Identifying Early Markers of Alzheimer’s Disease using Quantitative Multiplex Proteomic Immunoassay Panels

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Alzheimer’s disease (AD) is a debilitating neurodegenerative disorder with incidence expected to increase four-fold over the next decade. Extensive research efforts are focused upon identifying new treatments, and early diagnosis is considered key to successful intervention. Although imaging and cerebrospinal fluid biomarkers have shown promise in identifying patients in very early stages of the disease, more noninvasive cost-effective tools have remained elusive. Recent studies have reported that an 18-analyte multiplexed plasma panel can differentiate AD from controls suggesting plasma-based screening tools for early AD diagnosis exists. The current study tested the reproducibility of a subset of the original 18-analyte panel using a bead-based multiplex technology. Preliminary results suggest diagnostic accuracy using the subset was 61%. Multivariate analysis of an 89-analyte multivariate panel yielded a diagnostic accuracy of 70% suggesting a plasma-based AD signature that may be a useful screening tool.

Key words: biomarkers; Alzheimer’s disease; plasma; Luminex

Introduction
The Relevance of Early Diagnosis to Drug Development in Alzheimer’s Disease

Alzheimer’s disease (AD) currently affects 26.6 million patients worldwide with estimated incidence expected to increase four-fold during the next 50 years.1 Unfortunately, there are few effective treatments to halt AD’s debilitating neurodegenerative progression, and the majority of current treatment research strategies have focused upon targets that may have limited utility in reversing existing pathological damage. Based upon autopsy findings and amyloid positron emission tomography (PET) labeling, patients in even mild-moderate stages of the disease exhibit profound amyloid brain deposition and degenerative neuronal loss that may be irreversible.2–4 Patients with both memory complaints and evidence of high amyloid deposition are also at higher risk of dementia.4 Thus, the importance of early intervention cannot be overly emphasized.

Early diagnosis in stages of AD where frank dementia is not overt is quite challenging for the general practitioner and even for most specialists. Patients often present with a subjective memory complaint that can be confirmed with objective memory testing. Typically, several months of observation by both clinician and caregiver are required before a diagnosis of probable dementia of the Alzheimer’s type can be made. During the observation period, it is possible that profound neuropathological damage may occur. As a result, numerous studies are under way to identify more sensitive tools to identify those patients at greatest risk of
progressing to dementia following a subjective memory complaint. Much of the recent success in this field has been through a combined use of objective memory tests followed by imaging and cerebrospinal fluid (CSF) biomarkers.\textsuperscript{5}

**Current Tools for Early AD Diagnosis**

**PET**

The most promising imaging-based tools for early diagnosis include both fluorodeoxyglucose PET (FDG-PET) and amyloid PET labeling. Stereotypical patterns of regional glucose hypometabolism differentiate AD and mild cognitive impairment (MCI) patients from age-matched controls, and patterns of glucose hypometabolism are useful in differentiating AD from other forms of dementia.\textsuperscript{6,7} Interestingly, FDG-PET is emerging as a tool to identify patients at greater risk of progressing through various stages of disease. For example, recent studies have reported that decreased glucose metabolism in posterior cingulate, posterior precuneus, and temporal lobe in amnestic MCI patients with at least one ApoE4 allele are at greater risk of progressing to dementia suggesting some diagnostic utility of FDG-PET.\textsuperscript{8} In addition to glucose hypometabolism, \textit{in vivo} labeling of amyloid brain burden by PET has emerged as a powerful tool to assess risk in patients with a cognitive memory complaint.\textsuperscript{4,9}

Use of amyloid PET ligands as markers of disease progression have been complicated by unchanged amyloid PET labeling in AD patients who show progressive cognitive decline and by high amyloid brain load in cognitively normal individuals. Nevertheless, recent reports suggest that in patients with memory complaints, the presence of high brain amyloid load, as evidenced by amyloid PET imaging, is a significant risk factor for progression to dementia.\textsuperscript{4,9}

Indeed, the promise of PET as a biomarker of dementia risk is such that position papers have begun to emerge suggesting the use of amyloid PET labeling to identify patients in early stages of AD.\textsuperscript{5}

**CSF-based**

CSF biomarkers including amyloid beta 42 (Aß42) peptide fragments, total tau, and threonine 181 phosphorylated tau (pTau) are proving to be the most promising CSF candidates for the early detection of AD. Numerous labs using different variants of the assays have reported that AD patients exhibit elevated levels of pTau and total Tau and lower levels of Aß42 compared to cognitively normal controls.\textsuperscript{10–20} Furthermore, low Aß42 and high total Tau/pTau levels are endophenotypic traits of patients with a memory complaint who then progress to dementia. Interestingly, elevated levels of phosphorylated Tau appear to confer specificity over other forms of dementia.\textsuperscript{21} A recent large cross-site longitudinal study examining the utility of a combination of the CSF markers in diagnosing AD in predemented patients with a cognitive complaint suggest diagnostic sensitivity and specificity between 80 and 70\%, respectively.\textsuperscript{15}

From a diagnostic perspective, these numbers are relatively low. However, from a drug development perspective, the diagnostic accuracy is sufficient to enable patient enrichment for studies aimed at either modifying the time to progression to dementia of the Alzheimer’s type or cognitive and functional decline in a cognitively abnormal population at risk of progressing dementia. Recent studies have also suggested that low CSF levels do correlated with high amyloid brain load\textsuperscript{22,23} suggesting CSF Aß42 may be a surrogate marker for the degree of brain amyloid burden. Various labs have developed cutoff criteria for defining what constitutes “low” Aß42 and “high” Tau-based either upon CSF from autopsy-confirmed subjects\textsuperscript{17} or from large cohorts.\textsuperscript{15,18} The lack of standardized collection protocols, assay formats, and international calibrator standards have impeded the development of definitive diagnostic thresholds for CSF biomarkers. Despite these limitations, CSF Aß42, total Tau, and pTau remain the most promising CSF biomarkers for identifying nondemented patients with a memory
complaint who are at high risk of progressing to dementia.

**MRI-based**

Longitudinal assessment of brain atrophy is currently one of the few biomarkers in AD patients that shows change over time and good correlation with cognitive decline. Unlike CSF biomarkers, magnetic resonance imaging (MRI) measures of brain atrophy in AD patients are correlated with cognitive loss of function, and there are reports suggesting that increased rates of regional atrophy may be a risk factor for dementia. Recent studies have also suggested that multivariate analysis of baseline regional brain atrophy may be a useful diagnostic tool. Research using MRI measures of atrophy as a risk factor for dementia are still in early stages. Nevertheless, automated measures of MRI-based atrophy measures show promise as future tools to identify patients at risk of developing Alzheimer’s type dementia.

**Current Limitations for Widespread Implementation of Imaging and CSF Tools**

Despite the promising of imaging and CSF markers, there still remain limitations from a screening perspective. Currently, PET technologies are not readily available globally, and the tests can be cost prohibitive given the relatively modest diagnostic accuracy. Although some countries routinely collect CSF in AD patients as standard of care, the collection of CSF in most countries is not common in cases where dementia is suspected. Furthermore, CSF collection is often characterized as invasive and risky making justification of a CSF test with relatively modest diagnostic accuracy challenging. The global accessibility of MRI is actually quite good. However, the lack of standardization and availability of analysis algorithms have limited widespread implementation. Furthermore, the diagnostic performance of baseline MRI-atrophy measures remains under area investigation. As a result, the need for a noninvasive, cost-effective solution for early AD screening is high.

**The Search for Plasma Screening Tools**

**Plasma Proteomics in AD and Suitable Technologies for Clinical Trial Use**

A number of noninvasive screening tools for early AD have been reported in the literature including short psychometric tests, EEG tests, optical approaches, olfactory tests, and blood-based tests. Not all have been successfully established as reasonably accurate screening tools. Numerous proteomic studies have attempted to identify plasma-based diagnostic markers for AD with limited success. One of the major hurdles in plasma proteomics has been the application of qualitative rather than quantitative tools to proteomic discovery. Although such tools can be powerful in cases where the signal-to-noise ratio is dramatic, they have proven to be of limited utility in cases where the disease signal is much more subtle, as with AD. In most cases, identification of analytes using qualitative proteomic procedures has not yielded validated analytes following verification in more quantitative assays and larger independent clinical sample sets. Furthermore, qualitative tools can rarely be applied in the clinical trial arena, where regulatory requirements for validated quantitative tools and acceptance standards for batch runs using standard curves and quality control (QC) criteria have been clearly delineated.

An alternative strategy to identify plasma-based biomarkers for AD has relied on quantitative immunoassay-based multiplex panels. Although such platforms generally contain a very limited spectrum of the proteome, they have proved useful in identifying disease state and drug-response analytes that can be verified across multiple clinical data sets under clinical laboratory improvement amendments (CLIA) and good lab practice (GLP) standards.
TABLE 1. Summary of 18 Proteins Predicted to Differentiate AD from Controls and a Corresponding List of Analytes from the Commercially Available 89-analyte Luminex xMAP that were Tested in the AD Natural History Study

<table>
<thead>
<tr>
<th>Ray et al. 18-protein panel[^38]</th>
<th>Gene accession</th>
<th>Seven of 18 in AD natural history study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemokine (C-C motif) ligand 18-pulmonary and activation-regulated-(CCL18/PARC)</td>
<td>6362</td>
<td></td>
</tr>
<tr>
<td>Angiopoietin-2 (ANG-2)</td>
<td>285</td>
<td></td>
</tr>
<tr>
<td>Insulin-like growth factor binding protein 6 (IGFBP-6)</td>
<td>3489</td>
<td></td>
</tr>
<tr>
<td>Interleukin 8 (CXCL8/IL-8)</td>
<td>3576</td>
<td>IL-8</td>
</tr>
<tr>
<td>Interleukin 11 (IL-11)</td>
<td>3589</td>
<td></td>
</tr>
<tr>
<td>Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain (TRAIL R4)</td>
<td>8793</td>
<td></td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 5 (CCL5/RANTES)</td>
<td>6352</td>
<td>RANTES</td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 7 (CCL7/MCP-3)</td>
<td>6354</td>
<td></td>
</tr>
<tr>
<td>Epidermal growth factor (beta-urogastrone) (EGF)</td>
<td>1950</td>
<td>EGF</td>
</tr>
<tr>
<td>Chemokine (C-C motif) ligand 15 (CCL15/MIP-1d)</td>
<td>6359</td>
<td></td>
</tr>
<tr>
<td>Glial cell derived neurotrophic factor (GDNF)</td>
<td>2668</td>
<td></td>
</tr>
<tr>
<td>Colony stimulating factor 3 (granulocyte) (G-CSF)</td>
<td>1440</td>
<td>G-CSF</td>
</tr>
<tr>
<td>Colony stimulating factor 1 (macrophage) (M-CSF)</td>
<td>1435</td>
<td></td>
</tr>
<tr>
<td>Tumor necrosis factor-alpha (TNF superfamily, member 2) (TNF-α)</td>
<td>7124</td>
<td>TNF-α</td>
</tr>
<tr>
<td>Interleukin 3 (colony-stimulating factor, multiple) (IL-3)</td>
<td>3562</td>
<td>IL-3</td>
</tr>
<tr>
<td>Interleukin 1, alpha (IL-1alpha)</td>
<td>3552</td>
<td></td>
</tr>
<tr>
<td>Platelet-derived growth factor beta polypeptide (simian sarcoma viral (v-sis) oncogene homolog) (PDGF-BB)</td>
<td>5155</td>
<td></td>
</tr>
</tbody>
</table>

Note IL-1alpha levels were below limit of detection using Luminex technology. G-CSF and EGF had more than 20% of values below limit of detection.

**Quantitative Multiplex Noninvasive Technologies**

Recent targeted proteomic approaches to identify AD plasma-based biomarkers utilized a filter-based immunoassay technology representing 120 signaling proteins[^38]. A shrunken centroid algorithm developed for predictive analysis of microarrays was used to train data from an 83-patient set of archived plasma samples[^43] that yielded an 18-protein model (see Table 1 for summary of analytes). When the model was applied to a separate 92-AD-patient test set and a 47-patient MCI set, the overall diagnostic accuracy was reported to be 90% for AD and 81% for MCI patients who had progressed to dementia over a 7-year span[^38]. Under optimal conditions, a training set should include subjects that will be used in the future predictive model (e.g., MCI), and the test set should be a completely independent data, not a subset of the entire data set. Nevertheless, Ray et al.[^38] were among the first to advance the notion that a rationally designed proteomic multiplex plasma-based panel could be used to classify AD. Further analysis by an independent group suggested that the approach might have overfit the data set. Indeed, as few as five analytes from the original 18 panel could provide a greater than 90% diagnostic accuracy suggesting some redundancy among the original noninvasive panel[^44]. In an effort to reproduce initial findings reported by Ray et al.[^38], samples from a small 50-patient longitudinal natural history study were analyzed using a more quantitative Luminex immunoassay-based
technology. The 89-analyte Luminex xMAP multiplex panel is a commercially available platform qualified to CLIA standards amenable for clinical trial work. Seven of the original 18 analytes were present on the commercially available 89-analyte Luminex panel. Table 1 summarizes the seven of the 18 analytes analyzed in the AD natural history.

The Luminex xMAP technology (Austin, TX) uses a solid phase noninvasive approach to analyze multiplexed proteins. Figure 1 summarizes the technology. In brief, the xMAP technology is a flow cytometric-based platform that uses microspheres loaded with a ratio of two different fluorescent dyes. In theory, up to 100 differently colored beads can be generated with a theoretical multiplex capacity of up to 100 assays per well of a 96-well plate. The capture antibody is covalently coupled to the bead, and immunoassays run under standard sandwich immunoassay formats. Because the platform is based upon standard solid phase sandwich immunoassay format, it is amenable to CLIA/GLP validation, and numerous vendors have successfully validated assays on the platform in alignment with recent FDA guidance for biological assays. In practice, dynamic range and cross-reactivity limit the number of analytes that can be multiplexed (typically between three and two analytes per plex). Thus, the commercially available 89-analyte panel actually consists of several different multiplex panels. Detection involves two solid-state lasers, one that detects the fluorescent identity of the bead (e.g., assay identity), and the second that detects the molecules bound to the biological reactants at the microsphere surface (e.g., quantity of analyte within a complex matrix).

The current study enrolled 25 AD patients and 25 age-matched control subjects. AD subjects were allowed to enroll if National Institute of Neurological and Communicative Disorders and Stroke and the
Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA)-probable or possible AD and Diagnostic and Statistical Manual of Mental Disorders—version Four (DSM-IV) dementia criteria were met, baseline Mini-mental state exam (MMSE) scores were 15–27 inclusive, Hachinski scores were ≤ 4, geriatric depression scores ≤ 2, and patients were between the ages of 55 and 90. Known or suspected cases of Lewy body, frontotemporal, and/or vascular dementia were excluded. All subjects were required to have a caregiver able to provide support through the study and informed consent. All protocol procedures adhered to good clinical practices and were approved by the site institutional review board. Control healthy subjects were enrolled if MMSE ≥ 28, age was between 55 and 90 years, and subjects had no prior history of conditions that could lead to cognitive decline (e.g., multiple sclerosis, traumatic brain injury, etc.). Table 2 summarizes patient demographics and ApoE allele status. Interestingly, more than half the control subjects had one ApoE4 allele, which is greater than typical ApoE4 allele prevalence in the general population. 45

Plasma was collected at base line and at 3, 6, and 12 months following entry into the study. Plasma samples were sent for analysis in the 89-analyte Luminex multiplex panel. Figure 2 summarizes the 89 analytes in the panel and those analytes that were below the limit of detection in longitudinal AD natural history study.

Additional QC was conducted to allow multivariate analysis. In brief, analytes were excluded if there were more than 10% missing from the data set. With analytes that possessed more than 90% data, data points, reported as below lowest detectable limit, were imputed by dividing the lowest detectable limit of the assay by two. Finally, normalcy was calculated using the Anderson-Darling test. Analytes were log transformed if distribution was not normal. An outlier analysis was not conducted, as there were few outliers in analytes that had more than 90% of data in this data set. Table 3 summarizes univariate analysis of AUC 1yr of AD versus control. Univariate analysis of five of the Luminex-based analytes in the current data set did not detect significant differences when adjusted for age and gender.

Figure 3 illustrates expression levels of individual analytes.

A linear discriminant and random forest analysis using all five analytes reported an overall accuracy of between 58% and 67% suggesting performance was lower in the current data set than in the data set reported by Ray et al. 38 Inclusion of the additional two analytes did not improve model performance. Differences could be attributed to the relatively small sample size.

Additional univariate analysis identified other analytes in the panel that were significantly different between AD and controls (data not shown). However, the current data set is too small to draw any definitive conclusions, and additional studies to reproduce in larger data sets are ongoing. A linear discriminant analysis was completed using all 89 analytes with a diagnostic accuracy at 70% suggesting there may be a subtle signal in the 89-analyte panel (unpublished data).

**Population Cohort**

To test more thoroughly the performance of the original 18-analyte panel reported in Ray et al., 38 an additional 1200 subset of the Rotterdam cohort was analyzed using an expanded 151-analyte variant of the panel. The Rotterdam study is a large prospective population-based cohort study that is conducted among all

### Table 2. Demographics AD Longitudinal Natural History Study

<table>
<thead>
<tr>
<th></th>
<th>Alzheimer’s disease (n=19)</th>
<th>Healthy controls (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age years (mean ± SD)</td>
<td>81.0 ± 4.8</td>
<td>76.5 ± 7.5</td>
</tr>
<tr>
<td>Range</td>
<td>74–89</td>
<td>62–90</td>
</tr>
<tr>
<td>Gender female/male</td>
<td>12 F/7 M</td>
<td>14 F/8 M</td>
</tr>
<tr>
<td>ApoE status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% 4/4, 2/4, or 3/4</td>
<td>63%</td>
<td>61%</td>
</tr>
<tr>
<td>% 2/2, 2/3, or 3/3</td>
<td>38%</td>
<td>39%</td>
</tr>
<tr>
<td>MMSE (Mean ± SD)</td>
<td>22.5 ± 3.4</td>
<td>28.9 ± 1.4</td>
</tr>
</tbody>
</table>

Plasma was collected at base line and at 3, 6, and 12 months following entry into the study.
Figure 2. Human xMap ver 1.6. Analytes highlighted in dark gray were below the limit of detection in EDTA plasma from the 50-patient natural history study. Analytes in light gray had more than 20% missing values. (In color in Annals online.)

The third survey took place between 1997 and 1999. Of the 5990 participants that were still alive at the start of the third survey, 4797 participated, and 3795 participants had fasting blood samples drawn. From the larger cohort, a random subset of 970 participants was selected. An additional 43 participants that had

inhabitants of Ommoord (a district of Rotterdam, the Netherlands) aged 55 years and over. The diagnosis of dementia was made following a three-step protocol, and continuous monitoring for incident dementia, through medical records from general practitioners, was implemented, as described elsewhere.46
TABLE 3. Demographics AD Longitudinal Natural History Study

<table>
<thead>
<tr>
<th></th>
<th>Alzheimer’s disease</th>
<th>Healthy controls</th>
<th>P-value ADJUSTED</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-3</td>
<td>4.8 (4.0, 5.6)</td>
<td>3.8 (3.1, 4.6)</td>
<td>0.2068</td>
</tr>
<tr>
<td>RANTES</td>
<td>7967.1 (7075.9, 8858.2)</td>
<td>6893.6 (6030.7, 7756.4)</td>
<td>0.7280</td>
</tr>
<tr>
<td>IL-8</td>
<td>252.5 (216.6, 288.4)</td>
<td>232.3 (197.5, 267.1)</td>
<td>0.6226</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>1067.0 (924.8, 1209.1)</td>
<td>1104.7 (967.1, 1242.3)</td>
<td>0.3582</td>
</tr>
<tr>
<td>TNF-alpha</td>
<td>4.1 (3.9, 4.3)</td>
<td>3.9 (3.7, 4.1)</td>
<td>0.1722</td>
</tr>
</tbody>
</table>

Values reported as mean AUC_{1year} (lower and upper 95%).

Note: All analytes except IL-3 required log transformation for statistical analysis. Two of the analytes, G-CSF and EGF, had more than 20% missing data and were not included in the analysis. EGF, IL-3, IL-8, and RANTES showed a significant association with gender, and ICAM-1 was significantly associated with age.

prevalent AD at the time of sample draw were also included. This resulted in a total of 61 participants with prevalent AD and 952 participants that were free from dementia at the time of sample draw.

Eight of the original 18 analytes were represented in the updated 151-analyte Luminex xMAP panel (ANG-2, ICAM-1, IL-8, M-CSF, PDGF, PARC, RANTES, and TNF-α). When the original data set from Ray et al. was utilized for the analysis, diagnostic accuracy for predicting AD was 83%, which was very close to the original estimates using the full 18-analyte panel. However, when AD subjects were matched to randomly selected dementia-free subjects, only 42 of the 61 controls selected for the analysis were correctly classified, and only 32 of the 61 AD samples were correctly classified resulting in a test sensitivity and specificity of 63% and 59%, respectively (total diagnostic accuracy at 61%). Performance was slightly better when controls were carefully selected to represent a healthy population typical for clinical trial use. However, overall performance was not better than models that used well-known risk factors including age, ApoE, and education.

Despite an inability to repeat diagnostic performance of the analytes in the Ray et al. 18-analyte panel, there may yet be a plasma-based signature for AD. Indeed, recent promising reports suggest plasma Aß42 and Aß40 may have utility in predicting who will progress to dementia. Furthermore, utilization of other analytes from the 89-analyte panel did show a diagnostic accuracy of approximately 70%, which, if combined with other markers more closely linked to the pathology, could provide a useful screening tool.

Next Steps

Before data mining the Rotterdam cohort further, an 800-patient cohort (200 AD, 200 controls, and 400 amnestic MCI) of samples from a controlled natural history clinical trial called the Alzheimer’s Disease Neuroimaging Initiative (ADNI) is currently being analyzed by a precompetitive targeted proteomics team. The current plans include analyzing baseline and 1-year samples in the expanded Luminex xMAP panel to confirm preliminary findings from the natural history study and from other large AD-sample cohorts. This team is being sponsored by the biomarkers consortium of the Foundation for the National Institute of Health (fNIH) and includes both industry and academic participants with the intent that the data will be made freely available to the public through the ADNI consortium database.

Discussion and Summary

In summary, results using Luminex-based technology to confirm preliminary findings from initial reports suggesting diagnostic utility of an 18-analyte plasma-based panel were...
Figure 3. Protein expression levels of (A) EGF, (B) ICAM-1, (C) IL-3, (D) IL-8, (E) RANTES, and (F) TNF-alpha in AD and controls from the 50-patient natural history study.
unsuccessful. Confirmatory studies included a 50-patient, 1-year natural history study and a 1200 patient subset of the Rotterdam cohort. Neither a five-analyte nor an eight-analyte multivariate analysis could improve diagnostic accuracy above models that use well-known risk factors for AD, including ApoE allele, age, and education status. However, multivariate analysis of the full 89-analyte panel in the 50-patient natural history study suggests a plasma-based AD signature exists, which, when combined with markers more closely linked to the disease, such as Aβ, may provide sufficient accuracy to be useful for screening patients who might be eligible for more confirmatory CSF and imaging tests.

Given the difficulty in AD diagnostic markers, it is probable that AD plasma biomarker qualification will go the route of AD genetic candidates requiring large consortia approaches involving hundreds (if not thousands) of well-characterized samples that can be compiled across many sites. Any emergent plasma-based model would then need to be carefully confirmed in a prospective study. Requisite studies suitable for qualifying plasma biomarker candidates are being conducted on a precompetitive basis in the context of targeted proteomics subteam of the biomarkers consortium, and it is hoped the willingness to share the data will advance the field quickly to ensure patients and physicians have access to tools that can yield a more timely definitive diagnosis and access to treatment.

Acknowledgments


Conflicts of Interest

The authors declare no conflicts of interest.

References


