 − 80.1)
miR−19a−3p	0.61	0.72 (0.53 − 0.90)	3.01 (1.02 − 8.93)	2.64 (0.61 − 11.4)
miR−423−5p	0.58	0.77 (0.60 − 0.94)	6.41 (1.35 − 30.5)	12.6 (0.76 − 206.4)
let−7i−5p	0.58	0.73 (0.54 − 0.92)	3.90 (0.98 − 15.5)	3.75 (0.77 − 18.2)
miR−320d	0.57	0.80 (0.64 − 0.96)	13.0 (1.64 − 103.0)	22.4 (0.69 − 723.0)
miR−1202	0.54	0.77 (0.58 − 0.95)	4.37 (1.00 − 19.0)	2.71 (0.51 − 14.4)
miR−18b−5p	0.54	0.70 (0.50 − 0.90)	3.38 (0.99 − 11.5)	31.7 (0.88 − 1.1e3)
miR−22−5p	0.46	0.74 (0.55 − 0.93)	5.53 (1.02 − 30.1)	4.44 (0.60 − 33.1)
miR−590−5p	0.43	0.72 (0.53 − 0.91)	6.38 (1.12 − 36.5)	4.04 (0.44 − 36.7)
miR−345−5p	0.38	0.71 (0.52 − 0.90)	7.29 (1.07 − 49.7)	7.47 (0.44 − 126.7)
miR−128−3p	0.37	0.77 (0.58 − 0.96)	10.4 (1.08 − 99.8)	3.67 (0.40 − 34.1)
miR−27b−3p	−0.51	0.70 (0.51 − 0.89)	0.29 (0.08 − 1.06)	0.10 (0.01 − 1.14)
miR−423−3p	−0.52	0.74 (0.55 − 0.92)	0.20 (0.05 − 0.87)	0.24 (0.04 − 1.70)
miR−664a−3p	−0.6	0.77 (0.59 − 0.95)	0.16 (0.03 − 0.75)	0.28 (0.04 − 1.96)
miR−99b−5p	−0.68	0.72 (0.53 − 0.91)	0.37 (0.14 − 0.98)	0.21 (0.04 − 1.19)
let−7e−5p	−0.71	0.70 (0.51 − 0.90)	0.41 (0.16 − 1.01)	0.32 (0.08 − 1.19)
miR−26a−5p	−0.77	0.73 (0.55 − 0.92)	0.30 (0.10 − 0.91)	0.29 (0.06 − 1.32)
miR−448	−0.8	0.72 (0.53 − 0.91)	0.47 (0.22 − 1.02)	0.50 (0.18 − 1.39)
miR−181a−3p	−1	0.72 (0.53 − 0.91)	0.44 (0.20 − 0.95)	0.40 (0.15 − 1.05)
miR−199a−5p	−1.01	0.71 (0.51 − 0.90)	0.53 (0.28 − 1.01)	0.12 (0.01 − 1.13)
miR−190a−5p	−1.02	0.75 (0.57 − 0.93)	0.37 (0.16 − 0.90)	0.51 (0.17 − 1.54)
let−7f−5p	−1.03	0.79 (0.62 − 0.95)	0.30 (0.11 − 0.87)	0.50 (0.15 − 1.63)
miR−136−5p	−1.12	0.76 (0.58 − 0.94)	0.45 (0.22 − 0.91)	0.33 (0.11 − 0.98)
miR−124−3p	−1.12	0.68 (0.48 − 0.88)	0.52 (0.27 − 0.99)	0.41 (0.16 − 1.05)
miR−142−3p	−1.22	0.82 (0.66 − 0.97)	0.25 (0.09 − 0.73)	0.27 (0.07 − 1.07)
miR−339−5p	−1.33	0.77 (0.59 − 0.94)	0.36 (0.15 − 0.86)	0.46 (0.19 − 1.13)
miR−539−5p	−1.35	0.75 (0.57 − 0.93)	0.38 (0.15 − 0.95)	0.37 (0.12 − 1.12)



Significantly changed in patients with AD compared to those with NCI (two-tailed t-test, p<0.05)
Significantly associated with AD before covariate adjustment (univariate logistic regression, p<0.05)
* Significantly associated with AD after covariate adjustment (multiple logistic regression, p<0.05)

Fig. 2. Associations of serum-miRNAs and EV-miRNAs with AD. Forest plots showing univariate logistic regression analyses of (A) 25 serum-miRNAs and (B) 41 EV-miRNAs that were significantly altered in AD. Shown are odds ratio (OR) associations between miRNAs and AD and their 95% confidence intervals (CIs). Red lines indicate significant association before covariate adjustment ($p < 0.05$, univariate binary logistic regression), while asterisks indicate significant association after covariate adjustment for age, education and APOE $\varepsilon 4$, hypertension, and diabetes ($p < 0.05$, multiple binary logistic regression).

Table 2
Diagnostic performance of the individual and combined miRNAs panels identified by stepwise regression models in distinguishing AD from NCI ($n = 59$)

Sample	Panel	miRNA	OR (95% CI)	AIC	ROC analysis		
					AUC (95% CI)	Sensitivity % (95% CI)	Specificity % (95% CI)
Serum	Individual[a]	miR-431-3p	**4.22 (1.57–11.38)**	57.8	0.79 (0.66–0.91)	75.0 (45.8–100)	84.0 (40.0–100)
		miR-1290	**2.30 (1.15–4.60)**	64.4	0.70 (0.55–0.84)	70.8 (29.2–100)	72.0 (28.0–100)
		miR-330-5p	**0.65 (0.46–0.92)**	64.6	0.70 (0.54–0.85)	95.8 (50.0–100)	52.0 (32.0–88.0)
		miR-181a-2-3p	**0.38 (0.17–0.84)**	63.8	0.68 (0.53–0.83)	45.8 (25.0–100)	100 (32.0–100)
	Combined[b]	miR-431-3p	**18.4 (1.24–275.0)**	30.0	0.97 (0.94–100)	100 (91.6–100)	92.0 (76.0–100)
		miR-1290	**39.8 (2.6–603.4)**				
		miR-330-5p	**0.13 (0.03–0.58)**				
		miR-181a-2-3p	**0.02 (0.0004–0.8)**				
EV	Individual[a]	miR-1290	**32.4 (2.63–398.5)**	24.8	0.92 (0.83–100)	92.9 (64.3–100)	93.8 (62.5–100)
		miR-128-3p	**10.4 (1.08–99.8)**	39.8	0.77 (0.58–0.96)	100 (85.7–100)	68.8 (43.8–87.5)
	Combined[b]	miR-1290	1.17e4 (0.89–1.54e8)	14.0	0.98 (0.94–100)	92.9 (78.6–100)	100 (87.5–100)
		miR-128-3p	**1.77e4 (1.34–2.34e6)**				

Associations analyses were performed using [a]univariate or [b]stepwise multiple logistic regression models. Bold values indicate $p < 0.05$ using the respective binary logistic regression models. NCI, non-cognitive impairment; AD, Alzheimer's disease; EV, extracellular vesicle; OR, odds ratio; CI, confidence interval; AIC, Akaike's information criterion; ROC, receiver operating characteristic; AUC, area under curve.

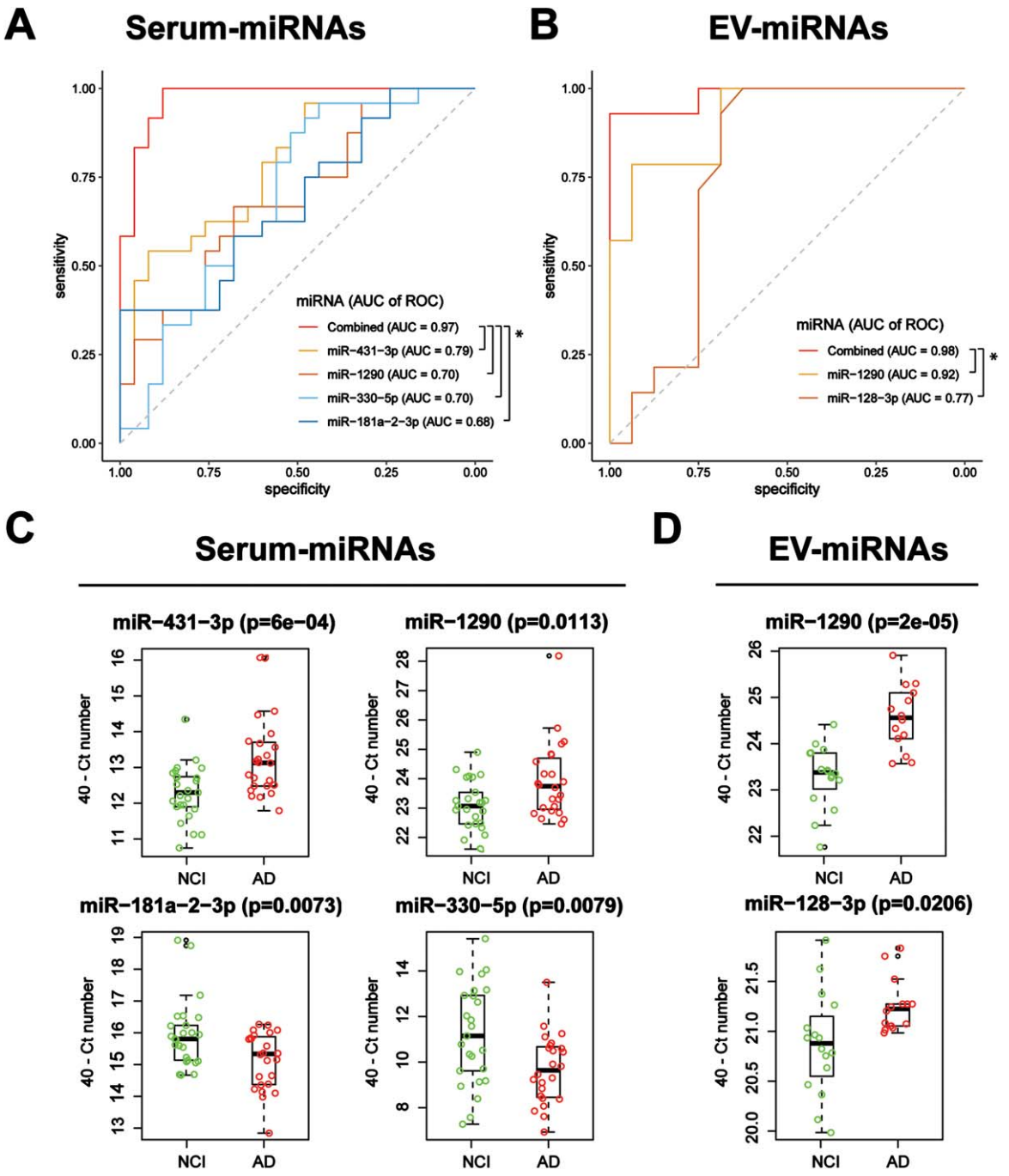


Fig. 3. Expression levels and AD classification potentials of serum- and EV-miRNAs. ROC analyses of AD versus NCI classification performance of logistic regression models obtained using (A) serum-miRNAs and (B) EV-miRNAs identified by stepwise regression. Performance improved when more than one miRNA was used, both for serum and EV miRNAs (*$p < 0.05$, likelihood ratio tests). Boxplots show 40 – Ct expression levels of (C) serum and (D) EV miRNAs in AD patients versus NCI controls.

0.92, the addition of hsa-miR-128-3p (individual AUC = 0.77) raised it to 0.98 ($p < 0.05$, likelihood ratio test; Fig. 3B). Both EV-miRNAs showed significant increases in expression in AD compared to that of the control (two-tailed t-tests; Fig. 3D).

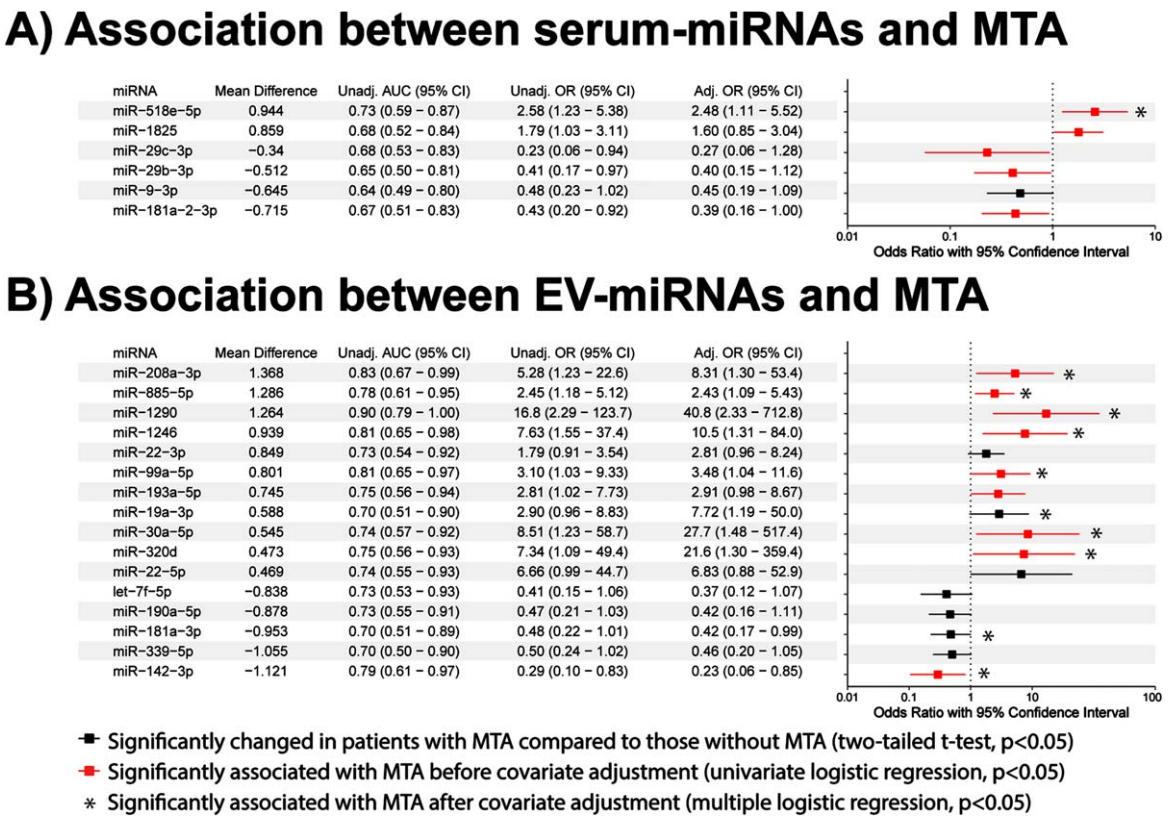


Fig. 4. Associations of serum and EV miRNAs with medial temporal atrophy. Univariate and multiple regression analyses of (A) 6 serum-miRNAs and (B) 16 EV-miRNAs significantly altered in medial temporal atrophy cases. Associations are expressed in terms of odds ratios (OR) and their 95% confidence intervals (CI). Red lines indicate significant association before covariate adjustment ($p < 0.05$, univariate binary logistic regression), while asterisks indicate significant association after covariate adjustment for age, gender, education, and APOE $\varepsilon 4$ ($p < 0.05$, multiple binary logistic regression).

Tissue source and gene enrichment analysis of miRNA combination panels

According to a human miRNA tissue atlas, the serum-miRNAs (hsa-miR-431-3p, hsa-miR-1290, and hsa-miR-181a-2-3p) and the EV-miRNAs (hsa-miR-1290 and hsa-miR-128-3p) are widely expressed in various human tissues, including the brain. The cellular origin of hsa-miR-330-5p was not examined due to a lack of data in the repository; however, its -3p counterpart was reported to be highly expressed in brain tissues [34].

GO enrichment analysis performed on experimentally validated targets of the four serum-miRNAs and the two EV-miRNAs: the former yielded cytoskeleton regulation involvement (FDR < 0.05; Supplementary Figure 3) and the latter yielded involvement in the regulation of membrane bound organelles, the nucleus, early endosomes (FDR < 0.05), dendrites (FDR < 0.1), and possibly other biological processes (Supplementary Figure 3) with potential relevance to hypoxic conditions, potentially implicating roles of these two miRNA in neuronal degeneration.

Associations of miRNA biomarkers with medial temporal atrophy

Biomarkers that can not only diagnose AD but also provide information on neuropathological characteristics of the AD brain have increased potential for clinical use [15, 19, 23]. Given that medial temporal atrophy is a common feature of AD, we investigated the associations between the significantly altered miRNAs (Fig. 2) and the presence of medial temporal atrophy. We identified six serum-miRNAs that were significantly altered ($p < 0.05$, two-tailed *t*-tests; Fig. 4A) and almost three times as many EV-miRNAs ($n = 16$, $p < 0.05$, two-tailed *t*-tests; Fig. 4B) in the presence of medial temporal atrophy. Of these miRNAs, five serum-miRNAs and nine EV-miRNAs were

significantly associated with medial temporal atrophy ($p < 0.05$, univariate logistic regression; Fig. 4, red bars). After covariate adjustment using multiple regression analyses, only one serum-miRNAs, but ten EV-miRNAs, remained significantly associated with medial temporal atrophy ($p < 0.05$, multiple logistic regression; Fig. 4A, B, asterisks). This comparative analysis highlights the increased potential of EV versus serum miRNAs in identifying the presence of AD and its associated medial temporal atrophy; EV-miRNA hsa-miR-1290's AUC of 0.90 (95% CI: 0.79 to 1) suggests it could be a clinically useful biomarker.

DISCUSSION

Our study yielded 25 serum-miRNAs and 41 EV-miRNAs significantly altered in a comparison of 25 AD patients to 34 NCI controls. Of these miRNAs, the expression level of one serum-miRNA (namely hsa-miR-29c-3p) was consistent with findings of previous studies [41]. In contrast, the literature supports many of our EV-miRNA observations: increased expression of miR-424-5p [16, 18], miR-423-5p [21], miR-18b-5p [16], miR-128-3p [21], and decreased expression of let-7e-5p [21] and miR-142-3p [15]. There were, however, also some discrepancies, specifically in miR-92b-3p [21], miR-185-5p [20] and let-7i-5p [17], between the literature and our EV-miRNAs expression level observations. Although these discrepancies remain to be resolved, on balance, our study suggests that EV-miRNAs contain more information relevant to identifying AD and medial temporal atrophy than serum-miRNAs.

ROC AUCs of predictions of patients being AD versus NCI were higher for EV-miRNAs than for serum miRNAs. In particular, EV-enriched hsa-miR-1290 showed the greatest diagnostic potential for identifying both AD (AUC of 0.92) and medial temporal atrophy (AUC of 0.90). There is thus clearly an advantage of isolating miRNAs from EVs rather than serum, as is often practiced in clinical settings. This advantage could be due to the protection of miRNAs from degradation via encapsulation in EVs before, during, and after blood collection [7–14], or due to the disproportionate sorting of miRNAs into EVs during disease pathogenesis [42].

Our study suggests that combining miRNAs could increase AD detection accuracy to a level that is even higher than the already high level obtained using hsa-miR-1290 alone. Combining miRNA from serum (hsa-miR-431-3p, hsa-miR-181-2-3p, hsa-miR-1290, and hsa-miR-330-5p) or EV (hsa-miR-1290 and hsa-miR-128-3p) could have clinical utility, as ROC AUC values then rise even further. As only two EV-miRNAs, as compared to four serum-miRNAs, were required to achieve an exceptionally high ROC AUC, EV-miRNAs may have stronger predictive potentials than the serum-miRNAs. Indeed, EV-enriched hsa-miR-1290 alone had exceptional AD diagnostic value with an ROC AUC of 0.92. Belief in hsa-miR-1290 as a true signal is further fortified by the fact that it was identified in both serum-miRNAs and EV-miRNAs, and in prior literature; there is evidence of hsa-miR-1290 involvement in neurogenesis and neuronal differentiation during early central nervous system development [43–47], and evidence of its utility as a biomarker for subarachnoid hemorrhage [48]. While the underlying mechanism of hsa-miR-1290 in subarachnoid hemorrhage is unclear, cell culture models suggest that it may be anti-apoptotic in ischemia-reperfusion [49, 50]. Given that ischemia stroke is an important risk factor of neurological abnormalities, including AD and medial temporal atrophy [51], the association of hsa-miR-1290 with AD observed in our study may be mediated by underlying pathophysiologies other than neurodegeneration, e.g., vascular pathologies. Additional studies are needed to better understand the role of hsa-miR-1290 in AD.

How other miRNAs identified here may impact neurological disorders and diseases is not known. Although hsa-miR-431-3p was, to our knowledge, never studied, its precursor, miR-431, was reported to exert a protective effect on neuronal cells by regulating axonal regeneration [52], as well as by preventing both amyloid-β-induced synapse loss [53] and cerebral ischemia-reperfusion injury [54]. Interestingly, its 5p counterpart (miR-431-5p) was reported to be upregulated in the brains of AD transgenic mice [55]. Another candidate in our serum-miRNA combination panel, miR-330-5p, was also suggested to play a role in neuronal plasticity and development in rat hippocampal neurons [56]; serum hsa-miR-330-5p was also found to be upregulated in the plasma of patients with Parkinson's disease [57]. Turning to our EV-miRNA panel, hsa-miR-128-3p is linked to cerebrovascular diseases, as higher expression is observed in the cerebrospinal fluid of acute ischemic stroke patients [58], and in rodent models of middle cerebral artery occlusion, miR-128-3p may suppress apoptotic processes and thus promote neuronal cell survival in ischemia-induced brain injuries [59]. Interestingly,

GO enrichment analysis search for biological processes influenced by our EV-miRNAs hsa-miR-1290 and hsa-miR-128-3p yielded cellular responses to hypoxia, which are crucial during ischemia and an important in vascular pathologies. While none of our miRNAs were previously reported in human clinical cohorts of AD, our findings provide new insights and thus contribute to AD research.

Our study has several limitations. First, as our analyses were cross-sectional, temporal associations between miRNAs and the progression of cognitive impairment was not assessed. Second, the sample size was small, and thus may have limited statistical power in detecting significantly altered miRNAs in AD. This sample size limitation also restricted the use of covariate adjustments in multiple regression models and multiple testing corrections. As such, validation with a bigger cohort is still needed to confirm our findings. Indeed, our AUCs are high partly due to the fact that they were assessed on the data to which the models were fitted; we expect smaller values in independent cohorts, though likely not much worse than for hsa-miR-1290 alone, as it was found reproducibly in both serum and EV. Although over-fitting is a concern, with ∼300 miRNAs and thus ∼15 false positives at an alpha of 0.05, there are 25 minus ∼15 = ∼10 and 41 minus ∼15 = ∼26 true positives in serum and EV, respectively, Thus, signals existed on both sides, and more so in EVs than in serum. Third, subjects with cerebrovascular disease were deliberately excluded in the analysis, so our results may not be generalizable to populations where cerebrovascular burdens are high [60, 61]; studies that include subjects with vascular cognitive impairment and dementia may thus broaden the clinical utilities of miRNAs as biomarkers. Our last caveat is that to make this study more clinically relevant, we collected blood samples in standard collection tubes for miRNA isolation [EDTA (EV-miRNAs) and SST (serum-miRNAs)]. Although blood was collected into both EDTA and SST tubes from each participant concurrently, caution needs to be taken while interpreting the data, as pre-analytic blood processing procedures may affect the expression of biomarkers in the blood [62–64].

We investigated the diagnostic potential of miRNAs isolated from AD-patient blood-derived EVs versus serum samples from a clinically and ethnically well-defined patient cohort and found twice as many altered miRNAs in EV than in serum. While the EV-miRNA hsa-miR-1290 alone may predict AD sufficiently, further including miRNA such as hsa-128-3p may further improve accuracy. This holds not only for AD but also for medial temporal atrophy, an important characteristic of AD brain pathology. In unpublished other work, we also found hsa-miR-1290 to be significantly enriched in EVs extracted from patients with vascular dementia. Its importance is thus well supported. Overall, our work suggests that there are diagnostic advantages of EV-miRNAs over serum-miRNAs. Further investigations of EVs as carriers of disease-predicting biomarkers for neurodegeneration are thus warranted.

AUTHOR CONTRIBUTIONS

Yuek Ling Chai (Data curation; Investigation; Methodology; Project administration; Writing – original draft; Writing – review & editing); Lea Strohm (Data curation; Formal analysis; Investigation; Methodology); Yanan Zhu (Data curation; Formal analysis; Investigation); Rachel Sher Li Chia (Data curation; Formal analysis; Investigation); Joyce Ruifen Chong (Data curation; Formal analysis; Investigation); Danesha Devini Suresh (Data curation; Formal analysis; Investigation); Li Han Zhou (Data curation; Formal analysis; Investigation); Heng Phon Too (Data curation; Formal analysis; Investigation); Saima Hilal (Data curation; Formal analysis; Investigation); Tomas Radivoyevitch (Data curation; Formal analysis; Writing – review & editing); Edward H. Koo (Funding acquisition; Project administration); Christopher P. Chen (Conceptualization; Funding acquisition; Methodology; Supervision); Gunnar Heiko Dirk Poplawski (Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Visualization; Writing – original draft; Writing – review & editing).

ACKNOWLEDGMENTS

The authors would like to thank the patients and their families for their participation in this study. Dr. Tan Boon Yeow, St Luke's Hospital, is acknowledged for his contributions to patient recruitment.

FUNDING

This work was supported by the National Medical Research Council (NMRC/CSA-SI/007/2016 to CPC; OFYIRG19MAY-0024 to GHDP), National University of Singapore Yong Loo Lin School of Medicine (Post-Doctoral Fellowship NUSMED/

2021/PDF/05 to YLC), and Lerner Research Institute, Center for Immunotherapy & Precision Immuno-Oncology, Cleveland Clinic, Cleveland Ohio (laboratory seed fund to GHDP).

CONFLICT OF INTEREST

The authors have no conflict of interest to report.

DATASETS/DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: https://dx.doi.org/10.3233/JAD-230572.

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