DOI:
10.1016/j.dadm.2014.11.005

Document Version
Peer reviewed version

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

Nicholas J. Ashton [1, 2#], Steven J. Kiddle [1, 3#], John Graf [4], Malcolm Ward [5], Alison Baird [1, 6], Abdul Hye [1, 2], Sarah Westwood [1, 2], Karyuan Vivian Wong [1], Richard J. Dobson [1, 2], Gil D. Rabinovici [7], Bruce L. Miller [7], Howard J. Rosen [7], Andrew Torres [4], Zhanpan Zhang [4], Lennart Thurfjell [8], Antonia Covin [9], Cristina Tan Hehir [4], David Baker [9], Chantal Bazenet [1, 2] and Simon Lovestone [1, 6] and the AIBL Research Group [10].

[1]Institute of Psychiatry, King's College London, London, UK

[2] NIHR Biomedical Research Centre for Mental Health and Biomedical Research Unit for Dementia at South London and Maudsley NHS Foundation, London, UK

[3] MRC Social, Genetic and Developmental Psychiatry Centre, King's College London, London, UK

[4] GE Global Research, Niskayuna, NY

[5] Proteomics Facility, Institute of Psychiatry, Kings College London

[6] Department of Psychiatry, University of Oxford, Oxford, UK.

[7] Memory & Aging Center, Dept. of Neurology, University of California San Francisco[7] GE Healthcare Life Sciences, Uppsala, Sweden.

[8] GE Healthcare Life Sciences, Uppsala, Sweden.

[9] Janssen R&D, Neurosciences, Titusville, NJ

[10] www.AIBL.csiro.au.

# these authors contributed equally to this manuscript

Abstract

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

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

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

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

Keywords: Plasma, β amyloid, proteomics, Alzheimer's disease, biomarker, fibrinogen gamma chain and clinical trials.

Background

The diagnosis of AD can only be confirmed, with certainty, by histological examination of brain tissue at autopsy. This inspection should demonstrate considerable evidence of the classical pathological hallmarks of AD; extracellular amyloid beta (Aβ) plaques and intracellular neurofibrillary tangles predominantly comprised of hyperphosphorylated Tau [1]. Although an age-related disease usually affecting people over the age of 65 it is believed the accumulation of Aβ plaques begins 15-20 years prior to clinical presentation [2] and reaches a plateau when cognitive, functional and behavioral decline occurs [3]. Existing treatments for AD are only capable of temporary symptomatic relief in a subset of patients [4]. As elevated brain Aβ is an important risk factor for eventual AD, it has become critical to identify individuals at the early stages of Aβ deposition to recruit into clinical trials of potentially disease-modifying therapeutics. Indeed, three prevention trials in asymptomatic individuals at the early stages of Aβ deposition have commenced recently [5].

At present neuroimaging and cerebrospinal fluid (CSF) biomarkers are the accepted standards used to provide evidence of on-going AD pathophysiology related to Aβ plaques. [11]C-Pittsburgh Compound B (PiB) coupled with Positron Emission Tomography (PET) is widely used in research in measuring *in vivo* Aβ deposition as its uptake in AD correlates with Aβ plaques measured neuropathologically in the same brains [6]. The availability of longer lived [18]F- labeled Aβ PET tracers, such as flutemetamol [7] and florbetapir [8] could foster wider utilization in clinical use [9]. Early "proof of concept" PiB-PET studies demonstrated an increase of Aβ deposition in a majority of individuals clinically diagnosed with AD as judged by visual assessment [10] or quantification of tracer uptake [11,12]. Two large studies, from Victoria

(Australia) and the University of California San Francisco Memory and Aging Center (UCSF, USA) have shown that PiB PET could discriminate between AD and non-Aβ dementias [11,13]. Some, but not all [14,15], studies also show that amyloid deposition as measured using PiB-PET either predicts decline in cognitive measures or tracks with such [2,16].

Many disease modifying therapeutics being developed target amyloid generation, deposition or clearance [17]. Recent phase III trials targeting amyloid reported that approximately 20% of trial participants actually had little or no Aβ when studied later using such PET imaging (Suspected Non Amyloid Pathology; SNAP) [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 harder when a large minority of trial subjects fail to have the primary target pathology.

A solution is to use amyloid-PET scans (~$3,000 per scan) to ensure primary target pathology. The first study to use this will be the Anti Amyloid in Asymptomatic AD (A4; n=1,000) prevention trial. In A4 the screen failure rate is anticipated to be even higher (~66%) due 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 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 tests are positive. This would reduce the screen failure rates, and save money if the blood test was inexpensive comparatively. Therefore, a blood-based measure that correlates with neocortical amyloid burden (NAB) would be of considerable value as an enrichment filter for clinical trials.

The obvious blood candidate biomarker of brain Aβ pathology would be Aβ itself. A systematic literature review and meta-analysis by Koyama et al [19], on 10,303 subjects, found that lower plasma Aβ42:Aβ40 ratios were significantly associated with development of AD. However, the estimates had wide confidence intervals, due to high inter-study differences. As such plasma Aβ42:Aβ40 ratios are unlikely to be useful by itself for the prediction of NAB. The same study found that individual Aβ42 and Aβ40 levels in blood were not significantly associated with AD. Clearly novel biomarkers are needed that reflect brain amyloid pathology in blood.

There has been considerable effort in the search for AD blood-based biomarkers. Most studies use a case-control design, based on a clinical diagnosis of AD as determined by medical history, cognitive assessments and clinical examination. This classical, case versus age-matched controls approach, has identified a large number of putative plasma biomarkers (reviewed in [20,21,22]). However, such approaches are intrinsically flawed in the context of AD where 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 non-apparent 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], APOE4 risk [25], brain atrophy [26,27] and hippocampal metabolism [28]. More recently, blood-based biomarkers of NAB, as measured by PiB PET, have been reported [29,30,31]. Both Kiddle et al and Burnham et al utilized the Rules Based Medicine panel of 190 analytes to discover plasma proteins that related to NAB, and proposed a thirteen and five analyte model respectively. These models both contained the protein pancreatic polypeptide.

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

In this study we employ a methodology 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 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].

Material and Methods

The Australian Imaging, Biomarkers and Lifestyle Flagship Study of Ageing (AIBL)

The AIBL study is a longitudinal study of ageing, neuroimaging, biomarkers, lifestyle, clinical and neuropsychological analysis with a focus on early detection and lifestyle intervention

(http://www.aibl.csiro.au/). Additional specifics regarding subject recruitment, diagnosis and study design have been described previously [32].

Discovery cohort; assessments, blood collection and processing

In total we examined 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 PiB+ and PiB- groups. To increase statistical power the subjects were selected to be enriched for clear cases of PiB negativity and positivity. Standardised clinical assessments included Mini-Mental State Examination (MMSE), and Apoliopoprotein E (APOE) genotypes were available.

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

AIBL PiB-PET

The PiB imaging methodology of the AIBL study is detailed elsewhere [33]. SUVR were generated using the cerebellar grey matter as the reference region as described in Burnham et al [31]. NAB was expressed as the average SUVR of the mean of frontal, superior parietal, lateral temporal, lateral occipital, and anterior and posterior cingulate regions.

Tandem Mass Tag (TMT) protein labeling, enzymatic digestion and peptide extraction

Each sample was randomly assigned and labeled with an Amine-Reactive TMT Reagent (TMT127-TMT131; Thermo Scientific #90064) with TMT126 being 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 (Supplementary Methods 1). Each TMT6Plex underwent 1D gel electrophoresis and excised into 10 fractions (Supplementary Methods 2). Gel pieces were then de-stained, digested, peptides extracted and lyophilised to completion prior to MS analysis (Supplementary Methods 3). LC-MS/MS data separated with 1D-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 (Supplementary Methods 5b).

Liquid chromatography – tandem mass spectrometry (LC-MS/MS)

Samples were analysed using an LTQ Orbitrap Velos instrument (Thermo Scientific) coupled to a Proxeon EASY-nLC II system (Thermo Scientific). Further details on chromatographic separation and MS data acquisition are outlined in Supplementary Methods 4.

Pre-processing of LC-MS/MS data

Raw data files produced in Excalibur software (Thermo Scientific) were processed using Proteome Discoverer (PD) V1.3 (Thermo Scientific) to determine peptide identification; the subsequent Mascot (v2.3; http://www.matrixscience.com) output file was used for further pre-processing and analysis (supplementary methods 5a). A script was written in R to complete the pre-processing taking into account the experimental setup described above (http://core.brc.iop.kcl.ac.uk/software/). We named the script Pre-processing for Relative

Quantification of LC-MS/MS data (PRQ; Supplementary Methods - PRQ). PRQ performs (1) median ratio normalisation [35], (2) calculates ratios for each peptide, (3) derives protein level data from peptide scores, (4) collects protein scores across all TMT6plex's.

The University of California San Francisco (UCSF) Memory and Aging Center cohort

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 genotyping, neurological and cognitive assessments [13], as well as plasma collection and storage [36] and as previously described. Clinical diagnoses of AD, FTD and MCI were made by consensus applying standard research criteria [37,38,39]. All subjects underwent PiB-PET at Lawrence Berkeley National Laboratory on a Siemens ECAT EXACT HR PET (n=69) or Biograph Truepoint 6 PET/CT (n=10) [13]. Scans were visually rated as PiB+ or PiB- by an experienced single rater blinded to clinical and plasma data [13]. Mean 50-70 min SUVR values were extracted from frontal, parietal, cingulate and lateral temporal cortex, using mean activity in the cerebellar gray matter as the reference tissue (for details of image processing see [40]).

Immunoassay – enzyme-linked immunosorbent assay (ELISA)

Single analyte sandwich ELISA was used to quantify candidate proteins and were performed as per manufacturer's instructions (Supplementary Methods 6).

Statistical analysis

All statistical analyses were performed in R (Supplementary Methods 7). For logistic and linear regression, age, gender and presence of APOE4 allele were used as covariates. For the ELISA analysis, data outliers were excluded (±3 standard deviations) and a fourth covariate, batch, was added. 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 pathway, regression and classification analyses are given in Supplementary Methods 7.

Results

LC-MS/MS performed on AIBL subjects

LC-MS/MS was performed on plasma samples from 78 AIBL subjects, whose demographics are shown in Table 1. Combining data from all MS/MS runs, we identified 4,518 unique peptides sequences that corresponded to 789 unique protein groups. PRQ was able to extract 2,319 unique TMT peptides, 1,139 MW isoforms and 379 unique protein groups (Supplementary Results 1a), which was reduced to 116 confidently annotated unique protein groups after post-PRQ data clean up; this consisted of 381 protein MW isoforms (Supplementary Results 1b).

Plasma protein markers of global PiB PET

Each protein MW isoform underwent Mann-Whitney U test and logistic regression to compare PiB+ and PiB- groups as well as Spearman Rank Correlation (SRC) and linear regression to associate protein MW isoform levels against PiB retention as a continuous measure. This was completed for both 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 $P < 0.05$) shown in Supplementary Results 1c. Pathway analysis (Supplementary Results 2) revealed that these protein groups were over-represented for involvement in complement and coagulation cascades ($p = 3.7 \times 10^{-22}$, $q = 3.3 \times 10^{-21}$), systemic lupus erythematosus ($p = 2.65 \times 10^{-4}$, $q = 0.15$) and prion diseases ($p = 5.9 \times 10^{-3}$, $q = 0.051$). Three albumin and 15 immunoglobulin MW isoforms were removed to leave 51 protein MW isoforms associated with PiB-PET retention (Table 2).

Subsequently, 17 proteins were selected for technical replication (Figure 1). In addition to statistical evidence we also considered the candidate's relationship with amyloid and/or AD Genome-Wide Association Studies results (Supplementary Results 3). We also chose to replicate Histidine-Rich Glycoprotein, the protein most associated with NAB, but had no prior evidence for a relationship with Aβ.

Technical Replication

We sought to translate our discovery findings to a simple-to-use commercially available ELISA format. The 17 proteins 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/ELISA plate as covariates) we found that two proteins - α-2m ($q = 0.076$) and FGG ($q = 0.076$), replicated our findings from the LC-MS/MS discovery study (Table 3). In the discovery study, FHR-1 was increased in the PiB+ group. Although FHR-1 ($q = 0.076$) was associated with NAB at 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.

Independent Replication

To verify the results from the AIBL samples, we measured the levels of the three proteins significantly associated with NAB (α-2m, FHR-1 and FGG) using samples from an independent cohort. These proteins were measured by ELISA in 79 samples from the UCSF cohort (Table 1). Table 4 shows that FGG was found to be significantly associated with PiB positivity, as determined both by visual examination of PiB-PET scans (q = 5.9 x10^{-3}) and by applying a threshold of 1.3 to SUVRs (q = 0.051). Despite not being significantly associated with NAB, α-2m correlated with SUVR positivity in the same direction as in the discovery study.

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+ based on SUVR > 1.3) and 78 subjects from UCSF (46 PiB-, 32 PiB+ based on 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/APOE4) was compared to a 'basic + proteins' model which also used the plasma concentration of FGG, α-2m and FHR-1. Figure 2a and 2b shows a Receiver Operator Characteristic (ROC) analysis, where Area Under the Curve (AUC) was shown to be higher for the 'basic + protein' model than for the 'basic' model in the test datasets. The highest test AUC was found using the Random Forest approach, where the 'basic +

protein' model (AUC = 0.70) outperformed the 'basic' model (AUC = 0.46) in the test dataset. The Random Forest 'basic + proteins' model gave a test set sensitivity of 50% and specificity of 85%. Additionally, a classification tree was fitted to the 'basic + proteins' model, to provide a simpler alternative with clear thresholds. The resulting classification tree used just two variables (age/plasma FGG level; Figure 2c) and achieved a comparable AUC to the Random Forest model (AUC 0.69, sensitivity 59%, specificity 78%). In the UCSF cohort, 23 out of 25 AD subjects are PiB+, it is noteworthy that the two PiB- subjects had plasma FGG levels above the threshold (Supplementary Results 4).

Discussion

With the failure of serial amyloid based therapeutics in clinical trials compromised by inclusion of substantial numbers of participants without the target pathology [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 CSF assays or PET scans, reducing screen failure rates. This could reduce recruitment time and costs, as well as allowing eligible subjects to be identified more readily, for example from biobanks with permission for re-contact.

This study has demonstrated that a simple blood test consisting of FGG plasma levels along with 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 may also have utility for distinguishing between amyloid

and non-amyloid 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, as the UCSF cohort contained only two cognitively normal individuals, further work will be needed to determine sensitivity and specificity in people 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 (Westwood et al., data not shown). Previously, Burnham et al [31] reported a blood test that achieved 79% sensitivity and 76% specificity in an independent test set. While our sensitivity is slightly lower, this is achieved by measuring a single plasma protein compared to 6 plasma proteins in the Burnham model.

While the sensitivity and specificity of these markers for predicting NAB are 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 looking for individuals with elevated NAB. Due to the relatively high cost of amyloid-PET scans (~$3,000) versus blood protein ELISAs, even a blood test without clinical utility could theoretically save millions of dollars from studies of the size of A4 (n=1,000).

APOE status is a substantial risk factor for AD [41] and amyloid [30,42]. While we took APOE4 into account during our analyses we were not surprised to find that APOE genotype markers did not improve our classification model as the study was designed to be independent of this effect. However, in a general population sample APOE genotype is likely to contribute to the prediction of NAB.

It is interesting that FGG, and to a lesser extent Complement C3 and Fibrinogen α chain, were associated with NAB in this study, as this has been previously found [29,30]. However in the study by Burnham et al [31] total fibrinogen was not found to associate with NAB, whereas Kiddle et al [30] showed it was negatively associated with NAB. Further to this, decreased levels of plasma FGG have been shown to be associated with a smaller whole brain volume in AD subjects [29] whereas measures of whole fibrinogen in plasma have shown an increase [43,44]. Discrepancies of these findings may be due to the platform used to measure total fibrinogen or highlight the importance of looking at 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 be due to 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, as smaller molecules are not BBB-permeable in AD [49]. Fibrinogen has been shown to accumulate over time as AD pathology progresses [46] and co-deposits with Aβ in brain tissue [50]. Ahn and colleagues [51] demonstrated that fibrinogen binds to Aβ, which enhances aggregation and increases Aβ fibrillisation. It is possible that decreased FGG levels associated with high NAB in our study is due to movement of fibrinogen across a compromised BBB in subjects with AD pathology.

After FGG, α-2m was the second most promising candidate, shown for the first time to associate with NAB. This is noteworthy because, α-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 higher sensitivity and specificity.

To our knowledge, this is the first study to apply an unbiased and non-targeted quantitative LC-MS/MS discovery approach, combining LC-MS/MS with TMT-labelling, 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 also describe a novel and automated bioinformatic pipeline - PRQ - to accurately pre-process TMT-MS data. PRQ not only conducts rigorous normalisation 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 positives and to ensure translation of LC/MS-MS findings using a platform more applicable to clinical setting. Using commercially available immunoassays, we confirmed that α-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 PTM, or different truncated forms of a same protein seen by LC/MS-MS. This could also explain the differences seen in 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 of investigating protein modifications as potential biomarkers in future studies.

The discrepancies between findings in AIBL and UCSF could be due to low statistical power, differences in disease stage or pre-analytical factors. The major difference in pre-analytical

factors is the centrifugation step of plasma collection: AIBL has a two-step centrifugation (200 x g, remove supernatant, then 800 x g), whereas UCSF has a single centrifugation step (1300 – 1800 x g). This highlights the importance of standardization of blood collection and preparation for biomarker studies.

While many agree that Aβ deposition is the earliest event in AD pathogenesis, one group has shown changes in episodic memory preceding changes in Aβ 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 summary, the current study presents a potential blood test, consisting of measuring FGG, which along with age has some ability to predict NAB in an independent sample. To ensure robustness and relevance of these findings, this test will need to be replicated in larger cohorts that are more representative of relevant clinical trial populations. This study adds further evidence that differences in the plasma proteome in relation to AD and its pathology do exist, and 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.

Acknowledgements

Steven Kiddle is supported by an MRC Career Development Award in Biostatistics (MR/L011859/1). We are grateful for grant funding from Alzheimer's Research UK, the

Alzheimer's Society (to SW) and the NIHR Biomedical Research Centre for Mental Health and Biomedical Research Unit for Dementia at the South London and Maudsley NHS Foundation Trust and Kings College London, and a joint infrastructure grant from Guy's and St Thomas' Charity and the Maudsley Charity.

This paper presents independent research part funded by the National Institute for Health Research (NIHR). A portion of this work was funded by GE Healthcare and Janssen R&D. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR, Department of Health, GEHC and Janssen.

UCSF work was funded by United States National Institute of Health K23-AG031861, R01-AG027859, R01-AG032306, R01-AG038791, P01-AG1972403 and P50-AG023501; State of California Department of Health Services; Alzheimer's Association and John Douglas French Alzheimer's Foundation. The authors would also like to thank William Jagust, MD, for PiB-PET imagery.

Finally, we wish to express our appreciation to all study participants of the AIBL and UCSF studies.

References

1. Blennow K, Hampel H, Weiner M, Zetterberg H (2010) Cerebrospinal fluid and plasma biomarkers in Alzheimer disease. Nat Rev Neurol 6: 131-144.
2. Villemagne VL, Pike KE, Chetelat G, Ellis KA, Mulligan RS, et al. (2011) Longitudinal assessment of Abeta and cognition in aging and Alzheimer disease. Ann Neurol 69: 181-192.
3. Villemagne VL, Burnham S, Bourgeat P, Brown B, Ellis KA, et al. (2013) Amyloid beta deposition, neurodegeneration, and cognitive decline in sporadic Alzheimer's disease: a prospective cohort study. Lancet Neurol 12: 357-367.
4. Corbett A, Smith J, Ballard C (2012) New and emerging treatments for Alzheimer's disease. Expert Rev Neurother 12: 535-543.
5. Aisen PS, Vellas B, Hampel H (2013) Moving towards early clinical trials for amyloid-targeted therapy in Alzheimer's disease. Nat Rev Drug Discov 12: 324.
6. Ikonomovic MD, Klunk WE, Abrahamson EE, Mathis CA, Price JC, et al. (2008) Post-mortem correlates of in vivo PiB-PET amyloid imaging in a typical case of Alzheimer's disease. Brain 131: 1630-1645.
7. Nelissen N, Van Laere K, Thurfjell L, Owenius R, Vandenbulcke M, et al. (2009) Phase 1 study of the Pittsburgh compound B derivative 18F-flutemetamol in healthy volunteers and patients with probable Alzheimer disease. J Nucl Med 50: 1251-1259.
8. Johnson KA, Sperling RA, Gidicsin CM, Carmasin JS, Maye JE, et al. (2013) Florbetapir (F18-AV-45) PET to assess amyloid burden in Alzheimer's disease dementia, mild cognitive impairment, and normal aging. Alzheimers Dement 9: S72-83.
9. Nordberg A, Rinne JO, Kadir A, Langstrom B (2010) The use of PET in Alzheimer disease. Nat Rev Neurol 6: 78-87.
10. Klunk WE, Engler H, Nordberg A, Wang Y, Blomqvist G, et al. (2004) Imaging brain amyloid in Alzheimer's disease with Pittsburgh Compound-B. Ann Neurol 55: 306-319.
11. Rowe CC, Ng S, Ackermann U, Gong SJ, Pike K, et al. (2007) Imaging beta-amyloid burden in aging and dementia. Neurology 68: 1718-1725.
12. Ng S, Villemagne VL, Berlangieri S, Lee ST, Cherk M, et al. (2007) Visual assessment versus quantitative assessment of 11C-PIB PET and 18F-FDG PET for detection of Alzheimer's disease. J Nucl Med 48: 547-552.
13. Rabinovici GD, Rosen HJ, Alkalay A, Kornak J, Furst AJ, et al. (2011) Amyloid vs FDG-PET in the differential diagnosis of AD and FTLD. Neurology 77: 2034-2042.
14. Jagust WJ, Landau SM, Shaw LM, Trojanowski JQ, Koeppe RA, et al. (2009) Relationships between biomarkers in aging and dementia. Neurology 73: 1193-1199.
15. Scheinin NM, Aalto S, Koikkalainen J, Lotjonen J, Karrasch M, et al. (2009) Follow-up of [11C]PIB uptake and brain volume in patients with Alzheimer disease and controls. Neurology 73: 1186-1192.
16. Villain N, Chetelat G, Grassiot B, Bourgeat P, Jones G, et al. (2012) Regional dynamics of amyloid-beta deposition in healthy elderly, mild cognitive impairment and Alzheimer's disease: a voxelwise PiB-PET longitudinal study. Brain 135: 2126-2139.

17. Blennow K, Hampel H, Zetterberg H (2014) Biomarkers in amyloid-beta immunotherapy trials in Alzheimer's disease. Neuropsychopharmacology 39: 189-201.
18. Salloway S, Sperling R, Fox NC, Blennow K, Klunk W, et al. (2014) Two phase 3 trials of bapineuzumab in mild-to-moderate Alzheimer's disease. N Engl J Med 370: 322-333.
19. Koyama A, Okereke OI, Yang T, Blacker D, Selkoe DJ, et al. (2012) Plasma amyloid-beta as a predictor of dementia and cognitive decline: a systematic review and meta-analysis. Arch Neurol 69: 824-831.
20. Kiddle SJ, Sattlecker M, Proitsi P, Simmons A, Westman E, et al. (2014) Candidate blood proteome markers of Alzheimer's disease onset and progression: a systematic review and replication study. J Alzheimers Dis 38: 515-531.
21. Lista S, Faltraco F, Prvulovic D, Hampel H (2013) Blood and plasma-based proteomic biomarker research in Alzheimer's disease. Prog Neurobiol 101-102: 1-17.
22. Zurbig P, Jahn H (2012) Use of proteomic methods in the analysis of human body fluids in Alzheimer research. Electrophoresis 33: 3617-3630.
23. Guntert A, Campbell J, Saleem M, O'Brien DP, Thompson AJ, et al. (2010) Plasma gelsolin is decreased and correlates with rate of decline in Alzheimer's disease. J Alzheimers Dis 21: 585-596.
24. Sattlecker M, Kiddle SJ, Newhouse S, Proitsi P, Nelson S, et al. (2014) Alzheimer's disease biomarker discovery using SOMAscan multiplexed protein technology. Alzheimers Dement.
25. Song F, Poljak A, Crawford J, Kochan NA, Wen W, et al. (2012) Plasma apolipoprotein levels are associated with cognitive status and decline in a community cohort of older individuals. PLoS One 7: e34078.
26. Thambisetty M, An Y, Kinsey A, Koka D, Saleem M, et al. (2012) Plasma clusterin concentration is associated with longitudinal brain atrophy in mild cognitive impairment. Neuroimage 59: 212-217.
27. Hye A, Riddoch-Contreras J, Baird AL, Ashton NJ, Bazenet C, et al. (2014) Plasma proteins predict conversion to dementia from prodromal disease *(In Press)*. Alzheimers Dement.
28. Thambisetty M, Hye A, Foy C, Daly E, Glover A, et al. (2008) Proteome-based identification of plasma proteins associated with hippocampal metabolism in early Alzheimer's disease. J Neurol 255: 1712-1720.
29. Thambisetty M, Tripaldi R, Riddoch-Contreras J, Hye A, An Y, et al. (2010) Proteome-based plasma markers of brain amyloid-beta deposition in non-demented older individuals. J Alzheimers Dis 22: 1099-1109.
30. Kiddle SJ, Thambisetty M, Simmons A, Riddoch-Contreras J, Hye A, et al. (2012) Plasma based markers of [11C] PiB-PET brain amyloid burden. PLoS One 7: e44260.
31. Burnham SC, Faux NG, Wilson W, Laws SM, Ames D, et al. (2013) A blood-based predictor for neocortical Abeta burden in Alzheimer's disease: results from the AIBL study. Mol Psychiatry.
32. Ellis KA, Bush AI, Darby D, De Fazio D, Foster J, et al. (2009) The Australian Imaging, Biomarkers and Lifestyle (AIBL) study of aging: methodology and baseline characteristics of 1112 individuals recruited for a longitudinal study of Alzheimer's disease. Int Psychogeriatr 21: 672-687.
33. Jagust WJ, Bandy D, Chen K, Foster NL, Landau SM, et al. (2010) The Alzheimer's Disease Neuroimaging Initiative positron emission tomography core. Alzheimers Dement 6: 221-229.

34. Dayon L, Hainard A, Licker V, Turck N, Kuhn K, et al. (2008) Relative quantification of proteins in human cerebrospinal fluids by MS/MS using 6-plex isobaric tags. Anal Chem 80: 2921-2931.
35. Carrillo B, Yanofsky C, Laboissiere S, Nadon R, Kearney RE (2010) Methods for combining peptide intensities to estimate relative protein abundance. Bioinformatics 26: 98-103.
36. Bettcher BM, Watson CL, Walsh CM, Lobach IV, Neuhaus J, et al. (2014) Interleukin-6, age, and corpus callosum integrity. PLoS One 9: e106521.
37. McKhann G, Drachman D, Folstein M, Katzman R, Price D, et al. (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: 939-944.
38. Petersen RC, Smith GE, Waring SC, Ivnik RJ, Tangalos EG, et al. (1999) Mild cognitive impairment: clinical characterization and outcome. Arch Neurol 56: 303-308.
39. Neary D, Snowden JS, Gustafson L, Passant U, Stuss D, et al. (1998) Frontotemporal lobar degeneration: a consensus on clinical diagnostic criteria. Neurology 51: 1546-1554.
40. Lehmann M, Ghosh PM, Madison C, Laforce R, Jr., Corbetta-Rastelli C, et al. (2013) Diverging patterns of amyloid deposition and hypometabolism in clinical variants of probable Alzheimer's disease. Brain 136: 844-858.
41. Blacker D, Haines JL, Rodes L, Terwedow H, Go RC, et al. (1997) ApoE-4 and age at onset of Alzheimer's disease: the NIMH genetics initiative. Neurology 48: 139-147.
42. Thambisetty M, An Y, Nalls M, Sojkova J, Swaminathan S, et al. (2013) Effect of complement CR1 on brain amyloid burden during aging and its modification by APOE genotype. Biol Psychiatry 73: 422-428.
43. van Oijen M, Witteman JC, Hofman A, Koudstaal PJ, Breteler MM (2005) Fibrinogen is associated with an increased risk of Alzheimer disease and vascular dementia. Stroke 36: 2637-2641.
44. Xu G, Zhang H, Zhang S, Fan X, Liu X (2008) Plasma fibrinogen is associated with cognitive decline and risk for dementia in patients with mild cognitive impairment. Int J Clin Pract 62: 1070-1075.
45. Cortes-Canteli M, Paul J, Norris EH, Bronstein R, Ahn HJ, et al. (2010) Fibrinogen and beta-amyloid association alters thrombosis and fibrinolysis: a possible contributing factor to Alzheimer's disease. Neuron 66: 695-709.
46. Ryu JK, McLarnon JG (2009) A leaky blood-brain barrier, fibrinogen infiltration and microglial reactivity in inflamed Alzheimer's disease brain. J Cell Mol Med 13: 2911-2925.
47. Paul J, Strickland S, Melchor JP (2007) Fibrin deposition accelerates neurovascular damage and neuroinflammation in mouse models of Alzheimer's disease. J Exp Med 204: 1999-2008.
48. Marques F, Sousa JC, Sousa N, Palha JA (2013) Blood-brain-barriers in aging and in Alzheimer's disease. Mol Neurodegener 8: 38.
49. Sagare A, Deane R, Bell RD, Johnson B, Hamm K, et al. (2007) Clearance of amyloid-beta by circulating lipoprotein receptors. Nat Med 13: 1029-1031.
50. Klohs J, Baltes C, Princz-Kranz F, Ratering D, Nitsch RM, et al. (2012) Contrast-enhanced magnetic resonance microangiography reveals remodeling of the cerebral microvasculature in transgenic ArcAbeta mice. J Neurosci 32: 1705-1713.

51. Ahn HJ, Zamolodchikov D, Cortes-Canteli M, Norris EH, Glickman JF, et al. (2010) Alzheimer's disease peptide beta-amyloid interacts with fibrinogen and induces its oligomerization. Proc Natl Acad Sci U S A 107: 21812-21817.
52. Jedynak BM, Lang A, Liu B, Katz E, Zhang Y, et al. (2012) A computational neurodegenerative disease progression score: method and results with the Alzheimer's disease Neuroimaging Initiative cohort. Neuroimage 63: 1478-1486.

Table 1 – Demographics of selected subjects from the AIBL and UCSF cohorts.

	AIBL Discovery Cohort			**UCSF Replication Cohort**		
	Low Neocortical SUVR (PiB-)	**High Neocortical SUVR (PiB+)**	**P-value**	**Low Neocortical Visual PiB read (PiB -)**	**High Neocortical Visual PiB read (PiB +)**	**P-value**
Number of subjects (*n*)	38	40		47	32	
SUVR (mean (s.d.) {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}
Gender; females (*n* (%))	18 (47%)	20 (50%)	0.83	18 (38%)	14 (44%)	0.65
Age in years (mean (s.d.))	75.8 (6.53)	80.9 (8.22)	0.0035	65 (8.8)	64 (8.4)	0.61
Clinical diagnosis (*n* (%))	HC: 13 (34%)	HC: 6 (15%)	0.0037	HC: 2 (4.3%)	HC: 1 (3.1%)	1.9×10^{-10}
	SMC: 18 (47%)	SMC: 13 (40%)		MCI: 1 (2.1%)	MCI: 1 (3.1%)	
	MCI: 7 (19%)	MCI: 16 (30%)		AD: 2 (4.3%)	AD: 23 (72%)	
	AD: 0 (0%)	AD: 6 (15%)		FTD: 42 (89.3%)	FTD: 7 (21.8%)	
APOE ε4 carrier (*n* (%))	14 (37)	25 (63)	0.36	8 (17%)	13 (41%)	0.036
MMSE (mean (s.d.))	28.3 (1.8)	26.8 (4.1)	0.038	26 (4.3)	21 (6.9)	0.0011

Table 2 – LC-MS/MS data; Protein MW isoforms significantly associated with NAB. (All multiple testing corrected Q-values were > 0.75). For regressions age, gender and presence of APOE4 was used as covariates.

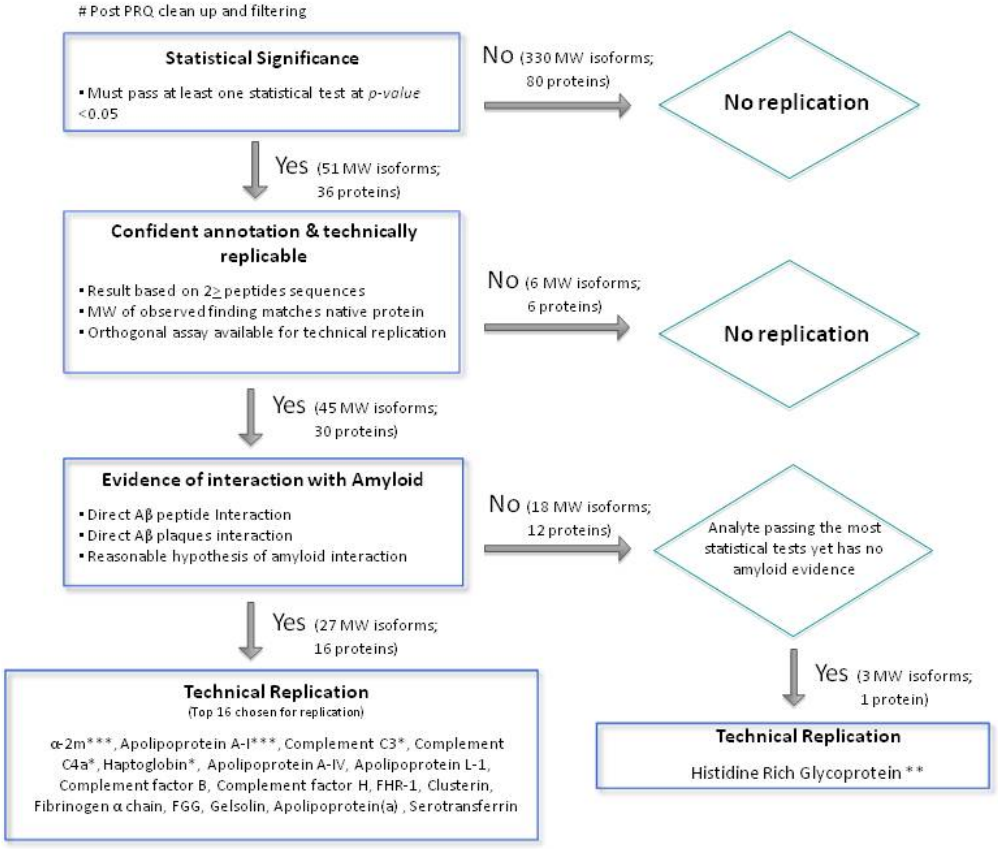

Figure 1 – Flow diagram to select LC-MS/MS plasma NAB candidate markers for technical replication. * Two protein MW isoforms associated with NAB; ** Three protein MW isoforms associated with NAB; *** Four protein MW isoforms.

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

			Logistic regression with SUVR > 1.3			Linear regression with SUVR		
UniProt ID	**Protein Name**	**Number of outliers excluded**	**Beta**	**P-value**	**Q-value**	**Beta**	**P-value**	**Q-value**
P01023	α-2-macroglobulin (α-2m)	10	1	$8.9x10^{-3}$	0.076	0.2	$7.9x10^{-3}$	0.068
Q03591	FHR-1	11	-1	$4.6x10^{-3}$	0.076	-0.22	$5.5x10^{-3}$	0.068
P02679	Fibrinogen γ chain (FGG)	0	-0.7	0.041	0.23	-0.2	0.014	0.081
P08519	Apolipoprotein(a)	21	0.48	0.13	0.34	0.18	0.042	0.18
P06396	Gelsolin	2	-0.48	0.11	0.34	-0.14	0.068	0.19
P00738	Haptoglobin	2	-0.38	0.18	0.39	-0.13	0.089	0.19

P04196	Histidine Rich Glycoprotein	2	0.48	0.14	0.34	0.14	0.081	0.19
P06727	Apolipoprotein A-IV	2	-0.63	0.083	0.34	-0.17	0.067	0.19
P01024	Complement C3	0	-0.61	0.25	0.47	-0.21	0.13	0.25
P0C0L4	Complement C4a	0	-0.55	0.51	0.66	-0.27	0.22	0.38
P10909	Clusterin	0	-0.27	0.36	0.51	-0.091	0.27	0.41
P02647	Apolipoprotein A-I	0	0.34	0.29	0.47	0.088	0.32	0.46
P02671	Fibrinogen α chain	6	-0.28	0.3	0.47	-0.064	0.39	0.52
P02787	Serotransferrin	1	-0.013	0.96	0.96	-0.041	0.6	0.73
O14791	Apolipoprotein L-1	0	-0.09	0.74	0.89	-0.026	0.73	0.77
P08603	Complement factor H	3	0.066	0.8	0.89	0.027	0.7	0.77
P00751	Complement factor B	2	0.053	0.84	0.89	0.018	0.81	0.81

Table 4 – Independent replication of plasma protein candidates discovered by LC-MS/MS and technically replicated. Only one outlier (> 3 standard deviations from mean) was excluded, which was detected for FGG. For regressions age, gender, presence of APOE4, ELISA plate and scanner type were used as covariates.

		Logistic regression to visual read			Logistic regression to SUVR > 1.3			Linear regression to SUVR		
UniProt ID	**Protein Name**	Beta	*P*-value	*Q*-value	Beta	*P*-value	*Q*-value	Beta	*P*-value	*Q*-value
P01023	α-2m	-0.013	0.96	0.96	0.27	0.29	0.44	0.075	0.22	0.33
P02679	FGG	-1.0	2.0×10^{-3}	5.9×10^{-3}	-0.74	0.017	0.051	-0.21	4.1×10^{-4}	1.2×10^{-3}
Q03591	FHR-1	-0.066	0.79	0.96	0.011	0.97	0.97	1.5×10^{-3}	0.98	0.98

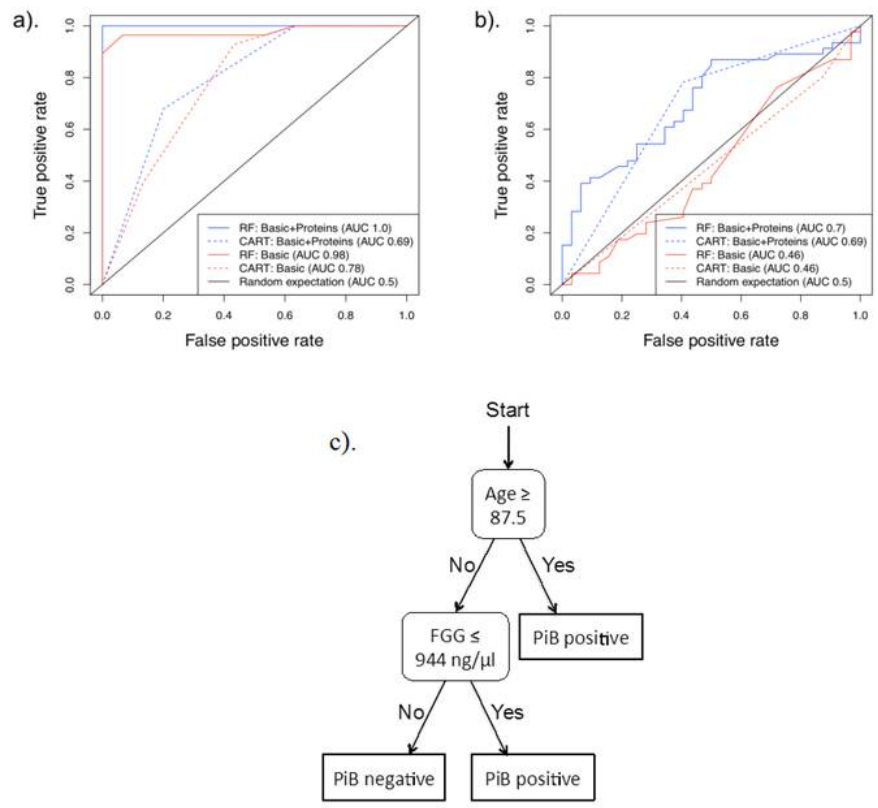

Figure 2 – Receiver Operator Characteristic (ROC) curves for the prediction of PiB positivity. A 'basic' model (age/gender/APOE4 presence) is compared to a 'basic + proteins' model also including the plasma levels of FGG, α-2M and FHR-1. Random Forest (RF) and Classification and Regression Trees (CART) were used to fit models in CARET using default parameters. Area Under the Curve (AUC) is given for each model. ROC curves are shown comparing predictive accuracy of models in (a) the training dataset (AIBL), and (b) the test dataset (UCSF). Classification tree trained on AIBL ELISA data to predict NAB positivity and estimated cut-off (c).