Target Genes of Circulating miR-34c as Plasma Protein Biomarkers of Alzheimer’s Disease and Mild Cognitive Impairment

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Abstract

MicroRNAs repress post-transcriptional expression of a variety of genes, several of which are involved in neurological development and Alzheimer’s disease (AD) pathology. In previous studies, we have shown that plasma miR-34c and miR-34a levels are increased in probable AD patients. In this study, we show that four key gene products silenced by these miRNAs, Onecut homeobox 2 (ONECUT2), B-cell lymphoma (BCL2), sirtuin 1 (SIRT1), and presenilin 1 (PSEN1), are all decreased in abundance in plasma specimens from mild cognitive impairment (MCI) and probable AD individuals. Additionally, there is a statistically significant inverse correlation between levels of miR-34c and expression of all four proteins, and between miR-34a and ONECUT2 expression. In particular, our results suggest that ONECUT2 plasma levels may act as a novel biomarker for early stages of cognitive decline. Additionally, we find statistically significant differences in expression levels of the other three targets, BCL2, SIRT1, and PSEN1, in circulating plasma, and levels of these proteins effectively distinguish among all three groups: normal elderly controls (NEC), MCI, and probable AD. Therefore, the inverse relationship between miR-34c and miR-34a and their targets may initially be detected by early decline in ONECUT2 expression, upon entry to the MCI stage, followed by decreased BCL2, SIRT1 and PSEN1 expression as patient’s transition to full-blown AD dementia.

Keywords: Alzheimer’s disease; BCL2; SIRT1; Presenilin-1; ONECUT2; Mild cognitive impairment

Abbreviations:
AD: Alzheimer’s Disease; MCI: Mild Cognitive Impairment; NEC: Normal Elderly Controls; Aβ42: Amyloid Beta 42; PSEN1: Presenilin-1; BCL2: B-cell Lymphoma 2; SIRT1: Sirtuin 1; ONECUT2: Onecut Homeobox 2; MMSE: Mini-Mental State Exam; LSD: Fisher’s Least Significant Difference Statistic; ROC: Receiver Operating Characteristic; AUC: Area Under the Curve

Introduction
An estimated 24.3 million people worldwide have AD, and it is projected that the number of AD sufferers will double every 20 years until 2040 [1]. AD pathology is characterized by the presence of extracellular plaques composed of amyloid beta fragments and intracellular neurofibrillary tangles composed of hyperphosphorylated tau [2]. Furthermore, mutations in amyloid precursor protein (APP) and its cleavage products, amyloid beta 40 (Aβ40) and amyloid beta 42 (Aβ42), and mutant PSEN1 and PSEN2, both involved in aberrant APP cleavage, are associated with development of familial AD [3]. Although the presence of plaques and tangles are indicative of AD, a variety of other mechanisms participate in disease pathogenesis, including inflammation, excitotoxicity, oxidative stress, synaptic dysfunction, and apoptosis [2,4]. The post-transcriptional repression by a particular microRNA of its target gene’s expression usually results in the decreased expression of the protein, i.e. increased abundance of a miRNA corresponds to decreased presence of its target protein(s). This effect is observed not only in in vitro functional assays in cultured cell systems, but also at the physiological level in numerous tissue and disease states. We have demonstrated such reciprocal expression with increased miR-34a expression and decreased abundance of its target genes in circulating mouse plasma during aging [5]. Recently, we demonstrated that miR-34a and its sister, miR-34c, are increased in abundance in plasma from patients with probable AD [6]. In particular, we showed that miR-34c is capable of repressing the expression of key target genes including ONECUT2, SIRT1, BCL2, and PSEN1, all of which are known to be involved in AD-related pathogenesis [6].

Target genes of miR-34c examined in our study are involved in the upstream regulation of neuronal development, apoptosis, and protection from oxidative stress, neuronal survival, and Aβ42 production. For example, ONECUT2, expressed in several cell types including neurons, is involved in development and differentiation of motor and sensory neurons, as well as the development of several brain regions [7-11]. SIRT1 has a protective, anti-apoptotic effect in cells, perhaps through protection from oxidative stress, and also provides protection from tau hyper phosphorylation [12-20]. BCL2, an anti-apoptotic, pro-survival cellular protein associated with tumor genesis [21], is elevated during brain development [22], remains relatively stable in certain areas of the brain during adulthood, and then declines during aging, leading to decreased neuronal survival...
Additionally, Aβ42 can suppress BCL2 activity, resulting in increased apoptosis [24,25], and overexpression of BCL2 results in suppression of Aβ42-induced apoptosis [26]. Finally, mutations in the protein PSEN1, a component of the machinery involved in APP cleavage [27], are associated with the development of early-onset familial AD [28,29] as well as late-onset sporadic AD [30].

Therefore, the paradigm for our study is to determine whether the observed increase of miR-34c in circulating plasma of AD patients is accompanied by the reduced presence of key target genes ONECUT2, SIRT1, BCL2, and PSEN1 in AD and MCI plasma, implicating these proteins as potential biomarkers for neurodegeneration. Additionally, a statistically significant inverse correlation exists between miR-34c plasma levels and levels of ONECUT2, SIRT1, BCL2, and PSEN1, and between miR-34a plasma levels and ONECUT2 plasma abundance over the course of disease progression. Statistical analysis indicates that ONECUT2 levels may be used to differentiate between NEC and MCI, and between NEC and probable AD, while SIRT1, BCL2, and PSEN1 levels effectively distinguish all three states: NEC, MCI, and AD. Overall, plasma levels of these four proteins may be excellent biomarkers for the transition from normalcy to mild cognitive impairment and finally progression to AD-associated dementia.

Materials and Methods

Subject identification and enrollment

Subjects recruited for this study consisted of individuals aged 60 to 85, with 10 or more years of education. Those diagnosed with AD or MCI were recruited to the study at the Memory Clinic of the Jewish General Hospital (JGH) in Montréal, and normal community elderly volunteers were recruited through advertising. Subjects received standardized clinical, mental status, neuropsychological, and cognitive evaluation. Depression was excluded via the Yesavage Geriatric Depression Scale (Geriatric Depression scale >15) [31]. None had evidence of cerebral infarct on CT, and all subjects scored less than 4 on the Hachinski ischemic scale [32]. No individuals accepted for the study had evidence of significant heart disease, alcoholism, or drug use, and all showed normal routine blood work.

Subjects were classified as normal if they lacked subjective memory complaints and scored at or above 26 on the Montreal Cognitive Assessment (MoCA) test [33] and above 25 on the Modified Wechsler Memory Scale-R subtests (digit symbol) [44], Wechsler Memory Scale-R subtests (Logical Memory, Digit Span) [45], Ravens Progressive Matrices [46,47] Visual perceptual tests, RAVLT [48,49], as well as Verbal Fluency.

RNA isolation, cDNA synthesis, and quantitative PCR (qPCR)

RNA was extracted using Qiagen’s miRNeasy serum/plasma kit (Qiagen, Venlo, Limberg, Netherlands). Aliquots of each sample were thawed on ice and spun in a microcentrifuge for 3 minutes at 3000 x g. The protocol for RNA extraction followed the vendor’s product handbook. The organic phase was saved for further protein extraction; the concentration of the resultant RNA was measured with a NanoVue (GE Healthcare Life Sciences, Pittsburgh, PA).

Complementary DNA specific to each target miRNA or endogenous control miRNA was synthesized by adding 100-200 ng of RNA to a cDNA reaction using the Taqman microRNA Reverse Transcription kit (Life Technologies, Carlsbad, CA) with the 5X primers provided in the Taqman small RNA assays (Life Technologies) for each miRNA. Reactions were placed in a Veriti 96-well Thermalcycler (Life Technologies), according to the protocol provided in the Taqman small RNA assay protocol handbook.

Complementary DNA template (1.33 ul) was added to each qPCR reaction well with 20X probes provided in the Taqman small RNA assays (Life Technologies) and real-time qPCR EvaGreen Master Mix (MiSci, St. Louis, MO) in triplicate. The plates were loaded into either an ABI7500 or ABI7500 Fast Real-time PCR system (Life Technologies).
Protein isolation, electrophoresis, and Western blotting

Using 100% isopropanol, proteins were precipitated from the organic phase left behind after removal of the aqueous phase during RNA isolation. The samples were spun and the pellet washed with 0.3 M guanidine hydrochloride in 95% ethanol three times. The pellets were then incubated for 20 minutes in 100% ethanol, and transferred to a new tube. Pellets were dried for 15 minutes, dissolved in 1% SDS, and incubated at 55°C for 2-3 hours [50].

Protein concentrations were determined by the Bradford method. Albumin and gamma-globulin proteins were then removed from 500-600 µg of protein using an albumin and IgG removal kit (Pierce, Rockford, IL). Protein concentrations were again determined by the Bradford method, and samples were stored at -80°C.

Following isolation, protein extracts were run on 10.5% polyacrylamide SDS-PAGE gels, and transferred to nitrocellulose membranes for 2-3 hours in 20% methanol transfer buffer. Membranes were stained with Ponceau Red to visualize total protein transfer and for normalization of the loaded protein samples. Membranes were then blocked in 5% milk or bovine serum albumin for 1 hour, incubated in the appropriate primary antibody overnight at 4°C, washed in Tris-Buffered Saline-Tween (Sigma-Aldrich, St. Louis, MO) and incubated in the secondary antibody for 1-2 hours at room temperature. Membranes were again washed and incubated in ECL solution (Pierce) for 2 minutes, and then imaged using autoradiography.

Data analysis

Relative expression values for qPCR data were calculated using the 1/A Ct method. Mean intensity values for Western blot data were calculated using ImageJ software (Public Domain, NIMH, NIH, Bethesda, MD) to find the intensity of each target protein band, which was then normalized by dividing this value by the intensity of the corresponding Ponceau-stained band. Although a housekeeping gene, such as β-actin or tubulin, is often used to normalize Western blot data from tissue or cultured cells, the abundance of these proteins is not well-characterized in cell-free plasma. Additionally, these housekeeping proteins may not be suitable for all systems or conditions, and may not always accurately reflect protein concentration [51-53]. Furthermore, it is unknown whether various disease states may induce changes in expression of these genes. A band recognized by Ponceau staining with consistent intensity across all lanes has been reported as a suitable alternative to the use of housekeeping genes for Western blot normalization [54]. All normalized values were then averaged, and the mean intensity was reported. Intensities were compared among the NEC, MCI, and AD cohorts using a Fisher’s Least Significant Difference (LSD) test [55]. Correlation analysis was performed using SPSS statistical software. Pearson correlation values (R) and p values were calculated. A p-value of less than 0.05 indicates a significant relationship.

Data were further subjected to Receiver Operating Characteristic (ROC) analysis to determine the sensitivity, specificity and accuracy of the biomarkers [56]. ROC curve analysis is used to compare results of a diagnostic laboratory test for two or more groups. The ROC curve is plotted using sensitivity (which represents true positive values) on the y-axis against 1-Specificity (which represents false positive values) on the x-axis. The resulting area under the curve (AUC) demonstrates the overall ability of the test to discriminate between the two groups compared. AUC values range from 0.5 to 1. A bad test has an area of 0.5, which is close to the diagonal line, and an area of 1.00 indicates perfect accuracy [56-58]. Calculation of all parameters is done using formulas described in our previous paper [6].

Results

Patient demographics and genetics

Ten samples were selected from each cohort (AD, MCI and NEC) to match age and years of education among the three groups. The mean age was 71.7 for the NEC samples, 74.3 for the MCI group, and 82.0 for the AD group (Table 1). Mean years of education were similar among the three groups, with an average of 14.3 years in the NEC group, 13.3 in the MCI group, and 13.0 in the AD subjects. There were differences in the percentage of participants possessing at least one ApoE4 allele: 36.4% of AD samples had either a 3/4 or 4/4 genotype; 8.3% of the MCI group had either a 3/4 or 4/4 genotype; and the NEC group contained no subjects with either genotype. This statistic reflects the composition of our bio bank: more AD and MCI patients possess an ApoE4 allele than participants from the NEC group, reflecting the important role of the ApoE4 allele as an AD risk factor [59].

Table 1: Characteristics of NEC, MCI, and AD subjects. Demographic parameters, including age, education, and ApoE allele status are presented for each cohort examined in the study.

<table>
<thead>
<tr>
<th></th>
<th>NEC</th>
<th>MCI</th>
<th>AD</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Mean Age</td>
<td>71.7 (3.1)</td>
<td>74.3 (9.0)</td>
<td>82.0 (6.9)</td>
</tr>
<tr>
<td>Mean Education</td>
<td>14.3 (3.8)</td>
<td>13.3 (3.8)</td>
<td>13 (4.7)</td>
</tr>
<tr>
<td>% ApoE 4/4 or 3/4 positive</td>
<td>0</td>
<td>8.3</td>
<td>36.4</td>
</tr>
</tbody>
</table>

Expression of ONECUT2, SIRT1, BCL2, and PSEN1 in AD and NEC plasma

Our previous data indicate that miR-34c constructs repress expression of ONECUT2, SIRT1, BCL2, and PSEN1, when transfected into HEK293 cells [6]. In the current study, we first measured levels of these four proteins in plasma from both AD and NEC participants, by Western blot (Figure 1A). We found the average expression of each protein to be significantly reduced in the AD cohort compared to the NEC subjects (Figure 1B and Table 2).

Table 2: Average relative intensities of protein expression in AD and NEC subjects. Relative intensities for each protein were averaged and standard deviations calculated for data presented in Figure 1, the average of the protein band intensities was normalized against a reference Ponceau Red band.

<table>
<thead>
<tr>
<th>Protein</th>
<th>AD</th>
<th>NEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCL2</td>
<td>1.259 (0.08)</td>
<td>1.522 (0.08)</td>
</tr>
<tr>
<td>PSEN1</td>
<td>0.767 (0.09)</td>
<td>0.955 (0.10)</td>
</tr>
<tr>
<td>ONECUT2</td>
<td>0.626 (0.02)</td>
<td>0.763 (0.02)</td>
</tr>
<tr>
<td>SIRT1</td>
<td>0.816 (0.01)</td>
<td>0.842 (0.02)</td>
</tr>
</tbody>
</table>
Expression of ONECUT2, SIRT1, BCL2, and PSEN1 in MCI plasma compared to NEC and AD plasma

Because we found a significantly decreased abundance of ONECUT2, SIRT1, BCL2, and PSEN1 in AD plasma compared to NEC plasma, we sought to determine whether these decreases in protein abundance could be detected earlier in disease progression, namely in the pre-dementia MCI phase. Therefore, we examined levels of these proteins in plasma from MCI participants, and compared expression to AD and NEC levels (Figure 3).

After normalization with Ponceau Red band intensities, all four proteins, BCL2, SIRT1, ONECUT2 and PSEN1, exhibited decreased expression when comparing AD or MCI individuals’ plasma with NEC samples (Figures 3A-3D). Interestingly, while measurement of ONECUT2 circulating levels revealed a significant decrease in expression between NEC and MCI samples (p<0.0001), there was no difference when comparing MCI and AD intensity levels (Figure 3E and Supplementary Table 1). This implies that decreased ONECUT2 may be manifested early in the disease process, and then may remain low as disease progresses.

Examination of SIRT1, BCL2, and PSEN1 plasma levels revealed that these proteins exhibit progressive decreases in expression from NEC to MCI, and again to the AD dementia stage of the illness (Figures 3A-3E and Supplementary Table 1). For both SIRT1 and PSEN1, p<0.0001 when comparing NEC and MCI, NEC and AD, or MCI and AD. For BCL2, p<0.0001 comparing NEC and MCI, p<0.001 comparing NEC and AD, and p=0.0169 comparing AD and MCI. These data indicate a pattern of stepwise repression of expression, in which these three proteins decrease in plasma during the transition from normalcy to mild cognitive impairment, and then again to the bona fide AD phase of cognitive decline.
Plasma ONECUT2, BCL2, SIRT1, and PSEN1 as biomarkers of AD and MCI

Because mean ONECUT2, BCL2, SIRT1, and PSEN1 levels are significantly lower in plasma from both MCI and AD patients (Figure 3) and inversely correlated with miR-34c levels (Figure 2), we next examined the ability of these proteins’ levels to distinguish between the AD and NEC, MCI and NEC, and AD and MCI cohorts. Receiver operating characteristic (ROC) analysis was used to determine the sensitivity of the four target genes as biomarkers for the two different cognitive decline states. Our results show that ONECUT2 separates the AD and NEC groups as well as the MCI and NEC groups, with an area under the curve (AUC) of 1.0, indicating that ONECUT2 may serve as an excellent biomarker for differentiating both AD and MCI from NEC (Figures 4A and 4B and Supplementary Table 2). However, because ONECUT2 levels remain low throughout progression from MCI to AD, this protein performs poorly as a biomarker to distinguish between these two disease states with an AUC value of only 0.65 (Figure 4C and Supplementary Table 2).

The same statistical analysis revealed a different pattern of biomarker efficacy for the other three proteins. Plasma levels of SIRT1, BCL2, and PSEN1 differentiate between NEC and AD, NEC and MCI, and AD and MCI, with excellent AUC values (Figures 5A-5C and Supplementary Table 2). When distinguishing between NEC and AD, all three target proteins demonstrated an AUC of 1.0. For differentiating between the MCI and NEC groups, BCL2 had an AUC of 0.96, SIRT1 an AUC of 0.99, and PSEN1 an AUC of 1.0. In separating the AD and MCI groups, the AUC for BCL2 was 0.91 and 1.0 for both SIRT1 and PSEN1. Together, these data indicate that all three of these proteins may serve as robust biomarkers for differentiating between the three stages of disease progression.

Inverse relationship between miR-34c and miR-34a and protein expression in AD, MCI, and NEC plasma

Finally, we examined linear trends to explore the correlation between expression of miR-34c and miR-34a and down-regulation of their protein targets in AD, MCI, and NEC plasma (Figures 6 and 7). Statistically significant correlations between miR-34c levels and each target protein were found, with p<0.001 for all four proteins examined (Figure 6). Analysis of the relationships between miR-34a and target proteins shows the inverse relationship to be significant only for
Onecut2 (p=0.005) (Figure 7A) but not the other three targets (Figures 7B-7D). This shows that among all four targets, decreased ONECUT2 level is most significantly correlated with the increase of both miR-34c and miR34a levels comparing the plasma specimens of all three groups.

### Figure 7: Relationships between miR-34a levels and protein expression.

miR-34a levels were measured in AD, MCI, and NEC plasma, and expressed as 1/ ΔCt. These values were plotted against (A) protein intensity for ONECUT2, (B) SIRT1, (C) BCL2, and (D) PSEN1. Pearson Correlation Value (R) and p values were calculated for each protein.

### Discussion

In this study, we show that known target genes, ONECUT2, BCL2, SIRT1, and PSEN1, of two microRNAs, miR-34a & -34c, are significantly reduced in abundance in plasma from AD and MCI patients as compared to NEC samples. Additionally, these reduced protein abundance levels correspond to the increased presence of the circulating miR-34c levels in examined plasma samples. Specifically, we demonstrate here that decreased ONECUT2 expression in plasma is a powerful and novel biomarker to distinguish normal elderly controls from MCI and AD patients, while BCL2, SIRT1, and PSEN1 distinguish among all three stages, from normalcy to the pre-dementia (MCI) and dementia (AD) stages of cognitive decline.

Selection of the four proteins included in our study follows the findings that they are targets of two microRNAs, miR-34a & -34c, as previously reported by cell-based functional assays [6]. Determination of a specific protein to be a good plasma biomarker is largely based on receiver operating characteristic (ROC) analysis. The specific area under the curve (AUC) calculated by the ROC method indicates whether a specific protein target of these two microRNAs is a good or poor protein biomarker. For example, Onecut2 is a powerful biomarker to distinguish the MCI state from normalcy, since the AUC value is 1.0 (Figure 4B). However, this protein’s abundance is not a good biomarker to distinguish MCI from the AD state, due to a low AUC value of 0.65 (Figure 4C). Figure 8 provides a flow chart outlining the identification and classification process from the qPCR analysis of the two microRNAs’ levels, to Western blotting of the abundance of their targets in the same individuals, to ROC analysis for the AUC values.

These decreases may have important physiological implications in MCI and AD (Figure 9). The inverse relationship between miR-34c abundance and levels of its protein targets may be thought of as a balance between two families of molecules, with various degrees of tilting between miR-34c and ONECUT2 in normal elderly controls, MCI, and AD stages (Figures 9A1-9A3). In this miRNA-target relationship, the degree of repression of the ONECUT2 gene reaches its maximal level at the transition between normal aging and MCI. In contrast, the other three targets of miR-34c, i.e. SIRT1, BCL2, and PSEN1, tilt more gradually from NEC to MCI, and then to AD, with the most severe tilting and lowest protein levels seen in the AD phase (Figures 9B1-9B3). Thus, the inverse relationship between miR-34c levels and expression of its targets, as represented here by four proteins, may point to a miR/target partnership in the control of AD pathogenesis, including the processes of amyloid plaque formation, synaptic decline, and neuronal apoptosis (Figures 9C1-9C3).

Our observation that increased circulating miRNA expression correlates with decreased circulating target protein expression suggests that plasma may provide a window into the etiopathogenesis of CNS disorders. Plasma, which is more readily available and easily accessible than other fluids such as cerebrospinal fluid, can therefore be reliably used to investigate interactions between miRNA and protein expression in neurodegeneration, and perhaps other diseases as well. Indeed, SIRT1 expression is down-regulated in both cortex of AD patients [60] and serum from AD and MCI patients [61], suggesting that circulating SIRT1 concentrations reflect levels in diseased tissue. Along with the current study, these results indicate that circulating protein and miRNA changes reflect pathological changes in tissue, and that plasma is a viable source of clinically-accessible protein biomarkers.
Intriguingly, our data point to ONECUT2 as a potentially important and novel circulating protein in neurodegenerative progression. Although several studies demonstrate a role for ONECUT2 as a transcription factor involved in liver [62] and neurological development [9,10], no link between this transcription factor and AD has previously been established. However, some brain and spinal structures influenced by ONECUT2 expression may also be involved in AD pathology. Of particular interest, the locus coeruleus is of particular interest, the locus coeruleus is involved in behavior and neurotransmitter regulation [7], significantly decreases fairly early in disease development [63-65]. Here we observe that ONECUT2, a driver of locus coeruleus development [7], significantly decreases fairly early in disease progression, i.e. at the MCI stage. This suggests that circulating ONECUT2 expression, regulated by both miR-34c and miR-34a, may serve as an early warning signal of AD-related processes in this key brain region.

Additionally, because ONECUT2 levels are expressed at significantly reduced levels early in disease progression, this protein may best serve as a prognostic indicator of very early cognitive decline. Indeed, it may point to the possibility of early therapeutic intervention and delay of disease progression. On the other hand, because BCL2, SIRT1, and PSEN1 expression are increased at a lower rate, leading to early stages of amyloid plaque deposition, synaptic decline, and neuronal apoptosis (A2, B2, and C2). Once the AD stage is reached, ONECUT2 is at similarly low levels as in the MCI phase, but miR-34c levels are even higher than in the MCI phase; SIRT1, BCL2, and PSEN1 expression has decreased from the MCI stage; amyloid plaque deposition, synaptic decline, and neuronal apoptosis continue (A3, B3, and C3).

In conclusion, we demonstrate decreased plasma expression of mir-34c target proteins, with ONECUT2 emerging as a potential candidate biomarker for transition from normal states to mild cognitive impairment (MCI), while the levels of SIRT1, BCL2, and PSEN1 may serve as diagnostic markers for further transition of decline from MCI to bonafide AD. Further work needs to be done to examine levels of other miR-34c-targeted proteins and to validate these proteins as prognostic and diagnostic biomarkers in large-cohort studies.

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Disclosure Statement

Eugenia Wang is on entrepreneurial leave from the University of Louisville School of Medicine, with 51% of her effort committed to Advanced Genomic Technology, LLC, a start-up company in Louisville, Kentucky; her other 49% is at the University of Louisville, as the Greens Endowed Chair on Aging and Professor of Biochemistry and Molecular Biology. Arin Zirnheld and Vikram Shetty are employees of Advanced Genomic Technology (AGT), and Erik L. Regalado was also an employee of AGT at the time the experiments were performed.

Howard Chertkow sits on an adjudication board for clinical trials for Bristol Myers Squibb, and has been a speaker and Advisory Board member for Pfizer Canada. Hyman Schipper has served as consultant to Caprinon Pharmaceuticals, Molecular Biometrics Inc., Osta Biotechnologies, TEVA Neurosciences, Immunotec, and Apopharma.

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