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RESEARCH ARTICLE

Preclinical Alzheimer's disease shows alterations in circulating neuronal-derived extracellular vesicle microRNAs in a multiethnic cohort

Paolo Reho[1] | **Vrinda Kalia**[1] | **Gabriela L. Jackson**[1] | **Fang Wang**[1] | **Erez Eiten**[2] | **Kasey Brennan**[3] | **Adam M. Brickman**[4,5,6] | **Richard Mayeux**[4,5,6,7] | **Gary W. Miller**[1] | **Badri N. Vardarajan**[4,5,6] | **Andrea Baccarelli**[3] | **Haotian Wu**[1]

[1]Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, New York, USA

[2]NeuroDex Inc., Natick, Massachusetts, USA

[3]Department of Environmental Health, Harvard T. H. Chan School of Public Health, Harvard University, Boston, Massachusetts, USA

[4]Taub Institute for Research on Alzheimer's Disease and the Aging Brain, College of Physicians and Surgeons, Columbia University, New York, New York, USA

[5]The Gertrude. H. Sergievsky Center, Vagelos College of Physicians and Surgeons, Columbia University, New York, New York, USA

[6]Department of Neurology, College of Physicians and Surgeons, Columbia University and the New York Presbyterian Hospital, New York, New York, USA

[7]Department of Epidemiology, Mailman School of Public Health, Columbia University, New York, New York, USA

Correspondence
Paolo Reho, Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, 630 W 168th St, New York, NY 10032, USA.
Email: pr2737@cumc.columbia.edu

Andrea Baccarelli and Haotian Wu, jointly shared supervisory role.

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Abstract

INTRODUCTION: Alzheimer's disease (AD) is the leading cause of dementia, affecting around 50 million individuals worldwide. Brain-derived extracellular vesicles (EVs) can cross the blood–brain barrier carrying neuron-specific molecules, such as microRNAs (miRNAs), which have potential as biomarkers of neurodegeneration.

METHODS: We explored the association between neuronal-derived EV miRNAs from serum and AD clinical status by performing a transcriptome-wide association study involving 46 participants with clinical AD, 14 participants with preclinical AD, and 60 neurologically healthy controls.

RESULTS: We identified 14 miRNAs associated with AD risk, with more pronounced transcriptional alterations in preclinical individuals compared to clinical AD individuals. Functional analysis revealed enrichment of miRNA-target genes in neurodegenerative pathways, highlighting synuclein alpha (*SNCA*), cytochrome c, somatic (*CYCS*), and microtubule associated protein tau (*MAPT*) as key targets.

DISCUSSION: Our results highlight the potential role of neuronal-derived EVs in neurodegeneration and suggest avenues for further research into brain-derived biomarkers.

KEYWORDS
ADRD, Alzheimer's disease, biomarker, EVs, exosomes, extracellular vesicles, microRNAs, multiethnic study, neurodegeneration, neuronal extracellular vesicles, plasma, preclinical stage

Highlights

- Neuronal-derived extracellular vesicles (NDEVs) carry potential brain biomarkers.
- We tested the association between NDEV microRNAs (miRNAs) and Alzheimer's disease (AD).
- Fourteen NDEV miRNAs were associated with AD.
- Preclinical AD displayed more pronounced transcriptional changes than clinical AD.
- miRNA-target genes were enriched in pathways associated with neurodegeneration.

1 | BACKGROUND

Neuropathological hallmarks of Alzheimer's disease (AD) include extracellular deposition of amyloid beta (Aβ) proteins forming senile plaques, neurofibrillary tangles of hyperphosphorylated tau protein within axons and dendrites, and neuronal loss.[1] Despite evidence suggesting that the amyloid system plays a role in the etiology of AD, the cause of the disease remains elusive.[2–5] Several risk factors have been suggested to modulate the risk of developing AD, with aging and apolipoprotein E (*APOE*) ε4 allelic variant being the foremost contributors.[6–10] Individuals with AD have access to few therapeutic options, which show mild efficacy, underscoring the need for new, easily accessible biomarkers that could improve population risk stratification, enhance diagnostic accuracy, and guide the development of more effective treatments.[11,12]

Clinically based diagnostic criteria of AD show sub-optimal diagnostic accuracy compared with neuropathological evidence of disease, with sensitivity ranging from 70.9% to 87.3% and specificity ranging from 44.3% to 70.8%.[13,14] The issue is even more pronounced when considering that over 80% of AD patients have co-pathologies, including cerebroascular disease, Lewy bodies, and TAR DNA-binding protein 43 (TDP-43) proteinopathies.[15] Recent advances in biomarker discovery showed that cerebrospinal fluid biomarkers and blood biomarkers such as Aβ and phosphorylated tau protein improve the accuracy of AD diagnosis.[16–21] Emerging evidence suggests that a preclinical phase of AD could last more than a decade, offering a significant window of time for biomarker screening that could possibly lead to an earlier diagnosis and opportunities for therapeutic intervention.[22,23]

Neurons are the primary targets of degeneration in AD, and gaining direct insights from these challenging-to-access cells is paramount to enhance our understanding of the pathological processes surrounding the disease. Extracellular vesicles (EVs) are small cell-specific membrane-bound cargos released by cells, carrying bioactive molecules such as nucleic acids, metabolites, and proteins, which play a crucial role in intercellular communication.[24] EVs are critical regulators of neurological health and function in the central nervous system.[25,26] Brain-derived extracellular vesicles are able to cross the blood–brain barrier (BBB), providing readily accessible, neuron-specific insights and offering a promising source of biomarkers for neurological health. Thus neuronal EVs quickly gained scientific interest in neurodegeneration. Recently, Kapogiannis and collaborators showed that plasma neuron-enriched EVs could be used to predict AD risk.[27] Among the cargo molecules transported by EVs are microRNAs (miRNAs), short non-coding single-stranded RNA molecules spanning 19 to 24 nucleotides that are actively involved in the regulation of gene expression. miRNAs act as post-transcriptional regulators, modulating gene expression in various physiological processes. In the central nervous system, they play a key role in neuron maturation and neurite growth, and are implicated in pathological processes, including neurological disorders. Recent studies have highlighted the diagnostic and prognostic role of tumor-derived EV miRNAs as circulating biomarkers in cancer, but their role in AD remains understudied.[28–31]

Growing evidence links plasma miRNAs with AD pathogenesis.[32–35] Little is known about the contribution of neuronal-derived EV (NDEV) miRNAs in AD, and studies are focused on patients with symptomatic AD.[36,37] There is a crucial need for accessible and non-invasive prognostic and diagnostic biomarkers that could detect early stages of the disease and enhance the diagnostic accuracy. For these reasons, we performed an miRNA transcriptome-wide association study (TWAS) in 44 clinically diagnosed participants with AD, 14 participants with preclinical AD who were subsequently diagnosed during follow-up, and 60 neurologically healthy controls within the Washington Heights-Hamilton Heights-Inwood Columbia Aging Project (WHICAP). We focused on NDEV miRNAs accessible via routine blood draw, highlighting NDEV miRNA biomarkers that preceded clinical diagnoses as well as molecular pathways that could be addressed in future research.

2 | METHODS

2.1 | Study cohort

Serum from 46 participants with AD, 14 participants with preclinical AD, and 60 age-matched controls collected between 1995 and 2018,

were obtained from the WHICAP study, a longitudinal multi-ethnic study that has been enrolling participants 65 years of age and older since 1992. All participants were interviewed and enrolled after providing informed consent. The study cohort was evenly divided among individuals self-identifying as non-Hispanic White, non-Hispanic Black, and Hispanic, following the classification guidelines of the 1990 U.S. Census. AD diagnosis follows the National Institute of Neurological and Communicative Disorders and Stroke (NINCDS) and the Alzheimer's Disease and Related Disorders Association (ADRDA) consensus criteria.[13] Participants were classified as having a clinical diagnosis of AD based on a neuropsychological battery and a neurological exam.[38,39] Preclinical participants were defined as neurologically healthy at blood draw and received a clinical diagnosis of AD during follow-up (following up time: 1.1–18.8 years). Healthy controls were defined as symptom-free at blood draw and were not diagnosed with AD during follow-up (following up time: 0–7.4 years). We provided a graphical overview of the workflow (Graphical Abstract, Figure 1). Demographic information about the study sample is shown in Table S1.

RESEARCH-IN-CONTEXT

1. **Systematic review**: The authors reviewed the literature using traditional sources such as PubMed. Limited information exists on the role of microRNAs (miRNAs) in neuronal-derived EVs (NDEVs) in Alzheimer's disease (AD), with most research focusing on clinically diagnosed patients. All relevant citations are appropriately cited in the manuscript.
2. **Interpretation**: Our study identified NDEV miRNAs and associated molecular pathways linked to AD, emphasizing transcriptional alterations that predominantly impact the preclinical stage of the disorder.
3. **Future directions**: Our findings offer support for further research into brain-derived biomarkers, emphasizing the potential of non-invasive methods to identify preclinical AD biomarkers. Expanding the sample size (from the longitudinal Washington Heights-Hamilton Heights-Inwood Columbia Aging Project [WHICAP] cohort) and investigating other brain-specific cell types, such as astrocytes and microglial cells, could strengthen the reliability of this approach and provide further evidence for the use of miRNAs as biomarkers for AD.

2.2 | Neuronal-derived extracellular vesicle isolation

NDEVs were isolated from 0.2 mL of blood serum using the ExoSORT isolation kit following the manufacturer's instructions (NeuroDex, Natick, MA, USA). In brief, ExoSORT follows an established immunoprecipitation protocol to capture NDEVs followed by incubation with antibodies to neuron-specific surface proteins, specifically neuroligin-3 (NLGN3) and growth associated protein 43 (GAP43).[40,41]

2.3 | MiRNA sequencing, quality control, and data processing

NDEVs were directly lysed on the magnetic beads using HTG biospecimen lysis buffer and miRNAs were sequenced using the HTG EdgeSeq miRNA Whole Transcriptome Assay that quantifies the expression of 2083 human miRNAs (HTG Molecular Diagnostics, Inc., Tucson, AZ, USA). Six quality control samples were analyzed in triplicates and used as internal technical replicates for quality control purposes. Raw read counts were converted to counts per million, normalized for library size using trimmed mean of M-values normalization method, and log transformed prior to analysis.[42] Only miRNAs that showed replicate coefficient of variation (CV) less than 0.20 were kept for analysis ($n = 383$).

2.4 | Transcriptome-wide association study

In the primary analysis, we tested the association between miRNAs and AD status, where a case was defined to include those diagnosed with

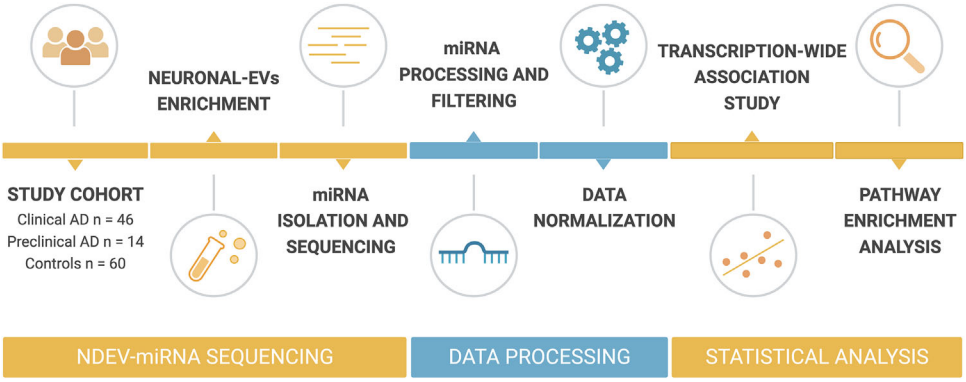

FIGURE 1 Study workflow. Schematic representation of the study workflow. Abbreviations: AD, Alzheimer's disease; EVs, extracellular vesicles; miRNA, microRNA. Figure created with BioRender.com.

AD at the time of blood draw and those diagnosed at follow-up ($n = 60$) compared to neurologically healthy controls ($n = 60$). We then separately tested the association between each miRNA and participants with AD status at blood draw ($n = 46$) and participants with preclinical AD ($n = 14$) compared to healthy controls ($n = 60$). Statistical modeling and data analysis were conducted using the R programming language (v4.2.0).[43] We used multivariable linear regression models adjusted for age, sex, and race/ethnicity of the participants. For interpretation, we exponentiated the regression coefficient to calculate fold-change (FC) differences between groups and applied a false discovery rate (FDR) threshold of 0.05 to adjust for multiple hypothesis testing. The dataset, including demographic information and log-transformed counts per million for the 383 miRNAs analyzed, has been deposited in Zenodo.org (accession # 14618591).

2.5 | Pathway enrichment analysis

We conducted a comprehensive functional enrichment analysis using the DIANA-miRPath v4.0 tool.[44] We obtained target genes from differentially expressed miRNAs in our TWAS using the TarBase v8 database, a collection of experimentally supported miRNA–gene interactions.[45] We performed an miRNA-centric analysis using the following settings: (1) long non-coding RNAs were included among targets; (2) miRbase-v22.1 was the annotation source, and Kyoto Encyclopedia of Genes and Genomes (KEGG) was the pathways database[46]; and (3) an FDR correction with corrected p-value threshold < .05. miR-6780b-5p was excluded from the analysis because no target genes were available for this miRNA with the selected resources. Our functional analysis included pathways enriched by genes targeted by all nine differentially expressed miRNAs. In addition, we performed a clustering of 109 common miRNA-target genes shared between "Pathways of neurodegeneration—multiple diseases" and "Parkinson disease" KEGG pathways using String Consortium database and the Markov Clustering Algorithm option.[47]

3 | RESULTS

3.1 | Study cohort

Almost two-thirds of the study participants were women. Non-Hispanic White ($n = 40$), non-Hispanic Black ($n = 40$), and Hispanic ($n = 40$) ethnicities were equally represented within the study cohort. The average age of participants with AD, participants with preclinical AD, and controls was 87.2 years (range 78.9–89.9), 82.4 years (range 71.2–88.6), and 86.4 years (range 78.7–90.0) respectively.

3.2 | Differentially expressed NDEV miRNAs are associated with AD risk

We first tested the association between each miRNA and all participants with AD (including clinical and preclinical AD cases) compared to neurologically healthy controls. Our TWAS identified nine downregulated miRNAs and one upregulated miRNA associated with AD: miR-6780b-5p (FC = 0.30, FDR = 0.01), miR-27b-3p (FC = 0.66, FDR = 0.01), miR-3940-5p (FC = 0.51, FDR = 0.01), miR-564 (FC = 0.79, FDR = 0.02), miR-29c-3p (FC = 0.68, FDR = 0.03), miR-223-3p (FC = 0.51, FDR = 0.04), miR-15b-5p (FC = 0.60, FDR = 0.04), miR-126-5p (FC = 0.70, FDR = 0.04), miR-19a-3p (FC = 0.65, FDR = 0.04), and miR_422a (FC = 1.36, FDR = 0.05) (Table 1, Figure 2A, Table S2).

TABLE 1 Neuronal-derived EV miRNAs associated with Alzheimer's disease.

	All AD vs Controls				Preclinical AD vs Controls				Clinical AD vs Controls			
miRNA	FC	95% CI	p-value	FDR	FC	95% CI	p-value	FDR	FC	95% CI	p-value	FDR
miR-6780b-5p	0.30	0.18–0.52	2.98×10^{-5}	0.01	0.15	0.06–0.40	1.84×10^{-4}	0.04	0.37	0.20–0.68	1.49×10^{-3}	0.15
miR-27b-3p	0.66	0.54–0.80	6.96×10^{-5}	0.01	0.51	0.36–0.72	2.02×10^{-4}	0.04	0.71	0.57–0.88	2.67×10^{-3}	0.15
miR-3940-5p	0.51	0.36–0.70	8.21×10^{-5}	0.01	0.37	0.20–0.65	9.47×10^{-4}	0.05	0.56	0.39–0.81	2.48×10^{-3}	0.15
miR-564	0.79	0.69– 0.89	2.41×10^{-4}	0.02	0.74	0.58–0.95	0.02	0.11	0.79	0.69–0.91	1.59×10^{-3}	0.15
miR-29c-3p	0.68	0.55–0.84	4.01×10^{-4}	0.03	0.54	0.37–0.77	1.08×10^{-3}	0.05	0.71	0.56–0.90	4.42×10^{-3}	0.15
miR-223-3p	0.51	0.35–0.75	6.81×10^{-4}	0.04	0.35	0.18–0.68	2.33×10^{-3}	0.07	0.57	0.37–0.87	9.18×10^{-3}	0.15
miR-15b-5p	0.60	0.45–0.81	8.79×10^{-4}	0.04	0.40	0.24–0.66	5.69×10^{-4}	0.04	0.66	0.48–0.91	0.01	0.15
miR-126-5p	0.70	0.57–0.86	9.76×10^{-4}	0.04	0.57	0.40–0.82	3.01×10^{-3}	0.08	0.75	0.60–0.94	0.01	0.15
miR-19a-3p	0.65	0.51–0.84	1.02×10^{-3}	0.04	0.51	0.33–0.78	2.21×10^{-3}	0.07	0.68	0.51–0.90	6.89×10^{-3}	0.15
miR-422a	1.36	1.13–1.63	1.21×10^{-3}	0.05	1.33	0.94–1.89	0.11	0.20	1.40	1.16–1.69	7.31×10^{-4}	0.15
miR-144-3p	0.68	0.53–0.88	3.19×10^{-3}	0.06	0.42	0.26–0.66	2.95×10^{-4}	0.04	0.76	0.58–1.00	0.05	0.15
miR-451a	0.44	0.25–0.75	2.74x10-3	0.06	0.19	0.07–0.47	5.80×10^{-4}	0.04	0.53	0.30–0.96	0.04	0.15
let-7f-5p	0.77	0.65–0.91	2.36x10-3	0.06	0.64	0.49–0.82	7.27×10^{-4}	0.04	0.81	0.67–0.97	0.02	0.15
let-7 g-5p	0.79	0.66–0.93	5.82x10-3	0.06	0.62	0.47–0.81	7.87×10^{-4}	0.04	0.85	0.71–1.02	0.09	0.18

Note: Statistically significant miRNAs associated with the risk of AD. "Clinical AD" refers to clinically diagnosed AD participants at blood draws. "All AD" groups clinical AD and preclinical AD. FC represents the miRNA expression ratio. 95% CI shows lower and upper intervals.
Abbreviations: AD, Alzheimer's disease; CI, confidence intervals; FC, fold change; FDR, false discovery rate; miRNA, microRNA.

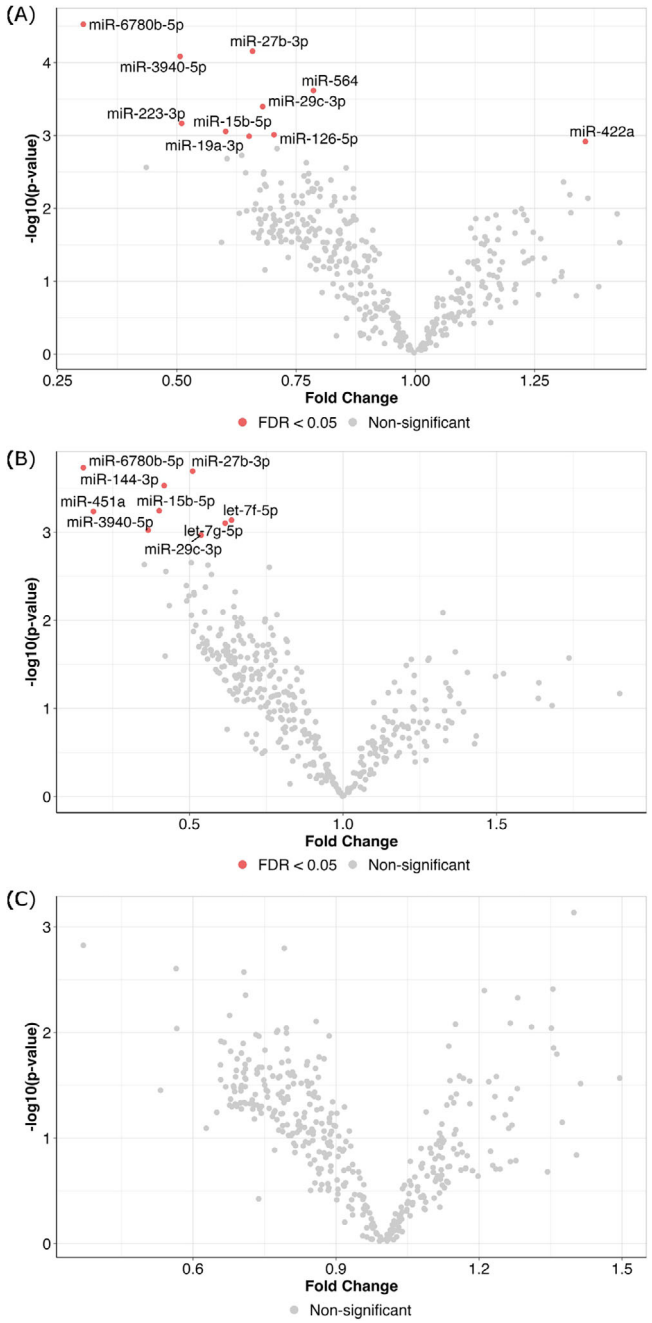

FIGURE 2 Differentially expressed miRNAs associated with Alzheimer's disease. Volcano plot showing the magnitude of change (FC) and statistical significance (–log10 *p*-value) of all miRNAs in participants with clinical and preclinical AD (A), participants with preclinical AD (B), and participants with clinical AD (C) compared to healthy aged controls. FC values below 1 indicate lower abundance in NDEVs of participants with AD (both clinical and preclinical) compared to controls, whereas FC values above 1 indicate higher abundance in NDEVs of participants with AD. Red dots show the statistically significant miRNAs surpassing the FDR threshold of 0.05. Gray dots identify miRNAs that showed non-significant different expression comparing cases and controls. Abbreviations: AD, Alzheimer's disease; FC, fold change; FDR, false discovery rate; miRNA, microRNA; NDEVs, neuronal-derived extracellular vesicles.

3.3 | TWAS identifies significant miRNAs in preclinical AD cases

To investigate a possible role of NDEV miRNAs in presymptomatic AD, we restricted our analyses to 14 participants with preclinical AD and 60 healthy controls. This analysis identified a lower expression of nine NDEV miRNAs associated with preclinical AD. Notably, five of these miRNAs, miR-6780b-5p (FC = 0.15, FDR = 0.04), miR-27b-3p (FC = 0.51, FDR = 0.04), miR-15b-5p (FC = 0.4, FDR = 0.04), miR-3940-5p (FC = 0.37, FDR = 0.05), and miR-29c-3p (FC = 0.54, FDR = 0.05), were previously associated with the risk of AD in the main TWAS (Table 1, Figure 2B, Table S2).

Finally, we performed TWAS with 46 participants with clinical AD and 60 controls. This approach did not identify any miRNA surpassing the FDR-adjusted sinificance threshold of 0.05 (Figure 2C). However, the comparisons between the estimates across the three models (all AD cases vs controls, clinical AD cases vs controls, and presymptomatic AD cases vs controls) revealed a consistent trend among the miRNAs identified, even if they did not survive FDR correction (Figure 3). We performed a sex-stratified sensitivity analysis and found no appreciable difference between men and women for the 14 significant miRNAs (Figure S1). Notably, we observed an expression pattern among the 14 previously identified miRNAs where participants with preclinical AD exhibited more pronounced modulations compared to participants with AD (Figure 4).

3.4 | Differentially expressed NDEV miRNAs are enriched in neurodegeneration pathways

We conducted a comprehensive functional enrichment analysis to find molecular pathways linked with the miRNAs identified in the TWAS. We identified 523 genes and seven molecular pathways targeted by the miRNAs associated with the risk of AD (Table 2, Figure S2, Table S3). Our analysis revealed pathways related to neurodegeneration, including "Parkinson disease" (miRNA-target genes $n = 121$, adjusted p-value $= 4.04 \times 10^{-5}$) and "Pathways of neurodegeneration—multiple diseases" (target genes $n = 219$, adjusted p-value $= 2.54 \times 10^{-6}$). We further performed a protein–protein interaction study among 109 target genes shared between "Pathways of neurodegeneration—multiple diseases" and "Parkinson disease" pathways. Our analysis identified eight functional groups, with *SNCA*, *CYCS*, and *MAPT* emerging as key genes, showing 59, 57, and 49 interactions, respectively (Figure 5, Table S4).

4 | DISCUSSION

There is a critical need to discover non-invasive biomarkers capable of crossing the BBB, providing brain-specific molecules that could aid in early detection, improve diagnosis, and enhance therapeutic efficacy in AD. Our study investigated the association between serum NDEV

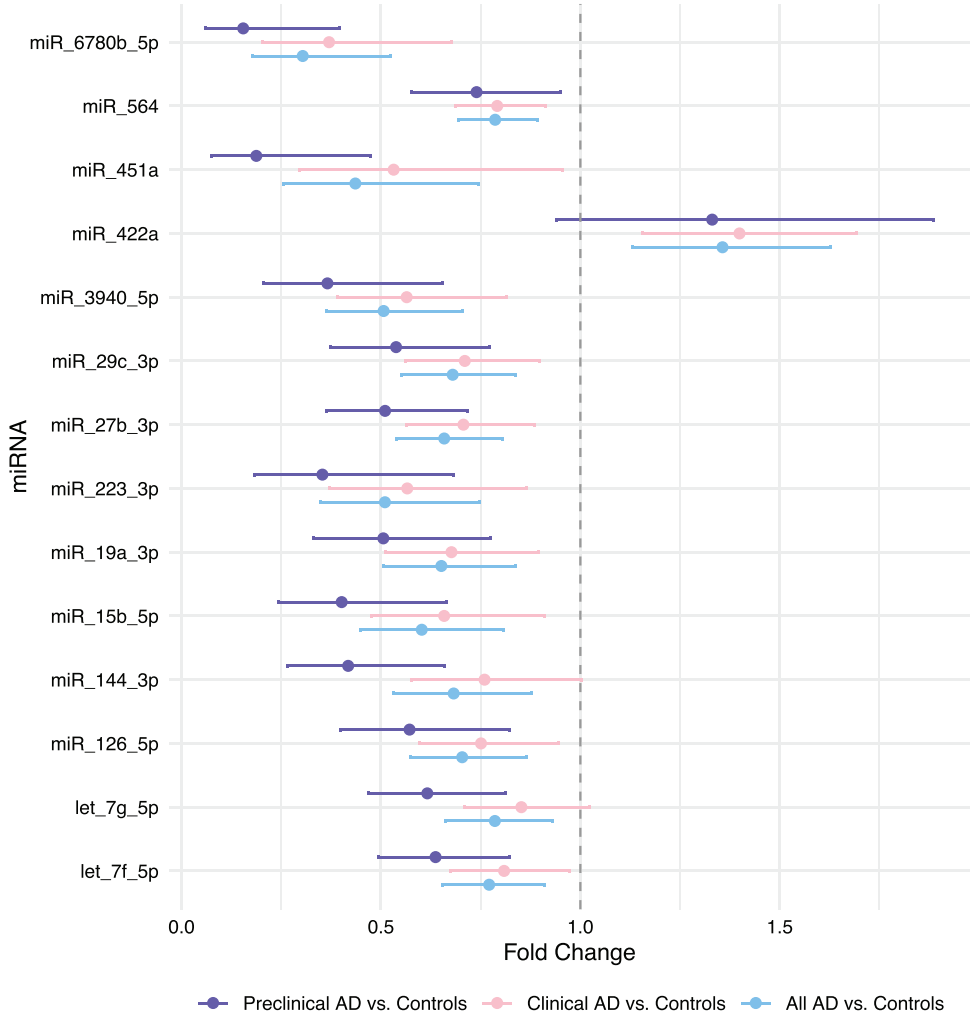

FIGURE 3 Estimates between different models. Estimates from three different models of the 14 miRNAs associated with an increased risk of AD. Purple dots and lines refer to participants with preclinical AD versus controls model, pink dots and lines refer to participatns with clinical AD versus controls, light blue data refer to all AD participants versus controls. Horizontal bars refer to lower (left end) and higher (right end) 95% confidence intervals. Abbreviations: AD, Alzheimer's disease; miRNA, microRNA.

TABLE 2 Biological pathways enriched by miRNA-target genes.

Pathway	Genes	Targets	*p*-value	Adjusted *p*-value
Ubiquitin-mediated proteolysis	142	85	4.69×10^{-13}	3.20×10^{-11}
Protein processing in endoplasmic reticulum	194	107	8.47×10^{-13}	4.82×10^{-11}
Cell cycle	129	75	8.05×10^{-11}	2.29×10^{-9}
Endocrine resistance	118	63	2.14×10^{-7}	1.81×10^{-6}
Pathways of neurodegeneration—multiple diseases	539	219	3.13×10^{-7}	2.54×10^{-6}
Parkinson's disease	282	121	7.59×10^{-6}	4.04×10^{-5}
Kaposi sarcoma–associated herpesvirus infection	245	100	4.34×10^{-4}	1.54×10^{-3}

Note: Biological pathways enriched by the target genes of the differentially expressed microRNAs (miRNAs) identified in the study. "Genes" refers to the number of genes annotated within each pathway. "Targets" shows the number of miRNA-target genes for each pathway. Adjusted *p*-value refers to a false discovery rate correction.

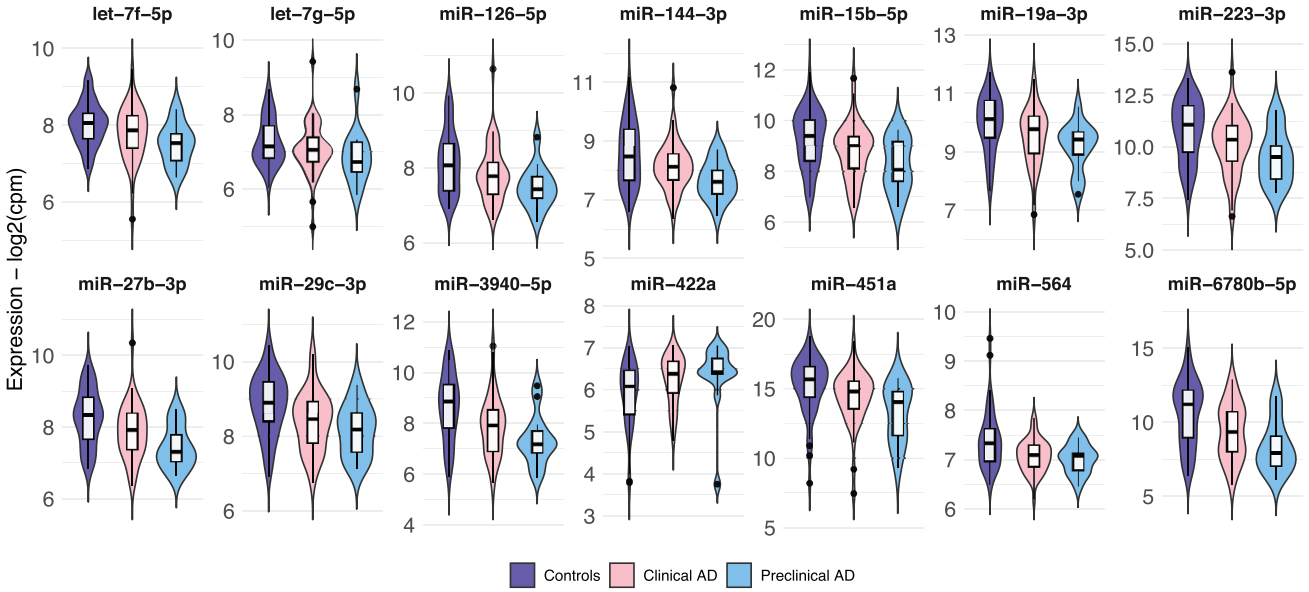

FIGURE 4 Differentially expressed miRNAs in preclinical and clinical AD. Violin plots showing the count per million of the differentially expressed miRNAs in neurologically healthy controls (purple), participants with clinical AD (pink), and participants with preclinical AD (light blue). The horizontal line of the boxplot refers to the median value, whereas the upper and lower limits of the boxplot show the first third quantiles, respectively. Significance between the groups is shown (one-way ANOVA). Abbreviations: AD, Alzheimer's disease; ANOVA, analysis of variance; cpm, count per million; miRNA, microRNA; NS, not significant.

miRNAs and clinical AD diagnosis. We found brain-specific miRNAs and molecular pathways that may modulate the risk of developing clinical AD, potentially representing therapeutic targets.

In a recent study, Jia and colleagues investigated cerebrospinal fluid and imaging biomarkers in cognitively unimpaired participants over a 20-year period, and they found that changes occurred as early as 18 years prior to the clinical diagnosis of AD.[22] Their findings reveal a preclinical AD phase that may span decades and reinforces the need for novel non-invasive predictive biomarkers that could improve the efficacy of therapeutic interventions. Within our study sample, 14 participants initially enrolled as healthy controls but progressed to clinical AD, enabling us to study the prodromal stage. Leveraging data from these individuals, we found a specific miRNA expression signature: participants with preclinical AD exhibited more pronounced modulation compared to participants with clinical AD (Figure 4). This finding suggests that specific miRNAs in NDEVs may reflect early biological changes before the onset of clinical symptoms. It is also possible that the milder miRNA modulations observed in later stages could result from neuronal compensation or reduced neuron availability due to neuronal death associated with disease progression. Alternatively, it is possible that once diagnosed with Alzheimer's disease, treatments or changes in lifestyle may ameliorate some of the alterations.

Among the miRNAs associated with increased AD risk, miR-27b-3p and miR-3940-5p stand out given their previous associations with neurodegenerative disorders. Aberrant expression of miR-27b-3p is implicated in neuroinflammation and associated with neurodegenerative disorders including AD, multiple system atrophy, and amyotrophic lateral sclerosis.[33,48–53] Of interest, a recent in vitro study showed that miR-3940-5p targets presenilin 1 (*PSEN1*), which encodes one of the catalytic subunits of γ-secretase, a crucial protein involved in the proteolytic cleavages of amyloid precursor protein (APP).[54] By targeting *PSEN1*, miR-3940-5p reduces the production of presenilin 1, resulting in a decreased function of γ-secretase and lower levels of the neurotoxic peptides Aβ40 and Aβ42.[31] Our analysis reveals a significant lower expression of NDEV miR-3940-5p in participants with AD compared to healthy controls, consistent with the hypothesis that downregulation of miR-3940-5p promotes amyloidogenic processing of *APP* and the pathogenesis of AD.

Our findings support growing evidence linking the dysregulation of miR-223-3p, miR-19a-3p, miR-29c-3p, and miR-15b-5p to neurodegeneration.[34,37,50,51,55] Notably, among the preclinical-specific miRNAs, miR-144-3p, miR-451a, let-7 g-5, and let-7f-5p are associated with an increased risk of Parkinson's disease (PD) and AD, further supporting their potential role in the prodromal stage of neurodegeneration.[34,36,56,57] The elevation of PD-associated miRNAs may not be surprising as recent advances in α-synuclein assay help identify that synuclein pathology in about 11% of preclinical AD and 25% of clinically diagnosed AD cases.[41] Comparing changes in NDEV miRNAs with cerebrospinal fluid biomarkers of synuclein pathology may help identify a less-invasive biomarker for this co-pathology. In addition, miR-144-3p and miR-451a co-localized upstream the gene *ERAL1* on the opposite strand, separated by 93 bps. *ERAL1* encodes a mitochondrial GTPase essential for the assembly of the 28S mitochondrial ribosomal subunit. Deletions or dysregulation of this gene have been associated with mitochondrial dysfunction, a well-established contributor to neurodegeneration.[58–61]

The functional enrichment analysis revealed a significant enrichment of miRNA-target genes within pathways related to

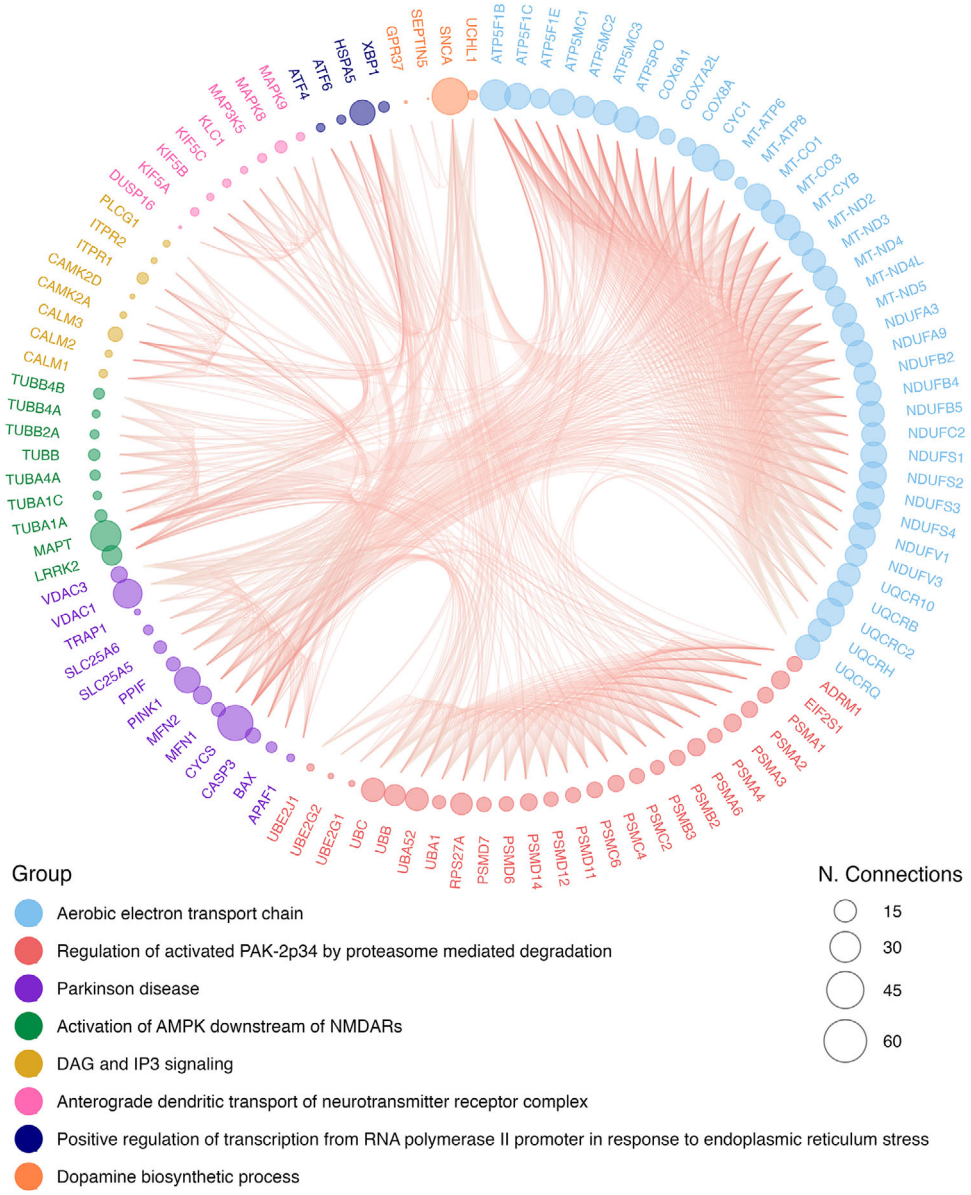

FIGURE 5 miRNA-target genes interactions. Circular plot showing the interconnections between 109 miRNA-target genes shared between "Parkinson disease" and "Pathways of neurodegeneration—multiple diseases" KEGG pathways. Color code refers to different functional clusters identified by the String Consortium database tool. The pink lines show the connections between miRNA-target genes. The size of the dots represents the number of interconnections between the genes. Abbreviations: KEGG, Kyoto Encyclopedia of Genes and Genomes; miRNA, microRNA.

neurodegeneration and PD. Among these genes, *SNCA*, *CYCS*, and *MAPT* emerged as genes of particular interest. *MAPT* encodes the major constituent of neurofibrillary tangles, a defining pathological feature of AD.[62] A recent study showed that an interaction between cytochrome C (*CYCS*) and mitochondrial metabolism with tau protein is associated with neurodegeneration.[63] In addition, mutations of *SNCA* cause PD,[64] and the intracellular aggregation of the misfolded protein into Lewy bodies is associated with synucleinopathies like PD, Lewy body dementia, and multiple system atrophy.[65] Of note, our findings align with increasing evidence linking *SNCA* to the etiology of AD.[66] However, these findings are exploratory, and additional evidence is necessary to confirm the role of these genes in neurodegeneration, particularly within NDEVs.

Emerging evidence shows that NDEVs are promising biomarkers for neurodegenerative diseases. Sinha and colleagues showed a significant enrichment of Aβ protein into post-mortem brain-specific exosomes of AD patients compared to controls, providing evidence of an exosome-mediated neuron-to-neuron propagation of toxic Aβ.[67] In addition, Ruan and collaborators showed the role of brain-derived EVs in the initiation and propagation of tau pathology in AD patients post-mortem, corroborating the involvement of NDEVs in the progression of the disease.[68] Although these findings are undoubtedly compelling and

expand our knowledge on the association of brain-specific EVs with pathological changes in AD, they highlight the pressing need for non-invasive approaches to effectively study and monitor these biomarkers in living patients.

Our study reveals several intriguing findings, but it does have limitations. Although the HTG EdgeSeq miRNA assay, coupled with stringent quality-check filtering, ensured high-quality data in our study, further research is needed to identify the most effective methodologies. The small sample size could affect the statistical robustness and expose our analysis to false findings. However, similar studies with comparable sample sizes have demonstrated that meaningful insights can still be generated under these conditions.[40,41,69] Even with such a small sample, we identified differentially abundant miRNAs in NDEVs between AD cases and controls that were consistent with prior evidence, but it is possible that we lacked the power to detect other differences. Although the study offers valuable insights into miRNAs as potential AD biomarkers, the confidence in our findings could have been enhanced with additional approaches, such as polymerase chain reaction (PCR) validation. However, the limited sample volume and low RNA yield from NDEVs restricted our ability to conduct complementary analyses. Our controls were healthy at the time of blood draw and were followed up for a short period; however, it is possible that some of them developed AD later. This possibility would likely result in bias toward the null and is unlikely led to spurious false-positive findings. Finally, although we were unable to adjust for co-morbidities, which may introduce residual confounding into our analysis, this limitation does not diminish the potential utility of NDEV miRNAs as biomarkers for AD.

In summary, our study identified several miRNAs that exhibit differential abundance in plasma NDEVs between participants with AD and healthy controls, including some differences that predate clinical diagnosis. Several of these miRNAs have been previously linked to AD, but the overall evidence on the role of these miRNAs in neurological health and pathology is scarce. Our findings provide valuable insights that could point to novel therapeutic strategies in AD.

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CONFLICT OF INTEREST STATEMENT

Dr Eitan is an employee and holds stocks of NeuroDex, a for-profit company. The remaining authors declare no conflicts of interest. Author disclosures are available in the Supporting Information.

CONSENT STATEMENT

This study was approved by the Columbia University Institutional Review Board, and was performed in accordance with the ethical standards (AAAT5194). All participants or their caregivers provided written informed consent.

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