Peripheral α-Defensins 1 and 2 are Elevated in Alzheimer’s Disease


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Abstract. Biomarkers enabling the preclinical identification of Alzheimer’s disease (AD) remain one of the major unmet challenges in the field. The blood cellular fractions offer a viable alternative to current cerebrospinal fluid and neuroimaging modalities. The current study aimed to replicate our earlier reports of altered binding within the AD-affected blood cellular fraction to copper-loaded immobilized metal affinity capture (IMAC) arrays. IMAC and anti-amyloid-β (Aβ) antibody arrays coupled with mass spectrometry were used to analyze blood samples collected from 218 participants from within the AIBL Study of Aging. Peripheral Aβ was fragile and prone to degradation in the AIBL samples, even when stored at −80°C. IMAC analysis of the AIBL samples lead to the isolation and identification of alpha-defensins 1 and 2 at elevated levels in the AD periphery, validating earlier findings. Alpha-defensins 1 and 2 were elevated in AD patients indicating that an inflammatory phenotype is present in the AD periphery; however, peripheral Aβ levels are required to supplement their prognostic power.

Keywords: α-defensins, Alzheimer’s disease, amyloid-β, biomarkers, blood, inflammation, mass spectrometry

INTRODUCTION

With underlying amyloid-β (Aβ) pathology preceding the clinical onset of Alzheimer’s disease (AD) by upwards of 20 years [1], it is recognized that to be effective, disease-specific therapeutic interventions should be implemented within the preclinical stages of the disease, before synaptic loss and neuronal degeneration are largely irreversible [2]. However in order to implement such a strategy, peripheral biomarkers allowing preclinical identification of at-risk individuals are needed.

To date, efforts to identify peripheral biomarkers for AD have focused on plasma Aβ levels; however, the inherent variability of these investigations (reviewed in [3]) have led investigators to turn their attention toward non-Aβ-centric blood-borne biomarkers. Previous investigations have reported that altered levels are present in AD for a number of non-Aβ related
biomarkers including plasma signaling and inflammatory proteins [4] and clusterin [5]. Over the last five years, longitudinal levels of plasma analytes have been monitored in two large-scale studies: The Alzheimer’s Disease Neuroimaging Initiative (ADNI) and The Australian Imaging Biomarker and Lifestyle Flagship Study of Aging (AIBL). Using serial annual measurements, the ADNI study reported that a number of pathology analytes, including creatinine, glucose, and cholesterol, may influence plasma Aβ expression [6]. Like ADNI, the AIBL study also focused on the influence of pathology analytes on Aβ levels, finding that inflammatory and renal function analytes were significant covariates [7] and that a panel of such markers were capable of distinguishing AD patients from controls [8] in addition to predicting neocortical Aβ burden [9]. Although a degree of overlap was observed between the two studies, there was also a large number of analytes that were unique to each cohort, highlighting the need to account for variations in sample processing [3] as well as other comorbidities, particularly those often associated with age.

While requiring further validation, these investigations highlight the potential of non-Aβ-centric biomarkers in the diagnosis of AD. However, they also highlight the proclivity for researchers to focus on plasma biomarkers at the expense of biomarkers in other blood fractions, despite a number of reports that Aβ is readily observable within the cellular blood fractions [10–12]. In 2010, using a technique previously used to identify potential biomarkers in cerebrospinal fluid (CSF) [13, 14], namely immobilized metal affinity capture (IMAC) in conjunction with SELDI-TOF mass spectrometric analysis [15], we identified three candidate biomarkers in the AD-affected blood cellular fractions [15]. These candidate markers were significantly correlated with clinical measures of disease, including Mini-Mental State Examination (MMSE), composite memory, brain Aβ burden, and hippocampal volume. Most pertinently, however, was the finding that a regression model combining levels of these candidate markers with peripheral Aβ levels [10] enabled the distinction of AD patients from healthy controls (HC) with high specificity (90%) and sensitivity (77%) and, furthermore, enabled the separation of individuals with mild cognitive impairment (MCI) who progressed to AD from those MCI that did not [15].

The present investigation aimed to replicate our earlier findings [15] using samples obtained from the AIBL. Blood cellular fraction samples were analyzed using copper-loaded IMAC in conjunction with SELDI-TOF MS.

MATERIALS AND METHODS

An outline of the materials and methodologies utilized in the current study, detailed methodologies are provided in the supplementary materials.

Human samples

Blood samples were collected from 218 participants in the AIBL study, 72 elderly subjects clinically diagnosed with mild to moderate AD, 113 age-matched cognitively unimpaired individuals, and 33 individuals classified as presenting with MCI [16]. Samples were collected at two time points: Baseline and 18-month follow-up.

Neuropsychological and neuroimaging assessments

All participants underwent a number of neuropsychological assessments which have previously been described [16] and a small subset of participants (nHC = 18, nMCI = 13, nAD = 9) also underwent Aβ imaging with [11]CJP3B-PET as previously described [17].

Preparation of human samples

Venipuncture was used to collect 4 mL samples of whole blood in EDTA vacutainers 1.6 mg/mL (C5675A, Greiner Bio-One) following overnight fasting. Blood processing commenced between 60 and 240 min of sample acquisition. Blood was separated into plasma, cellular fraction, red blood cells (RBC) and white blood cell (WBC) fractions. All samples were stored −80°C until required, thus ensuring that all samples only underwent a single freeze/thaw cycle. Full blood processing details are provided in the supplementary materials.

SELDI-TOF MS analysis

Mass spectrometric analysis of the blood cellular fractions were carried out using either ProteinChip® PS10 Arrays (Bio-Rad; CAT #C55-30044) loaded with W02 (2 μL at 0.25 mg/mL) or ProteinChip® IMAC30 Arrays (Bio-Rad, CAT # C57-30078) charged with CuSO₄ (100 μL at 0.1 M Chem-Supply; Gillman, South Australia). All samples were analyzed blinded to diagnostic status, using a ProteinChip SELDI System Enterprise Edition (BioRad). Full methodological details of this
Isolation and purification of candidate markers

Candidate markers were isolated following a combination of peak intensities from the three HPLC columns. Subsequent to HPLC, matrix-assisted laser desorption/ionization (MALDI)-TOF MS was used to assess the fractions of interest.

Identification of candidate markers

Samples underwent tryptic digestion before undergoing analysis by liquid chromatography-mass spectrometry (LC-MS) using a LTQ Orbitrap Elite (Thermo Scientific) with a nanoelectrospray interface coupled to an Ultimate 3000 RSLC nanosystem (Dionex).

Statistical analysis

Continuous variables including peak intensities and measures of disease severity were tested for normality using the Shapiro-Wilk test. P-values were corrected for multiple testing controlling the false discovery rate [18]. Partial Least squares (PLS) regression was performed to generate predictive models using a combination of peak intensities from the three candidate markers and other clinical variables in accordance with our previous study [15]. Data are presented as mean ± standard deviation (SD) unless otherwise stated. All analyses were undertaken using Graphpad Prism® for Windows (Version 5.03, 2009), XLSTAT (Version 2010.5.05) and R (Version 2.9.2. The R Foundation for Statistical Computing. (2009)).

RESULTS

Participant demographics

Demographic, neuropsychological and neuroimaging information pertaining to the 218 AIBL participants are outlined in Supplementary Table 1.

Blood-borne Aβ was not reliably observed in the AIBL samples

Cursory examination of the resulting WO2 spectra confirmed the inherent variability of the samples outlined in the CV data (Supplementary Table 2); however, it also revealed an almost complete absence of detectable Aβ within the AIBL samples (Fig. 1). The absence of detectable Aβ was a remarkable finding as, not only did it contradict our earlier findings [10], it contradicted the preparatory stages of the current investigation where abundant levels of both monomeric and dimeric Aβ were observed. Further investigation established that while Aβ levels were readily observable in the month following sample collection, analysis of the same samples that were stored at −80°C for ~12 months revealed that Aβ in the samples was no longer present at detectable levels (Supplementary Figure 1). These observations were inconsistent with our experiences working with cellular fraction samples from the Healthy Aging Study, where Aβ levels were observed at stable levels over multiple years, and led us to question whether differences in the pre-analytical aspects of the studies had inadvertently influenced the stability of Aβ in the samples.

Comparisons between the pre-analytical protocols of AIBL and the Healthy Aging Study

Comparisons between the pre-analytical protocols of AIBL and the Healthy Aging Study revealed consensus between the two studies with regard to anti-coagulant, tube type, centrifugation parameters and storage times. However, there were discrepancies between the two studies regarding the time taken to process the samples (Supplementary Table 3).

Identifying potential biomarkers using immobilized metal affinity capture

Three robust peaks were identified which were able to significantly differentiate between AD patients and controls, following adjustments for multiple testing (Supplementary Table 4). The candidate markers 3370Da and 3440Da, were consistent with those observed in our previous study [15] and were both elevated in the AD cellular fraction compared to control. The candidate biomarker at 5352Da was observed at decreased levels in AD compared to control. The three markers will henceforth be referred to as candidate markers (CM; CM3370, CM3440, and CM5352 respectively (Fig. 2). Ratios between...
Fig. 1. Representative Aβ profiles of the AIBL cellular fraction samples. (a) Representative WO2 SELDI-TOF MS spectra of the AD-affected blood cellular fraction showing the lack of both monomeric (b) and dimeric (c) Aβ present in the AIBL samples. (d) Representative WO2 SELDI-TOF MS spectra of the AD-affected blood cellular fraction from the earlier Healthy Aging Study [10], showing both monomeric and dimeric Aβ. Note that this analysis was conducted using a Ciphergen PBS IIC.

CM3370/CM5352 (p = 0.0001) and CM3440/CM5352 (p = 0.0005) resulted in significant differences between AD patients and HC, but no significant differences were observed between either AD patients or HC and MCI participants (Supplementary Figure 2). No significant differences were observed across the groups for CM3370/CM3440 (p = 0.2).

Effect of APOE genotype on candidate biomarkers levels

Peak intensities of CM5352 were significantly higher in apolipoprotein E (APOE) ε4 non-carriers compared to ε4 heterozygotes (p = 0.002), but not compared to ε4 homozygotes (Supplementary Figure 3).

No significant differences were observed across the groups for CM3370 or CM3440.

Correlations between candidate biomarkers and measures of disease severity

Correlations between the peak intensities of the candidate biomarkers and clinical measures of disease severity were conducted to ascertain the level of overlap between AD progression and the underlying pathological processes in the blood (Table 1). These comparisons revealed that both CM3370 and CM3440 were significantly associated with the three measures of disease severity: MMSE (CM3370 rS = –0.239, p < 0.001; CM3440 rS = –0.203, p < 0.01), Clinical...
Fig. 2. Representative SELDI-TOF MS spectra arising from the copper-loaded IMAC analysis of AIBL blood cellular fraction samples. (a) Spectra illustrate elevated levels of candidate markers 3370 Da and 3440 Da in AD blood compared to control and (b) decreased levels of candidate marker 5352 Da. Scatterplots for candidate markers (c) 3370 Da ($p = 0.019$), (d) 3440 Da ($p = 0.038$), and (e) 5352 Da ($p = 0.023$), respectively.
Table 1

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<td>0.154</td>
<td>-0.449***</td>
<td>0.271</td>
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* p<0.05, ** p<0.01, *** p<0.001.  † nHC = 18, nMCI = 13, nAD = 9.

Dementia Rating (CDR) score (CM3370 rS = 0.171, p<0.05; CM3440 rS = 0.144, p<0.05) and CDR Sum of Boxes (CM3370 rS = 0.196, p<0.01; CM3440 rS = 0.164, p<0.05). These associations indicated that as levels of the candidate biomarkers increased there was a corresponding decrease in cognitive function as measures by these clinical measures. However, no significant correlations were observed between CM3370 and CM5352 and neocortical Aβ burden in a subset of participants.

Similar correlations were observed between measures of disease severity and CM5352 and the ratio CM3370/CM5352 and CM3440/CM5352. However, only CM3532 was found to be significantly negatively associated with neocortical Aβ burden, as measured by PiB PET SUVR (rS = -0.448, p<0.01), suggesting that CM3532 may be related to the AβPP or Aβ processing pathways.

Correlations between candidate markers

Analysis using Spearman’s correlation between the candidate markers revealed a very strong positive correlation between CM3370 and CM3440 (rS = 0.964, p<0.0001), suggesting that the two markers may be monotonically related (Supplementary Figure 4). A weak negative correlation was observed between CM3370 and CM5352 (rS = -0.137, p=0.046), while no association was observed between CM3440 and CM5352.

Isolation and identification of candidate biomarkers

Isolation of RBCs, platelets, and WBCs from the cellular fraction revealed that both CM3370 and CM3440 were enriched within the WBC fraction in AD patients, observed as clean peaks unimpeded by adjacent peptides in the sample (Fig. 3a). CM3532 on the other hand, appeared to be predominantly located within the RBC fraction (Fig. 3b); however, the relative intensity of the peak and the presence of neighboring peptides made further purification of this candidate marker difficult.

Purification and identification of CM3370 and CM3440 from WBCs

CM3370 and CM3440 were isolated and purified using ProteinChip IMAC Spin Columns in conjunction with HPLC (Fig. 3c-d). A trypsin digest of the HPLC fraction was followed by LC MS/MS in an effort to identify the resulting tryptic peptides (Supplementary Table 5). This analysis identified three tryptic peptides belonging to α-Defensin-1, also known as human neutrophil peptide-1 (HNP-1) and enabled near full sequence coverage of the peptide (Supplementary Figure 5). These findings enabled the unambiguous assignment of peak 3440 Da observed in the IMAC analysis as HNP-1. Furthermore, the identical HPLC retention times of CM3440 and CM3370 was considered sufficient evidence to assign the 3701 Da IMAC peak as HNP-2. The sequence of HNP-2 is identical to HNP-1 with the exception of a single N-terminal alanine which is missing from the truncated HNP-2.

Confirmation analysis was performed using electrospray ionization (ESI) fragmentation coupled with high accuracy measurements. HPLC purified fractions of CM3440 were compared to synthetic HNP-1 on a nanoscale HPLC coupled to an Orbitrap Elite MS, with both fractions showing matching retention times and mass (Fig. 4). Mass alignment between CM3440 and synthetic HNP-1 showed differences of less than 3.4 ppm for the [M+5H]+ peptide and less than 1.7 ppm for the [M+4H]+ peptide. Further isotopic modelling using the mMass software on the [M+5H]+.
Fig. 3. Isolation and purification of candidate biomarkers from AIBL blood fractions. (a) Representative SELDI-TOF MS spectra arising from copper-loaded IMAC analysis of the cellular fraction (top), plasma (second), red blood cell (RBC; third), and white blood cell (WBC; bottom) fractions of AD-affected blood, indicates that CM3370 and CM3440 are enriched within WBCs. (b) CM5352 appears to be predominantly found within the RBC fraction. Pooled WBCs were isolated using copper-loaded IMAC spin column analysis followed by HPLC purification. (c) Full MALDI-TOF MS spectra and (d) zoomed spectra.
Fig. 4. Identification of CM3440 as human neutrophil peptide-1. Comparative analysis of HPLC purified CM3440 against synthetic human neutrophil peptide (HNP-1) enabled the identification of CM3440 as HNP-1. (a) Total ion chromatogram (top panel) and extracted ion chromatogram (lower panel) of the [M+5H]$^+$ peptide indicates that both samples display near identical HPLC retention time (lower panel). (b) MS spectra of the [M+5H]$^+$ and [M+4H]$^+$ peptide showing differences of less than 3.4 ppm for the H$^+$ peptide and less than 1.7 ppm for the H$^+$ peptide. (c) Enhanced zoom of the [M+5H]$^+$ spectra demonstrates that isotopic clustering of the two fractions resulted in similar masses between both samples. Z = charge state and R = resolution.

Alpha-defensins and white blood cell fractions

The identification of HNP-2 and HNP-1 raised the question of whether the elevation of these biomarkers in the periphery of AD patients was an indication of aberrant WBC levels. Spearman’s correlation was used to analyze the association between HNP levels and levels of WBCs, neutrophils and lymphocytes (Fig. 5). These analyses revealed strong positive correlations between α-defensin levels and both white cell count (HNP-2 $r_s = 0.377$, $p < 0.0001$; HNP-1 $r_s = 0.354$, $p < 0.0001$) and neutrophil count reference interval (HNP-2 $r_s = 0.426$, $p < 0.0001$; HNP-1 $r_s = 0.402$, $p < 0.0001$). However, no correlation was observed between α-defensin levels and lymphocyte count reference intervals (HNP-2 $r_s = 0.053$, $p > 0.05$; HNP-1 $r_s = 0.044$, $p > 0.05$). Further analysis revealed that α-defensin levels remained elevated in AD-affected blood following normalization to neutrophil levels (HNP-2 $p = 0.008$, HNP-1 $p = 0.032$; Fig. 5).

Using Partial Least Squares regression to generate predictive models

In order to enhance the distinction between the spectral profiles of AD patients and their healthy counterparts, PLS regression was utilized to investigate which combination of the candidate peaks, if any, would allow for the optimal separation of the clinical diagnoses. In accordance with our earlier investigation [15], data for these models included peak intensities of the candidate peaks, ratios between candidate markers and participants’ age, gender and APOE status. However, only participant’s age and APOE e4 were found to have variable importance of the projection (VIP) values 1 standard deviation above 0.8 (Supplementary Table 6). These findings indicated that the candidate markers were not able to produce a robust diagnostic model and thus no subsequent PLS analyses were
Fig. 5. Correlations between candidate biomarkers and WBC components and comparative analyses of α-defensin levels normalized to neutrophil levels. a-f) Spearman’s correlations revealed strong positive correlations between α-defensin levels and both white cell count (HNP-2 rS = 0.377, p < 0.0001; HNP-1 rS = 0.354, p < 0.0001) and neutrophil count reference interval (HNP-2 rS = 0.426, p < 0.0001; HNP-1 rS = 0.402, p < 0.0001). No correlation was observed between α-defensin levels and lymphocyte count reference intervals. Comparative analyses demonstrated that following normalization to neutrophil levels, α-defensin levels, both ((g) HNP-2 and (h) HNP-1 remained significantly elevated in AD-affected blood (p = 0.008 and p = 0.032, respectively).
performed. It should be noted that the success of HNP-2 (CM3370) and HNP-1 (CM3440) in earlier PLS models was reliant on monomeric and dimeric Aβ levels [15]; however, the instability of Aβ levels in the AIBL samples rendered current attempts to produce predictive models ineffective. Attempts to generate predictive models using HNP levels in association with previously reported plasma Aβ levels [19]; however, these values were not able to add to the predictive power of the model (data not shown).

An additional attempt to generate a predictive PLS model was undertaken by combining α-defensin levels with the panel of 18 biomarkers identified in the AIBL cohort by Doecke et al. [8]. This analysis indicated that participant’s age, APOE e4, insulin-like growth factor binding protein 2, pancreatic polypeptide and interleukin 17 had VIP values 1 standard deviation above 0.8 (Supplementary Table 7) and indicated that levels of the α-defensins and CM5352, in addition to ratios between these marker levels, as measured by IMAC, were not able to provide additional predictive power to the model produced by Doecke et al. [8].

**DISCUSSION**

The identification of a panel of biomarkers capable of identifying preclinical AD remains one of the major unmet goals in the field. The measurement of cortical Aβ burden using PIB-PET and CSF measurements of Aβ and tau remain the most clinically effective diagnostic markers of AD [20–22], with both showing strong potential as measures of preclinical disease [1, 23]. However, given the goal of screening asymptomatic individuals, PET imaging and CSF sampling remain logistically and economically impractical [21, 24]. Such obstacles could be overcome by utilizing a more readily accessible biological sample, such as blood. As in previous studies [18, 5], the fractionation protocol utilized in the current investigation was kept purposely minimalistic; reflective of the standard protocols utilized in clinical laboratories worldwide. The plasma and cellular fractions were separated before the cellular fraction was analyzed using IMAC in conjunction with SELDI-TOF MS, leading to the identification of three candidate biomarkers of AD: HNP-1, HNP-2 and CM5352.

CM5352 levels were lower in the AD cellular fraction and were significantly associated with cognitive performance and cortical amyloid burden. Unfortunately, the low levels of CM5352 observed in the cellular fraction combined with the abundance of neighboring peaks prevented further identification of this marker. This highlights a limitation of the combined use of IMAC and SELDI-TOF MS in that the peptide or protein of interest may not be suitable for identification due to low peak intensity/poor relative abundance or the presence of extraneous peaks in a similar mass range. Such peaks are often not resolvable using electrophoresis and subsequent MS identification.

The observation of elevated levels of HNP-1 and HNP-2 in the AD cellular fraction was of particular importance as it corroborated our earlier findings in an independent cohort [15]. As in the earlier study, these were observed to be elevated in AD patients through processes independent of age, gender and APOE status; indicating that the elevation of these blood markers was likely driven by underlying pathogenic processes.

Defensins are a family of mammalian peptides found in a number of human biological fluids including blood, milk, saliva, tears, and urine [25–27]. Three α-defensins, HNP-1, HNP-2, and HNP-3, are cationic antimicrobial peptides that are produced predominantly by human neutrophils and are active components of the innate immune system [26, 28–30]. The sequence of the three peptides is identical with the exception of a single N-terminal residue, which is an alanine in HNP-1, an aspartate in HNP-3 and which is missing from the truncated HNP-2 [31]. α-defensins are released in biological fluids during inflammation [26] and elevated levels of the peptides have previously been reported as markers of cancer [32–34], schizophrenia [35], HIV [36], and herpes simplex virus [37].

The role of inflammation in AD remains somewhat controversial, with researchers continuing to question whether microglial activation in the AD brain has a neuroprotective or neurodegenerative function [38]. However, regardless of their exact role in AD progression, neuropathological and neuroimaging studies have consistently reported that microglial activation accompanies Aβ deposition in AD and genes encoding inflammatory proteins are often upregulated in AD, even at the early stages of the disease [39–43]. Reports of elevated inflammatory markers in the AD periphery have also been well established [8, 44–46], leading to speculation regarding the presence of an inflammatory endophenotype in the lead up to a dementia diagnosis [44, 47, 48]. The findings of elevated α-defensins provide further support for this notion and indicate that a more thorough understanding of systemic inflammation may help to elucidate the preclinical stages of AD pathogenesis.
Our previous investigation indicated that it was the combination of blood borne Aβ and HNP levels that provided the most robust predictive model, while on their own the respective marker lacked the predictive power to be of significant prognostic value [15].

In the present study, it is thought that the extended sample processing time in the AIBL study resulted in decreased sample durability and a consequential lack of data pertaining to levels of monomeric and dimeric Aβ. As a result of this, attempts to generate predictive models using levels of HNP-1 and HNP-2 alone were unsuccessful, with all markers failing to meet the requisite VIP levels for inclusion in the model.

It is important to note however, that the failure of HNP-1 and HNP-2 to meet the requisite VIP levels for model inclusion is based on the measurement of their levels using broad-based IMAC analysis, rather than a more targeted antibody-based approach; something to be considered in future investigations. However, regardless of the analytical techniques employed to investigate markers of peripheral inflammation, such as the α-defensins, the ubiquitous elevation of such markers across a wide range of disease states (cancer [32-34], schizophrenia [35], HIV [36], and herpes simplex virus [37]) necessitates the parallel observation of aberrations in Aβ, tau, or other AD-specific protein levels. Attempts to measure Aβ in these samples were undertaken, albeit unsuccessfully, in the current study; however, peripheral tau levels were not assessed and data pertaining to these levels was not available from the AIBL database.

The deteriorating Aβ signal observed in the AIBL samples over time is of considerable concern and raises the question of whether biofluid samples held within the AIBL, and potentially the ADNI and DIAN, biobanks will contain viable levels of the peptide in the years to come. This finding, whilst requiring further investigation, is testament to the fragile nature of the Aβ peptide and highlights the logistical difficulties of analyzing longitudinal Aβ levels in complex samples. Finally, despite the aforementioned issues with sample durability, both HNP-1 and HNP-2 remained at significantly elevated levels in the AD periphery; suggesting that these markers offer a more robust measure of disease state than the more fragile Aβ peptides.

The finding that α-defensins are elevated in the AD-affected periphery reaffirms, not only our earlier investigation [15], but also the notion that the identification of peripheral inflammation may be a key step toward identifying AD in its earlier stages. While it is not being asserted that additional investigation into the underlying causes of elevated α-defensin levels will further our understanding of AD pathogenesis; it is clear that non-amyloidogenic biomarkers offer an alternative pathway in the diagnosis and monitoring of AD.

DISCLOSURE STATEMENT

Authors’ disclosures available online (http://www.j-alz.com/disclosures/viewdoc.php?id=2600).

SUPPLEMENTARY MATERIAL

The supplementary material is available in the electronic version of this article: http://dx.doi.org/10.3233/JAD-142286.

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