

## RESEARCH ARTICLE

# Plasma microRNA vary in association with the progression of Alzheimer's disease

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## Abstract

**Introduction:** Early intervention in Alzheimer's disease (AD) requires the development of an easily administered test that is able to identify those at risk. Focusing on microRNA robustly detected in plasma and standardizing the analysis strategy, we sought to identify disease-stage specific biomarkers.

**Methods:** Using TaqMan microfluidics arrays and a statistical consensus approach, we assessed plasma levels of 185 neurodegeneration-related microRNA, in cohorts of cognitively normal amyloid  $\beta$ -positive (CN-A $\beta$ +), mild cognitive impairment (MCI), and Alzheimer's disease (AD) participants, relative to their respective controls.

**Results:** Distinct disease stage microRNA biomarkers were identified, shown to predict membership of the groups (area under the curve [AUC] >0.8) and were altered dynamically with AD progression in a longitudinal study. Bioinformatics demonstrated that these microRNA target known AD-related pathways, such as the Phosphoinositide 3-kinase (PI3K-Akt) signalling pathway. Furthermore, a significant correlation was found between miR-27a-3p, miR-27b-3p, and miR-324-5p and amyloid beta load.

**Discussion:** Our results show that microRNA signatures alter throughout the progression of AD, reflect the underlying disease pathology, and may prove to be useful diagnostic markers.

## KEYWORDS

Alzheimer's disease, biomarker, disease progression, early diagnostic, microRNA, plasma

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## 1 | BACKGROUND

Early detection of Alzheimer's disease (AD) is critical to the delivery of effective treatment strategies. Although it is feasible to identify known mutations in family pedigrees with a history of AD, no such routine genetic test is available to detect the sporadic, late-onset form of the disease. The apolipoprotein E (APOE)- $\epsilon$ 4 genotype is a long-established risk factor for late-onset AD, but by itself it is not strongly predictive of progression to AD<sup>1</sup> and no other single mutation has demonstrated a stronger predictive value.<sup>2</sup> By contrast, although it is possible to quantify levels of amyloid beta (A $\beta$ ), one of the diagnostic features of AD, in cerebrospinal fluid and brain,<sup>3</sup> as well as anatomical changes in cortical structures,<sup>4</sup> it is not possible to measure these early changes without repeated use of positron emission tomography (PET) scans, magnetic resonance imaging (MRI) scans, or lumbar puncture. These expensive and/or invasive procedures are available only in highly specialized centers and are not currently suitable for population screening.<sup>5</sup>

It is therefore vital to identify robust, easily monitored biomarkers that are accurate indicators of incipient AD and to understand how they change through the entire course of the disease. Attention has focused on blood-borne biomarkers of AD<sup>6</sup>; yet no such biomarker is available in clinical practice. Indeed, despite the strong association between amyloidosis and cognitive decline, there is much debate as to whether amyloid levels in blood plasma correlate well with the disease.<sup>6,7</sup> Direct measurement of A $\beta$  (or phospho-tau) has been immensely technically challenging, despite development of new technologies.<sup>5,8</sup> This is likely due to the highly aggregative nature of A $\beta$ , low levels within blood, and a lack of knowledge regarding how export of A $\beta$  from the brain changes with disease.

Recently a growing body of evidence suggests that microRNA, a class of non-coding RNA that function by regulating gene expression at the post-transcriptional level,<sup>9</sup> are dysregulated in AD<sup>10</sup> and that blood-borne microRNA may be good candidate biomarkers of the disease.<sup>11</sup> Of interest, although microRNA can be detected in cerebrospinal fluid,<sup>12</sup> microRNA crosses the blood-brain barrier and are protected from degradation by association with protein complexes and sequestration into membrane bound vesicles, such as exosomes.<sup>13</sup> Indeed, recent evidence suggest that exosomes may be involved in the propagation of neurodegenerative disease<sup>14,15</sup> and that exosome-derived microRNA can transduce recipient cells.<sup>16</sup> Thus circulating levels of microRNA may not only accurately reflect neuronal function and dysfunction, but may represent novel therapeutic targets for the treatment of dementia. Several recent meta-analyses have sought to identify a consensus microRNA signature for AD. For example, Swarbrick et al.<sup>17</sup> identified a 10-peripheral blood microRNA signature putatively associated with Braak Stage III. By contrast Bottero and Potashkin<sup>18</sup> predicted microRNA that were likely to influence the expression of genes known to be differentially expressed in MCI and AD. However, to date there is little concordance in the microRNA species contributing to the putative Alzheimer's associated panels.<sup>11</sup>

Alongside heterogeneity between study cohorts, variation in the blood fractions collected, the processing or storage of the blood and analytical and statistical platforms used to assess biomarker levels

### RESEARCH IN CONTEXT

- 1. Systematic review:** A comprehensive review of the literature and conference proceedings shows that despite much research, there is currently no universally agreed early diagnostic microRNA-based biomarker for Alzheimer's disease (AD).
- 2. Interpretation:** We hypothesized that this was a result of variation in microRNA levels as the disease progresses, alongside heterogeneity within study cohorts and pre-analytical variation in the collection, processing, and storage of blood. By using a standardized biofluid (plasma) and robust microRNA analysis platform (quantitative polymerase chain reaction [PCR] TaqMan microfluidics arrays), we have identified distinctive microRNA that effectively reflect the progression of the disease from amyloid positive to mild cognitive impairment (MCI) and AD.
- 3. Future directions:** Although our MCI and AD biomarkers are derived from more than one cohort, the next most important steps are to extend the cohort size of the amyloid-positive group, to validate these microRNA as being able to identify individuals at risk of cognitive decline, and to determine whether these biomarkers are specific to AD or are universal markers of neurodegeneration.

### HIGHLIGHTS

- Plasma microRNA are dynamically expressed during the progression of Alzheimer's disease (AD).
- Specific microRNA are altered at specific diseases stages and have potential diagnostic utility.
- miR-195-5p and miR-324-5p are consistently regulated throughout disease progression.
- miR-27a-3p, miR-27b-3p, and miR-324-5p are correlated with amyloid beta (A $\beta$ ) load.
- Target analysis of biomarker microRNA reflects underlying neuropathology of AD.

are mooted as crucial limiting factors in the search for blood-based biomarkers of AD.<sup>19</sup> We reasoned that for a biomarker to be clinically relevant, it should be independent of cohort-cohort variation, but may vary according to biofluid or method chosen for microRNA analysis. Furthermore, we predicted that plasma microRNA will vary with the progression of AD, as we found to occur during the development of amyloidosis in a mouse model of AD.<sup>20</sup> Here, using a standardized biofluid (plasma) and robust microRNA analysis platform

(TaqMan microfluidics arrays), we identified distinctive microRNA-based biomarkers that effectively reflect the progression of AD.

## 2 | METHODS

For further details refer to Supplementary Information.

### 2.1 | Cohorts

*Otago Alzheimer's disease (Otago-AD)*: probable AD ( $n = 44$ ); cognitively normal age and sex matched controls (CN;  $n = 49$ ). *PrecisionMed Inc* (Solano Beach, CA, USA; [www.precisionmed.com/](http://www.precisionmed.com/)): MCI ( $n = 36$ ) and CN ( $n = 40$ ). *Australian Imaging, Biomarker & Lifestyle Flagship Study of Ageing* (AIBL; [www.aibl.csiro.au](http://www.aibl.csiro.au)): probable AD ( $n = 21$ ), MCI ( $n = 38$ ), cognitively normal  $A\beta$ -positive (CN- $A\beta+$ ;  $n = 21$ ), and cognitively normal  $A\beta$ -negative (CN- $A\beta-$ ) ( $n = 20$ ). All participants gave written informed consent.

### 2.2 | MicroRNA expression profiling

We created custom-designed microfluidics arrays representing 185 microRNA correlated with neurological disease and controls (U6 snRNA and ath-miR-159a).<sup>20</sup> RNA was isolated from plasma (MirVana Paris; Life Technologies, Cat # AM1556M), converted to complementary DNA, and pre-amplified (12 cycles) before qPCR (ViiA-7; Applied Biosystems). MicroRNA that were not expressed in all samples or had Ct <12 and >33 were excluded. All samples passed the miR-23a/miR-451 test of hemolysis.<sup>21</sup>

### 2.3 | Statistical analysis

*Cross-sectional studies*: Following data normalization using Norm Rank Invariant, differentially expressed microRNA were identified using empirical-Bayes moderated  $t$ -tests (case/matched cohort control;  $P < .05$ ). Outliers were identified using Grubb's test. Normal distribution of the data was confirmed using the D'Agostino and Pearson omnibus normality test ( $P > .05$ ). Data were processed and heatmap generated using GraphPad Prism (v8).

Meta-analyses were conducted using the R package "metaphor" (v2.0-0; <http://CRAN.R-project.org/package=metaphor>). A fixed-effect model was chosen for CN- $A\beta+$  group, whereas a random-effect model was chosen for the MCI and AD groups (DerSimonian and Laird method) to allow for the different group sizes. Heterogeneity of the mean estimates (pooled estimated effect size) was assessed using Cochran's Q and the  $I^2$  statistic; where  $Q_{ep} < 0.1$ ;  $I^2$  (%) >75% was considered significantly heterogeneous in the AD group and excluded from the analysis. Results were visualized with Forest Plots, showing the the pooled effect size estimate, along with their confidence intervals (95% CI).

Logistic regressions were performed with the Forward: Wald method (MedCalc, v15.11.4). The goodness of fit for each logistic regression model was evaluated using the Hosmer-Lemeshow test ( $P > .05$ ). Receiver-operating characteristic (ROC) curve, area under the curve (AUC) was evaluated for the overall model fit ( $P < .05$ ). Log-rank test were performed using the Mantel-Cox method to compare expression (normalized Ct) of the diseased and control groups ( $P < .05$ ).

### 2.3.1 | Longitudinal studies

A subgroup of longitudinal samples from the AIBL cohort (CN- $A\beta+$  to MCI,  $n = 21$ ; MCI to AD,  $n = 18$ ) were used to identify change in microRNA expression with disease progression. Box and Whisker plots (median normalized Ct values and 95% CI) were constructed to display the data and generalized estimating equations (GEEs; SPSS, v25.0) used to determine significant effects. The dependent variable was the microRNA expression studied (normalized Ct). Compound symmetry was used for the working correlation matrix structure and the Wald chi-square tested for the effect of group, followed by pairwise comparisons of the estimated marginal means at each set ( $P > .05$ ). AUC were determined as above; 95% CIs are reported. Pearson's correlation coefficient  $r$  with  $P$ -value were generated for multiple variables, including normalized Ct, which could possibly explain the different expression in microRNA using method using MedCalc, v15.11.4.

### 2.4 | Bioinformatics analysis

DIANA-microT v3.0 (Tarbase v7.0) and miRTarBase 7.0 <http://mirtarbase.mbc.nctu.edu.tw/php/index.php> were employed using the most stringent algorithm parameters to identify validated targets of the candidate microRNA biomarker for each disease stage (Figure 3), as well as the microRNA, which correlated with amyloidosis. Using DAVID 6.7 (<http://david.ncifcrf.gov>), we focused on genes expressed in brain and blood. Biological pathways enriched within this group were identified using the Enrichr tool ([amp.pharm.mssm.edu/Enrichr](http://amp.pharm.mssm.edu/Enrichr)<sup>22,23</sup>) to search Wikipathways. Kegg Mapper (<https://www.genome.jp/kegg/mapper.html>) was used to color the genes associated with each disease stage.

## 3 | RESULTS

### 3.1 | Identification of differentially expressed microRNA: cross-sectional analysis

To identify microRNA-based biomarker panels representing CN- $A\beta+$  (AIBL), MCI (PMed, AIBL), and AD (Otago-AD, AIBL) cohorts, we used qPCR TaqMan microfluidics arrays to quantify microRNA in plasma and expressed these relative to their respective CN controls (Table 1 and Table S1). The heatmap (Figure 1) provides an overview of the data and includes 32 microRNA that were found to be significantly differentially

**TABLE 1** Demographic characterization of cohorts

Cross-sectional study							
Otago-AD Cohort (NZ)	CN	AD	<i>p</i> -value	PMed Cohort (USA)	CN	MCI	<i>P</i> -value
Number	49	44		Number	40	36	
Age (mean ± SD)	74.0 ± 5.9	75.5 ± 8.3	0.332	Age (mean ± SD)	71.5 ± 3.9	73.2 ± 5.0	0.109
Sex (F: M)	(25: 24)	(23: 21)	0.905	Sex (F: M)	(25: 24)	(18: 18)	1
MMSE (mean ± SD)	28.7 ± 1.1	19.0 ± 6.2	<0.0001	MMSE (mean ± SD)	29.6 ± 0.7	24.9 ± 1.4	<0.0001
APOE ε4- : APOE ε4+ (%)	73: 27	40: 60	0.003	APOEε4- : APOEε4+ (%)	n/a	n/a	
AIBL cohort (Australia)	CN	CN Aβ+	<i>p</i> -value	MCI	<i>p</i> -value	AD	<i>p</i> -value
Number	20	21		38		21	
Age (mean ± SD)	74.7 ± 6.4	74.3 ± 5.7	0.851	75.8 ± 6.2	0.448	75.9 ± 7.8	0.616
Sex (F: M)	(10: 10)	(11: 10)	0.883	(17: 21)	0.711	(10: 11)	0.883
MMSE (mean ± SD)	29.3 ± 0.9	28.6 ± 1.3	0.989	26.4 ± 3.2	<0.0001	19.5 ± 6.2	<0.0001
Image.PET.Centiloid (mean ± SD)	-0.5 ± 5.9	70.0 ± 29.8	<0.0001	83.8 ± 33.2	<0.0001	102.2 ± 23.1	<0.0001
APOE ε4- : APOE ε4+ (%)	80: 20	33: 67	<0.0001	32: 68	<0.0001	29: 71	<0.0001
Longitudinal study							
AIBL Converter cohort (Aus)	CN Aβ+ to MCI	MCI to AD					
Number	21	18					
Age (mean ± SD)	(74.3 ± 5.7): (77.9 ± 5.5)	(74.7 ± 7.1): (77.6 ± 6.9)					
Sex (F: M)	(11: 10)	(8: 10)					
MMSE (mean ± SD)	(28.6 ± 1.3): (26.4 ± 1.5)	(26.3 ± 3.1): (23.3 ± 3.4)					

\*For full details refer Supplementary Table x.

Abbreviations: AD, Alzheimer's disease; Aβ<sup>-</sup>, cognitively normal amyloid negative; CN-Aβ<sup>+</sup>, cognitively normal amyloid positive; F, female; M, male; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination, APOE ε4, apolipoprotein E ε4 variant; *P* < .05; Participants: CN, cognitively normal control; *P*-value: Student *t*-test, compared to CN.

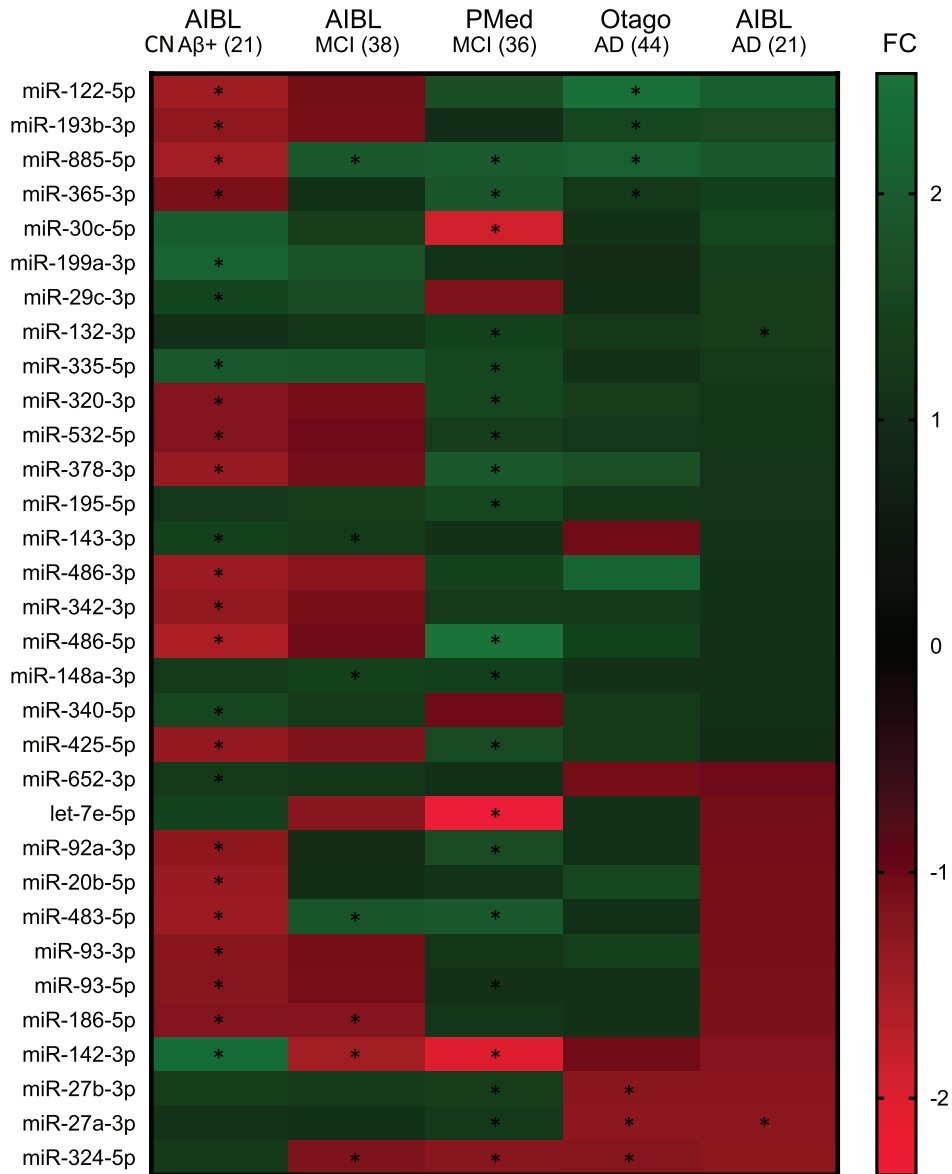
expressed within at least one group (see Table S3 for fold change; FC). A flow chart summarizing our study is presented in Figure S1.

This mode of analysis not only identified microRNA previously related to MCI or AD (eg, miR-27a-3p, miR-29c-3p, miR-132-5p, miR-142-3p, miR-195-5p, miR-885-3p<sup>24</sup>), but also showed considerable similarities in microRNA levels in like-cohorts and variation with disease progression. Within the AD cohorts, 75% appeared to be altered in the same direction (59% up; 16% down). It appears that the microRNA that were not validated between the AD cohorts were largely heterogeneous in the MCI group as well. Indeed, the MCI group overall showed greater heterogeneity, with only 50% of microRNA being altered in the same direction (39% up; 9% down). Of interest, of the microRNA consistently upregulated in the MCI group, 62% were also upregulated in the CN-Aβ<sup>+</sup> group, and 75% of the microRNA regulated in the CN-Aβ<sup>+</sup> group were also consistently regulated in the AIBL-MCI group, giving weight to the conclusion that these microRNA are altered early in the progression of AD.

To interrogate these findings more rigorously, we pooled the data between like-cohorts by meta-analysis. This produced a weighted fold change (pooled estimated effect size) and 95% confidence intervals for each of the 32 microRNA. Assuming that there would be substantial heterogeneity within the MCI group, we next filtered the data by sig-

nificant heterogeneity in the AD group (Log-rank; Qep test, *I*<sup>2</sup> statistics). This resulted in a set of 16 putative biomarker microRNA (Table S4). These microRNA are dynamically expressed across the CN-Aβ<sup>+</sup>, MCI, or AD groups relative to CN groups (Figure 2A). For example, three microRNA (miR-27a-3p, miR-27b-3p, miR-324-5p) were downregulated in the AD and upregulated in the CN-Aβ<sup>+</sup> group. By contrast, six microRNA were upregulated in AD (miR-122-5p, miR-132-3p, miR-193b-3p, miR-320a-3p, miR-365-3p, and miR-885-5p) and two of these were downregulated in the CN-Aβ<sup>+</sup> group (miR-122-5p and miR-885-5p). Only miR-195-5p and miR-335-5p showed consistent upregulation in all three disease stages but showed substantial heterogeneity in the CN-Aβ<sup>+</sup> group. The Venn diagram (Figure 2B) summarizes the association of specific microRNA and disease stage, with miR-27b-3p and miR-885-5p significantly, yet dynamically, regulated in all disease groups. Thus, drawing on data from three independent cohorts, these analyses confirm that specific microRNA are dynamically expressed across the CN-Aβ<sup>+</sup>, MCI, or AD groups relative to CN groups.

To prioritize candidate microRNA biomarkers from this 16-microRNA set, we ranked the importance of the individual microRNA using a statistical consensus approach.<sup>25,26</sup> This involved combining the output of the differential expression analysis (*P*-value), the distribution of normalized Ct values (log-rank *P*-value), and the ability of



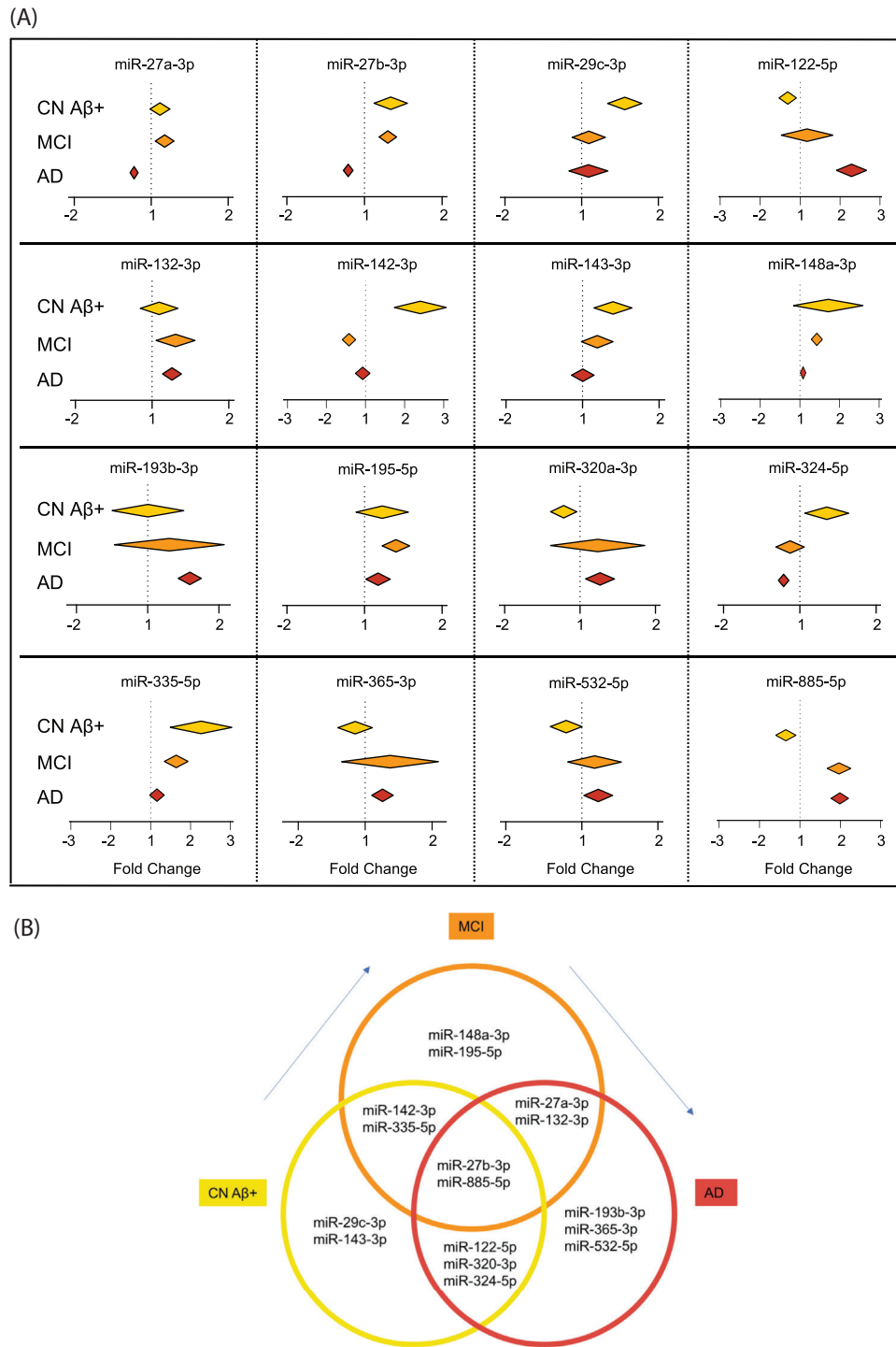
**FIGURE 1** Heat map showing microRNA expression profiles in cognitively normal amyloid positive (CN-Aβ+), mild cognitive impairment (MCI), and Alzheimer's disease (AD) cross-sectional cohorts. A statistically significant expression of microRNA was identified in each cohort using empirical-Bayes moderated *t*-tests (\*; *P* < .05), based on log2 mean-fold changes relative to CN. The microRNA that were differentially expressed in at least one cohort are presented in the heat map as green/upregulated or red/downregulated. Each column represents a different disease stage or cohort (number of participants in parenthesis) and each row represents a single microRNA. Data were processed using GraphPad Prism v8.

the microRNA to predict membership of the disease groupings (AUC). The prioritized list of the putative biomarkers for each disease stage obtained is shown in Figure 3 (and Table S5). In the AD group, the top ranked microRNA had the highest fold change (Table S3); however, this was not true for the CN-Aβ+ or MCI groups, thus supporting the use of this consensus approach. Next we determined the ability of combinations of the top-ranked microRNA to predict membership of the disease groups by ROC analysis. To avoid over-fitting the model, the number of microRNA were constrained to comply with the recommendation of Peduzzi et al. (1996).<sup>27</sup> Derived AUCs were CN-Aβ+: 0.857 (miR-29c-3p and miR-335-5p); MCI: 0.823 (miR-142-3p, miR-324-5p, miR-195b-5p, miR-148a-3p); and AD: 0.817 (miR-27a-3p, miR-27b-3p,

miR-122-5p, miR-193b-3p, miR-324-5p and miR-885-5p). This analysis suggests that unique microRNA biomarker signatures reflect each disease stage and may have potential diagnostic utility.

### 3.2 | MicroRNA expression: longitudinal analysis

To further explore the association of our candidate microRNA biomarkers with disease progression, we extracted a subgroup of longitudinal samples from the AIBL cohort comprising *n* = 21 individuals who donated samples when CN-Aβ+ as well as when classified as MCI and *n* = 18 individuals who donated samples when MCI as well as when

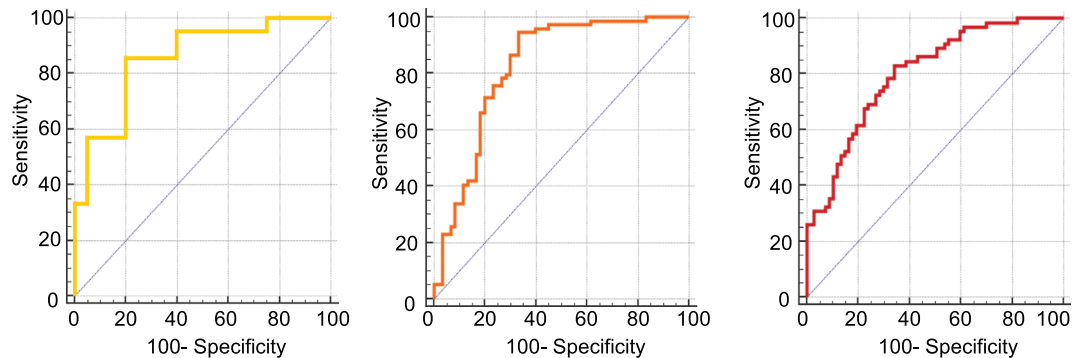


**FIGURE 2** Forest plots showing the weighted fold-change of 16 microRNA highlighted following meta-analysis as potential biomarkers in the CN-A $\beta$ +, MCI, and AD cross-sectional cohorts. (A) The linear mixed-effects model included CN-A $\beta$ +, MCI, and AD cross-sectional cohorts. Observed outcomes for each disease stage are represented with a diamond (CN-A $\beta$ + = gold, MCI = orange, AD = crimson). The width of the diamond reflects the precision of the estimate (95% CI); the weights correspond to the inverse standard deviations of the effect size estimates from the studies; the position on the x-axis represents the measure estimate, with the vertical line indicating “no change” in microRNA expression. A positive effect size represents upregulation and a negative effect size represents downregulation. Data are relative to CN groups. Summary estimates are provided in Table S4. (B) Venn showing the association of the 16 microRNA retained after the meta-analyses with disease stage.

(A)

	CN A $\beta$ +	MCI	AD
Signature	miR-29c-3p, miR-335-5p	miR-142-3p, miR-324-5p, miR-195-5p, miR148a-3p	miR-27a-3p, miR-27b-3p, miR-122-5p, miR-193b-3p, miR-324-5p, miR-885-5p
AUC	0.857	0.823	0.817
SE	0.0587	0.0386	0.0375
95% CI	0.712 - 0.947	0.748 - 0.884	0.732 - 0.873
z statistic	6.051	8.334	8.407
p-value	<0.0001	<0.0001	<0.0001
Youden index	0.6571	0.5991	0.5124
Sensitivity	86	95	83
Specificity	80	67	66

(B)



**FIGURE 3** Consensus ranking of microRNA and diagnostic value of disease-stage-specific putative biomarker signatures. Each of the 16 microRNA identified in the meta-analysis were ranked using three independent criteria. The three rankings per microRNA were then summed to provide a final rank. Lower total rank sums were given the highest ranking. The three ranking criteria used were (1) differential expression ( $P$ -value; refer Table S3), (2) distribution of normalized Ct values (log-rank tests;  $P$ -values; refer Table S4), and (3) predictive power (AUC from logistic regression). The signature and results of each ROC analysis are shown in (A). The diagnostic ability of each derived signature was assessed by computing the AUC value of the ROC curve (logistic regression with normalized Ct values), compared to the CN group (B).

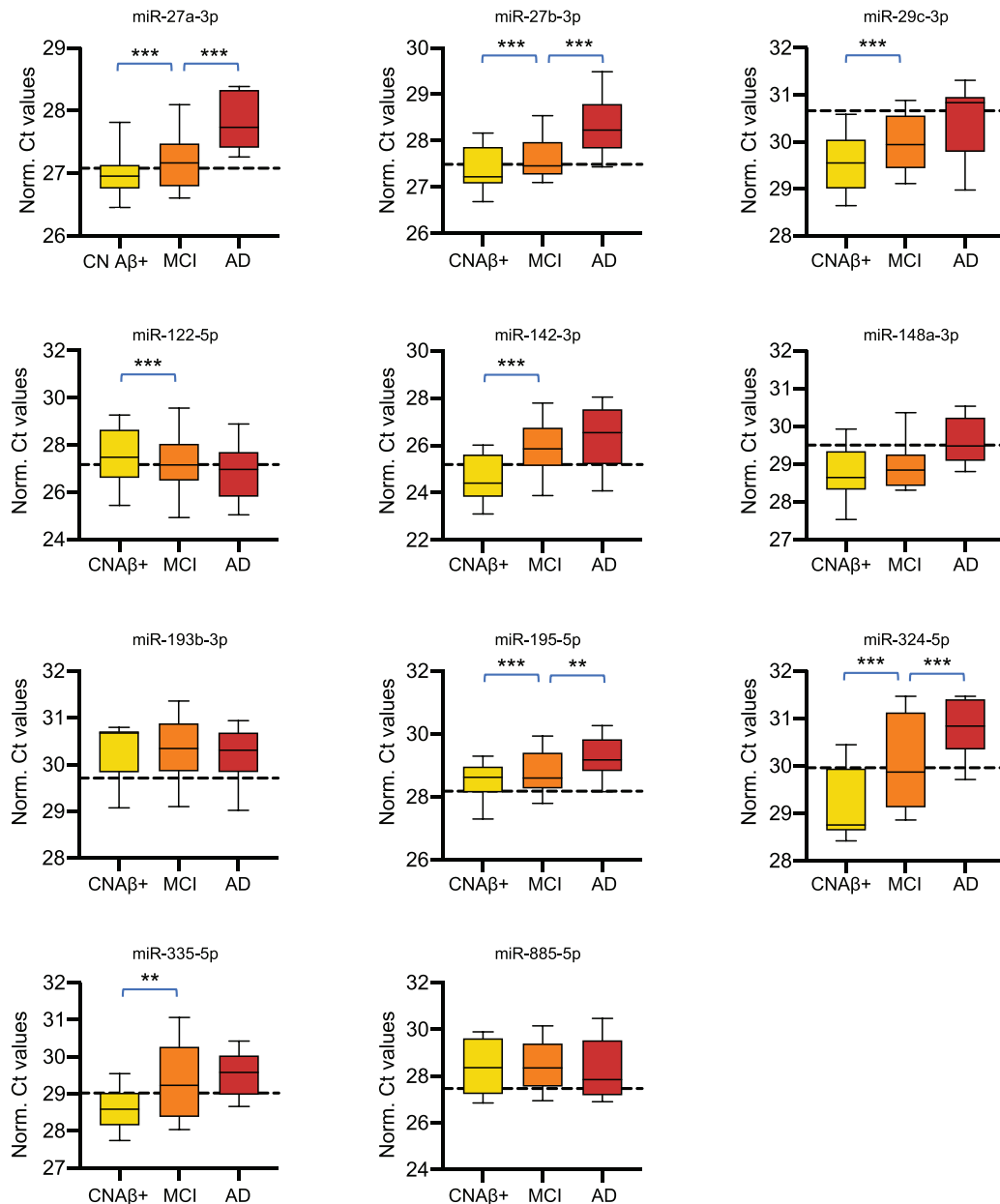
classified as AD (Table 1; Figure S1). We assessed the trends in expression of our candidate biomarkers with the progression of disease using a generalized estimating equation (or GEE) (Figure 4; Table S6), to account for the longitudinal nature of the samples and the resulting lack of independence of the data sets. This analysis confirmed dynamic regulation of this group of microRNA within the individuals in this longitudinal cohort. Eight microRNA were shown to be significantly alter in the transition from CN-A $\beta$ + to MCI (up: miR-27a-3p, miR-27b-3p, miR-122-5p; down: miR-29c-3p, miR-142-3p, miR-195-5p, miR-324-5p, miR-335-5p), and four microRNA were shown to be significantly downregulated in the transition from MCI to AD. This group included miR-27a-3p, miR-27b-3p, which were both upregulated in the CN-A $\beta$ + to MCI transition, and miR-195-5p, miR-324-5p, which were both downregulated in the MCI to AD transition. These results reinforce that changes in microRNA levels occur early in the disease and can be dynamic. However, this also shows that particular microRNA, such as miR-195-5p, miR-324-5p, are consistently regulated throughout the disease. In addition, these results show that the observed variation in microRNA levels (Figure 1,2) is not simply a result of variation in preanalytical processing and indeed reflects the progression of disease.

To probe the diagnostic utility of the microRNA, we assessed the ability of each to predict membership of the disease groups by ROC analysis within this longitudinal cohort. Resulting AUCs ranged from

0.51 to 0.76 and were pronouncedly increased to 0.8 to 0.95 by inclusion of APOE  $\epsilon$ 4 as a factor (Table S7). AUCs for each microRNA were assessed singly in line with the Peduzzi constraints. miR-29c-3p and miR-335-5p showed the equal strongest AUCs in the CN-A $\beta$ + group, whereas, miR-142-3p, miR-148a-3p, and miR-27b-3p were strongest in the MCI group and miR-27a-3p showed the strongest in the AD group. Furthermore, we found using Pearson correlations that A $\beta$  load (centiloid values; Table 1) in the AD group was significantly correlated with miR-27a-3p ( $r = 0.466$ ;  $P = .002$ ), miR-27b-3p ( $r = 0.391$ ;  $P = .012$ ), and miR-324-5p ( $r = 0.406$ ;  $P = .009$ ). Together these analyses reinforce that these microRNA may have prognostic utility.

### 3.3 | Relationship between putative biomarker microRNA and AD: Biological relevance

Using a bioinformatic approach, we probed the association of the candidate microRNA biomarkers with AD molecular pathology. Focusing on the disease stage-related signatures (Figure 3), we identified validated target mRNA and interpreted the resulting lists using the gene set enrichment tool, Enrichr (Kuleshov et al., 2016). Targets for the candidate CN-A $\beta$ +, MCI, and AD biomarker microRNA groups were significantly enriched in AD-relevant Wikipathways, including the *Phosphoinositide 3-kinase (PI3K-Akt) Pathway* (Figure S2), the highest



**FIGURE 4** Box and whisker plots showing microRNA expression in the AIBL longitudinal cohort. Expression of biomarker microRNA (Figure 3) was studied in the AIBL longitudinal cohort ( $n = 21$ : CN- $A\beta+$  to MCI stage and  $n = 18$  MCI to AD stage; total MCI = 39). Y-axis shows the normalized Ct values where high values = low expression. The lines within the boxes show the median microRNA expression (normalized Ct values) and the whiskers represent the 95% CI. Statistically significant differences were identified using generalized estimating equations (\*  $P < .05$ ; \*\*  $P < .01$ ; \*\*\*  $P < .001$ ). The hashed line indicates the median values in the AIBL CN group, and these data were not included in this longitudinal analysis.

overrepresented pathway identified for each of the disease stages and in combination (Table 2). Pathway analysis of all microRNA targets combined additionally identified *Neurotrophin*, *Mitogen-activated protein kinase (MAPK)*, and *mechanistic target of rapamycin (mTOR) signaling*. *Neurotrophin signaling* was also highlighted in pathway analysis of the microRNA correlated with centiloid values, alongside *Insulin Resistance* and *Long-term potentiation*. Alongside the observations that the candidate biomarker microRNA have been shown previously to be altered in either AD plasma and/or post-mortem tissue (Table S8), this

analysis reinforces the connection between the candidate microRNA biomarkers and the pathology underlying the progressive stages of AD.

#### 4 | DISCUSSION

Small non-coding RNA, in particular microRNA, are a central focus both as biomarkers of neurodegenerative diseases and novel therapeutic agents. To date there is no agreed blood biomarker for AD, despite



**TABLE 2** Candidate biomarker microRNA target AD-relevant pathways: (A) Pathways targeted by CN-Aβ+, MCI, and AD-related microRNA (refer Figure 3); (B) combined candidate biomarker microRNA and (C) those correlated with centiloid values (amyloidosis)

A) Pathways enriched at specific disease stages	
<b>CN-Aβ+</b>	
1	PI3K-Akt signaling pathway
2	Focal adhesion
3	Focal adhesion-PI3K-Akt-mTOR-signaling pathway
4	Breast cancer pathway
5	Signaling pathways in glioblastoma
6	miRNA targets in ECM and membrane receptors
7	Inflammatory response pathway
8	Metastatic brain tumor
9	Overview of nanoparticle effects
10	Somatroph and its relationship to dietary restriction and aging
<b>MCI</b>	
1	PI3K-Akt signaling pathway
2	DNA damage response
3	Pancreatic adenocarcinoma pathway
4	Leptin signaling pathway
5	Focal adhesion
6	Integrated breast cancer pathway
7	Breast cancer pathway
8	VEGFA-VEGFR2 signaling pathway
9	Colorectal cancer
10	AGE/RAGE pathway
<b>AD</b>	
1	PI3K-Akt signaling pathway
2	Pathways in cancer
3	Colorectal cancer
4	FoxO signaling pathway
5	AGE-RAGE signaling pathway in diabetic complications
6	Human cytomegalovirus infection
7	Proteoglycans in cancer
8	Kaposi sarcoma-associated herpesvirus infection
9	Hepatitis B
10	Hepatocellular carcinoma
B) Pathways enriched by combined candidate microRNA biomarkers	
Combined Signature	
1	Lysine degradation
2	MAPK signaling pathway
3	FoxO signaling pathway
4	mTOR signaling pathway
5	Long-term potentiation
6	HIF-1 signaling pathway
7	Insulin resistance

(Continues)

**TABLE 2** (Continued)

8	Oocyte meiosis
9	Neurotrophin signaling pathway
10	Progesterone-mediated oocyte maturation
C) Pathways enriched by centiloid-correlated microRNA	
<b>Centiloid</b>	
1	PI3K-Akt signaling pathway
2	Focal adhesion
3	Neurotrophin signaling pathway
4	Ras signaling pathway
5	Human papillomavirus infection
6	FoxO signaling pathway
7	Pathways in cancer
8	MAPK signaling pathway
9	T cell receptor signaling pathway
10	mTOR signaling pathway

more than 30 studies proposing 100 candidate microRNA.<sup>17</sup> This failure in replication may be due to inconsistent blood-fraction methods and variation in microRNA analysis.<sup>19</sup> Using an innovative statistical consensus approach and holding the blood fraction, technical platform, and mode of analysis constant, we have determined a unique set of plasma microRNA associated with AD, which rise above innate heterogeneities found within populations. It is notable that we have shown for the first time that plasma microRNA levels are altered before symptoms manifest and vary dynamically with disease progression. The individual microRNA that we have highlighted have all been associated previously with AD, and using bioinformatics, we have shown that our candidate microRNA converge on *PI3K-Akt signalling*, a pathway with a well-established relationship with the molecular pathology underlying AD, including neurofibrillary tangles and microglial and astroglial inflammasome regulation.<sup>28</sup> Thus the microRNA compiled in our study are likely a valid set of biomarkers reflecting the progression of AD.

Our work particularly highlights elevated miR-29c-3p and miR-335-5p levels as novel biomarkers of early amyloidosis. Levels of both microRNA have been shown previously to be altered in AD biofluids (refer Table S8), and miR-29c-3p and BACE1 as well as miR-335-5p and Aβ levels are inversely correlated,<sup>29,30,31</sup> suggesting that they both contribute directly to cerebral amyloid levels. Furthermore, both miR-29c-3p and miR-335-5p have been shown to enhance memory performance in the Morris water maze.<sup>31,32</sup> miR-335-5p is a neuronally enriched microRNA and a proposed key regulator of AD-related gene networks.<sup>33</sup> Our bioinformatic analysis showed that together these microRNA map to *Inflammatory Response* and *Glioblastoma* as well PI3K and mTOR pathways. Indeed, miR-29c-3p is known to protect against inflammasome activation in microglia,<sup>34</sup> suggesting a role in neuroprotection. Our finding that miR-335-5p is upregulated in plasma supports the findings of Cheng et al., who showed this microRNA was upregulated in extracellular vesicles isolated from plasma from the AIBL-AD

cohort.<sup>35</sup> Of interest, two previous studies have shown that miR-335-5p is downregulated in post-mortem brain, thus suggesting that AD is associated with an increase in the export of miR-335-5p into extracellular vesicles.

Within our biomarker signature we found that expression of most microRNA varied across the progression of the disease. However, our cross-sectional study showed that miR-195-5p and miR-335-5p were consistently elevated, but it is notable that we found that their levels were reducing with the progression of the disease (while remaining above control levels), in the AIBL longitudinal sub-group. Upregulation of miR-195-5p may also be part of a neuroprotective response, as this microRNA inhibits the expression of both BACE1 and APP<sup>36</sup> and apoptosis.<sup>37</sup> Indeed, overexpression of miR-195-5p has recently been shown to alleviate cognitive deficits and reduce A $\beta$  load and tau hyperphosphorylation in APOE  $\epsilon$ 4<sup>+/+</sup> mice.<sup>38</sup> Furthermore, knock down of miR-195-5p has been shown to decrease dendritic length and number<sup>39</sup> and the synaptically located molecule, neurogranin, is a validated target of miR-195-5p, the levels of which are reduced in AD post-mortem tissue and neural-derived exosomes.<sup>40</sup>

Concomitant with change in the composition of the disease stage-related biomarker microRNA, we observed additional pathways mapping to the MCI and AD groups. In particular the MCI analysis identified the AGE/RAGE Pathway and VEGFA-VEGFR2 signaling Pathway. This is interesting because both pathways are also linked to neuroprotection.<sup>41</sup> Indeed, inhibition of advanced glycation end products and its receptor has been mooted as a potential AD therapy,<sup>42</sup> and miR-142, our most highly ranked microRNA in the MCI group and highlighted by Kumar et al., 2013,<sup>43</sup> targets the RAGE pathway.<sup>44</sup> Because miR-142-3p directly targets inflammatory pathways,<sup>45</sup> this leads to the suggestion that de-repression of miR-142-3p targets may be associated initially with curtailing a neuroinflammatory response. Although there is much debate over the role of neuroinflammation in AD, it is intriguing that recently a genetic variation in the promoter of miR-142 (rs2526377:A > G), which results in reduced expression, was shown to be significantly associated with a reduced risk of AD.<sup>46</sup> Thus further investigations into the role of miR-142 in AD are warranted.

The microRNA correlated with the amyloid load (centiloid values) in individuals with advanced AD mapped to the HIF-1 Signaling pathway. This pathway is interlinked with VEGF, MAPK, and PI3K signaling and promotes amyloidogenic processing of APP.<sup>47</sup> The plasticity-related pathways *Neurotrophin Signaling and Long-term potentiation* were also mapped to this group. miR-27a-3p, miR-27b-3p, and miR-324-5p have been shown previously to be altered in blood or post-mortem brain tissue (Table S8). Indeed downregulation of miR-324-5p has been mooted to contribute to synaptic loss during aging,<sup>48</sup> whereas miR-27b-3p is considered a proinflammatory microRNA.<sup>49,50</sup> Of interest, miR-27a-3p targets SERPINA3, which encodes a serine protease inhibitor associated with the APOE  $\epsilon$ 4 genotype, inflammation, and amyloid polymerization.<sup>51</sup> Together these bioinformatic analyses highlight a strong relationship between our candidate biomarker microRNA inflammation and amyloidosis, lending weight to the conclusion that these plasma biomarkers reflect the disease processes occurring within the brain.

In summary, the plasma microRNA highlighted by our studies, derived by using a statistical consensus approach using multiple cohorts, vary with disease progression in the AIBL longitudinal study and reflect known steps underlying AD neuropathology; therefore, may be useful in disease risk prediction in clinical practice. Our early signature likely predicts underlying pathology before individuals become symptomatic. These data are unique and need to be strengthened by further in-depth analysis of pre-symptomatic individuals,<sup>52</sup> validation in other cohorts, and by analysis of neuronal exosome-derived microRNA in plasma or CSF.<sup>53</sup> It will also be important to understand the influence of other endophenotypes such as APOE  $\epsilon$ 4 status on the plasma levels of these microRNA<sup>54</sup> as well as sex and ethnicity of the study cohorts.<sup>55</sup> Furthermore, it will be important to explore the specificity of the biomarker by testing whether the same or different microRNA are useful in the early diagnosis of other neurodegenerative diseases. These data will be highly valuable for improved criteria of inclusion into clinical trials where currently available cognitive behavioral and drug therapies can be further tested<sup>56,57,58</sup> and thus the onset of disease can be delayed. Overall, we have shown that biomarkers are dynamic, altering with disease progression, thus emphasizing the need for longitudinal biomarker testing. The transition to our later signature may further identify at-risk individuals and be useful in prioritizing individuals for more highly specialized testing.

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## OTHER RELATIONSHIPS

Warren P. Tate: 2012-2021 Governance Board member of the Maurice Wilkins Centre of Excellence - support to travel to governance and scientific meetings.

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### SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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