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Discovery and Subsequent Confirmation of Novel Serum Biomarkers Diagnosing Alzheimer's Disease

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Abstract.
Background: Alzheimer's disease (AD) remains challenging to diagnose, especially early disease. Having serum AD biomarkers would be of significant interest both in the clinical setting and in drug development efforts.
Objective: We applied a novel serum proteomic approach to interrogate the low-molecular weight proteome for serum AD biomarkers.
Methods: A discovery study used sera from 58 any-stage AD cases and 55 matched controls analyzed by capillary liquid chromatography-electrospray ionization-tandem mass spectrometry. Candidate biomarkers were statistically modeled and promising biomarkers were retested in a second, blinded confirmatory study (AD cases = 68, controls = 57). Biomarkers that replicated in the second study were modeled for the diagnosis of any-stage and very early stage AD. Further, they were chemically identified by tandem MS.
Results: The initial discovery study found 59 novel potential AD biomarkers. Thirteen recurred in more than one multi-marker panel. In a second, blinded, confirmatory study, these same biomarkers were retested in separate specimens. In that study, four markers validated comparing controls to patients with any-stage AD and also with very early AD. The four biomarkers with replicable ability to diagnose AD were then chemically identified.
Conclusion: These results suggest novel serum AD diagnostic biomarkers can be found using this approach.

Keywords: Alzheimer's disease, diagnosis, lipidomics, mass spectrometry, proteomics, serum biomarkers

INTRODUCTION

Alzheimer's disease (AD) is the sixth leading cause of death in the US and is predicted to become three times more prevalent by 2050 [1]. AD is a fatal disease without cure or treatment [1, 2]. This absence of therapy is largely due to the inability to diagnose AD early. Therapeutics are less likely to be useful as the characteristic tissue pathology becomes more extensive. These changes include amyloid-β protein (Aβ) plaque development and often neurofibrillary tangles of tau protein [3].

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At present, diagnosis of early stage AD remains problematic [4]. The most promising biomarkers have been those identified in cerebrospinal fluid (CSF) and include reduced levels of $A\beta_{42}$ found in many but not all individuals with AD [5, 6]. Also, increased CSF levels of tau protein (tau) or phosphorylated tau (P-tau) are observed in many subjects with AD, more often with advanced disease. These changes are not entirely specific to AD [7]. The combination of low CSF $A\beta_{42}$ and increased tau or P-tau may predict disease progression [8]. Yet, levels of $A\beta_{42}$, tau, and P-tau in patients with very mild AD are frequently indistinguishable from controls and their use in predicting or diagnosing very early AD would be challenging [9].

There have been improvements in CSF-introduced, PET ligands that bind Aβ aggregates in the brain, diagnosing AD better and earlier [10]. These ligands seem to overcome much of the insensitivity of CSF-based biochemical markers. Their ability to detect very early AD universally remains to be established. There is still debate as to whether aggregating $A\beta_{42}$ is the initiating pathological event or the sole mediator of AD progression [11]. However, even if CSF-introduced imaging agents are reliable, screening the extraordinarily large population of aging, and hence at-risk, individuals to assess asymptomatic disease would be difficult and expensive. For this, a blood test for AD would be valuable [12].

Evidence suggests AD patients have some disruption of the blood-brain barrier, enabling Aβ to enter the peripheral circulation [13–15]. This may be important in that alterations in the brain may be reflected in blood biomarkers. Not surprisingly, research seeking blood AD biomarkers has focused on $A\beta_{42}$, tau, and P-tau. Unfortunately, existing studies find changes in these infrequently and to a lesser degree in serum compared with their performance in CSF [16].

Using an unbiased proteomics approach that considers all observable proteins to find serum or plasma AD biomarkers has been attempted [17–19]. With few exceptions approaches pooled serum from individuals with AD and pooled specimens from controls without AD and then performed two-dimensional gel electrophoresis (2DGE) on each pool then compared [20–29]. Spots that appeared quantitatively different were typically identified by matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS). In a number of these reports, a second confirmatory study was conducted using immunoassays to measure the candidate biomarkers. However, 2DGE is frequently limited to high abundance proteins and method reproducibility is difficult [30, 31]. The results of AD biomarker studies using 2D-GE proteomics studies can be summarized as follows: The markers found in the 2DGE discovery phase with few exceptions were no longer different in the validation phase. Further, there were not consistent AD markers across studies [17–25]. These results should not be interpreted as an absence of blood AD biomarkers, but rather a reflection of the insensitivity and poor reproducibility of 2DGE.

Top-down 'global' or 'shotgun' proteomic approaches, e.g., Multidimensional Protein Identification Technology (MudPIT), are used in detailing the complement of proteins present in a tissue or cell [32], but do not work well for quantitative comparisons.

Recently, a metabolomics/lipidomics approach was used as part of a partially prospective study of AD [33]. In this group's discovery phase, metabolites, including amino acids, serotonin, DOPA, certain acyl-carnitines and certain phosphatidylcholines were found to be different in controls versus a group of 18 non-cognitively impaired subjects that later converted to MCI or mild AD. They developed a separate panel of 10 lipids that were significantly different between non-converting controls versus phenotype-converting subjects. This was tested in a validation study of 10 converters and the panel continued to discriminate well between normal controls and mild cognitive impairment or mild AD patients. Results for the metabolites and amino acids that had useful markers in the discovery set were not reported. This study seems promising but the numbers were few and the results unconfirmed by others.

Here we applied another global serum proteomics approach that interrogates the low abundance, low molecular weight molecules in serum [34–36]. This approach has been successfully applied to other clinical indications [32]. When applied to serum, it routinely surveys ~8,000 species [37]. We hypothesized that this method, in conjunction with appropriate biostatistical analyses, would provide useful panels of serum biomarkers for AD diagnosis.

METHODS

Study population

For both the initial discovery and later confirmatory studies, serum specimens were obtained from the Knight Alzheimer's Disease Research Center (Knight ADRC) at the Washington University School of Medicine, St. Louis, MO. These specimens had been obtained as part of previous studies. No patient was enrolled and consented for the current study. Those earlier studies had received approval from the Institutional

Review Board of the Washington University School of Medicine and all subjects gave signed, written consent prior to blood being drawn. As part of that consent, permission was granted to allow those specimens to be further used for later laboratory analysis provided no personal information was disclosed. Prior to specimens being sent to Brigham Young University (BYU) for laboratory analysis, approval was obtained from the Institutional Review Board at BYU for these studies prior to specimen analysis. Specimens analyzed at BYU had been alpha-numerically coded without any personal information provided by personnel at Knight ADRC prior to their being sent to BYU.

In the initial, discovery study, sera from 58 cases and 55 controls were analyzed. Specimens were selected randomly by personnel at Knight ADRC. AD cases were randomly selected and represented different clinical dementia ratings (CDR): mild cognitive impairment (MCI) or very mild AD (CDR 0.5, $n = 7$), mild AD (CDR 1, $n = 4$), moderate AD (CDR 2, $n = 19$), and severe AD (CDR 3, $n = 28$). However, in the initial study a greater focus was placed on patients with more severe AD. Controls (CDR 0, $n = 55$) were matched to AD cases for gender, age and, as possible, ApoE4 status. No subject whose specimen was part of either study had major, known co-morbidities, including diabetes or any other neurological disorder. All individuals were screened using the Mini-Mental State Exam (MMSE) and CDR. Cases had a clinical diagnosis of dementia of the Alzheimer's type (probable/definite) according to NINCDS-ADRDA [38]. Non-demented controls were screened for dementia (MMSE >27, CDR = 0) and sampled from the same source population as their matched cases. Descriptive statistics are found in the Supplementary Material.

Specimens when originally collected were allowed to clot for 30 min, then immediately centrifuged and the serum aliquoted and frozen. Specimens were maintained at –80°C until analyzed at BYU. At BYU specimens were stored at –80°C, pre and post processing.

A second, blinded, confirmatory study of promising biomarkers from the discovery study was carried out with additional specimens provided by Knight ADRC. A set of 125 previously unanalyzed serum samples was provided, including 68 AD cases and 57 controls from non-demented subjects matched for age and gender. The 68 cases included $n = 24$ CDR 0.5, $n = 23$ CDR 1, and $n = 21$ CDR 2. The choice to include more subjects with early stage disease was by design to allow assessment of biomarkers for very early stage AD. This comparison was not possible in the discovery study due to small numbers. An independent arbiter, not part of the analysis team but aware of case/control status, created analysis sets that included both cases and controls randomized and submitted to our laboratory for analysis in a blinded fashion. In all, 11 sets of 10–13 sera each were analyzed. Each set had approximately the same number of cases and controls as well as a similar number of men and women to avoid batch effects. All MS results were provided to the arbiter for statistical analysis without breaking the blind.

Specimen processing

Serum contains ~30 highly abundant proteins which mask other molecules through ion suppression when analyzed by MS. To markedly increase the number of biomolecules in serum interrogated by MS, high abundance proteins were removed as described previously [39, 40]. This sacrifices most proteins but allows several thousand otherwise unobservable small proteins, peptides, lipids and other biomolecules to be studied by MS.

Proteomic analysis

Capillary liquid chromatography (cLC) was used to fractionate protein-depleted serum by gradient elution using an aqueous phase of 98% H_2O, 2% acetonitrile, 0.1% formic acid, and an organic phase of 2% H_2O, 98% acetonitrile, 0.1% formic acid. A 1 mm (16.2 μL) microbore guard column (Upchurch Scientific, Oak Harbor, WA) and a 15 cm × 250 μm i.d. capillary column were employed in the cLC step. The columns were packed with POROS-R1 reversed-phase media (Applied Biosystems, Framingham, MA). Using a flow rate of 5.0 μL/min, the gradient began with 3 min 95% aqueous/5% organic phase, followed by an increase in organic phase to 60% over 24 min. The organic phase was then increased to 95% over 7 min, held at 95% for 7 min, and returned to initial conditions over 5 min and maintained there to re-equilibrate the column.

The cLC was interfaced through an IonSpray source to a QSTAR Pulsar I quadrupole, time-of-flight tandem mass spectrometer used in positive ion mode (Applied Biosystems). MS data was collected from m/z 500–2500 from 5–55 min elution at a scan rate of 1 spectrum/s. Analyst QS® allowed for data collection, mass spectra comparison, and analysis. Each set was processed and analyzed by MS during a single day.

Tandem MS with fragmentation to chemically identify biomarkers in the final panel was accomplished using the same tandem MS as described previously

[41]. Stability of the final biomarkers in the protein-depleted specimen was determined before and after 24 h at room temperature with no change in abundance.

Between the first and second studies, the mass spectrometer required replacement. For the blinded, follow-up study specimens were processed as described but analyzed using an Agilent 6530 Accurate-Mass Q-TOF LC/MS system. Both instruments are ESI-Q-TOF-MS-MS instruments. Again one set of specimens was analyzed in a single day.

For the second study, an Agilent 1260 Infinity Series HPLC system was used, equipped with guard columns and analytical columns that employed the same packing. The loaded amount (5 μg), flow rate, and gradient were identical to those used in the initial study. The ESI source was operated in positive ion mode and MS scans were collected at a rate of 8 spectra/s from m/z 400–3200. MassHunter Data Acquisition B.05.01 [42] and MassHunter Qualitative Analysis B.06.00 permitted data collection, specific ion extraction, and *post hoc* analysis.

MS data analysis

Ten endogenous species, occurring every 2-3 min in the cLC chromatogram, were used for normalizing chromatographic times [36]. In the discovery study, spectra were aligned using these peaks and 2 min windows created around each time marker. Then comparison groups were color coded, overlaid, and analyzed for peaks differing in abundance (>1.5×) [32]. Differences were determined first visually and then using instrument software to provide peak height in ion counts which were evaluated statistically between cases and controls using Student's *t*-test. Molecular species that were statistically significant (alpha = 0.05) were considered candidate biomarkers.

Potential biomarkers were submitted to additional statistical analysis. To compensate for the non-biologic variability between samples, the peak intensity of a candidate species was ratioed to the intensity of a co-eluting species that was quantitatively comparable in specimens of cases and controls to reduce variability due to sample processing, loading, ionization efficiencies and instrument performance. Raw (un-normalized) and log-normalized values were calculated for each of the potential biomarkers. Those candidate biomarkers that continued to demonstrate statistical significance after normalization were further evaluated by receiver operator curve characteristic analysis to determine individual sensitivity and specificity. Statistical analysis was conducted using the R statistical programming language. All analysis code is freely available for download at: https://github.com/wevanjohnson/alzheimers.

Combinations of candidate AD biomarkers were modeled. Candidates with *p*-values less than 0.10 were also included because they may provide complementary diagnostic utility. Development of panels was accomplished using logistic regression analysis to model the log-odds of developing AD. A forward-selection procedure was used where each marker was considered, testing the effects of combining other markers one at a time. The marker that increased the area under the curve (AUC) the most was then included in the model, hence a 'leave-one out' prediction approach [37]. The algorithm stopped adding markers when no additional marker increased the AUC of the combination by more than 0.03. Receiver operator characteristic (ROC) curves were again plotted and AUCs were calculated for the multi-marker combinations using the 'ROCR' R package [43]. In addition to requiring that candidate markers demonstrate statistical significance in both the discovery and confirmation studies, a Bonferonni cutoff was applied to validating markers. R code for the step-wise regression procedure is given in GitHub repository mentioned above. Given that each candidate was considered as the index marker and additional candidate markers were added to form one panel for each index candidate, several of the panels included the same candidate. Only those candidate markers that occurred in 10% of all panels were considered in the second, confirmatory study.

RESULTS

Initial discovery of candidate serum AD biomarkers

The hypothesis predicted serum biomarkers useful in the diagnosis of AD could be found. A novel serum proteomic approach was employed that focused on the low molecular weight components in serum. Using raw, un-normalized mass spectral data, 44 candidate serum AD biomarkers were found in the initial study that were statistically significant comparing any stage AD (CDR 0.5–3) to non-demented controls (CDR 0). Another 25 biomarkers showed quantitative differences between cases and controls having near-significant *p*-values between 0.05 and 0.10. After log normalization of each candidate to an endogenous control, the number of biomarkers was reduced to 38 significant and 21 near-significant candidates ($p = 0.05$ to <0.10). All biomarkers significant after

normalization had been significantly or near significantly different in the un-normalized results. Candidate biomarkers were further evaluated by logistic regression analysis, leading to ROC curves, providing AUCs, sensitivities, and specificities. Some biomarkers were increased in AD while others decreased. The best serum AD biomarker provided an AUC of 0.645.

Samples from cases and controls were always run together, although batches were run on different days. When the data were clustered using a hierarchical algorithm, there were no batch effects. Likewise, cluster analysis did not find any gender bias.

Evaluation of the panels of biomarkers in initial study

Combinations of serum biomarkers, evaluated as described above, identified markers that appeared in multiple models, suggesting they may be more promising. Thirteen biomarkers appeared in 10% or more of all multi-marker models (Table 1). When reanalyzed as a subset, the best combination provided an AUC of 0.912 (Fig. 1) with a sensitivity of 88% at a specificity of 87%. Five additional multi-marker sets provided AUCs >0.80.

Confirmation study of previously found biomarkers

The second confirmation study focused on how these 13 biomarkers would perform in a separate set of specimens analyzed in a blinded fashion. This set included

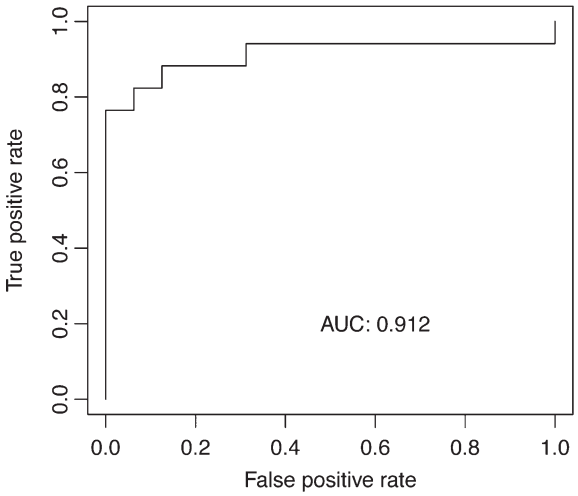

Fig. 1. Best performing multimarker model from the initial study. Shown is an ROC curve produced for biomarkers 531.3, 1568.2, 804.6, 602.3, and 708.3 by logistic regression analysis. The AUC was 0.912 with a sensitivity of ∼88% at a specificity of ∼87%.

Table 1

Candidate serum AD biomarkers from initial study. Values were compared after log normalization to an endogenous species

Marker m/z	cLC Retention time (min)	Charge	*p*-value
921.4	8–15	1	0.035*
1091.4	8–15	1	0.019*
515.3	11–15	1	0.048*
531.3	11–15	1	0.041*
602.3	11–15	1	0.079
701.8	11–15	1	0.095
821.3	11–15	1	0.045*
971.4	11–15	1	0.099
1107.5	11–15	1	0.022*
1194.5	11–15	1	0.085
1396.6	11–17	1	0.086
587.3	13–19	1	0.014*
583.3	16–22	1	0.017*
616.4	16–22	1	0.038*
660.5	16–22	1	0.053
704.5	16–22	1	0.040*
734.5	16–22	1	0.028*
778.6	16–22	1	0.021*
822.5	16–22	1	0.058
880.6	16–22	1	0.073
542.4	17–25	1	0.098
560.4	17–25	1	0.022*
564.3	17–25	1	0.023*
614.4	17–25	1	0.067
687.4	17–25	1	0.020*
701.5	17–25	1	0.086
804.6	17–25	1	0.019*
938.7	17–25	1	0.046*
1126.8	17–25	1	0.015*
1156.9	17–25	1	0.066
1170.9	17–25	1	0.021*
513.3	22–32	1	0.016*
660.4	22–32	1	0.011*
530.4	25–34	1	0.018*
790.6	25–34	1	0.051
804.6	25–34	1	0.082
830.6	25–34	1	0.013*
848.6	25–34	1	0.005*
856.6	25–34	1	0.003*
878.6	25–34	1	0.009*
730.6	29–37	1	0.097
874.6	29–37	1	0.004*
1516.2	29–37	1	0.016*
1540.2	29–37	1	0.027*
675.6	31–38	1	0.071
784.6	31–38	1	0.041*
808.6	31–38	1	0.006*
810.6	31–38	1	0.005*
834.6	31–38	1	0.042*
1568.2	31–38	1	0.002*
1576.2	31–38	1	0.009*
1592.2	31–38	1	0.010*
1616.2	31–38	1	0.066
1618.2	31–38	1	0.011*
1642.2	31–38	1	0.061

Significant and near significant markers were considered when developing multi-marker models. *37 markers with $p < 0.05$, 18 markers with $p > 0.05$ to <0.10.

68 cases, many having a CDR of 0.5, and 57 controls, matched for age and gender. Of the 13 AD biomarker candidates, the biomarkers m/z 708.3 and 660.4 could not be clearly evaluated due to ion suppression and/or unrelated overlapping peaks.

Table 2
Biomarkers that appeared in >10% of all the multi-marker models in initial study

Biomarker Number	Biomarker (m/z ratio)	AUC
1	1568.2	0.654
2	874.6	0.640
3	810.6	0.630
4	1618.2	0.599
5	660.4	0.582
6	804.6	0.568
7	531.3	0.550
8	583.3	0.539
9	701.8	0.519
10	602.3	0.507
11	989.3	0.487
12	892.4	0.431
13	708.3	0.425

The raw extracted MS data obtained for the remaining biomarkers was sent to the independent arbiter who conducted statistical analyses and maintained the blind. The data was tested in four ways: i) controls versus any-stage AD cases; ii) controls versus CDR >0.5; iii) controls versus moderate AD (CDR 2); and finally iv) controls versus very early AD only.

Among the remaining 11 biomarkers, m/z 602.3, 804.55, and 874.6 were significantly different between cases and controls for all four comparisons, using a conservative Bonferroni cutoff, and with the same trend as observed in the original study (Table 2).

As we reviewed data from the initial study, we found that there were two candidate biomarkers with the same nominal m/z of 804.5, but offset somewhat in elution time. To insure that there was no misinterpretation of the data, both species were included. The second

Table 3
Promising initial study biomarkers performance in the second study

Window	m/z	z	Retention	*P*-values for CDR comparisons				Trend	Verified?
			(min)	0 versus >0	0 versus >0.5	0 versus 2	0 versus 0.5		
1	531.3	1	19–21	0.5669	0.3645	0.6203	0.1037	NA	No
2	602.3	1	19.5–22.5	0.0014	0.0584	0.0465	<0.0001	Same	Passed
3	701.8	2	19–21	0.3251	0.2837	0.8336	0.6766	NA	No
4	892.4	1	18.5–20.5	0.5819	0.6021	0.5393	0.7325	NA	No
5	989.3	4	21.5–23.5	0.0586	0.0691	0.0787	0.2012	Opposite	No
5	1318.7	3	21.5–23.5	0.4336	0.39	0.3264	0.6888	NA	No
5	989.3 + 1318.7		21.5–23.5	0.0709	0.0787	0.0877	0.2321	NA	No
6	583.3	1	28–30	0.101	0.2172	0.02	0.0727	Opposite	No
7	804.55	1	33–35	<0.0001	<0.0001	<0.0001	<0.0001	Same	Passed
8	874.6	1	40–44	<0.0001	0.0006	0.0025	<0.0001	Same	Passed
9	810.6	1	45–48	0.4717	0.4593	0.9367	0.68	NA	No
9	832.6	1	43–46	0.2827	0.5921	0.4973	0.0489	NA	No
9	810.6 + 832.6		43–46	0.3948	0.4181	0.9792	0.5536	NA	No
10	1568.2	1	44–47	<0.0001	0.0001	0.0142	0.0115	Mixed	No
11	1618.2	1	44–47	0.9239	0.6117	0.0835	0.3607	Same	No
12	804.53	1	39.5–41.5	<0.0001	<0.0001	<0.0001	<0.0001	Same	Passed

Different stages were compared and *p*-values for them are listed. A biomarker 'Passed' if in this confirmatory study, quantitative differences were still statistically significant as determined by a two-tailed Student's *t*-test with a stringent Bonferroni cutoff and follow the same trend.

Table 4
Raw and normalized results for the 4 markers with continued utility in the second study

m/z	*p*-values all stage AD versus CTL		*p*-values CDR 0.5 versus CTL		higher in
	raw	log normalized	raw	log normalized	
602.3	0.0014	8.23×10^{-5}	8.41×10^{-5}	0.0013	cases
ref 857	0.0057	NA	0.222	NA	controls
804.53	1.80×10^{-6}	2.02×10^{-7}	1.37×10^{-6}	0.0034	cases
ref 566	0.0126	NA	0.118	NA	controls
804.55	1.89×10^{-6}	6.06×10^{-5}	1.75×10^{-8}	0.0013	cases
ref 830	0.88	NA	0.74	NA	cases
874.6	6.37×10^{-5}	5.30×10^{-5}	3.00×10^{-5}	0.0074	cases
ref 830	0.88	NA	0.74	NA	cases

P-values are provided for statistical comparisons of all stage AD versus controls and CDR 0.5 versus controls. The reference used for normalization is also shown.

species had an m/z of 804.53 (compared with 804.55) and eluted somewhat later. This species had been a significant biomarker in the initial study but was not part of the subset of 13. This candidate also validated. A summary of the performance of these four biomarkers is provided in Table 3.

In the initial study candidate peaks had been normalized to endogenous controls. These four validating candidates were normalized to endogenous controls used in the first study. All four candidates were still significantly different (Table 4).

Identification of the validated biomarkers

The four biomarkers that validated in the confirmation study were characterized by tandem MS. One of these was a peptide and three were glycerophosphatidylcholines. Structural features obtained from MS-MS studies are summarized as follows:

Biomarker 602.3 ($z = 1$) was a peptide with an amino acid sequence of L/IAENR. The parent protein cannot be unambiguously identified as this sequence is found in a few proteins.

Biomarker 804.55 ($z = 1$, elution 33–35 min) was a phosphatidylcholine with two fatty-acyl side chains, one with 18 carbon atoms and 2 double bonds (18:2) and the other having 20 carbon atoms and 5 double bonds (20:5). Positions of double bonds cannot be determined by MS. A fragment at m/z = 542.321 suggests the loss and hence presence of a fatty acid (18:2) and a fragment at m/z = 520.334 affirms the presence of a fatty acid (20:5). The molecular formula was $[C_{46}H_{78}NO_8P]+H^+$.

Biomarker 874.6 ($z = 1$) was a peroxidated phosphatidylcholine. Previous research determined this molecule to be a PC (38:4)-$(OOH)_2$ [44]. It was oxidatively modified to contain a bis-peroxide of one fatty acid (20:4) plus an unmodified second fatty acyl group (18:0). The elemental composition was $C_{46}H_{84}NO_{12}P$. Given the saturated nature of the one fatty acid (18:0), it is almost certain that the second, oxidized fatty acid was arachidonic acid (20:4).

Biomarker 804.53 ($z = 1$) elution 39.5–41.5 min was an oxidized glycerophosphatidylcholine of formula $C_{42}H_{78}NO_{11}P+H^+$. There were three primary possible structures: 1-palmitoyl-2-(14,15-di-hydroxy-9-keto-10, 12-octadecadienoyl) glycerophosphatidyl choline; 1-palmitoyl-2-(13,14-di-hydroxy-10-keto-8, 11-octadecadienoyl) glycerophosphatidyl choline; or 1-palmitoyl-2-(13-hydroperoxy-8-keto-9, 11-octadecadienoyl) glycerophosphatidyl choline. Again, palmitic acid is one of the fatty acyl groups and less prone to oxidation. The second fatty acid (18:2) has likely been oxidized [45].

Constructing multi-marker diagnostic panels for AD

For the four validated markers, logistic regression analysis was sufficient to test multi-marker sets diagnosing AD. Statistics were carried out for controls

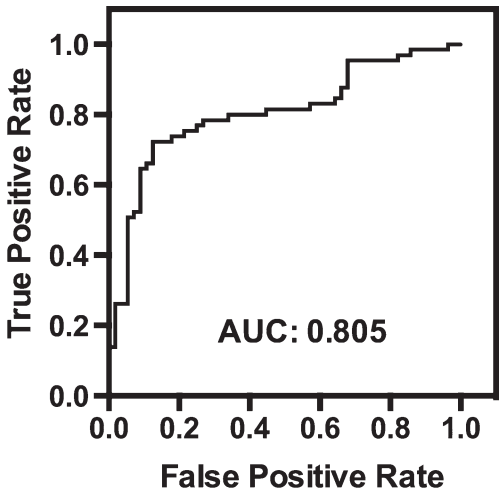

Fig. 2. Receiver operator characteristic curve modeled on the four validated biomarkers m/z 602.3, 804.6, 874.6, and 804.5 using logistic regression analysis. The analysis compared controls (CDR = 0) to any stage AD (CDR = 0.5, 1.0, 2.0). The AUC was 0.805 with a sensitivity of ∼78% at a specificity of 80%.

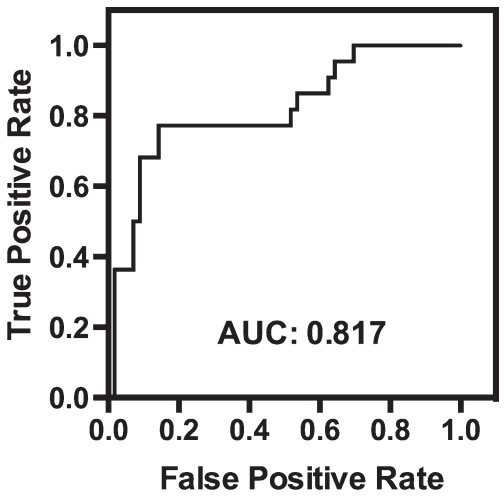

Fig. 3. Receiver operator characteristic curve modeled on three of the four validated markers, m/z 602.3, 804.53, and 874.59. The analysis compared controls with very early AD (CDR 0.5). The AUC was 0.817 providing a sensitivity of ∼78% at a specificity of ∼77%.

versus any-stage AD and for controls versus very early AD (CDR = 0.5). All possible combinations were considered. Analysis provided ROC curves, AUCs, sensitivities, and specificities. When comparing controls (CDR 0) versus any-stage AD (CDR 0.5, 1.0, 2.0), the best model included all four biomarkers (AUC = 0.805, see Fig. 2). When comparing controls to very mild AD (CDR 0.5), the best combination of biomarkers included m/z 602.3, 804.53, and 874.6 (AUC = 0.817, Fig. 3).

DISCUSSION

The hypothesis was that this analytical approach, focused on low molecular weight serum components, in conjunction with appropriate statistical modeling could find panels of biomarkers that capable of diagnosing individuals with AD. Our initial study found several species significantly different between cases and controls. Combinations of these provided a model with 88% sensitivity and 87% specificity. These results were promising.

Any proteomic approach is dependent on the quality of specimens available for analysis. Specimens were obtained from the Knight ADRC at Washington University School of Medicine which has stringent criteria for AD diagnosis and classification. Cases and controls were matched for age, gender, the proportion of subjects carrying the ApoE4 allele (as best as possible), and study center in the initial study. The second study included more subjects with very early AD (CDR 0.5) and subjects were matched for age and gender. In the follow up study, 56.6% of cases had at least one copy of the ApoE4 allele compared with 31.8% of controls. This may have introduced differences. Similar ApoE4 prevalence was seen in the first study. Cases and controls for both studies were without co-morbidities, making biomarkers more representative of AD and not of other diseases. While the candidate biomarkers likely reflect changes occurring in AD, we cannot, at this time, rule out the possibility that some of these serum markers might be changed in other neurologic or more general medical conditions.

In our approach we deliberately excluded highly abundant, higher molecular weight proteins that mask smaller and less abundant species. We recognize that potentially informative species may have been lost in this approach. Nevertheless, our approach allowed for the survey of >8000 low molecular weight species. These are endogenous, previously undescribed molecules that would not be surveyed using other published MS-based approaches. Although our findings are limited to this subset of serum molecules, they appear to provide many candidate biomarkers for AD. This same serum proteomic method has been successful in defining candidate biomarkers for other medical indications [33].

Of the 13 most promising biomarkers in the first study, five appeared to be peptides, the balance lipids. Of these, four were validated in the second study. Extensive investigation of their chemical nature was conducted. The sequence of the one peptide was determined but was too small to assign to a parent protein unambiguously. The other three biomarkers were glycerophosphatidyl cholines. The fatty acid side chain carbon number and double bond number cannot be specified by MS and comparison databases for lipid fragmentation data do not exist. This is the current state of the art and all MS-based lipidomic methods suffer from this same limitation. Alternative methods, e.g., NMR, to completely characterize lipid structure are unusable at the concentrations available. Two of the lipids represented oxidized species, suggesting strongly the action of reactive oxygen species. The observation of differences in these lipids in both very early AD and in later stages suggests that reactive oxygen species-related changes arise very early in AD pathology. This might provide a link between pro-inflammatory diseases, e.g., diabetes, and AD. None of these lipids was described in the recent work of Mapstone et al. [29] but given the different processing and the use of cLC-tandem MS here, this is not surprising.

We performed simple statistical analyses on both normalized and un-normalized sets of data to determine all the significant and near significant biomarkers that might be considered. Near significant species were considered because they might be diagnostically useful for a subset of patients and would be complementary to other markers as part of a set. We found more potential markers when using the raw, un-normalized data. All of the potential biomarkers found after normalization to an endogenous species in the serum were represented among the un-normalized biomarker candidates. The rationale was that such a species would be subject similarly to fluctuations in ionization efficiency, sensitivity, baseline noise, processing inconsistencies, separation column performance, etc. The use of the reference should reduce variability. Previous studies had shown this approach to be somewhat superior to normalization to total ion counts, a common normalization strategy [33]. Normalization to reference standards was not an option given that none of the relevant biomarkers were identified at the outset of the experiments. In these

studies, it was difficult to find an ideal endogenous reference species and sometimes the reference did not reduce the relative variability. Nevertheless, we consistently applied this approach across both studies, but for this reason we have presented both normalized and un-normalized data.

In the initial study combined sets or panels of biomarkers were constructed using a 'leave one out' forward selection model in conjunction with logistic regression analyses. This allowed for combinations of the many candidate biomarkers to be modeled. This employed normalized data and identified several combinations of markers that provided improved sensitivities over those of single markers. Four of these provided sensitivities of 95% or better with a specificity of at least 80% in the initial study. However, thereafter, we limited additional analyses to those markers that appeared in at least 10% of all the models. Considering just these 13 biomarkers, there were still six panels with AUCs >0.80, two having AUCs above 0.90, providing ∼85% sensitivity with ∼85% specificity. In the replication study, it was unnecessary to repeat the leave-one-out approach because there were just four candidates to be modeled. Using logistic regression analyses on those four markers, three combinations provided AUC values greater than 0.80 with two additional having AUCs of 0.79 comparing controls to any stage AD and very similar findings for controls versus CDR 0.5 patients. Sensitivities were ∼75–78% with specificities of 80–88%. These confirmatory results suggest that this approach was successful.

Only 13 of 59 candidate biomarkers were considered in the replication study. It is possible that some of the 46 additional markers will validate and contribute to improved diagnostic sensitivity in combination with the four currently validated markers. This will require substantial additional analysis. Further, it would be interesting to include data on CSF $A\beta_{42}$, tau protein, or P-tau protein levels or ligand studies to determine if the biomarkers found here correlate with or are complementary to those. However, it is unknown if the subjects studied here have existing CSF markers. The designation of CDR 0.5 is used very conservatively at the Knight ADRC and is considered very early AD. Nevertheless, at other sites and in other studies the conversion rate from MCI to AD has varied dramatically from 25% to 100% [46]. Still, the markers described here were able to predict about 80% of those individuals with CDR 0.5, with any stage AD, but also for those with CDRs 0.5 and greater.

In summary, this serum proteomic approach appeared capable of finding novel candidate biomarkers for AD. Some of these markers individually appeared to be useful diagnostically, but multi-marker models appeared to diagnose a larger portion of AD patients, including very early AD subjects. The markers characterized and identified provide some potential biological insights into early events in AD. While the discovery and validation results are promising, additional studies representing other centers with a broader range of patients would be required to confirm the clinical utility of these biomarkers.

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SUPPLEMENTARY MATERIAL

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REFERENCES

[1] Alzheimer's Association (2015) Alzheimer's disease facts and figures. *Alzheimers Dement* **9**, 208.

[2] Williamson J, Goldman J, Marder KS (2009) Genetic aspects of Alzheimer disease. *Neurologist* **15**, 80.

[3] Iqbal K, Grundke-Iqbal I (2008) Alzheimer neurofibrillary degeneration: Significance, etiopathogenesis, therapeutics and prevention. *J Cell Mol Med* **12**, 38-55.

[4] Goldman JS, Hahn SE, Catania JW, Larusse-Eckert S, Butson MB, Rumbaugh M, Strecker MN, Roberts JS, Burke W, Mayeux R (2011) Genetic counseling and testing for Alzheimer disease: Joint practice guidelines of the american college of medical genetics and the national society of genetic counselors. *Genetics Med* **13**, 597-605.

[5] Sunderland T, Linker G, Mirza N, Putnam KT, Friedman DL, Kimmel LH, Bergeson J, Manetti GJ, Zimmermann M, Tang B (2003) Decreased β-amyloid1-42 and increased tau levels in

cerebrospinal fluid of patients with Alzheimer disease. *JAMA* **289**, 2094-2103.

[6] Hu Y, Hosseini A, Kauwe JS, Gross J, Cairns NJ, Goate AM, Fagan AM, Townsend RR, Holtzman DM (2007) Identification and validation of novel CSF biomarkers for early stages of Alzheimer's disease. *Proteomics Clin Appl* **1**, 1373-1384.

[7] Blennow K (2004) Cerebrospinal fluid protein biomarkers for Alzheimer's disease. *Neuro Rx* **1**, 213-225.

[8] Hansson O, Zetterberg H, Buchhave P, Londos E, Blennow K, Minthon L (2006) Association between CSF biomarkers and incipient Alzheimer's disease in patients with mild cognitive impairment: A follow-up study. *Lancet Neurol* **5**, 228-234.

[9] Ringman JM, Coppola G, Elashoff D, Rodriguez-Agudelo Y, Medina LD, Gylys K, Cummings JL, Cole GM (2012) Cerebrospinal Fluid Biomarkers and Proximity to Diagnosis in Preclinical Familial Alzheimer's Disease. *Dement Geriatr Cogn Dis* **33**, 1-5.

[10] Weiner MW, Veitch DP (2015) Introduction to special issue: Overview of Alzheimer's disease neuroimaging initiative. *Alzheimers Dement* **11**, 730-733.

[11] Selkoe DJ (2000) Toward a comprehensive theory for Alzheimer's Disease. Hypothesis: Alzheimer's disease is caused by the cerebral accumulation and cytotoxicity of amyloid β-protein. *Ann NY Acad Sci* **924**, 17-25.

[12] Sano M, Bell K, Galasko D, Galvin J, Thomas R, van Dyck C, Aisen P (2011) A randomized, double-blind, placebo-controlled trial of simvastatin to treat Alzheimer disease. *Neurologist* **77**, 556-563.

[13] Deane R, Zlokovic BV (2007) Role of the blood-brain barrier in the pathogenesis of Alzheimer's disease. *Curr Alzheimer Res* **4**, 191-197.

[14] Zipser B, Johanson C, Gonzalez L, Berzin T, Tavares R, Hulette C, Vitek M, Hovanesian V, Stopa E (2007) Microvascular injury and blood–brain barrier leakage in Alzheimer's disease. *Neurobiol Aging* **28**, 977-986.

[15] Lue L-F, Walker DG, Jacobson S, Sabbagh M (2009) Receptor for advanced glycation end products: Its role in Alzheimer's disease and other neurological diseases. *Future Neurol* **4**, 167-177.

[16] Watt AD, Perez KA, Faux NG, Pike KE, Rowe CC, Bourgeat P, Salvado O, Masters CL, Villemagne VL, Barnham KJ (2011) Increasing the predictive accuracy of amyloid-β blood-borne biomarkers in Alzheimer's disease. *J Alzheimers Dis* **24**, 47-59.

[17] Zürbig P, Jahn H (2012) Use of proteomic methods in the analysis of human body fluids in Alzheimer research. *Electrophoresis* **33**, 3617-3630.

[18] Ghidoni R, Paterlini A, Benussi L (2013) Translational proteomics in Alzheimer's disease and related disorders. *Clin Biochem* **46**, 480-486.

[19] Lista S, Faltraco F, Prvulovic D, Hampel H (2013) Blood and plasma-based proteomic biomarker research in Alzheimer's disease. *Prog Neurobiol* **101**, 1-17.

[20] Zhang R, Barker L, Pinchev D, Marshall J, Rasamoelisolo M, Smith C, Kupchak P, Kireeva I, Ingratta L, Jackowski G (2004) Mining biomarkers in human sera using proteomic tools. *Proteomics* **4**, 244-256.

[21] Lopez MF, Mikulskis A, Kuzdzal S, Bennett DA, Kelly J, Golenko E, DiCesare J, Denoyer E, Patton WF, Ediger R (2005) High-resolution serum proteomic profiling of Alzheimer disease samples reveals disease-specific, carrier-protein–bound mass signatures. *Clin Chem* **51**, 1946-1954.

[22] Liu H-C, Hu C-J, Chang J-G, Sung S-M, Lee L-S, Yuan R-Y, Leu S-J (2006) Proteomic identification of lower apolipoprotein AI in Alzheimer's disease. *Dement Geriatr Cogn Dis* **21**, 155-161.

[23] Hye A, Lynham S, Thambisetty M, Causevic M, Campbell J, Byers H, Hooper C, Rijsdijk F, Tabrizi S, Banner S (2006) Proteome-based plasma biomarkers for Alzheimer's disease. *Brain* **129**, 3042-3050.

[24] German DC, Gurnani P, Nandi A, Garner HR, Fisher W, Diaz-Arrastia R, O'Suilleabhain P, Rosenblatt KP (2007) Serum biomarkers for Alzheimer's disease: Proteomic discovery. *Biomed Pharmacother* **61**, 383-389.

[25] Liao PC, Yu L, Kuo CC, Lin C, Kuo YM (2007) Proteomics analysis of plasma for potential biomarkers in the diagnosis of Alzheimer's disease. *Proteomics Clin Appl* **1**, 506-512.

[26] Akuffo EL, Davis JB, Fox SM, Gloger IS, Hosford D, Kinsey EE, Jones NA, Nock CM, Roses AD, Saunders AM (2008) The discovery and early validation of novel plasma biomarkers in mild-to-moderate Alzheimer's disease patients responding to treatment with rosiglitazone. *Biomarkers* **13**, 618-636.

[27] Cutler P, Akuffo EL, Bodnar WM, Briggs DM, Davis JB, Debouck CM, Fox SM, Gibson RA, Gormley DA, Holbrook JD (2008) Proteomic identification and early validation of complement 1 inhibitor and pigment epithelium-derived factor: Two novel biomarkers of Alzheimer's disease in human plasma. *Proteomics Clin Appl* **2**, 467-477.

[28] Thambisetty M, Simmons A, Velayudhan L, Hye A, Campbell J, Zhang Y, Wahlund L-O, Westman E, Kinsey A, Güntert A (2010) Association of plasma clusterin concentration with severity, pathology, and progression in Alzheimer disease. *Arch Gen Psych* **67**, 739-748.

[29] Henkel AW, Müller K, Lewczuk P, Müller T, Marcus K, Kornhuber J, Wiltfang J (2012) Multidimensional plasma protein separation technique for identification of potential Alzheimer's disease plasma biomarkers: A pilot study. *J Neural Transmission* **119**, 779-788.

[30] Gygi SP, Corthals GL, Zhang Y, Rochon Y, Aebersold R (2000) Evaluation of two-dimensional gel electrophoresis-based proteome analysis technology. *Proc Natl Acad Sci U S A* **97**, 9390-9395.

[31] Zhang G, Annan RS, Carr SA, Neubert TA (2010) Overview of peptide and protein analysis by mass spectrometry. *Curr Protocols Protein Sci* 16.11. 11-16.11. 30.

[32] Vaudel M, Sickmann A, Martens L (2012) Current methods for global proteome identification. *Expert Rev Proteomics* **9**, 519-532.

[33] Mapstone M, Cheema AK, Fiandaca MS, Zhong X, Mhyre TR, MacArthur LH, Hall WJ, Fisher SG, Peterson DR, Haley JM (2014) Plasma phospholipids identify antecedent memory impairment in older adults. *Nature Med* **20**, 415-418.

[34] Merrell K, Southwick K, Graves SW, Esplin MS, Lewis NE, Thulin CD (2004) Analysis of low-abundance, low-molecular-weight serum proteins using mass spectrometry. *J Biomol Tech* **15**, 238-248.

[35] Merrell K (2009) *Mining the Serum Proteome for Novel Biomarkers of Pregnancy Related Disease*. Dissertation, Brigham Young University, pp, 141.

[36] Esplin MS, Merrell K, Goldenberg R, Lai Y, Iams JD, Mercer B, Spong CY, Miodovnik M, Simhan HN, van Dorsten P (2011) Proteomic identification of serum peptides predicting subsequent spontaneous preterm birth. *Am J Obstet Gynecol* **204**, 391. e391-e398.

[37] Alvarez MTB, Shah DJ, Thulin CD, Graves SW (2013) Tissue proteomics of the low-molecular weight proteome using an integrated cLC-ESI-QTOFMS approach. *Proteomics* **13**, 1400-1411.

[38] McKhann G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM (1984) Clinical diagnosis of Alzheimer's disease Report of the NINCDS-ADRDA Work Group* under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease. *Neurology* **34**, 939-939.

[39] Merrell K, Thulin CD, Esplin MS, Graves SW (2009) An integrated serum proteomic approach capable of monitoring the low molecular weight proteome with sequencing of intermediate to large peptides. *Rapid Comm Mass Spectrom* **23**, 2685-2696.

[40] Merrell K, Thulin CD, Esplin MS, Graves SW (2008) Systematic internal standard selection for capillary liquid chromatography–mass spectrometry time normalization to facilitate serum proteomics. *J Biomol Tech* **19**, 320-327.

[41] Alvarez MTB (2013) *Exploration of the Low-Molecular Weight Proteome of Tissue and Serum, With Applications on Disease Biomarker Discovery*. Dissertation, Brigham Young University, Provo, UT, pp 182.

[42] Agresti A (2002) *Categorical Data Analysis*, John Wiley & Sons, Inc., Hoboken, NJ, USA.

[43] Sing T, Sander O, Beerenwinkel N, Lengauer T (2005) ROCR: Visualizing classifier performance in R. *Bioinformatics* **21**, 3940-3941.

[44] Ingram PR, Homer NZ, Smith RA, Pitt AR, Wilson CG, Olejnik O, Spickett CM (2003) The interaction of sodium chlorite with phospholipids and glutathione: A comparison of effects *in vitro*, in mammalian and in microbial cells. *Arch Biochem Biophys* **410**, 121-133.

[45] Pereira ALdRP (2006) *Identificação de radicais livres e produtos de oxidação de biomoléculas por MS*. Dissertation, Universidade de Aveiro, Aveiro, Portugal.

[46] Smailagic N, Vacante M, Hyde C, Martin S, Ukoumunne O, Sachpekidis C (2015) 18F-FDG PET for the early diagnosis of Alzheimer's disease dementia and other dementias in people with mild cognitive impairment (MCI). *Cochrane Database Systematic Rev* 1, Art. No.: CD010632 1-106.