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Blood-Based Biomarkers

Blood protein predictors of brain amyloid for enrichment
in clinical trials?

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Abstract

Background: Measures of neocortical amyloid burden (NAB) identify individuals who are at substantially greater risk of developing Alzheimer's disease (AD). Blood-based biomarkers predicting NAB would have great utility for the enrichment of AD clinical trials, including large-scale prevention trials.

Methods: Nontargeted proteomic discovery was applied to 78 subjects from the Australian Imaging, Biomarkers and Lifestyle Flagship Study of Ageing with a range of NAB values. Technical and independent replications were performed by immunoassay.

Results: Seventeen discovery candidates were selected for technical replication. α 2-Macroglobulin, fibrinogen γ -chain (FGG), and complement factor H-related protein 1 were confirmed to be associated with NAB. In an independent cohort, FGG plasma levels combined with age predicted NAB had a sensitivity of 59% and specificity of 78%.

Conclusion: A single blood protein, FGG, combined with age, was shown to relate to NAB and therefore could have potential for enrichment of clinical trial populations.

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Keywords:

Plasma; β amyloid; Proteomics; Alzheimer's disease; Biomarker; Fibrinogen γ -chain; Clinical trials

1. Background

The diagnosis of Alzheimer's disease (AD) can only be confirmed, with certainty, by histologic examination of the brain tissue at autopsy. This inspection should demonstrate considerable evidence of the classic pathologic hallmarks of AD: extracellular amyloid β ($A\beta$) plaques and intracellular neurofibrillary tangles predominantly composed of

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hyperphosphorylated tau [1]. Although an age-related disease usually affecting people older than age 65, it is believed the accumulation of A β plaques begins 15 to 20 years before clinical presentation [2] and reaches a plateau when cognitive, functional, and behavioral decline occurs [3]. The existing treatments of AD are only capable of temporary symptomatic relief in a subset of patients [4]. Because elevated brain A β is an important risk factor for eventual AD, it has become critical to identify individuals at the early stages of A β deposition to recruit into clinical trials of potentially disease-modifying therapeutic agents. Three prevention trials of asymptomatic individuals at the early stages of A β deposition have recently begun [5].

At present, neuroimaging and cerebrospinal fluid biomarkers are the accepted standards used to provide evidence of ongoing AD pathophysiology related to A β plaques [6]. ¹¹C-Pittsburgh compound B (PiB), coupled with positron emission tomography (PET), is widely used in research in measuring in vivo A β deposition, because its uptake in AD correlates with A β plaques measured neuropathologically in the same brains [7]. The availability of longer lived [18] F- labeled A β PET tracers, such as flutemetamol [8] and florbetapir [9] could foster wider usage in the clinic [10]. Early “proof of concept” PiB-PET studies demonstrated an increase of A β deposition in most individuals clinically diagnosed with AD, as judged by visual assessment [11] or quantification of tracer uptake [6,12]. Two large studies, from Victoria (Australia) and the University of California, San Francisco, Memory and Aging Center (UCSF), have shown that PiB-PET could discriminate between AD and non-A β dementia [6,13]. Some, but not all [14,15], studies have also shown that amyloid deposition as measured using PiB-PET either predicts a decline in cognitive measures or tracks with such declines [2,16].

Many disease-modifying therapeutic agents being developed target amyloid generation, deposition, or clearance [17]. Recent phase III trials targeting amyloid have reported that approximately 20% of trial participants actually had little or no A β when studied later using such PET imaging (suspected nonamyloid pathologic findings) [18]. This is a very serious problem for such trials—success is hard to find in the field of neurodegeneration but likely to be significantly more difficult when a large minority of trial subjects fail to have the primary target pathologic entity.

A solution is to use amyloid-PET scans (~\$3000 per scan) to ensure the presence of the primary target pathologic entity. The first study to use this will be the Anti Amyloid in Asymptomatic AD (A4; n = 1000) prevention trial. In A4, the screen failure rate is anticipated to be even greater (~66%) owing to the use of asymptomatic subjects. The great expense of the anticipated ~20% and ~66% amyloid-PET screen failure rates for clinical and prevention anti-amyloid trials means that a blood test with even relatively low predictive accuracy for neocortical amyloid burden (NAB) has the potential to greatly reduce costs. This would work by applying the blood tests to large

numbers of potentially eligible subjects and only performing PET scans on those whose blood test results are positive. This would reduce the screen failure rates and save money if the blood test was comparatively inexpensive. Therefore, a blood-based measure that correlates with the NAB would be of considerable value as an enrichment filter for clinical trials.

The obvious blood candidate biomarker of brain A β pathology would be A β itself. A systematic review of the published data and meta-analysis by Koyama et al. [19] of 10,303 subjects found that lower plasma A β 42/A β 40 ratios were significantly associated with the development of AD. However, the estimates had wide confidence intervals because of the high interstudy differences. As such, plasma A β 42/A β 40 ratios are unlikely to be useful by themselves for the prediction of NAB. The same study found that the individual A β 42 and A β 40 levels in blood were not significantly associated with AD. Clearly, novel biomarkers are needed that reflect the brain amyloid pathologic features in the blood.

There has been considerable effort in the search for AD blood-based biomarkers. Most studies have used a case-control design, with a clinical diagnosis of AD, determined by the medical history, cognitive assessment findings, and clinical examination results. This classic, case versus age-matched controls approach has identified a large number of putative plasma biomarkers [20–22]. However, such approaches are intrinsically flawed in the context of AD, because a considerable proportion of cognitively unimpaired controls will be in the prodromal phase of AD (e.g., asymptomatic but with elevated NAB).

An approach to overcome this is to use a nonapparent measure of disease activity (endophenotype paradigm). The endophenotype approach is increasingly being adopted, for example, to study blood-based biomarkers of cognitive decline [23,24], apolipoprotein E ϵ 4 (*APOE* ϵ 4) carrier status [25], brain atrophy [26,27], and hippocampal metabolism [28]. More recently, blood-based biomarkers of NAB, as measured by PiB-PET, have been reported [29–31]. Both Kiddle et al. [30] and Burnham et al. [31] used the Rules Based Medicine panel of 190 analytes to discover plasma proteins related to NAB and proposed a 13- and 5-analyte model, respectively. These models both contained the protein pancreatic polypeptide.

In a different approach, Thambisetty et al. [29] used two-dimensional gel electrophoresis (2D-GE), coupled with mass spectrometry (MS), to identify protein spots associating with NAB in an unbiased fashion. That study identified 6 proteins for spots associated with NAB, including *APOE* and complement C3, which were independently replicated in the study by Kiddle et al. [30]. 2D-GE is a well-established technique for blood biomarker research and offers many advantages. However, it is restricted by the lengthy procedure with poor reproducibility that can only identify a small number of “candidate spots” in limited sample sets.

In the present study, we used a method that combines the unbiased approach of gel-based proteomics with

high-throughput multiplex technology and the latest in MS instrumentation. This has enabled the identification and quantification of several hundred proteins, comparable to some panel-based arrays, without losing the key advantages of unbiased gel-based discovery. This is the first application of this approach to identify blood-based biomarkers of NAB and was applied to a subset of patients from the Australian Imaging, Biomarkers and Lifestyle Flagship Study of Ageing (AIBL) cohort with either high or low NAB. Promising markers were then replicated using immunoassays, first in the same cohort and then in an independent cohort [13].

2. Material and methods

2.1. Australian Imaging, Biomarkers and Lifestyle Flagship Study of Ageing

The AIBL study is a longitudinal study of ageing, neuroimaging, biomarkers, lifestyle, and clinical and neuropsychological analysis, with a focus on early detection and lifestyle intervention (available at: <http://www.aibl.csiro.au/>). Additional specifics regarding subject recruitment, diagnosis, and study design have been previously described [32].

2.2. Discovery cohort: assessments, blood collection, and processing

We examined the plasma samples from a subset of 78 subjects from the AIBL study, who had undergone PiB-PET scans. A standardized uptake value ratio (SUVR) cutoff of 1.3 was used to classify subjects as belonging to the PiB+ and PiB- groups. To increase the statistical power, the subjects were selected to be enriched for clear cases of PiB negativity and positivity. The standardized clinical assessments included the Mini-Mental State Examination, and *APOE* genotypes were available.

The details of blood collection and sample processing have been previously reported [31]. Plasma proteomic analysis and immunoassay measures were undertaken at King's College London.

2.3. AIBL PiB-PET methods

The PiB imaging method of the AIBL study has been previously reported [33]. SUVRs were generated using the cerebellar gray matter as the reference region, as described in the study by Burnham et al. [31]. NAB was expressed as the average SUVR of the mean of the frontal, superior parietal, lateral temporal, lateral occipital, and anterior and posterior cingulate regions.

2.4. Tandem mass tag protein labeling, enzymatic digestion, and peptide extraction

Each sample was randomly assigned and labeled with an amine-reactive tandem mass tag (TMT) reagent (TMT127-

TMT131; catalog no. 90064, Thermo Scientific), with TMT126 used to label the study reference, an equal pool of the plasma obtained from all 78 subjects. A complete TMT6Plex combined five labeled plasma samples with a labeled study reference. In general, sample preparation and TMT labeling was performed as previously described [23,34], with some minor modifications (see [Supplemental Methods 1](#), available online). Each TMT6Plex underwent one-dimensional gel electrophoresis and excised into 10 fractions (see [Supplemental Methods 2](#), available online). The gel pieces were then destained and digested and the peptides extracted and lyophilized to completion before MS analysis ([Supplemental Methods 3](#), available online). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) data separated with one-dimensional GE can show a single protein in multiple fractions. Therefore, identical protein identifications observed in different fractions were considered as separate entities defined as protein molecular weight (MW) isoforms (see [Supplemental Methods 5b](#), available online).

2.5. LC-MS/MS data acquisition

The samples were analyzed using an LTQ Orbitrap Velos instrument (Thermo Scientific) coupled to a Proxeon EASY-nLC II system (Thermo Scientific). Additional details on chromatographic separation and MS data acquisition are outlined in [Supplemental Methods 4](#) (available online).

2.6. Preprocessing of LC-MS/MS data

Raw data files produced in Excalibur software (Thermo Scientific) were processed using Proteome Discoverer, version 1.3 (Thermo Scientific) to determine peptide identification; the subsequent Mascot (version 2.3; available at: <http://www.matrixscience.com>) output file was used for additional preprocessing and analysis (see [Supplemental Methods 5a](#), available online). A script was written in R to complete the preprocessing, taking into account the experimental setup described (available at: <http://github.com/KHP-Informatics/PRQ>). We named the script Preprocessing for Relative Quantification of LC-MS/MS data (PRQ; see [Supplemental Methods-PRQ](#), available online). PRQ (1) performs median ratio normalization [35], (2) calculates ratios for each peptide, (3) derives protein level data from peptide scores, and (4) collects the protein scores across all TMT6Plexs.

2.7. UCSF Memory and Aging Center cohort

2.7.1. Replication cohort: assessments, blood collection, and processing

The replication cohort consisted of samples from 79 participants enrolled in the UCSF Alzheimer's Disease Research Center ([Table 1](#)). All subjects underwent *APOE*

Table 1
Demographics of selected subjects from AIBL and UCSF cohorts

Variable	AIBL discovery cohort			UCSF replication cohort		
	Low neocortical SUVR (PiB–)	High neocortical SUVR (PiB+)	<i>P</i> value	Low neocortical visual PiB read (PiB–)	High neocortical visual PiB read (PiB+)	<i>P</i> value
Subjects (n)	38	40		47	32	
SUVR (missing)	1.11 ± 0.06	2.34 ± 0.33	2.4 × 10 ^{−25}	1.2 ± 0.12 (1)	2.2 ± 0.35 (2)	4.2 × 10 ^{−16}
Female gender	18 (47)	20 (50)	.83	18 (38)	14 (44)	.65
Age (y)	75.8 ± 6.53	80.9 ± 8.22	.0035	65 ± 8.8	64 ± 8.4	.61
Clinical diagnosis			.0037			1.9 × 10 ^{−10}
HC	13 (34)	6 (15)		2 (4.3)	1 (3.1)	
SMC	18 (47)	13 (40)		1 (2.1)	1 (3.1)	
MCI	7 (19)	16 (30)		0 (0)	0 (0)	
AD	0 (0)	6 (15)		2 (4.3)	23 (72)	
FTD	0 (0)	0 (0)		42 (89.3)	7 (21.8)	
<i>APOE</i> ε4 carrier	14 (37)	25 (63)	.36	8 (17)	13 (41)	.036
MMSE	28.3 ± 1.8	26.8 ± 4.1	.038	26 ± 4.3	21 ± 6.9	.0011

Abbreviations: AD, Alzheimer's disease; AIBL, Australian Imaging, Biomarkers and Lifestyle Flagship Study of Ageing; *APOE*, apolipoprotein E; FTD, frontotemporal dementia; HC, healthy control; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; PiB, Pittsburgh compound B; SUVR, standardized uptake value ratio; UCSF, University of California San Francisco.

NOTE. Data presented as mean ± standard deviation or n (%).

genotyping, neurologic and cognitive assessments [13], and plasma collection and storage [36], as previously described. AD, frontotemporal dementia, and mild cognitive impairment were diagnosed clinically by consensus applying standard research criteria [37–39]. All subjects underwent PiB-PET at Lawrence Berkeley National Laboratory on a Siemens ECAT EXACT HR PET (n = 69) or Biograph Truepoint 6 PET/computed tomography (n = 10) [13]. The scans were visually rated as PiB+ or PiB– by an experienced single rater, who was unaware of the clinical and plasma data [13]. The mean 50- to 70-minute SUVR values were extracted from the frontal, parietal, cingulate, and lateral temporal cortex, using the mean activity in the cerebellar gray matter as the reference tissue (for details of image processing, see Lehmann et al. [40]).

2.7.2. Immunoassay–enzyme-linked immunosorbent assay

Single analyte sandwich enzyme-linked immunosorbent assay (ELISA) was used to quantify the candidate proteins and were performed as per the manufacturer's instructions (see Supplemental Methods 6, available online).

2.8. Statistical analysis

All statistical analyses were performed in R (see Supplemental Methods 7, available online). For logistic and linear regression analysis, age, gender, and the presence of an *APOE* ε4 allele were used as covariates. For the ELISA analysis, data outliers were excluded (±3 standard deviations) and a fourth covariate, batch, was added. The PET scanner type was added as a covariate for the UCSF data. Benjamini-Hochberg *q* values were calculated as a multiple testing correction. Details of the pathway, regression, and classification analyses are given in Supplemental Methods 7 (available online).

3. Results

3.1. LC-MS/MS performed on AIBL subjects

LC-MS/MS was performed on plasma samples from 78 AIBL subjects, whose demographic data are listed in Table 1. Combining the data from all MS/MS runs, we identified 4518 unique peptide sequences that corresponded to 789 unique protein groups. PRQ was able to extract 2319 unique TMT peptides, 1139 MW isoforms, and 379 unique protein groups (see Supplemental Results 1a, available online), which was reduced to 116 confidently annotated unique protein groups after post-PRQ data clean up. This consisted of 381 protein MW isoforms (see Supplemental Results 1b, available online).

3.2. Plasma protein markers of global PiB-PET

Each protein MW isoform underwent Mann-Whitney *U* test and logistic regression analysis to compare PiB+ and PiB– groups and the Spearman rank correlation and linear regression to associate the protein MW isoform levels against PiB retention as a continuous measure. This was completed for the mean and median protein roll-up methods separately, giving a total of eight statistical tests per protein. One protein MW isoform, complement C4a, passed all eight statistical tests. A total of 69 protein MW isoforms passed at least one statistical test (uncorrected *P* < .05; see Supplemental Results 1c, available online). Pathway analysis (see Supplemental Results 2, available online) revealed that these protein groups were overrepresented for involvement in the complement and coagulation cascades (*P* = 3.7 × 10^{−22}, *q* = 3.3 × 10^{−21}), systemic lupus erythematosus (*P* = 2.65 × 10^{−4}, *q* = 0.15), and prion diseases (*P* = 5.9 × 10^{−3}, *q* = 0.051). Three albumin and 15 immunoglobulin MW isoforms were removed, leaving 51

Table 2

LC-MS/MS data: Protein MW isoforms significantly associated with NAB

		Protein level data (roll up method indicated; i.e., mean or median)																	
		Mean for all peptides mapping to protein						Median over all peptides mapping to protein											
		Logistic regression	Mann-Whitney U test	Linear regression	Spearman's rank correlation	Logistic regression	Mann-Whitney U test	Linear regression	Spearman's rank correlation	Logistic regression	Mann-Whitney U test	Linear regression	Spearman's rank correlation	Tests with P < .05 (n)					
UniProt ID	Protein name	ID-GE fraction	β	P value	Median difference	β	P value	ρ	P value	β	P value	Median difference	β	P value	ρ	P value			
P0C0L4	Complement C4a	7	-0.656	.038*	-0.235	.027*	-0.162	.019*	-0.302	.007*	-0.592	.049*	-0.224	.037*	-0.143	.042*	-0.229	.044*	8
P00738	Haptoglobin	8	-0.932	.015*	-0.331	.011*	-0.170	.026*	-0.248	.047*	-1.048	.018*	-0.190	.030*	-0.157	.043*	-0.226	.070	7
P02647	Apolipoprotein A-I	10	-0.877	.014*	-0.173	.070	-0.211	.013	-0.204	.136	-0.872	.025*	-0.087	.087	-0.183	.035*	-0.266	.049*	5
O14791	Apolipoprotein L-1	5	0.829	.048*	0.731	.004*	0.165	.035*	0.308	.014*	0.578	.091	0.795	.007*	0.120	.145	0.238	.061	5
P00738	Haptoglobin	5	-0.728	.023*	-0.521	.017*	-0.198	.005*	-0.280	.013*	-0.446	.109	-0.367	.134	-0.149	.041*	-0.181	.112	5
Q03591	FHR-1	8	1.667	.120	0.313	.008*	0.155	.178	0.483	.007*	1.414	.187	0.186	.019*	0.144	.217	0.444	.014*	4
P25311	Zinc- α 2-glycoprotein	7	0.140	.681	0.327	.043*	0.076	.461	0.327	.021*	0.140	.681	0.327	.043*	0.076	.461	0.327	.021*	4
O43866	CD5 molecule like	5	-0.929	.110	-0.398	.013*	-0.140	.088	-0.288	.028*	-0.596	.080	-0.307	.015*	-0.165	.042*	-0.235	.076	4
P04196	Histidine rich glycoprotein	5	-0.996	.031*	-0.093	.150	-0.228	.011*	-0.238	.096	-0.996	.031*	-0.093	.150	-0.228	.011*	-0.238	.096	4
P02747	C1q subcomponent subunit	2	-0.746	.043*	-0.460	.095	-0.201	.024*	-0.210	.165	-0.746	.043*	-0.460	.095	-0.201	.024*	-0.210	.165	4
P00450	Ceruloplasmin	6	-2.088	.032*	-0.222	.019*	-0.105	.169	-0.154	.247	-2.088	.032*	-0.222	.019*	-0.105	.169	-0.154	.247	4
P04196	Histidine rich glycoprotein	3	1.205	.039*	0.334	.034*	0.120	.169	0.106	.449	1.149	.024*	0.611	.030*	0.114	.199	0.084	.548	4
P00751	Complement factor B	1	0.341	.419	0.065	.657	0.164	.081	0.310	.038*	1.239	.371	0.058	.188	0.189	.042*	0.363	.014*	3
P04003	C4b-binding protein α chain	4	-0.405	.189	-0.108	.174	-0.127	.076	-0.248	.035	-0.466	.106	-0.473	.038*	-0.105	.154	-0.275	.018*	3
P02647	Apolipoprotein A-I	6	-0.487	.080	-0.074	.310	-0.157	.023*	-0.189	.098	-0.403	.137	-0.156	.109	-0.143	.038*	-0.236	.038*	3
P02679	FGG	6	0.108	.789	-0.038	.081	0.044	.613	-0.331	.021*	-0.671	.085	-0.206	.164	-0.191	.021*	-0.298	.039*	3
Q92620	DEAH box protein 38	2	-0.383	.351	0.046	.044*	-0.095	.207	0.223	.067	-0.380	.351	0.046	.031*	-0.095	.208	0.243	.046*	3
Q06033	ITI heavy chain H3	2	1.525	.027*	0.297	.099	0.148	.144	0.170	.265	1.267	.019*	0.422	.013*	0.187	.061	0.225	.137	3
P06727	Apolipoprotein A-IV	6	-1.837	.206	-0.100	.087	-0.144	.115	-0.389	.010*	-1.432	.271	-0.093	.107	-0.139	.128	-0.387	.010*	2
O43866	CD5 molecule like	6	0.556	.275	0.277	.147	0.077	.400	0.347	.022*	0.260	.484	0.623	.201	0.090	.313	0.369	.015*	2
P08603	Complement factor H	6	0.031	.917	0.147	.409	-0.001	.989	0.128	.299	0.190	.490	0.243	.041*	-0.001	.987	0.286	.018*	2
P01023	α 2m	3	0.046	.853	0.331	.130	0.048	.497	0.290	.010*	0.034	.893	0.351	.223	0.042	.559	0.254	.025*	2
P07357	Complement C8 α chain	5	-0.303	.346	-0.294	.111	-0.105	.229	-0.197	.137	-0.539	.104	-0.474	.032*	-0.153	.073	-0.261	.048*	2
P00739	Haptoglobin-related protein	5	0.960	.076	0.190	.072	0.209	.016*	0.232	.104	0.960	.076	0.190	.072	0.209	.016*	0.232	.104	2
P08519	Apolipoprotein(a)	1	0.929	.062	0.315	.030*	0.115	.212	0.237	.105	0.869	.064	0.222	.045*	0.111	.226	0.221	.131	2
P04003	C4b-binding protein α chain	7	-0.602	.064	-0.198	.238	-0.153	.028*	-0.156	.173	-0.648	.052	-0.199	.151	-0.158	.023*	-0.150	.189	2
P19823	ITI heavy chain H2	4	-0.762	.067	-0.294	.026*	-0.159	.061	-0.267	.039*	-0.510	.105	-0.412	.125	-0.134	.112	-0.158	.227	2
P01024	Complement C3	6	-0.374	.171	0.034	.783	-0.148	.035*	-0.107	.351	-0.445	.116	-0.115	.475	-0.156	.025*	-0.119	.300	2
Q92620	DEAH box protein 38	5	0.449	.199	0.123	.576	0.197	.038*	0.144	.318	0.449	.199	0.123	.576	0.197	.038*	0.144	.318	2
Q14624	ITI heavy chain H4	1	1.244	.041*	0.187	.118	0.251	.013*	0.222	.142	0.651	.220	0.127	.281	0.152	.139	0.136	.373	2
P00747	Plasminogen	1	0.901	.026*	0.610	.078	0.151	.112	0.154	.295	0.786	.049*	0.409	.102	0.128	.182	0.119	.420	2
P06396	Gelsolin	5	1.018	.251	0.174	.033*	0.095	.275	0.171	.198	1.149	.202	0.151	.042*	0.095	.275	0.088	.509	2
P01023	α 2m	9	-0.749	.035*	-0.246	.112	-0.196	.037*	-0.092	.482	-0.555	.078	-0.165	.230	-0.139	.113	-0.044	.737	2
O75636	Ficolin-3	6	-0.372	.249	0.043	.629	-0.152	.044*	0.034	.798	-0.372	.249	0.043	.629	-0.152	.044*	0.034	.798	2
P19827	ITI heavy chain H4	8	-0.396	.253	-0.252	.301	-0.155	.115	-0.265	.069	-0.337	.373	-0.196	.282	-0.164	.113	-0.313	.030*	1
P01023	α 2m	2	-0.044	.861	-0.190	.823	0.000	.996	0.013	.912	0.108	.659	0.259	.171	0.048	.495	0.237	.037	1

(Continued)

Table 2

LC-MS/MS data: Protein MW isoforms significantly associated with NAB (Continued)

		Protein level data (roll up method indicated; i.e., mean or median)																	
		Mean for all peptides mapping to protein								Median over all peptides mapping to protein									
UniProt ID	Protein name	1D-GE fraction	Logistic regression		Mann-Whitney U test		Linear regression		Spearman's rank correlation		Logistic regression		Mann-Whitney U test		Linear regression		Spearman's rank correlation		
			β	<i>P</i> value	Median difference	<i>P</i> value	β	<i>P</i> value	ρ	<i>P</i> value	β	<i>P</i> value	Median difference	<i>P</i> value	β	<i>P</i> value	ρ	<i>P</i> value	
P02790	Hemopexin	5	0.255	.341	0.018	.368	0.080	.252	0.152	.184	0.424	.158	0.164	.123	0.105	.134	0.228	.045*	1
P01023	α 2m	1	0.325	.196	0.198	.133	0.057	.420	0.118	.303	0.340	.181	0.469	.046*	0.063	.380	0.184	.107	1
P13671	Complement C6	2	-0.454	.132	-0.338	.064	-0.089	.288	-0.167	.190	-0.430	.145	-0.662	.048*	-0.085	.310	-0.203	.110	1
P02675	Fibrinogen β chain	6	-0.441	.241	0.042	.869	-0.113	.117	-0.082	.504	-0.539	.090	-0.213	.341	-0.172	.016*	-0.188	.125	1
P02790	Hemopexin	6	0.587	.049*	0.216	.053	0.090	.203	0.230	.059	0.429	.147	0.234	.082	0.041	.562	0.166	.176	1
P01024	Complement C3	8	-0.569	.058	-0.480	.030*	-0.129	.101	-0.166	.161	-0.422	.132	-0.401	.081	-0.091	.249	-0.135	.255	1
P02787	Serotransferrin	1	0.123	.658	0.205	.170	0.046	.544	0.093	.450	0.249	.372	0.352	.040*	0.068	.370	0.133	.281	1
P02647	Apolipoprotein A-I	5	-0.458	.221	-0.293	.126	-0.101	.229	-0.263	.046*	-0.237	.484	-0.099	.333	-0.026	.762	-0.129	.335	1
P0C0L4	Complement C4a	1	0.735	.117	0.058	.937	0.241	.009*	0.160	.293	0.156	.631	-0.004	.847	0.064	.513	0.124	.419	1
P02647	Apolipoprotein A-I	9	-0.599	.039*	-0.061	.255	-0.134	.060	-0.033	.780	-0.414	.134	0.036	.812	-0.090	.211	0.067	.566	1
P00450	Ceruloplasmin	2	0.543	.136	-0.097	.638	0.167	.029*	0.000	.998	0.177	.524	0.038	.946	0.082	.302	0.058	.639	1
P00734	Prothrombin	6	1.356	.402	0.004	.409	0.190	.027*	0.208	.135	-0.053	.879	-0.001	.908	-0.002	.983	0.056	.689	1
P10909	Clusterin	6	-0.480	.134	0.036	.422	-0.159	.037*	-0.102	.408	-0.434	.179	-0.150	.646	-0.140	.068	-0.033	.789	1
P02671	Fibrinogen α chain	4	-0.698	.122	-0.175	.107	-0.142	.052	-0.284	.012*	-0.054	.835	0.065	.700	-0.054	.472	-0.028	.809	1
P04196	Histidine rich glycoprotein	6	-0.459	.343	0.005	.447	-0.185	.049*	-0.194	.214	-0.315	.485	0.251	.895	-0.163	.083	-0.021	.895	1

Abbreviations: 1D-GE, one-dimensional gel electrophoresis; α 2m, α 2-macroglobulin; FGG, fibrinogen γ -chain; FHR-1, factor H-related protein 1; ID, identification; ITI, inter- α -trypsin inhibitor; LC-MS/MS, liquid chromatography tandem mass spectrometry; UniProt, Universal Protein Resource.

NOTE. All multiple testing corrected *q* values were >0.75 ; for regression analysis, age, gender, and presence of apolipoprotein E ϵ 4 were used as covariates.

*Statistically significant.

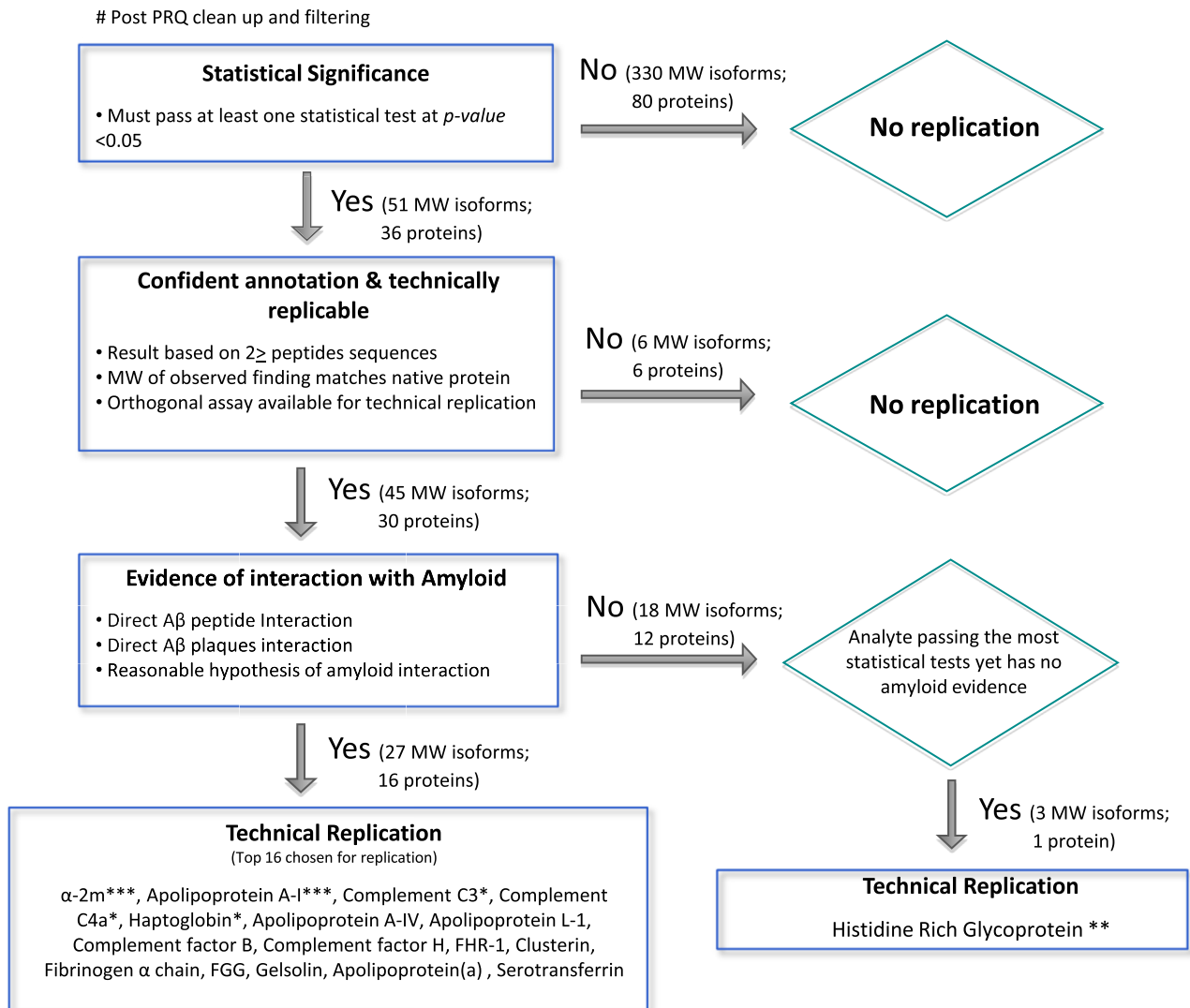


Fig. 1. Flow diagram to select liquid chromatography tandem mass spectrometry plasma neocortical amyloid burden (NAB) candidate markers for technical replication. *Two protein molecular weight (MW) isoforms associated with NAB; **three protein MW isoforms associated with NAB; ***four protein MW isoforms. Abbreviations: Aβ, amyloid β; α2m, α2-macroglycane; FGG, fibrinogen γ-chain; FHR-1, factor H-related protein 1.

protein MW isoforms associated with PiB-PET retention (Table 2).

Subsequently, 17 proteins were selected for technical replication (Fig. 1). In addition to the statistical evidence, we also considered the candidate's relationship with the amyloid and/or AD Genome-Wide Association Studies results (see Supplemental Results 3, available online). We also chose to replicate histidine-rich glycoprotein, the protein most associated with NAB, but that had no previous evidence for a relationship with Aβ.

3.3. Technical replication

We sought to translate our discovery findings to a simple-to-use commercially available ELISA format. The 17 protein candidates from MS were measured in plasma samples from the 78 AIBL subjects in the discovery cohort. Using

linear regression models (including age, gender, *APOE* ε4, and ELISA plate as covariates), we found that two proteins, α2-macroglobulin (α2m) ($q = 0.076$) and fibrinogen γ-chain (FGG) ($q = 0.076$), replicated our findings from the LC-MS/MS discovery study (Table 3). In the discovery study, factor H-related protein 1 (FHR-1) was increased in the PiB+ group. Although FHR-1 ($q = 0.076$) was associated with NAB at the 0.1 q value in the ELISA technical replication, an opposite trend was observed. Apolipoprotein A-IV, gelsolin, histidine-rich glycoprotein, haptoglobin, and apolipoprotein(a) all showed the same directional change as in the LC-MS/MS discovery.

3.4. Independent replication

To verify the results from the AIBL samples, we measured the levels of the three proteins significantly

Table 3
Technical replication of plasma protein candidates discovered by LC-MS/MS

UniProt ID	Protein name	Outliers excluded (n)	Logistic regression with SUVR >1.3			Linear regression with SUVR		
			β	P value	q value	β	P value	q value
P01023	α2m	10	1	8.9 × 10 ⁻³	0.076	0.2	7.9 × 10 ⁻³	0.068
Q03591	FHR-1	11	-1	4.6 × 10 ⁻³	0.076	-0.22	5.5 × 10 ⁻³	0.068
P02679	FGG	0	-0.7	.041	0.23	-0.2	.014	0.081
P08519	Apolipoprotein(a)	21	0.48	.13	0.34	0.18	.042	0.18
P06396	Gelsolin	2	-0.48	.11	0.34	-0.14	.068	0.19
P00738	Haptoglobin	2	-0.38	.18	0.39	-0.13	.089	0.19
P04196	Histidine rich glycoprotein	2	0.48	.14	0.34	0.14	.081	0.19
P06727	Apolipoprotein A-IV	2	-0.63	.083	0.34	-0.17	.067	0.19
P01024	Complement C3	0	-0.61	.25	0.47	-0.21	.13	0.25
POC0L4	Complement C4a	0	-0.55	.51	0.66	-0.27	.22	0.38
P10909	Clusterin	0	-0.27	.36	0.51	-0.091	.27	0.41
P02647	Apolipoprotein A-I	0	0.34	.29	0.47	0.088	.32	0.46
P02671	Fibrinogen α chain	6	-0.28	.3	0.47	-0.064	.39	0.52
P02787	Serotransferrin	1	-0.013	.96	0.96	-0.041	.6	0.73
O14791	Apolipoprotein L-1	0	-0.09	.74	0.89	-0.026	.73	0.77
P08603	Complement factor H	3	0.066	.8	0.89	0.027	.7	0.77
P00751	Complement factor B	2	0.053	.84	0.89	0.018	.81	0.81

Abbreviations: 1D-GE, one-dimensional gel electrophoresis; α2m, α2-macroglobulin; FGG, fibrinogen γ-chain; FHR-1, factor H-related protein 1; ID, identification; ITI, inter-α-trypsin inhibitor; LC-MS/MS, liquid chromatography tandem mass spectrometry; UniProt, Universal Protein Resource.

associated with NAB (α2m, FHR-1, and FGG) using samples from an independent cohort. These proteins were measured using ELISA in 79 samples from the UCSF cohort (Table 1). FGG was significantly associated with PiB positivity, as determined both by visual examination of PiB-PET scans ($q = 5.9 \times 10^{-3}$) and by applying a threshold of 1.3 to SUVRs ($q = 0.051$; Table 4). Despite not being significantly associated with NAB, α2m correlated with SUVR positivity in the same direction as in the discovery study.

3.5. Multivariate analysis

Subjects with any missing covariates or protein measurements were excluded from the multivariate analysis, leaving 58 subjects from AIBL (28 PiB-, 30 PiB+ using an SUVR >1.3) and 78 subjects from UCSF (46 PiB-, 32 PiB+ determined from visual inspection). Classification models were trained in the AIBL ELISA data to predict SUVR positivity (>1.3) and tested in the UCSF ELISA

data to predict PiB positivity, determined by visual inspection (more robust across multiple scanners). A “basic” model (age, gender, APOE ε4) was compared with a “basic + protein” model, which also used the plasma concentration of FGG, α2m, and FHR-1. Fig. 2A and 2B shows a receiver operating characteristic analysis, in which area under the curve (AUC) was shown to be greater for the “basic + protein” model than for the “basic” model in the test data sets. The highest test AUC was found using the random forest approach, in which the basic + protein model (AUC 0.70) outperformed the basic model (AUC = 0.46) in the test data set. The random forest basic + protein model gave a test set sensitivity of 50% and specificity of 85%. Additionally, a classification tree was fitted to the basic + protein model to provide a simpler alternative with clear thresholds. The resulting classification tree used just two variables (age/plasma FGG level; Fig. 2C) and achieved a comparable AUC to that of the random forest model (AUC 0.69, sensitivity 59%, specificity 78%). In the UCSF cohort, 23 of 25 AD subjects were PiB+, and two

Table 4
Independent replication of plasma protein candidates discovered by LC-MS/MS and technically replicated

UniProt ID	Protein name	Logistic regression to visual read			Logistic regression to SUVR >1.3			Linear regression to SUVR		
		β	P value	q value	β	P value	q value	β	P value	q value
P01023	α2m	-0.013	.96	0.96	0.27	.29	0.44	0.075	.22	0.33
P02679	FGG	-1.0	2.0 × 10 ⁻³	5.9 × 10 ⁻³	-0.74	.017	0.051	-0.21	4.1 × 10 ⁻⁴	1.2 × 10 ⁻³
Q03591	FHR-1	-0.066	.79	0.96	0.011	.97	0.97	1.5 × 10 ⁻³	.98	0.98

Abbreviations: α2m, α2-macroglobulin; FGG, fibrinogen γ-chain; FHR-1, factor H-related protein 1; ID, identification; LC-MS/MS, liquid chromatography-tandem mass spectrometry; SUVR, standardized uptake value ratio; UniProt, Universal Protein Resource.

NOTE. Only one outlier (>3 standard deviations from mean) was excluded, which was detected for FGG; for regression analysis, age, gender, presence of apolipoprotein E ε4, enzyme-linked immunosorbent assay plate, and scanner type were used as covariates.

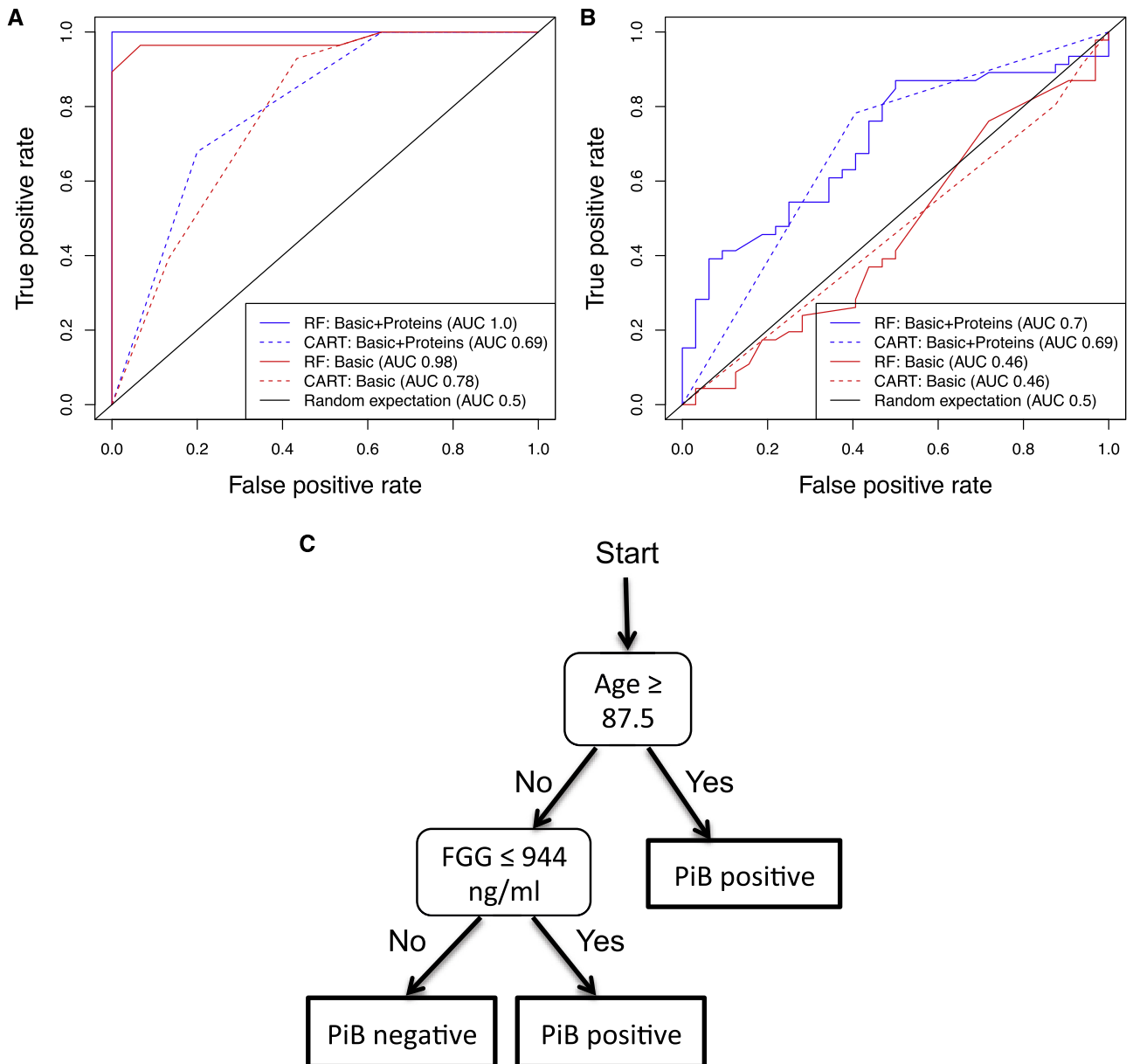


Fig. 2. Receiver operating characteristic (ROC) curves for the prediction of Pittsburgh compound B (PiB) positivity. A “basic” model (age, gender, *APOE* $\epsilon 4$ presence) was compared with a “basic + protein” model that also included the plasma levels of fibrinogen γ -chain (FGG), $\alpha 2$ -macrophage, and factor H-related protein 1. Random forest (RF) and classification and regression trees (CARTs) were used to fit models in classification and regression training using default parameters. The area under the curve (AUC) is given for each model. ROC curves are shown comparing the predictive accuracy of the models in (A) the training data set (Australian Imaging, Biomarkers and Lifestyle Flagship Study of Ageing [AIBL]) and (B) the test data set (University of California, San Francisco). Classification tree trained on AIBL enzyme-linked immunosorbent assay data to predict neocortical amyloid burden positivity and estimated cutoff (C).

PiB— subjects had plasma FGG levels greater than the threshold (see [Supplemental Results 4](#), available online).

4. Discussion

With the failure of serial amyloid-based therapeutic agents in clinical trials compromised by the inclusion of substantial numbers of participants without the target pathologic entity [18], and with the prospect of very large trials in pre-

symptomatic AD such as the A4 trial and others [5], the need for blood-based markers of NAB has never been greater. Blood-based biomarkers could be used to screen large numbers of potential participants, and only those predicted to have abnormally high NAB would be retested using cerebrospinal fluid assays or PET scans, reducing the screen failure rates. This could reduce recruitment time and costs and allow eligible subjects to be identified more readily (e.g., from biobanks with permission for recontact).

The present study has demonstrated that a simple blood test consisting of FGG plasma levels and patient age could have some potential for predicting NAB, achieving a test set sensitivity, specificity, and AUC of 59%, 78%, and 69% respectively, highlighting its potential use in stratifying patients for anti-amyloid trials. This independent replication was performed in a mixed dementia cohort (UCSF), suggesting that FGG and age could also have utility for distinguishing between amyloid and nonamyloid dementias. Additionally, because the classification model was trained in a subset of the AIBL cohort containing very few AD subjects, it is more likely that FGG will be able to predict PiB positivity in non-AD subjects. However, because the UCSF cohort contained only two cognitively normal individuals, additional work is needed to determine the sensitivity and specificity in those who are cognitively normal. These measures will determine the cost-saving potential of this blood test for prevention trials. Preliminary data generated from a cognitively normal cohort in our laboratory supports this (data not shown). Previously, Burnham et al. [31] reported a blood test that achieved 79% sensitivity and 76% specificity in an independent test set. Although our sensitivity was slightly lower, this was achieved by measuring a single plasma protein compared with the 6 plasma proteins used in the Burnham model.

Although the sensitivity and specificity of these markers for predicting NAB was not high enough to use clinically, they would be useful for enrichment of clinical trials if they performed at this level in relevant populations. The strongest case can be made for prevention trials in asymptomatic subjects because of the large expected screen failure rate (~66% or higher) when searching for individuals with elevated NAB. Because of the relatively high cost of amyloid-PET scans (~\$3000) versus blood protein ELISAs, even a blood test without clinical utility could theoretically save millions of dollars for studies the size of A4 ($n = 1000$).

The *APOE* $\epsilon 4$ allele is a substantial risk factor for AD [41] and amyloid [30,42]. Although we considered *APOE* $\epsilon 4$ during our analyses, we were not surprised to find that the *APOE* genotype markers did not improve our classification model, because the study was designed to be independent of this effect. However, in a general population sample, the *APOE* genotype is likely to contribute to the prediction of NAB.

It was interesting that FGG, and to a lesser extent complement C3 and fibrinogen α -chain, were associated with NAB in our study, which has been previously found [29,30]. However, in the study by Burnham et al. [31], total fibrinogen was not associated with NAB. In contrast, Kiddle et al. [30] showed that it was negatively associated with NAB. Furthermore, decreased levels of plasma FGG have been shown to be associated with a smaller whole brain volume in AD subjects [26] and measures of whole fibrinogen in plasma have shown an increase [43,44].

Discrepancies in these findings might have resulted from the platform used to measure total fibrinogen or might highlight the importance of studying specific fibrinogen chains.

FGG is normally rejected from the brain by the blood-brain barrier (BBB), yet has still been detected in mice and human brain tissue [45,46]. This could result from the reported dysfunction of the BBB in mice [47] and humans in AD [48]. However, the movement of fibrinogen across a defected BBB seems to be molecule-specific, because smaller molecules are not BBB permeable in AD [49]. Fibrinogen has been shown to accumulate over time as the AD pathology progresses [46] and codeposits with $A\beta$ in brain tissue [50]. Ahn et al. [51] demonstrated that fibrinogen binds to $A\beta$, which enhances aggregation and increases $A\beta$ fibrillization. It is possible that the decreased FGG levels associated with high NAB in our study resulted from movement of fibrinogen across a compromised BBB in the subjects with AD pathologic features.

After FGG, $\alpha 2m$ was the second most promising candidate, shown for the first time to associate with NAB. This is noteworthy, because $\alpha 2m$ has been found to be one of the most replicable markers of other AD-related phenotypes, including diagnosis, hippocampal metabolism, and response to treatment with divalproex sodium [20]. Future studies should aim to replicate all previously discovered markers of NAB and investigate which combination of analytes would achieve greater sensitivity and specificity.

To our knowledge, this is the first study to apply an unbiased and nontargeted quantitative LC-MS/MS discovery approach, combining LC-MS/MS with TMT labeling, for the investigation of plasma proteins related to NAB. Furthermore, this method will allow the unprecedented exploration of plasma peptide and modified proteins as markers of NAB. We have also described a novel and automated bioinformatic pipeline, PRQ, to accurately preprocess the TMT-MS data. PRQ not only conducts rigorous normalization of MS data [35] but also automates the calculation of peptide/protein ratios against the study reference.

Subsequently, technical replication was performed to reduce the number of false-positive results and to ensure translation of LC/MS-MS findings using a platform more applicable to the clinical setting. Using commercially available immunoassays, we confirmed that $\alpha 2m$, FGG, and FHR-1 significantly predicted NAB, with a 0.1 q -value significance level. All except FHR-1 displayed a similar direction of association between discovery and replication. Immunoassays cannot always distinguish between sequence variants, proteins modified with different post-translational modification, or different truncated forms of a same protein seen by LC/MS-MS. This could also explain the differences seen in the association trend between discovery and replication in some cases (e.g., FHR-1); therefore, these candidates should not necessarily be discounted. The discrepancies observed between the two platforms point to the need for

investigation of protein modifications as potential biomarkers in future studies.

The discrepancies between the findings from the AIBL and UCSF studies could have resulted from the low statistical power, differences in disease stage, or differences in pre-analytical factors. The major difference in the pre-analytical factors is the centrifugation step of plasma collection. AIBL used a two-step centrifugation (200g, remove supernatant, then 800g), and UCSF used a single centrifugation step (1300–1800g). This highlights the importance of standardization of blood collection and preparation for biomarker studies.

Although many have agreed that A β deposition is the earliest event in AD pathogenesis, one group has shown changes in episodic memory preceding the changes in A β levels [52]. If confirmed in other cohorts, it would be interesting to compare the ability of episodic memory and our blood test to predict NAB in asymptomatic individuals.

In conclusion, the present study reports a potential blood test, consisting of measuring FGG, which, along with age, showed some ability to predict NAB in an independent sample. To ensure the robustness and relevance of these findings, this test must be replicated in larger cohorts that are more representative of the relevant clinical trial populations. Our results provide additional evidence that differences in the plasma proteome in relation to AD and its pathology do exist. Therefore, such changes could be used to stratify patients for anti-amyloid treatment trials. This could lower barriers to the development of an effective treatment to combat the increasing concern of dementia.

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Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.dadm.2014.11.005>.

RESEARCH IN CONTEXT

1. Systematic review: We searched PubMed up to June 2014 using the keywords, Alzheimer's disease (AD), plasma biomarkers, pathology, fibrinogen gamma chain (FGG), beta amyloid.
2. Interpretation: Previous targeted studies have demonstrated that the combination of several blood proteins had the potential to predict the NAB. Our study is the first to apply an unbiased and nontargeted quantitative LC-MS/MS discovery approach, combined with isobaric labeling, to investigate the plasma proteins related to NAB. Our findings showed a prediction of NAB, with comparable accuracy to previous studies, which used a single plasma protein. This highlights the potential use of a simple blood-based screen to stratify patients for anti-amyloid trials.
3. Future directions: To ensure the robustness and reliability of these findings, this test should be replicated in larger cohorts more representative of relevant clinical trial populations.

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