

A Blood-Based Screening Tool for Alzheimer's Disease That Spans Serum and Plasma: Findings from TARC and ADNI

Sid E. O'Bryant^{1*}, Guanghua Xiao², Robert Barber³, Ryan Huebinger⁴, Kirk Wilhelmsen⁵, Melissa Edwards⁶, Neill Graff-Radford⁷, Rachele Doody⁸, Ramon Diaz-Arrastia⁹, for the Texas Alzheimer's Research & Care Consortium^{†a}, for the Alzheimer's Disease Neuroimaging Initiative^{†b}

1 Department of Neurology, F. Marie Hall Institute for Rural and Community Health, Garrison Institute on Aging, Texas Tech University Health Sciences Center, Lubbock, Texas, United States of America, **2** Department of Clinical Sciences, University of Texas Southwestern Medical Center, Dallas, Texas, United States of America, **3** Department of Pharmacology and Neuroscience, Institute for Aging and Alzheimer's Disease Research, University of North Texas Health Science Center, Fort Worth, Texas, United States of America, **4** Department of Surgery, University of Texas Southwestern Medical Center, Dallas, Texas, United States of America, **5** Department of Genetics, University of North Carolina School of Medicine, Chapel Hill, North Carolina, United States of America, **6** Department of Psychology, Texas Tech University, Lubbock, Texas, United States of America, **7** Department of Neurology, Mayo Clinic, Jacksonville, Florida, United States of America, **8** Department of Neurology, Alzheimer's Disease and Memory Disorders Center, Baylor College of Medicine, Houston, Texas, United States of America, **9** Center for Neuroscience and Regenerative Medicine, Uniformed Services University of the Health Sciences, Rockville, Maryland, United States of America

Abstract

Context: There is no rapid and cost effective tool that can be implemented as a front-line screening tool for Alzheimer's disease (AD) at the population level.

Objective: To generate and cross-validate a blood-based screener for AD that yields acceptable accuracy across both serum and plasma.

Design, Setting, Participants: Analysis of serum biomarker proteins were conducted on 197 Alzheimer's disease (AD) participants and 199 control participants from the Texas Alzheimer's Research Consortium (TARC) with further analysis conducted on plasma proteins from 112 AD and 52 control participants from the Alzheimer's Disease Neuroimaging Initiative (ADNI). The full algorithm was derived from a biomarker risk score, clinical lab (glucose, triglycerides, total cholesterol, homocysteine), and demographic (age, gender, education, *APOE***E4* status) data.

Major Outcome Measures: Alzheimer's disease.

Results: 11 proteins met our criteria and were utilized for the biomarker risk score. The random forest (RF) biomarker risk score from the TARC serum samples (training set) yielded adequate accuracy in the ADNI plasma sample (training set) (AUC = 0.70, sensitivity (SN) = 0.54 and specificity (SP) = 0.78), which was below that obtained from ADNI cerebral spinal fluid (CSF) analyses (t-tau/ $A\beta$ ratio AUC = 0.92). However, the full algorithm yielded excellent accuracy (AUC = 0.88, SN = 0.75, and SP = 0.91). The likelihood ratio of having AD based on a positive test finding (LR+) = 7.03 (SE = 1.17; 95% CI = 4.49–14.47), the likelihood ratio of not having AD based on the algorithm (LR-) = 3.55 (SE = 1.15; 2.22–5.71), and the odds ratio of AD were calculated in the ADNI cohort (OR) = 28.70 (1.55; 95% CI = 11.86–69.47).

Conclusions: It is possible to create a blood-based screening algorithm that works across both serum and plasma that provides a comparable screening accuracy to that obtained from CSF analyses.

Citation: O'Bryant SE, Xiao G, Barber R, Huebinger R, Wilhelmsen K, et al. (2011) A Blood-Based Screening Tool for Alzheimer's Disease That Spans Serum and Plasma: Findings from TARC and ADNI. PLoS ONE 6(12): e28092. doi:10.1371/journal.pone.0028092

Editor: Ashley I. Bush, Mental Health Research Institute of Victoria, Australia

Received: August 26, 2011; **Accepted:** November 1, 2011; **Published:** December 7, 2011

Copyright: © 2011 O'Bryant et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This study was made possible by the Texas Alzheimer's Research Consortium funded by the state of Texas through the Texas Council on Alzheimer's Disease and Related Disorders. Investigators at the UTSW acknowledge NIH, NIA grant P30AG12300. The investigations at Baylor's Alzheimer's Disease and Memory Disorders Center were supported by the Cynthia and George Mitchell Foundation. Investigators at Texas Tech University Health Sciences Center were supported by The CH Foundation. ADNI. Data collection and sharing for this project was funded by the Alzheimer's Disease Neuroimaging Initiative (ADNI) (National Institutes of Health Grant U01 AG024904). ADNI is funded by the National Institute on Aging, the National Institute of Biomedical Imaging and Bioengineering, and through generous contributions from the following: Abbott, AstraZeneca AB, Bayer Schering Pharma AG, Bristol-Myers Squibb, Eisai Global Clinical Development, Elan Corporation, Genentech, GE Healthcare, GlaxoSmithKline, Innogenetics, Johnson and Johnson, Eli Lilly and Co., Medpace, Inc., Merck and Co., Inc., Novartis AG, Pfizer Inc, F. Hoffman-La Roche, Schering-Plough, Synarc, Inc., as well as non-profit partners the Alzheimer's Association and Alzheimer's Drug Discovery Foundation, with participation from the U.S. Food and Drug Administration. Private sector contributions to ADNI are facilitated by the Foundation for the National Institutes of Health (www.fnih.org). The grantee organization is the Northern California Institute for Research and Education, and the study is coordinated by the Alzheimer's Disease Cooperative Study at the University of California San Diego. ADNI data are disseminated by the Laboratory for Neuro Imaging at the University of California Los Angeles. This research was also supported by NIH grants P30 AG010129, K01 AG030514, and the Dana Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have the following competing interest: In the TARC, a patent has been submitted on this blood-based screener. There are no other products in development or marketed products to declare. This does not alter the authors' adherence to all PLoS ONE policies on sharing data and materials, as detailed online in the guide for authors. ADNI has received funding from the following commercial sources: Abbott, AstraZeneca AB, Bayer Schering Pharma AG, Bristol-Myers Squibb, Eisai Global Clinical Development, Elan Corporation, Genentech, GE Healthcare, GlaxoSmithKline, Innogenetics, Johnson and Johnson, Eli Lilly and Co., Medpace, Inc., Merck and Co., Inc., Novartis AG, Pfizer Inc, F. Hoffman-La Roche, Schering-Plough, Synarc, Inc. This does not alter the authors' adherence to all PLoS ONE policies on sharing data and materials, as detailed online in the guide for authors. ADNI data is freely available to any interested scientists.

* E-mail: Sid.Obryant@ttuhsc.edu

☉ These authors contributed equally to this work.

▫a For a full list of the investigators from the Texas Alzheimer's Research Consortium please see the Acknowledgments section

▫b For more information about the Alzheimer's Disease Neuroimaging Initiative please see the Acknowledgments section

Introduction

Alzheimer's disease (AD) is a devastating disease affecting millions of people worldwide. While a Food and Drug Administration (FDA) working group recently provided preliminary approval for a beta amyloid (A β) neuroimaging technique as a biological marker (Amyvid[®], Elli Lilly), no blood-based biomarker screening tool has received approval to date. However, blood-based biomarkers present significant advantages over neuroimaging modalities. For example, blood-based screenings offer a cost effective method of screening candidates for therapeutic trials [1], provide a rapid, cost-effective means of screening for AD at the population level [2,3,4,5], and provide an optimal starting point for a multi-stage assessment process that can be followed-up by clinical modalities (i.e. medical exam, neuropsychological testing, standard neuroimaging, clinical blood-work), specialized neuroimaging (i.e. A β imaging, fMRI, volumetric MRI analyses), and/or CSF (i.e. t-tau, A β ₁₋₄₂, and/or t-tau/A β ₁₋₄₂ ratio score) analyses [4] for screen positive cases. The 2009 U.S. Census estimates suggested that there were nearly 40 million Americans age 65 and above with an additional 34 million reaching 65 within 10 years; there are many more world-wide. Given their cost and limited availability, available imaging, clinical, and CSF modalities are not reasonable first-line approaches for screening all elders at risk of having AD or that have concerns about having the disease. The purpose of this study was to generate and cross-validate a blood-based screener for AD that can be incorporated into the existing medical infrastructure with additional assessments (e.g. clinical, imaging, CSF analysis) to confirm those who screen positive.

In the last several years, there have been significant advancements in the search for blood-based biomarkers for Alzheimer's disease (AD). In 2007, Ray and colleagues [6] analyzed a panel of plasma-based proteins among samples from 259 controls, AD and mild cognitive impairment (MCI) cases and generated a biomarker algorithm that accurately identified 89% of those with and without the disease; however, this work has not been replicated [7]. Buerger and colleagues [8] examined blood-based microcirculation markers as possible diagnostic markers for AD (AD n = 94, controls n = 53). These authors found that a ratio score of pro-atrial natriuretic peptide (MR-proANP) to C-terminal endothelin-1 precursor fragment (CT-proET-1)(MR-proANP/CT-proET-1 ratio) from plasma yielded a sensitivity of 0.81 and specificity of 0.82 in discriminating probable AD from healthy controls. More recently, we created a biomarker risk score from serum proteins (AD n = 197, controls n = 203) that yielded a 91% overall accuracy [2]. Our approach took the algorithm a step further by combining both demographic (i.e. age, gender, education, and *APOE*E4* status) and clinical lab values (i.e. cholesterol, triglycerides, high density lipoproteins, low density lipoproteins, lipoprotein-associated phospholipase, homocysteine, and C-peptide) into the algorithm, which improved the overall accuracy to 95% [5]. Analyzing samples from 22 AD cases, 22 controls, and 12 non-AD disease comparison subjects, Reddy and colleagues [9] took a novel approach by examining serum IgG antibodies as potential biomarkers of AD

status obtaining impressive results (AUC = 0.99); however, the sample size was very small (n = 15 AD cases in test set) limiting the generalizability of the findings at this point. Together, these studies suggest that a blood-based screening tool for AD is on the horizon.

Although this work is promising, there is little consistency as to what biological fluid is used for biomarker assays (i.e. serum versus plasma), which may explain many inconsistent findings found in the literature. While some assays must be conducted in one medium or another, there are numerous studies linking a variety of blood-based markers to AD from both mediums. Mayeux and colleagues [10] analyzed *plasma* amyloid β (A β) peptides A β ₁₋₄₀ and A β ₁₋₄₂ on 530 participants and found that A β ₁₋₄₂ (but not A β ₁₋₄₀) levels were higher among baseline AD cases as well as those who developed AD over a three-year period as compared to those who did not. Luis et al. [11] analyzed *serum* A β ₁₋₄₀ and A β ₁₋₄₂ levels among a sample of 87 AD and MCI cases as well as controls. In that study, serum A β ₁₋₄₀ levels did not differ between groups whereas serum A β ₁₋₄₂ levels were highest among MCI cases (versus AD cases and controls) and controls and AD levels were intermediate between those of the MCI cases and controls. The *serum* A β _{1-42/1-40} ratio was also highest among the MCI group. In a sample of 40 AD cases and controls, Laske et al. [12] found that *serum* brain derived neurotrophic factor (BDNF) levels varied according to AD severity, suggesting BDNF as a potential biomarker for AD, though we failed to cross-validate these findings in a sample of 198 AD cases and controls from the Texas Alzheimer's Research Consortium (TARC) cohort [13]. In a follow-up study of 399 AD cases and controls, *elevated* serum BDNF was found to be specifically related to poorer memory performance among AD cases [14] whereas Komulainen and colleagues [15] found that *lower plasma* BDNF levels were significantly related to poorer scores on tests of language and memory among women in a population based sample of aging men and women (n = 1389).

To date, we are aware of no prior work that has explicitly sought to find blood-based biomarkers of AD across both serum and plasma and with no previous attempts at identifying blood-based screening tools utilizing markers across blood fractions. Additionally, no previously created blood-based tools have been cross-validated in independent cohorts. The current study was designed to (1) identify blood-based proteins that were highly correlated across both serum and plasma that also were significantly related to AD status, and (2) generate a screening algorithm for AD utilizing those markers from serum in the TARC cohort and validate that algorithm in the Alzheimer's Disease Neuroimaging Initiative (ADNI) plasma-samples. We hypothesized that, as with our prior work, we would be able to generate a screening algorithm that accurately identified AD across cohorts.

Methods

Participants

Texas Alzheimer's Research Consortium (TARC). Serum protein data were analyzed from 396 participants (197 AD

subjects, 199 controls) from the TARC longitudinal cohort. In addition, plasma protein data were analyzed on a matched sample of 40 AD cases from the TARC. Blood samples for comparison of plasma and serum proteins were drawn concurrently from the same individuals. The methodology of the TARC project has been described in detail elsewhere [2,16]. Briefly, each participant undergoes a standardized annual examination at the respective sites, which includes a medical evaluation, neuropsychological testing, interview, and blood draw for storage of samples in the TARC biobank. Diagnosis of AD was based on NINCDS-ADRDA criteria [17] utilizing consensus review. Institution Review Board approval was obtained for this study with each participant (or caregiver) providing written informed consent. The Institution Review Board (IRB) at Texas Tech University Health Sciences Center, Baylor College of Medicine, University of North Texas Health Science Center, the University of Texas Southwestern Medical Center, and the University of Texas Health Science Center - San Antonio approved this research.

Alzheimer's Disease Neuroimaging Initiative (ADNI). Data used in the preparation of this article were obtained from the ADNI database (adni.loni.ucla.edu). The ADNI was launched in 2003 by the National Institute on Aging (NIA), the National Institute of Biomedical Imaging and Bioengineering (NIBIB), the Food and Drug Administration (FDA), private pharmaceutical companies and non-profit organizations, as a \$60 million, 5-year public-private partnership. The primary goal of ADNI has been to test whether serial magnetic resonance imaging (MRI), positron emission tomography (PET), other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of mild cognitive impairment (MCI) and early Alzheimer's disease (AD). The Principal Investigator of this initiative is Michael W. Weiner, MD, VA Medical Center and University of California – San Francisco. ADNI is the result of efforts of many co-investigators from a broad range of academic institutions and private corporations, and subjects have been recruited from over 50 sites across the U.S. and Canada. For up-to-date information, see www.adni-info.org. Data from 170 participants from ADNI (58 controls and 112 AD cases) for whom plasma-based protein results were available were utilized in this study.

Blood Assays. In TARC, non-fasting samples were collected whereas ADNI utilized a fasting blood collection procedure. Serum blood samples were collected in serum-separating tubes during clinical evaluations, allowed to clot at room temperature for 30 minutes, centrifuged, aliquoted, and stored in polypropylene tubes at -80°C . In both TARC and ADNI, plasma samples were collected in lavender-top tubes and gently mixed 10–12 times. Next tubes were centrifuged at room temperature and plasma extracted and frozen until assay. In both studies, serum and plasma samples were sent to Rules Based Medicine (RBM, www.rulesbasedmedicine.com, Austin, TX) for assay on the RBM multiplexed immunoassay human Multi-Analyte Profile (human-MAP). Individual proteins were quantified with immunoassays on colored microspheres. Information regarding the least detectable dose (LDD), inter-run coefficient of variation, dynamic range, overall spiked standard recovery, and cross-reactivity with other humanMAP analytes can be readily obtained from RBM. **Clinical lab data.** Homocysteine, hemoglobin A1c, c-peptide, and lipoprotein-associated phospholipase A2 (Lp-PLA2) was provided by the Ballantyne laboratory at Baylor College of Medicine. Sample collection and storage was as described above. Lipids were measured using a AU400e automated chemistry analyzer (Olympus America; Center Valley, PA), serum total homocysteine (tHcy) by recombinant enzymatic cycling assay (Roche Hitachi

911), c-peptide by enzyme-linked immunosorbent assay (ELISA), HbA1c measurement by turbidimetric inhibition immunoassay (TINIA) for hemolyzed whole blood and Lp-PLA2 levels by diaDexus PLAC[®] test (diaDexus, Inc, San Francisco, CA). Clinical lab data from ADNI was conducted using kits provided by Covance. **ADNI CSF Biomarkers.** Our blood-based algorithm was compared to the diagnostic accuracy of the total tau (t-tau) to beta amyloid ($\text{A}\beta_{1-42}$) ratio (t-tau/ $\text{A}\beta_{1-42}$) previously completed as part of the ADNI protocol. The CSF methods for ADNI have been described in detail elsewhere [18]. Lumbar punctures were conducted with a median of one day after baseline clinical visit. Once CSF was transferred into polypropylene tubes it was frozen and shipped to the ADNI Biomarker Core laboratory at the University of Pennsylvania Medical Center where biomarker assays were conducted [18].

Statistical Analyses. Analyses were performed using R (V 2.10) statistical software [19]. Biomarker data were transformed using Box-Cox [20] transformation so that the distribution of each protein is approximately normal. Analyses took place in a series of steps. **Identification of proteins across serum and plasma.** Pearson correlations were conducted in the TARC sub-sample across serum and plasma proteins to determine which markers were comparable across mediums. Model-based clustering algorithm [21] (Mclust package in R) was used to empirically determine the optimal correlation cut-off that separated the highly correlated versus weakly correlated proteins. The optimal cut-score was 0.75, which identified 33 proteins with high correlation (≥ 0.75) between serum and plasma (see Figure 1). T-test analyses comparing the abundance of proteins between AD and controls identified 29 that were differentially expressed between groups ($p < 0.05$) in full the TARC cohort (training set). Eleven proteins were significantly different between AD and control participants and were found to be correlated ≥ 0.75 across serum and plasma. These 11 proteins are defined as protein biomarkers in this study. Figure 2 reflects a graphic representation of the methods. **Development of Biomarker Diagnostic Model.** Next, we used the 11 protein biomarkers to develop our prediction model using random forest (RF) method [22,23], implemented using R package *randomforest* (V 4.5) [22]. The TARC cohort was designated as the training sample in which the prediction model was derived. **Validation of the Prediction Model.** The protein biomarker-based RF prediction model derived from the TARC serum-based biomarker training set (TARC) was applied to the ADNI plasma-based dataset (test sample) to predict the risk score for each patient in the ADNI cohort. Of note, no ADNI data were utilized in (1) identification of serum-plasma comparable proteins or (2) development of the RF prediction model. This was done to avoid the overfitting or other possible confounds across medium and/or cohorts. **Diagnostic Accuracy.** Diagnostic accuracy was evaluated by examining the area under the receiver operating characteristic (ROC) curves (AUC). Our approach to creating a blood-based diagnostic algorithm for AD is to combine the predicted biomarker risk score from the RF model with demographic and clinical lab data via a multivariate logistic regression model. Demographic data incorporated into the algorithm was age, gender, level of education, and presence of *APOE*E4* genotype (homozygous or heterozygous) while clinical lab data included glucose, triglycerides, total cholesterol, and homocysteine. These variables were included as they were (1) available from both cohorts and (2) have been linked to AD. Lastly, the likelihood ratios of having AD based on a positive test finding (LR+), the likelihood ratio of not having AD based on the algorithm (LR-) and the odds ratio of AD were calculated in the ADNI cohort.

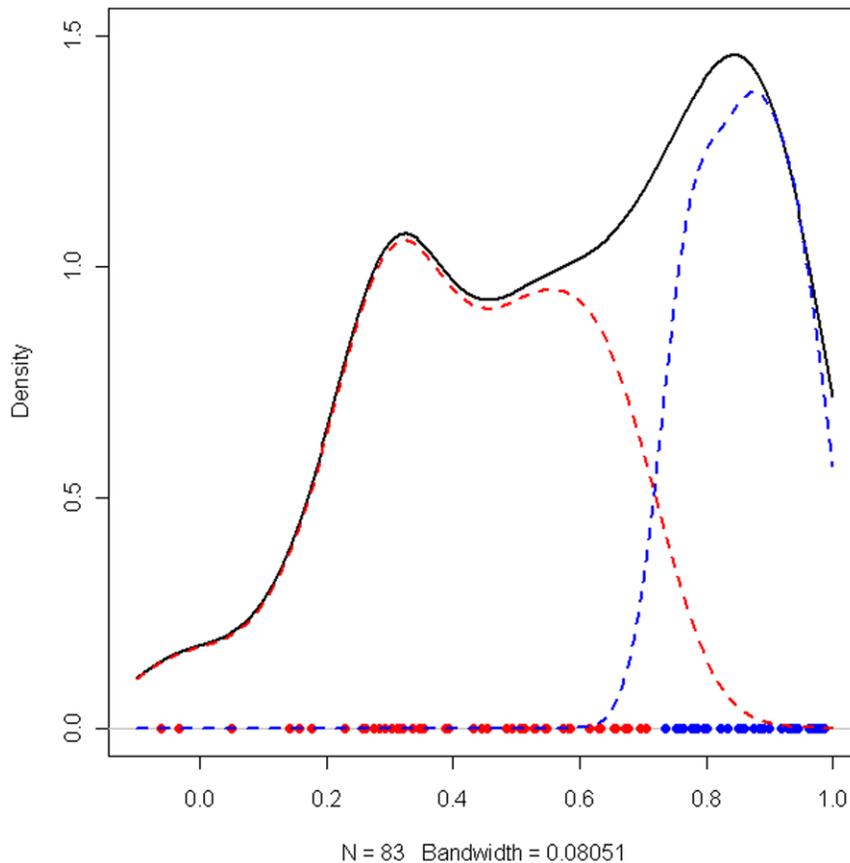


Figure 1. The density plot the Pearson's correlation coefficients between serum and plasma in TARC cohort. We used Mclust (model-based clustering algorithm [21]) package in R to fit the data and discovered two clusters in the correlation coefficients: one (red) corresponding to low correlation and the other (blue) corresponding to high correlation. The threshold value that separated these two clusters most effectively is 0.75. The black line is the density plot of all biomarkers. The dots represent the correlation coefficients of the biomarkers and the color indicates the cluster membership.

doi:10.1371/journal.pone.0028092.g001

Results

Demographic characteristics of the samples are provided in Table 1. Eleven proteins met our criteria of (1) having a correlation coefficient ≥ 0.75 between serum and plasma in the same participant and (2) being associated with disease status $p < 0.05$. The 11 proteins were as follows: C-reactive protein, adiponectin, pancreatic polypeptide, fatty acid binding protein, interleukin 18, beta 2 microglobulin, tenascin C, T lymphocyte secreted protein 1.309, factor VII, vascular cell adhesion molecule 1, and monocyte chemoattractant protein 1. See Table 2 for correlations among serum and plasma for these 11 proteins as well as the mean differences between cases and control groups of these biomarkers and clinical lab data across cohorts.

The optimal cut-score for the RF biomarker risk score from the test sample (ADNI) was 0.51 which obtained AUC of 0.70 with a sensitivity (SN) and specificity (SP) of 0.54 and 0.78, respectively. For comparison purposes, the ADNI CSF $t\text{-tau}/A\beta_{1-42}$ ratio yielded a superior diagnostic accuracy with an observed AUC = 0.92, SN = 0.84, and SP = 1.00. However, as with our prior approach, when the biomarker risk score was combined with demographic and clinical lab data [2,5], the precision improved substantially. Our combined algorithm yielded a much better diagnostic accuracy with an observed AUC = 0.88, SN = 0.75, and SP = 0.91. Of note, the diagnostic accuracy of our serum-plasma based algorithm was comparable to that

obtained from ADNI CSF analyses. See Table 3 and Figure 3. The likelihood ratio positive (LR+) was 7.03 (SE = 1.17; 95% CI = 4.49–14.47), the likelihood ratio negative (LR-) was 3.55 (SE = 1.15; 2.22–5.71), and the odds ratio (OR) was 28.70 (1.55; 95% CI = 11.86–69.47). The misclassification rate was 14% (95% CI = 9–21%). If we set SN at 0.80 for our full algorithm, the resulting SP was 0.81, which also meets the criteria for the Consensus Report of the Working Group on Molecular and Biochemical Markers of AD [24].

Discussion

In the current study we demonstrate that (1) there are proteins that are highly correlated in plasma and serum and are associated with AD status across blood fractions, (2) these findings are replicable across independent cohorts, and (3) using these proteins, we generated a prediction model in the TARC cohort that, when combined with demographic and clinical lab data, yielded clinically significant classification accuracy in the ADNI cohort. To date, this is the first blood-based screener for AD developed that has been cross-validated in an independent large-scale cohort that also works across blood fractions. This work not only further supports the notion that an accurate blood-based screening tool for AD can be generated, but also that such an algorithm can be applied across serum and plasma mediums. Our 11-protein serum-plasma risk score alone yielded an AUC of 0.70 accuracy that was

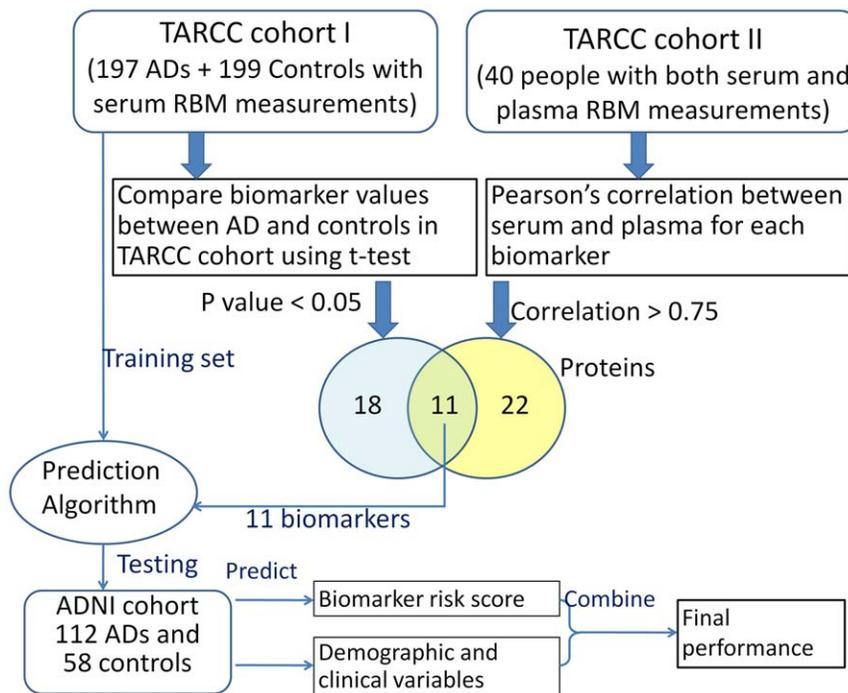


Figure 2. Outline of methods.
doi:10.1371/journal.pone.0028092.g002

enhanced by the addition of demographic (i.e. age, gender, education, *APOE*E4* status) and clinical lab (i.e. glucose, triglycerides, total cholesterol, and homocysteine) data. In Table 3, the addition of clinical lab data did not improve the overall accuracy of the algorithm beyond demographic information, which is largely driven by the *APOE*E4* rates in the ADNI cohort. However, in our prior work [5], the use of clinical lab data improved overall accuracy and will likely contribute to the robustness of our approach as it is applied to other cohorts. It is certainly possible that inclusion of additional markers, not available in the current analyses, would increase the accuracy of that risk score, which is an additional advantage of our approach as it can be expanded or reduced as necessary to support the accuracy and cost-effectiveness of the algorithm. A single biomarker algorithm that works across both serum and plasma will offer laboratories options that may be preferable for a variety of reasons.

There are several implications for the current findings. There are a number of previously conducted research projects with stored blood biospecimens; however, there is little consistency between what medium was stored. The current findings open up the possibility of utilizing samples from such studies to further validate and refine our algorithm. Additionally, it is likely that the components of diagnostic algorithms will be different from the components of algorithms for progression and different from those predicting long-term risk. Our findings offer a novel approach to each of these questions as well. These findings also support the need for standard protocols to be generated for blood-based AD biomarker research as is currently underway for the CSF markers.

These results also support the robustness of our methodological approach. In our initial serum-based algorithm, the biomarker risk score alone yielded an AUC of 0.91 whereas the serum-plasma algorithm in the current study yielded an AUC of 0.70. While impressive, this overall accuracy is not clinically adequate.

Table 1. Demographic characteristics of the cohorts.

	TARC – serum sample			TARC – plasma sample		ADNI	
	AD (N = 197)	Control (N = 198)	p-value	AD (n = 40)	AD (n = 112)	Control (n = 58)	p-value
Gender (male)	34.5%	31.3%	0.52	40%	42%	48%	0.52
Age (years, mean/sd)	77.4(8.3)	70.4(8.9)	<0.001	75.7(1.6)	75.2(8.1)	75.5(5.8)	0.63
Education (years, mean/sd)	14.0(3.5)	15.5(2.7)	<0.001	14.5(0.6)	15.1(3.2)	15.6(2.7)	0.38
<i>APOE*E4</i> positive	59.3%	26.5%	<0.001	50%	68%	9%	<0.001

Note: TARC = Texas Alzheimer’s Research Consortium; ADNI = Alzheimer’s Disease Neuroimaging Initiative. Fisher exact test was used for categorical outcomes (Gender, *APOE*E4* positive) and Wilcoxon test was used for continuous outcomes (Age, Education).
doi:10.1371/journal.pone.0028092.t001

Table 2. Biomarkers and Clinical Labs Across Cohorts.

Marker	Pearson correlation for serum vs. plasma (TARC cohort)	Mean difference in TARCC	Mean difference in ADNI
C Reactive Protein	0.97	-3.35	-2.07
Adiponectin	0.95	1.88	1.79
Pancreatic polypeptide	0.89	4.29	2.78
Fatty Acid Binding Protein	0.88	1.72	-0.79
IL 18	0.86	-1.87	0.51
Beta 2 Microglobulin	0.85	3.14	2.09
Tenascin C	0.85	4.56	2.93
I.309	0.8	1.12	-1.68
Factor VII	0.8	-2.78	-1.26
VCAM 1	0.78	3.00	2.82
MCP 1	0.75	-2.74	-0.30
Total Cholesterol	-	0.13	0.78
Triglycerides	-	-0.63	1.59
Homocysteine	-	3.99	1.06

Note: Mean difference reflects the mean difference between cases and controls divided by the its standard deviation.
doi:10.1371/journal.pone.0028092.t002

However, as with our prior approach, the combination of clinical lab data and demographic variables into the algorithm increased the precision substantially (AUC = 0.88). In our prior work, the training and test sample were both based on serum assays and were from the larger TARC cohort; however, the derivation of the algorithm in the TARC cohort and validation in the ADNI cohort supports the robustness of this method. As we have previously argued, using only age, gender, education and *APOE*E4* status, one can accurately classify a large number of AD cases when compared to controls. Therefore, consideration of such factors should be considered when examining biomarkers of AD status. We are not the first to demonstrate that inclusion of these factors into an algorithm can improve overall accuracy as others have suggested that a multi-marker approach is superior to single-marker approaches [25,26]. As an example, Vemuri and colleagues found that including demographic factors with structural MRI added to the overall accuracy of disease-prediction models even when cases and controls were matched by these variables [27]. This is important given that the TARC cohort did not match cases and controls whereas ADNI samples were matched. The robustness of our methodology may also provide an

explanation for the lack of cross-validation of prior work [6,7]. The utility of our algorithm for separating MCI cases from normal controls (and/or AD) remains unknown at present.

The current markers overlap with our prior serum-only based algorithm [2,5] though they do not overlap with those found by Ray and colleagues [6], which may be due to the significant differences in assay platforms utilized. However, there is an existing literature directly or indirectly linking each of the 11 proteins identified in this study to AD. As with our prior work, many of the markers in the algorithm are inflammatory in nature, which we propose as evidence of an inflammatory endophenotype of AD [2,28]. We, and others, have documented a link between CRP and AD [28]. Based on the available data, we proposed that the link between CRP and the risk of AD changes over the life course with midlife elevations in CRP increasing risk for AD, but that this risk declines as one ages with decreased CRP related to AD status though elevations in CRP are still related to increased disease severity among cases [28]. Adiponectin, an adipocytokine, is related to obesity, insulin resistance, metabolic syndrome, type 2 diabetes, and cardiovascular disease [29] and was recently found to be elevated in plasma among MCI and AD cases [30].

Table 3. Diagnostic accuracy of the serum-plasma algorithm.

	AUC (95% CI)	SN (95% CI)	SP (95% CI)
biomarker + clinical + demographic	0.88 (0.83–0.93)	0.75 (0.67–0.83)	0.91 (0.80–0.96)
biomarker + demographic	0.88 (0.83–0.93)	0.79 (0.71–0.86)	0.87 (0.75–0.93)
Biomarker + clinical	0.71 (0.63–0.79)	0.73 (0.64–0.81)	0.60 (0.47–0.72)
biomarker risk score alone	0.70 (0.62–0.78)	0.54 (0.45–0.63)	0.78 (0.65–0.87)
clinical variables alone	0.59 (0.50–0.68)	0.53 (0.43–0.62)	0.72 (0.58–0.82)
demographic variables alone	0.81 (0.75–0.88)	0.70 (0.61–0.78)	0.92 (0.82–0.97)
CSF tau/abeta ratio	0.92 (0.87–0.96)	0.84 (0.76–0.90)	1.00 (0.93–1.00)

Note: AUC = area under the receiver operating characteristic curve; SN = sensitivity; SP = specificity; CI = confidence interval; demographic = age, gender, education, *APOE*E4* status (presence/absence); clinical = glucose, triglycerides, total cholesterol, homocysteine.
doi:10.1371/journal.pone.0028092.t003

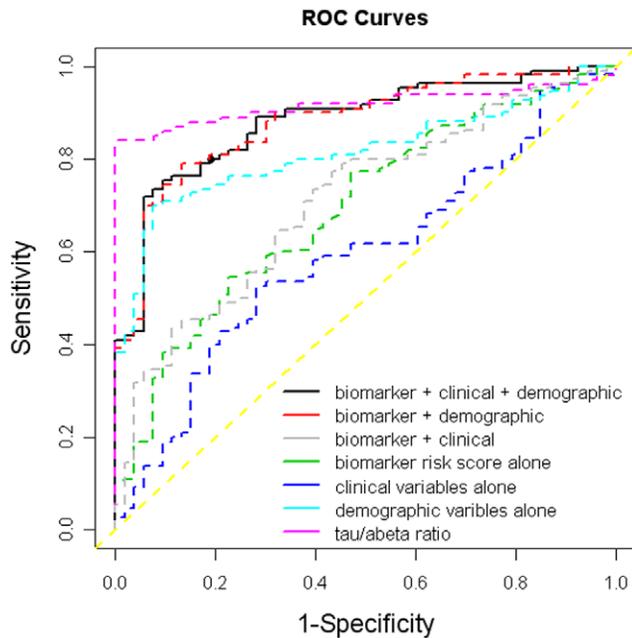


Figure 3. ROC curve for serum-plasma based biomarker algorithm. Each line represents the AUC of the respective portions of the algorithm with the yellow line reflecting chance. doi:10.1371/journal.pone.0028092.g003

Therefore, adiponectin levels may be related to the documented links between changes in body composition (e.g. weight loss) seen in prodromal and early stage AD. Pancreatic polypeptide is also linked with diabetes and obesity [31,32] and may provide a clue into the biological link between these conditions and AD. Fatty acid binding proteins, cytosolic proteins found in all cells utilizing fatty acids, are rapidly released into circulation following cell damage [33]. Serum levels fatty acid binding proteins have been shown to be elevated among AD and other dementia cases as compared to normal controls [33,34]. A recent meta-analysis showed a significant up-regulation in blood concentrations of IL-18 (as well as IL-6, TNF α , IL1, transforming growth factor, IL-12) among AD cases [35]. β 2 microglobulin is an amyloid protein [36] that has been found to be elevated in the CSF of AD cases [37,38]. Tenascin-C, an extracellular matrix glycoprotein, is involved in a number of biological processes that have been linked to AD including inflammation and angiogenesis [39], which may provide a biological mechanism linking AD to a broad spectrum of cardiovascular diseases and risk factors. The human cytokine I-309, a small glycoprotein, was recently found to be elevated in a proteomic study of CSF among AD cases and was also related to scores on a test of global cognitive functioning (i.e. Mini Mental State Examination [MMSE]) [40]. Factor VII is a protein in the coagulation cascade that is required for thrombin generation, which has also been linked to AD [41]. VCAM-1 is a member of the immunoglobulin superfamily that has been found elevated in plasma of AD cases [42]. It has been proposed that MCP-1 plays a dominant role in the chronic inflammation seen in AD [43] and has been found to be elevated in serum of patients diagnosed with MCI and mild AD [44].

Given the sheer volume of elders worldwide who are at risk for AD, there is an urgent need for a multi-stage approach to screening and diagnosis. There are insufficient numbers of

dementia experts to meet the needs of all individuals at risk for the disease and prior work has demonstrated that non-experts are not completely accurate in diagnosing the disease [45], particularly in the earlier stages [46]. Our blood-based screener fits into the existing medical infrastructure where screen positives can be referred for confirmatory diagnosis using clinical, imaging, and/or CSF analysis. As with any screening measure, one must consider acceptable levels of false positive and false negative rates of the instrument as well as overall disease base rates of the setting when deciding on appropriate cut-scores on any instrument [47]. Therefore it is important that additional work be conducted to determine how this algorithm (and other previously published biomarkers) performs in community-based settings (e.g. primary care offices) as both the TARC and ADNI are clinic-based cohort studies. While sensitivity and specificity are not base rate dependent, accuracy of diagnosis (prediction of disease status present/absent) is a function of base rates of the disease within a given population therefore, overall accuracy of AD presence (i.e. true positives) will increase with advancing age while accuracy of AD absence (i.e. true negatives) will be higher with younger ages. As with age, *APOE*E4* genotype, gender, and/or years of education are also important considerations, which is why these variables are included in the algorithm itself.

The independent cohorts strongly support the validity of the findings. These observations also justify further analysis examining a broader range of markers across serum and plasma to determine if the biomarker risk score can be further refined. Our results also suggest that further work in the field should specifically examine the performance of blood-based protein panels across serum and plasma.

Acknowledgments

TARC. We would like to thank Dr. Christie Ballantyne and his lab at Baylor College of Medicine for measuring the clinical lab data of glucose, tryglicerides, total cholesterol, and homocysteins. We also would like to thank the people of Texas and the research participants for making this work possible. Funding acknowledgments are available online.

Investigators from the Texas Alzheimer's Research Consortium: Baylor College of Medicine: Susan Rountree, Christie Ballantyne, Evleen Darby, Aline Hittle, Aisha Khaleeg; Texas Tech University Health Science Center: Paula Grammas, Benjamin Williams, Andrew Dentino, Gregory Schrimsher, Kuo Chuang Wu, Parastoo Momeni, Larry Hill; University of North Texas Health Science Center: Janice Knebl, Lisa Alvarez, Douglas Mains, Thomas Fairchild, James Hall; University of Texas Southwestern Medical Center: Joan Reisch, Perrie Adams, Roger Rosenberg, Ryan Huebinger, Janet Smith, Mechelle Murray, Tomequa Sears; University of Texas Health Sciences Center – San Antonio: Donald Royall, Raymond Palmer.

Data used in the preparation of this article were obtained from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (www.loni.ucla.edu/ADNI). As such, the investigators within the ADNI contributed to the design and implementation of ADNI and/or provided data, but did not participate in analysis or writing of this report. ADNI investigators include (complete listing available at www.loni.ucla.edu/ADNI/Collaboration/ADNI_Manuscript_Citations.pdf).

Author Contributions

Conceived and designed the experiments: SEO GX RB RD RDA. Performed the experiments: SEO GX RB KW RD RDA. Analyzed the data: SEO GX RB RH KW ME NGR RD RDA. Contributed reagents/materials/analysis tools: SEO GX RB KW RD RDA. Wrote the paper: SEO GX RB RH KW ME NGR RD RDA.

References

1. Thal LJ, Kantarci K, Reiman EM, Klunk WE, Weiner MW, et al. (2006) The role of biomarkers in clinical trials for Alzheimer disease. *Alzheimer Disease & Associated Disorders* 20: 6–15.
2. O'Bryant SE, Xiao G, Barber R, Reisch J, Doody R, et al. (2010) A serum protein-based algorithm for the detection of Alzheimer disease. *Archives of Neurology* 67: 1077–1081.
3. O'Bryant S, Xiao G, Barber R, Reisch J, Doody R, et al. for the Texas Alzheimer's Research Consortium (in press) A serum protein-based algorithm for the detection of Alzheimer's disease. *Arch Neurol*.
4. Schneider P, Hampel H, Buerger K (2009) Biological marker candidates of Alzheimer's disease in blood, plasma, and serum. *CNS Neuroscience and Therapeutics* 15: 358–374.
5. Ray S, Britschgi M, Herbert C, Takeda-Uchimura Y, Boxer A, et al. (2007) Classification and prediction of clinical Alzheimer's diagnosis based on plasma signaling proteins. *Nature Medicine* 13: 1359–1362.
7. Soares HD, Chen Y, Sabbagh M, Rohrer A, Schrijvers E, et al. (2009) Identifying early markers of Alzheimer's disease using quantitative multiplex proteomic immunoassay panels. pp 56–67.
8. Buerger K, Ernst A, Ewers M, Uspenskaya O, Omerovic M, et al. (2009) Blood-Based Microcirculation Markers in Alzheimer's Disease-Diagnostic Value of Midregional Pro-atrial Natriuretic Peptide/C-terminal Endothelin-1 Precursor Fragment Ratio. *Biological Psychiatry* 65: 979–984.
9. Reddy MM, Wilson R, Wilson J, Connell S, Gocke A, et al. (2011) Identification of candidate IgG biomarkers for Alzheimer's disease via combinatorial library screening. *Cell* 144: 132–142.
10. Mayeux R, Honig LS, Tang MX, Manly J, Stern Y, et al. (2003) Plasma A β [40] and A β [42] and Alzheimer's disease: relation to age, mortality, and risk. *Neurology* 61: 1185–1190.
11. Luis CA, Abdullah L, Paris D, Quadros A, Mullan M, et al. (2009) Serum β -amyloid correlates with neuropsychological impairment. *Aging, Neuropsychology, and Cognition* 16: 203–218.
12. Laske C, Stransky E, Leyhe T, Eschweiler GW, Wittorf A, et al. (2006) Stage-dependent BDNF serum concentrations in Alzheimer's disease. *Journal of Neural Transmission* 113: 1217–1224.
13. O'Bryant SE, Hobson V, Hall JR, Waring SC, Chan W, et al. (2009) Brain-Derived Neurotrophic Factor Levels in Alzheimer's Disease. *Journal of Alzheimer's Disease* 17: 1051–1055.
14. O'Bryant SE, Hobson VL, Hall JR, Barber RC, Zhang S, et al. (2010) Serum Brain-Derived Neurotrophic Factor Levels Are Specifically Associated with Memory Performance among Alzheimer's Disease Cases. *Dementia and Geriatric Cognitive Disorders* 31: 31–36.
15. Komulainen P, Pedersen M, Hanninen T, Bruunsgaard H, Lakka TA, et al. (2008) BDNF is a novel marker of cognitive function in ageing women: the DR's EXTRA Study. *Neurobiology of Learning & Memory* 90: 596–603.
16. Waring S, O'Bryant SE, Reisch JS, Diaz-Arrastia R, Knebl J, et al. (2008) , for the Texas Alzheimer's Research Consortium (2008) The Texas Alzheimer's Research Consortium longitudinal research cohort: Study design and baseline characteristics. *Texas Public Health Journal* 60: 9–13.
17. McKhann D, Drockman D, Folstein M, et al. (1984) Clinical diagnosis of Alzheimer's disease: Report of the NINCDS-ADRDA Work Group. *Neurology* 34: 939–944.
18. Vemuri P, Wiste HJ, Weigand SD, Shaw LM, Trojanowski JQ, et al. (2009) MRI and CSF biomarkers in normal, MCI, and AD subjects: Diagnostic discrimination and cognitive correlations. *Neurology* 73: 287–293.
19. R Development Core Team (2009) R: A language and environment for statistical computing. Vienna, Austria.
20. Osborne J (2010) Improving your data transformations: Applying the Box-Cox transformation. *Practical Assessment Research Evaluation* 15: 2.
21. Fraley CR, AE (2002) Model-based clustering, discriminant analysis, and density estimation. *Journal of the American Statistical Association* 97: 611–631.
22. Breiman L (2001) Random forests. *Machine Learning* 45: 5–32.
23. Breiman L Manual on setting up, using, and understanding random forests V3.1.
24. Anonymous (1998) Consensus report of the Working Group on: "Molecular and Biochemical Markers of Alzheimer's Disease". The Ronald and Nancy Reagan Research Institute of the Alzheimer's Association and the National Institute on Aging Working Group.[see comment][erratum appears in *Neurobiol Aging* 1998 May–Jun;19(3):285]. *Neurobiology of Aging* 19: 109–116.
25. Zhang D, Wang Y, Zhou L, Yuan H, Shen D (2011) Multimodal classification of Alzheimer's disease and mild cognitive impairment. *NeuroImage*.
26. Brys M, Glodzik L, Mosconi L, Switalski R, De Santi S, et al. (2009) Magnetic resonance imaging improves cerebrospinal fluid biomarkers in the early detection of Alzheimer's disease. *Journal of Alzheimer's Disease* 16: 351–362.
27. Vemuri P, Gunter JL, Senjem ML, Whitwell JL, Kantarci K, et al. (2008) Alzheimer's disease diagnosis in individual subjects using structural MR images: Validation studies. *NeuroImage* 39: 1186–1197.
28. O'Bryant SE, Waring SC, Hobson V, Hall JR, Moore CB, et al. (2010) Decreased C-reactive protein levels in Alzheimer disease. *Journal of Geriatric Psychiatry and Neurology* 23: 49–53.
29. Gustafson DR (2010) Adiposity hormones and dementia. *Journal of the Neurological Sciences* 299: 30–34.
30. Une K, Takei YA, Tomita N, Asamura T, Ohru T, et al. (2011) Adiponectin in plasma and cerebrospinal fluid in MCI and Alzheimer's disease. *European Journal of Neurology* 18: 1006–1009.
31. Cui Y, Andersen DK (2011) Pancreatogenic diabetes: Special considerations for management. *Pancreatology* 11: 279–294.
32. Zhang L, Bijker MS, Herzog H (2011) The neuropeptide γ system: Pathophysiological and therapeutic implications in obesity and cancer. *Pharmacology and Therapeutics* 131: 91–113.
33. Steinacker P, Mollenhauer B, Bibl M, Cepek L, Esselmann H, et al. (2004) Heart fatty acid binding protein as a potential diagnostic marker for neurodegenerative diseases. *Neuroscience Letters* 370: 36–39.
34. Teunissen CE, Veerhuis R, De Vente J, Verhey FRJ, Vreeling F, et al. (2011) Brain-specific fatty acid-binding protein is elevated in serum of patients with dementia-related diseases. *European Journal of Neurology* 18: 865–871.
35. Swardfager W, Lanctt K, Rothenburg L, Wong A, Cappell J, et al. (2010) A meta-analysis of cytokines in Alzheimer's disease. *Biological Psychiatry* 68: 930–941.
36. Gejyo F, Yamada T, Odani S, Nakagawa Y, Arakawa M, et al. (1985) A new form of amyloid protein associated with chronic hemodialysis was identified as β 2-microglobulin. *Biochemical and Biophysical Research Communications* 129: 701–706.
37. Abdi F, Quinn JF, Jankovic J, McIntosh M, Leverenz JB, et al. (2006) Detection of biomarkers with a multiplex quantitative proteomic platform in cerebrospinal fluid of patients with neurodegenerative disorders. *Journal of Alzheimer's Disease* 9: 293–348.
38. Zellner M, Veitinger M, Umlauf E (2009) The role of proteomics in dementia and Alzheimer's disease. *Acta Neuropathologica* 118: 181–195.
39. Midwood KS, Hussenet T, Langlois B, Orend G (2011) Advances in tenascin-C biology. *Cellular and Molecular Life Sciences* 68: 3175–3199.
40. Hu WT, Chen-Plotkin A, Arnold SE, Grossman M, Clark CM, et al. (2010) Novel CSF biomarkers for Alzheimer's disease and mild cognitive impairment. *Acta Neuropathologica* 119: 669–678.
41. Akiyama H, Ikeda K, Kondo H, McGeer PL (1992) Thrombin accumulation in brains of patients with Alzheimer's disease. *Neuroscience Letters* 146: 152–154.
42. Ewers M, Mielke MM, Hampel H (2010) Blood-based biomarkers of microvascular pathology in Alzheimer's disease. *Experimental Gerontology* 45: 75–79.
43. Sokolova A, Hill MD, Rahimi F, Warden LA, Halliday GM, et al. (2009) Monocyte chemoattractant protein-1 plays a dominant role in the chronic inflammation observed in Alzheimer's disease. *Brain Pathology* 19: 392–398.
44. Galimberti D, Fenoglio C, Lovati C, Venturelli E, Guidi I, et al. (2006) Serum MCP-1 levels are increased in mild cognitive impairment and mild Alzheimer's disease. *Neurobiology of Aging* 27: 1763–1768.
45. Boustani M, Callahan CM, Unverzagt FW, Austrom MG, Perkins AJ, et al. (2005) Implementing a screening and diagnosis program for dementia in primary care. *Journal of General Internal Medicine* 20: 572–577.
46. Doody R, Ferris S, Salloway S, Meuser TM, Murthy A, et al. (in press) Inter-rater reliability between expert and nonexpert physicians in the diagnosis of amnesic MCI in the community setting. *Clinical Drug Investigation*.
47. O'Bryant SE, Humphreys JD, Smith GE, Ivnik RJ, Graff-Radford NR, et al. (2008) Detecting dementia with the mini-mental state examination in highly educated individuals. *Archives of Neurology* 65: 963–967.