Distinguishing mild cognitive impairment from Alzheimer’s disease by increased expression of key circulating microRNAs.

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Abstract

Levels of key microRNAs circulating in blood have recently emerged as promising noninvasive diagnostic markers for neurodegenerative disorders. We previously demonstrated increased levels of miR-34a in peripheral blood mononuclear cells (PBMC), and of this microRNA and its sister, miR-34c, in plasma, of probable Alzheimer’s disease (AD) patients. In the present study, we expand this work to demonstrate that other plasma microRNAs, miR-181c and miR-411, can distinguish subjects of all three stages of AD dementia from normal elderly controls (NEC), as well as from their mildly cognitively impaired (MCI) counterparts. The area under the curve (AUC) value of miR-411 levels in circulating blood for all these comparisons is >90; thus it may represent a powerful biomarker for dementia progression. Our findings suggest that: a. inclusion of an MCI cohort, and sub-cohort analysis, are essential in biomarker discovery; and b. when this cohort is included, miR-411 is a far more powerful biomarker than miR-34c or -181c to distinguish MCI from the mild, moderate and severe stages of AD dementia.

Keywords: Alzheimer’s disease, Circulating microRNA, Mild cognitive impairment, Biomarker.

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Introduction

Individuals with mild cognitive impairment (MCI) perform below normal age-matched controls on tests of short-term memory or other cognitive abilities, but are functionally intact and not as cognitively impaired as individuals with Alzheimer's disease (AD) [1,2]. Within a cohort of MCI patients, some remain stable, with non-progressive memory deficit over a long period of time, while others advance to a state of AD dementia, exhibiting progressive impairment in multiple cognitive domains [3]. Thus, MCI is in many cases prodromal AD, with gradual and progressive memory deficits occurring over a span of many years [2,4].

Within the AD cohort, a spectrum of disease states ranges from mild dementia to severe impairment. The Mini-Mental State Examination (MMSE) is a commonly used tool for estimating the severity of cognitive decline [5,6]. Scores range from 0-30, with lower scores indicating higher levels of impairment. Probable AD patients can be categorized as having mild (MMSE scores of 21-24), moderate (MMSE scores of 10-20), or severe (MMSE scores of 0-9) dementia, while normal subjects generally score above 24 [5,6]. Despite the MMSE’s usefulness in estimating the severity of disease, some patients score outside of the commonly accepted cut-off values for normalcy, mild, moderate, and severe stages of disease [7]. Additionally, no routinely used noninvasive blood biomarkers are presently in use to determine disease stage in MCI individuals and their probable AD counterparts. Therefore, the discovery of biological markers able to distinguish MCI individuals from normal elderly and probable AD individuals, as well as biomarkers to separate mild, moderate, and severe AD cases, represents a significant achievement in the evaluation and management of this debilitating neurodegenerative spectrum.

MicroRNA biomarker discovery has progressed from documentation of tissue-specific signatures to their presence in circulating blood, where they may indicate...
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We previously demonstrated enhanced expression of miR-34a, miR-579, miR-181b, miR-520h, miR-155, miR-517*, let-7f, miR-200a, and miR-371 in the PBMC compartment of blood samples from individuals with probable sporadic AD [12,17]. Among these lead microRNAs, miR-34a affects the highest number of predicted target genes vital to cellular defense and neuronal survival, such as BCL2, SIRT1, and ONECUT2 [18-20]. Moreover, increased levels of miR-34a have recently been reported in human AD temporal cortex [21] and both miR-34a and its sister, miR-34c, are elevated in the brains of AD-transgenic mice [22,23]. Furthermore, inhibitors of the seed sequence of miR-34c de-repress SIRT1 expression and functionally rescue cognitive deficits in a learning impairment mouse model [23]. Our recent work shows that miR-34c, and to a lesser degree its sister, miR-34a, are significantly elevated in circulating probable AD plasma, relative to normal elderly controls [24].

Despite our reports of miR-34a and its sister -34c as biomarkers to distinguish AD from normal elderly controls (NEC), other candidate plasma microRNAs are yet to be identified as biomarkers. There is a particular need to identify microRNAs that signal the progression from MCI to mild, moderate, and severe Alzheimer dementia, and distinguish the transitions between these stages of cognitive decline, because they (1) are readily identifiable in plasma, and (2) indicate dysregulated signaling pathways pointing to potential intervention. Toward this end, we expanded the focus of our study to include a comparison of four cohorts: those with moderate or severe probable AD, mild probable AD, MCI, and NEC. In addition to examining miR-34a and miR-34c, we also measured miR-181b, miR-181c, let-7d, let-7f, let-7e, miR-200b, miR-141, miR-144, and miR-411, chosen for their reported roles in defense against oxidative stress, apoptosis, proliferation, and regulation of expression of key AD-related proteins such as Bcl-2 and Sirt1 [17,25-37].

The data presented here suggest that while miR-34c and miR-181c may be good biomarkers to differentiate severe or moderate probable AD from MCI and/or NEC cohorts, they are only fair biomarkers to differentiate mild AD from MCI. On the other hand, miR-411 emerges not only as an excellent biomarker to identify moderate to severe AD patients from their MCI counterparts, but also as a powerful probe to distinguish MCI from the mild stage of Alzheimer’s disease.

Materials and Method

Subjects

This study was approved by the Institutional Review Board of the Jewish General Hospital (JGH) in Montréal. Written informed consent was obtained from the participants themselves, if competent, or their caregivers. Patients diagnosed with either probable sporadic Alzheimer’s disease (AD) or mild cognitive impairment (MCI) was recruited from the Memory Clinic of the Jewish General Hospital (JGH), a teaching hospital of McGill University. Volunteer normal elderly controls (NEC) were recruited from the community through public lectures and newspaper advertisements. All subjects underwent thorough neurological and cognitive assessment, with a full neuropsychological battery. Individuals who were depressed, as determined by the Yesavage Geriatric Depression Scale (>15), were excluded [38]. Subjects accepted into the study are mostly non-smoker had no evidence of cerebral infarct or ischemia [39], heart disease, alcoholism, or drug use, and routine blood work was within normal limits.

In order to be classified as a normal control subject, individuals had to report no subjective memory complaints, score equal to or above 25 on the MMSE [5], and test within normal limits on the neuropsychological battery. MCI and probable AD participants were assessed in the Memory Clinic by a neurologist or geriatrician skilled in evaluating memory-impaired elderly individuals. Diagnosis of probable AD and MCI was based on elements of the following assessments: Modified WAIS subtests (digit symbol) [40], Wechsler Memory Scale-R subtests (Logical Memory, Digit Span) [41], Ravens Progressive Matrices [42,43], Visual perceptual tests, RAVLT [44,45], as well as Verbal Fluency. All subjects classified as MCI met the clinical criteria for MCI defined by Winblad’s group [46]. All had a history of memory decline in the last 1-4 years. All MCI subjects were found to have objective memory impairment on the JGH Memory Clinic Assessment (unpublished), which contains elements of the CERAD, CDR, and Toronto Behavioral Neurology Assessment batteries appropriate for mild dementia subjects [47]. The Montréal Cognitive Assessment (MoCA) [48] exam was also administered to these individuals, as the MMSE score lacks sensitivity for detecting cognitive impairment at the MCI stage; all MCI individuals scored below 26 out of 30 on the MoCA assessment. The MCI subjects did not meet NINCDS-ADRDA criteria for the diagnosis of probable AD, or the DSM-III criteria for dementia [49]. None had significant impairment in activities of daily living.
In addition to the clinical diagnosis of MCI, all subjects underwent neuropsychological evaluation which included the Logical Memory I and II components of the Weschler Memory Scale-Revised [41]. A shortened version of the Boston Naming test [50], letter and category fluency, tests of block design [40] and clock drawing [51] as well as the digit symbol and digit span sub-tests of the WAIS-R verbal intelligence scale [40] were administered. The diagnosis of MCI in our cohort was supported by a neuropsychological evaluation establishing that there was episodic memory performance at least 1 S.D. below the age-normed mean on one of the Logical Memory (paragraph recall) subtests of the Wechsler Memory Scale – Revised, or the delayed verbal memory score from the Rey Auditory Verbal Learning test [52], or below 25 on the Montréal Cognitive Assessment (MoCA). Episodic memory performance based on these results was rated as either intact (within 1 S.D. of age and education norms), >1 S.D. below normal, >1.5 S.D. below normal, or >2 S.D. below normal, again using age and education norms for the tests. A similar procedure was used for each subject in rating their performance on executive function, language function, and visuospatial functions. Of the 34 MCI individuals, 22 had additional cognitive domains >1 S.D. below normal. These would be classified as multi-modal MCI, all with predominant amnesia as the most striking problem. The remaining 11 had impairments limited to the memory domain, and would have been classified as purely amnestic MCI. There was no evidence of clinical differentiation between these subgroups.

Subjects diagnosed as probable AD met clinical criteria for dementia and probable AD [49]. The Mini-Mental State Examination (MMSE) [5] was administered to all subjects, and scores were used as inclusion criteria for the study.

**Collection of Blood and Isolation of RNA**

Ficoll-Paque Plus (GE Healthcare, Piscataway, New Jersey) was used to isolate the plasma fraction from 30 mL blood samples collected in EDTA Vacutainers®. RNA was extracted from plasma samples using the miRNasy Serum Plasma Kit (Qiagen, Venlo, Limberg, Netherlands) following the manufacturer’s instructions, with the addition of a second extraction step following the initial chloroform extraction, adapted from a previous study [53]. For quality control of extraction efficiency and cDNA synthesis in qPCR assays, 33 fmol of both cel-miR-39 and cel-miR-54 (Qiagen) were added to the samples before adding chloroform; the RNA concentrations of the samples were quantified using the Epoch Spectrophotometer (Biotek, Winooski, Vermont), and cellular contamination was assessed on an Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany).

**Sample Selection**

Samples were selected based on a number of factors, including age, MMSE and MoCA scores, and the absence of hemolysis in the samples. MMSE scores for AD samples were required to be below 25; for NEC participants, MMSE scores were equal to or higher than 27. Samples from MCI participants scoring in the 17-23 range on the MoCA were included. Additionally, probable AD samples were classified as mild, moderate, and severe based on the following MMSE scores: 0-9 indicated severe AD, 10-20 moderate AD, and 21-24 mild AD [5,6]. Plasma samples with an absorbance reading above 0.25 at 414 nm were excluded, to avoid interference from hemolysis [54].

**Real Time qPCR using the TaqMan microRNA Assays**

RNA isolated from the aqueous phase of plasma was used to generate cDNA by means of the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, California). Purified RNA was used to synthesize first strand cDNA, using specific miRNA stem-loop primers (Life Technologies, Thermo Fisher, Grand Island, New York) for each microRNA target (catalog number 4427975 and assay IDs: miR-34c: 000428; miR-181c: 000482; miR-411: 001610; cel-miR-39: 002000; cel-miR-54: 001361) to determine these microRNAs’ expression levels by real time quantitative PCR. The reactions were carried out on an ABI 7500 or ABI 7500 Fast real-time PCR system (Applied Biosystems) with Bulseye TaqProbe qPCR 2x Mastermix (ABI, Foster City, California); all reactions were performed in triplicate to control variation. Data normalization was carried out as described below.

**Statistical Analyses**

All statistical analyses were conducted using MS Excel 2010 and SPSS 23.0 statistical software (IBM). For multiple group comparisons, a one-way ANOVA followed by Fisher’s Least Significant Difference (LSD) test and the Bonferroni post hoc test, to control for familywise type 1 error, were applied [55]. Statistical significance was defined as a p-value of p<0.05 for all analyses. For determining relative expression, the ΔΔCt method was utilized, where ΔCt=(normalized Ct value of target microRNA – Ct value of spike-in reference microRNA). We excluded any sample for which the extraction or cDNA step was inefficient, as indicated by high Ct values for cel-miR-39 or cel-miR-54.

Receiver operating characteristic (ROC) analyses were performed with the results of the qPCR data after the above calculations. In general, these analyses involve three parameters: sensitivity, specificity, and accuracy. All three are denoted by numerical indices less than 1; the closer to 1, the better the score for biomarkers to differentiate between two states. The numerical values for sensitivity represent the ability of a test to identify individuals within a given cohort exhibiting a specific state. The numerical values for specificity represent the power to distinguish a specific state from others. ROC analysis also provides numerical values for the area under the curve (AUC), on a scale from 0.5-1.0: the higher the value, the more accurate the test. AUC values between 0.9 and 1.0 are considered...
to be excellent; values between 0.8 and 0.89 are good; values between 0.7 and 0.79 are fair; values between 0.6 and 0.69 are poor; values below 0.6 indicate no usefulness as a biomarker [56]. Calculation of sensitivity, specificity and accuracy follows published methods [57,58] using the same parameters and formulas as previously described [24].

Spearman correlation (ρ) was used to measure the strength of linear association between MMSE scores and relative expression levels of miRNAs studied. The ρ (rho) value ranges from 0 to 1, with a positive or negative relationship. The closer the value of rho to 1, the stronger the association between the two variables [59].

Results

Selection of Plasma Samples for Quantitative PCR Assays and Identification of Expressed microRNA Targets

Plasma samples used for this study were selected from four cohorts, which include individuals with probable moderate or severe sporadic AD, probable mild sporadic AD, MCI, or NEC. Average ages of the cohorts were 75.3 for NEC, 78.9 for MCI, 78.2 for mild AD, and 77.7 for moderate-severe AD. In addition, probable AD patients with MMSE scores of 25 or higher were excluded from our study, as were NEC volunteers scoring below this cutoff. Moderate and severe AD patients were identified as having MMSE scores of 18 or less. Table 1 shows that the overall average MMSE is 13.3 for moderate and severe AD patients, 23.1 for mild AD patients, and 28.7 for NEC, with the MCI group’s cognitive ability determined by the MoCA scoring matrix. The average MoCA score for the MCI group is 20.7. The MMSE scores of the NEC, MCI, mild AD, and moderate-severe AD cohorts are compared with education level, age, and the distribution of ApoE genotypes (Figure 1).

Following the initial selection of samples by cognitive assessment scores for each group, additional acceptance criteria involving RNA sample quality were also considered [60]. Samples with no contaminating cellular

<table>
<thead>
<tr>
<th>Cohort</th>
<th>N</th>
<th>Age Range</th>
<th>Average Age (S.D.)</th>
<th>MMSE Range</th>
<th>MMSE Average (S.D.)</th>
<th>MoCA Range</th>
<th>MoCA Average (S.D.)</th>
<th>% Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEC</td>
<td>37</td>
<td>67-92</td>
<td>75.3 (5.4)</td>
<td>25-30</td>
<td>28.7 (1.2)</td>
<td>N/A</td>
<td>N/A</td>
<td>84</td>
</tr>
<tr>
<td>MCI</td>
<td>34</td>
<td>63-87</td>
<td>78.9 (6.7)</td>
<td>N/A</td>
<td>N/A</td>
<td>17-23</td>
<td>20.7 (1.7)</td>
<td>44</td>
</tr>
<tr>
<td>Mild AD</td>
<td>16</td>
<td>73-84</td>
<td>78.2 (3.5)</td>
<td>21-24</td>
<td>23 (1.1)</td>
<td>N/A</td>
<td>N/A</td>
<td>44</td>
</tr>
<tr>
<td>Moderate-Severe AD</td>
<td>20</td>
<td>63-88</td>
<td>77.7 (6.8)</td>
<td>2-20</td>
<td>13.3 (4.0)</td>
<td>N/A</td>
<td>N/A</td>
<td>70</td>
</tr>
</tbody>
</table>

Figure 1. Demographic distribution of MMSE scores and ApoE status of patients in NEC, MCI and AD cohorts. (A) The MMSE score distribution for each cohort, the MMSE scores for each participant with respect to (B) education level and (C) age, and (D) the ApoE status for each participant are presented.
ribosomal RNA, as measured by the Agilent Bioanalyzer, were considered non-contaminated and selected for use in the study. Additionally, we employed the use of spiked-in synthetic *C. elegans* microRNAs to monitor the consistency of RNA extracted and the quality of cDNA synthesized. The consistent expression of cel-miR-39 and cel-miR-54 between samples and across cohorts suggests that these assays are stringent, and the cDNA synthesized is of optimal quality with minimal putative RNA inhibitors present (Figures 2A and 2B).

**MicroRNA-34c as a Good Biomarker for Distinguishing Probable Moderate-Severe AD from MCI, but a Fair Biomarker for Distinguishing Mild AD from MCI**

We have previously shown that miR-34c is increased in plasma from probable AD patients, compared to plasma from NEC volunteers [24]. Furthermore, we demonstrated that miR-34c is equally abundant in plasma from mild and moderate AD patients. In the current study, we examined miR-34c levels in MCI plasma as well, to test its ability to act as an MCI biomarker.

First, we measured miR-34c levels in moderate-severe AD, mild AD, MCI, and NEC cohorts (Figure 3A). We found that miR-34c is more highly expressed in the moderate-severe and mild AD groups than in MCI or NEC. However, there is no significant difference between the moderate-severe AD and mild AD groups (LSD p-value=0.392; Bonferroni p-value=0.999), or between the MCI and NEC groups (LSD p-value=0.711; Bonferroni p-value=0.999).

Next, we performed ROC analysis to determine the efficacy of miR-34c as a biomarker to distinguish the four cohorts from one another (Figures 3B-3E). The ROC data indicate that miR-34c is a fair test to differentiate moderate-severe AD from MCI (AUC=0.78); a good test to distinguish moderate-severe AD from NEC (AUC=0.82); a fair test to differentiate mild AD from NEC (AUC=0.71); and a poor test to distinguish mild AD from MCI (AUC=0.68).

**MiR-181c as a Biomarker for Probable Moderate-Severe and Mild AD**

In addition to our examination of miR-34c, we also expanded our focus to include additional miRNAs. Members of the miR-181 family are implicated in neuroinflammation [61] and Alzheimer’s disease [17,62]. In the current study, we found that miR-181c is more highly abundant in plasma samples from moderate-severe AD patients than from MCI patients (LSD p-value<0.001; Bonferroni p-value=0.004) or NEC volunteers (LSD p-value=0.004; Bonferroni p-value=0.022) (Figure 4A). Additionally, miR-181c is more highly expressed in the mild AD group than in MCI (LSD p-value<0.001; Bonferroni p-value=0.004) and NEC (LSD p-value=0.005; Bonferroni p-value=0.027) cohorts. However, there are similar levels of miR-181c in plasma from moderate-severe and mild AD groups.
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There are also similar levels in the MCI and NEC cohorts (LSD p-value=0.493; Bonferroni p-value=0.999). ROC analysis shows that miR-181c effectively differentiates moderate-severe AD samples from both MCI and NEC cohorts, with AUC values of 0.86 and 0.79, respectively (Figures 4B and 4C). This indicates that miR-181c serves as a good biomarker for separation of moderate-severe AD and MCI, and a fair biomarker for separation of moderate-severe AD and NEC. However, miR-181c is a poor biomarker for distinguishing between mild AD and either MCI (AUC=0.63) or NEC (AUC=0.60) (Figures 4D and 4E).

miR-411 Distinguishes MCI and NEC from Mild, Moderate and Severe AD

Although neither miR-34c nor miR-181c perform well as biomarkers for mild AD compared to MCI and NEC cohorts, qPCR results for plasma miR-411 indicate that this microRNA may be a promising biomarker to distinguish between all stages of probable AD and both MCI and NEC groups. There are significant differences when comparing moderate-severe AD to either MCI or NEC (LSD p-values<0.001; Bonferroni p-values<0.001), and when comparing mild AD to either MCI or NEC (LSD p-values<0.001; Bonferroni p-values<0.001) (Figure 5A). However, no difference is detected between either the moderate-severe and mild AD groups (LSD p-value=0.136; Bonferroni p-value=0.818), or between the MCI and NEC cohorts (LSD p-value=0.551; Bonferroni p-value=0.999) (Figure 5A).

Next, we applied ROC analysis to determine the effectiveness of miR-411 in separating the four cohorts examined. We calculated AUC values of 0.92 when comparing moderate-severe and MCI groups, and 0.99 when comparing moderate-severe and NEC groups (Figures 5B and 5C). For the mild AD comparisons, we calculated AUC values of 0.93 when compared to the MCI cohort, and 0.98 when compared to the NEC cohort (Figures 5D and 5E). These values indicate that miR-411 is an excellent biomarker to separate all stages of AD from both MCI and NEC. All ROC values, including AUC, 95% confidence interval, sensitivity, and specificity are summarized for miR-34c (Table 2A), miR-181c (Table 2B), and miR-411 (Table 2C).
Correlation between microRNA Biomarker Expression and MMSE Scores

Finally, correlation analysis was used to examine the relationship between MMSE scores and microRNA expression (Figure 6). Interestingly, we found statistically significant inverse relationships for all three microRNAs: p=0.001 for miR-34c; p=0.011 for miR-181c; and p<0.001 for miR-411. However, for miR-34c and miR-181c the ρ-values of -0.37 for miR-34c and -0.3 for miR-181c are relatively low, indicating that high expression of these microRNAs is not a strong predictor of a low MMSE score or more severe cognitive decline (Figures 6A and 6B). On the other hand, the ρ-value for miR-411 is -0.75, which shows a strong negative correlation between levels of this microRNA and MMSE score, i.e., the higher miR-411 levels in the plasma, the lower the MMSE scores (Figure 6C).

Discussion

We have previously demonstrated overexpression of miR-34c in plasma of Alzheimer’s disease patients of all three stages, mild, moderate and severe, compared to plasma from normal elderly controls [24]. The current study replicates this result, with the important additional measurement of miR-34c in plasma from MCI patients, which is found at levels comparable to the NEC cohort. Additionally, another of our previous studies indicated that miR-181b is upregulated in blood mononuclear cells from probable AD patients [12]. In the present study, we examined levels of the related miR-181 family member, miR-181c, and found this microRNA also overexpressed in plasma from probable AD patients, but not MCI patients. Most importantly, we found that miR-411, a novel AD-associated microRNA, may serve as a far more powerful plasma biomarker based on AUC values, which are greater than 0.90 comparing all stages of probable AD with MCI individuals and normal elderly controls. These results suggest the importance not only of including MCI patients in all dementia biomarker discovery endeavours, but also of the possibility of identifying a new class of biomarkers which differentiate cognitively-impaired, but functionally intact (MCI), persons from functionally impaired individuals in the earliest stages of Alzheimer dementia.
Figure 5. Expression levels and Receiver Operating Characteristic curves for miR-411. (A) Relative expression levels of miR-411 in moderate-severe AD, mild AD, MCI, and NEC cohorts, along with the sample distributions within each cohort, are shown. (B-E) ROC curve plot results for miR-411 analyzing (B) moderate-severe AD vs. MCI, (C) moderate-severe AD vs. NEC, (D) mild AD vs. MCI, and (E) mild AD vs. NEC, with AUC values, are depicted. The dotted line for each plot represents the area under the curve, with the solid line representing the reference line. The ROC curve plots sensitivity (true positive) against 1 – specificity (false positive).

Table 2. AUC, Sensitivity, Specificity, for miRNA biomarkers in combined cohorts. ROC analysis parameters are summarized for (A) miR-34c, (B) miR-181c and (C) miR-411

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Comparison</th>
<th>Area under the curve</th>
<th>95% Confidence Interval</th>
<th>Sensitivity</th>
<th>Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) miR-34c</td>
<td>Sev/Mod AD vs. MCI</td>
<td>0.77 (p&lt;0.005)</td>
<td>0.61–0.93</td>
<td>0.64</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>Sev/Mod AD vs. NEC</td>
<td>0.82 (p&lt;0.001)</td>
<td>0.67–0.95</td>
<td>0.71</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>Mild AD vs. MCI</td>
<td>0.68 (p=0.051)</td>
<td>0.48–0.88</td>
<td>0.60</td>
<td>0.89</td>
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<td></td>
<td>Mild AD vs. NEC</td>
<td>0.71 (p=0.025)</td>
<td>0.51–0.90</td>
<td>0.73</td>
<td>0.68</td>
</tr>
<tr>
<td>(B) miR-181c</td>
<td>Sev/Mod AD vs. MCI</td>
<td>0.86 (p&lt;0.001)</td>
<td>0.74–0.97</td>
<td>0.69</td>
<td>0.85</td>
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<td>Sev/Mod AD vs. NEC</td>
<td>0.79 (p&lt;0.004)</td>
<td>0.64–0.93</td>
<td>0.69</td>
<td>0.73</td>
</tr>
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<td></td>
<td>Mild AD vs. MCI</td>
<td>0.63 (p=0.187)</td>
<td>0.39–0.86</td>
<td>0.57</td>
<td>0.96</td>
</tr>
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<td></td>
<td>Mild AD vs. NEC</td>
<td>0.60 (p=0.321)</td>
<td>0.36–0.83</td>
<td>0.57</td>
<td>0.88</td>
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<tr>
<td>(C) miR-411</td>
<td>Sev/Mod AD vs. MCI</td>
<td>0.92 (p&lt;0.001)</td>
<td>0.84–1.00</td>
<td>0.77</td>
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<td>Sev/Mod AD vs. NEC</td>
<td>0.99 (p&lt;0.001)</td>
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<td>Mild AD vs. MCI</td>
<td>0.93 (p&lt;0.001)</td>
<td>0.86–1.00</td>
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<td></td>
<td>Mild AD vs. NEC</td>
<td>0.98 (p&lt;0.001)</td>
<td>0.94–1.00</td>
<td>0.93</td>
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</tr>
</tbody>
</table>

Of note, the miRNAs found in this study to be over-represented in plasma of probable AD patients are present in the CNS. The expression of members of the miR-34 family in brain is widely documented and linked to aging in mice [63], learning impairment in mice [23], and AD pathology in human hippocampus [64]. MicroRNA-181
family members are dysregulated in a mouse model of AD [62], involved in neuroprotection following stroke [65], protection from neuroinflammation [61] and neuronal apoptosis [66], and related to aberrant gene expression in schizophrenia [67]. miR-411 has also been reported in human brain, although its function(s) within the CNS remain unknown [68,69].

To our knowledge, PubMed contains no literature reporting differences in gender specificity in plasma microRNA expression. Moreover, in both animal and human studies, none of the above mentioned microRNAs exhibit gender-specific differences in expression in circulating blood samples. Therefore, even though our normal elderly control (NEC) cohort is predominantly female (84%), and the MCI/Mild-AD cohorts only 44% female, the impact of this gender difference on our data analysis is likely to be negligible. However, we shall address this possibility of gender difference as a potential confounding factor impacting our data analysis when future whole genome analysis by Next Generation Sequencing of large scale human populations of both genders becomes available.

Previously, we and others have shown that miR-34a and miR-34c repress BCL2, SIRT1, and ONECUT2 translational expression, vital to signaling pathways for neuronal survival, antioxidant defense, and synaptic maintenance [18-21,24]. Interestingly, according to targetscan.org, members of the miR-181 family are also predicted to target and repress all three of these proteins as well [70]. The underlying functional impact of increases in these two families of microRNAs is a weakening of antioxidant defences within the CNS, one of the prominent signalling networks compromised in AD [71]. Furthermore, this suggests that upregulation of microRNAs in the miR-34 and miR-181 families may work together to repress expression of key AD-associated genes, resulting in AD pathology and symptomatology.

miR-411, however, targets a different set of proteins, involved in separate AD-related signaling pathways. Although little is known about the role of miR-411 in AD, targetscan.org and DAVID Bioinformatics Resources [72,73] reveal that this microRNA is expected to repress expression of several known AD-related genes. Two of these genes, neprilysin and insulin-degrading enzyme, have
important roles in the degradation of beta-amyloid [74-78]. Other putative AD-related target genes include beta-secretase-1, NMDA receptor, nitric oxide synthase, and several mitochondrial complexes, including cytochrome b and c, ATP synthase, and NADH dehydrogenase [79-82].

In conclusion, we present a trio of microRNAs, miR-34c, miR-181c, and miR-411, which may aid in the diagnosis of AD by differentiating AD patients, most importantly those in the early, mild phase of the disease, from those with MCI or normal elderly individuals. Clearly, the usefulness of increases in these microRNAs, especially miR-411, needs to be validated by studies with larger cohort sizes. Additionally, measuring these miRNAs in cerebrospinal fluid may provide a broader picture of miRNA expression in AD and MCI. Dementia of the Alzheimer type is a chronic, progressive, and variable disease, whether characterized in terms of clinical assessment or in terms of biomarkers for beta-amyloid plaques and neurofibrillary tangles [3,83-87]. However, utilization of a non-invasive blood-based biomarker test that can clearly distinguish between MCI and AD, specifically those at the mild stage, would be highly beneficial not only for diagnosis and prognosis of mild cognitive impairment, but also as a tool to assess the transition from mild memory loss without functional impact, to a clinical stage characterized by loss of daily functional capability. Future experiments using RNAseq may yield other candidate microRNAs in addition to miR-411 which mark this critical transition to dementia. Finally, it will be essential to ascertain the accuracy of miR-411 and other lead miRNAs in distinguishing AD from other neurocognitive disorders, such as dementia with Lewy bodies, frontotemporal dementia, normal pressure hydrocephalus and depression.

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Distinguishing mild cognitive impairment from Alzheimer’s disease by increased expression of key circulating microRNAs.


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