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# Ptau-A$\beta_{42}$ ratio as a continuous trait for biomarker discovery for early stage Alzheimer's disease in multiplex immunoassay panels of Cerebrospinal fluid

**Oscar Harari**[1], **Carlos Cruchaga**[1,7], **John S.K. Kauwe**[9], **Benjamin J. Ainscough**[8], **Kelly Bales**[10], **Eve H. Pickering**[10], **Sarah Bertelsen**[1], **Anne M. Fagan**[2,6,7], **David M. Holtzman**[2,5,6,7], **John C. Morris**[2,3,6,7], and **Alison M. Goate**[1,2,4,6,7,†] **for the Alzheimer's Disease Neuroimaging Initiative**

[1]Department of Psychiatry, Washington University School of Medicine, St Louis, MO 63110, USA

[2]Department of Neurology, Washington University School of Medicine, St Louis, MO 63110, USA

[3]Department of Pathology and Immunology, Washington University School of Medicine, St Louis, MO 63110, USA

[4]Department of Genetics, Washington University School of Medicine, St Louis, MO 63110, USA

[5]Department of Developmental Biology, Washington University School of Medicine, St Louis, MO 63110, USA

[6]Knight Alzheimer's Disease Research Center, Washington University School of Medicine, St Louis, MO 63110, USA

[7]Hope Center for Neurological Disorders, Washington University School of Medicine, St Louis, MO 63110, USA



[†]Corresponding author: Alison Goate, D.Phil., Samuel & Mae S. Ludwig Professor of Genetics in Psychiatry, Professor of Neurology, Professor of Genetics, T: 314-362-8691, F: 314-747-2983, goatea@psychiatry.wustl.edu.

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[8]Bio & Biomed Science Grad Affairs, Washington University School of Medicine, St Louis, MO 63110, USA

[9]Department of Biology, Brigham Young University, Provo, UT, USA

[10]Neuroscience Research Unit, Worldwide Research and Development, Pfizer, Inc., Groton, CT, USA

## Abstract

**Background—**Identification of the physiological changes that occur during the early stages of Alzheimer's disease (AD) may provide critical insights for the diagnosis, prognosis and treatment of disease. Cerebrospinal fluid (CSF) biomarkers are a rich source of information that reflect the brain proteome.

**Methods—**We applied a novel approach to screen a panel of ~190 CSF analytes quantified by multiplex immunoassay and detected common associations in the Knight- Alzheimer's Disease Research Center (ADRC;N=311) and the Alzheimer's Disease Neuroimaging Initiative (ADNI;N=293) cohorts. CSF $ptau_{181}$-$A\beta_{42}$ ratio was used as a continuous trait, rather than case control status in these analyses.

**Results—**We demonstrate the $ptau_{181}$-$A\beta_{42}$ ratio has more statistical power than traditional modeling approaches and that the levels of CSF Fatty Acid Binding Protein (H-FABP) and 12 other correlated analytes increase as the disease progresses. These results were validated using the traditional case control status model. Stratification of our dataset demonstrated that increases in these analytes occur very early in the disease course and were apparent even in non-demented individuals with AD pathology (low $ptau_{181}$, low $A\beta_{42}$) compared to pathology-negative elderly control subjects (low $ptau_{181}$, high $A\beta_{42}$). FABP-$A\beta_{42}$ ratio demonstrates a similar hazard ratio for disease conversion to $ptau_{181}$-$A\beta_{42}$ even though the overlap in classification is incomplete suggesting that FABP contributes independent information as a predictor

**Conclusions—**Our results clearly indicate that the approach presented here can be employed to correctly identify novel biomarkers for AD, and that CSF H-FABP levels start to increase at very early stages of the disease.

### Keywords

Alzheimer's disease; Biomarkers; cerebrospinal fluid (CSF); Ptau-Aβ42 ratio; Heart Fatty Acid binding protein; Brain Proteome - Rules Based Medicine Discovery Multi-Analyte Profile 1.0

## Introduction

There is accumulating evidence that the clinical symptoms of Alzheimer's disease (AD) are preceded by a long preclinical phase in which pathological protein aggregation occurs in the brain (1–4). β-amyloid plaques are estimated to develop ~15–20 years before the onset of cognitive impairment and neurofibrillary tangles begin to accumulate at least 5 years before symptom onset (2; 3). Furthermore, substantial neurodegeneration is apparent in even mildly symptomatic individuals (1). These observations, together with the failure of clinical trials in symptomatic individuals illustrate the urgent need for additional biomarkers that

characterize the preclinical stage of disease and enable treatment before the brain undergoes irreversible neurological damage (2–4).

Analytes in cerebrospinal fluid (CSF) reflect the brain proteome and are a rich source of biomarkers. In fact, CSF levels of $A\beta_{42}$, tau, and tau phosphorylated at threonine 181 ($ptau_{181}$) are related to AD by their association with the presence of β-amyloid deposition, neurofibrillary tangles and neuronal cell death (5–8). Similarly, changes in CSF Visinin-like protein-1 (VILIP-1), a neuronal calcium-sensor protein employed as a marker of neuronal injury (2; 9), and and chitinase-3 like-1, and chitinase 3-like 1, cartilage glycoprotein-39 (YKL-40), an inflammatory biomarker (10), have also been associated with AD. Additionally, both $ptau_{181}$-$A\beta_{42}$ ratio ($ptau_{181}/A\beta_{42}$) and YKL-40-$A\beta_{42}$ ratio (YKL-40/$A\beta_{42}$) have been shown to be strong predictors of conversion from cognitively normal to very mild/mild cognitive impairment over a 3~4 year period (l; 10; 11). Despite this, there is a need for additional biomarkers to identify and characterize the “preclinical” stage (pathology present with cognition intact) of the disease and to track the effectiveness of therapies. A challenge for clinical trials is to identify a target population of individuals with a high risk for converting from cognitively normal to impaired over a relatively short period of time. Currently elevated $ptau_{181}$-$A\beta_{42}$ ratio is used in some clinical trials to select such individuals. Other predictive biomarkers are needed both for the selection of patient cohorts for clinical trials and for monitoring disease progression and the success of disease-modifying treatment outcomes. Ultimately, biomarkers will enable early intervention in presymptomatic individuals with the goal of delaying the onset or preventing the cognitive decline seen in AD before the brain is irreversibly injured (2).

Two features of AD result in misclassification of subjects and severely reduce the power of traditional clinical measures in the search for novel biomarkers: 30% of cognitively normal individuals show Alzheimer’s-type neuropathology by age 75yrs (2; 3; 12; 13) and current clinical diagnostic methods are only 83% accurate as defined by neuropathological confirmation at autopsy (14). To identify new CSF analytes associated with AD we took a novel approach by developing a quantitative measure of “caseness”, employing the CSF $ptau_{181}$-Aβ42 ratio ($ptau_{181}/A\beta42$) as an endophenotype for AD. This status independent criterion avoids the misclassification of controls and cases, noted above and is a continuous quantitative trait. Both of these characteristics improve the power of this approach over traditional clinical measures.

## Methods and Materials

### Subjects

Participants enrolled in the Knight-ADRC (N=311) were diagnosed based on criteria from the National Institute of Neurological and Communicative Diseases and Stroke-Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) (15). CSF collection, processing and assessment is described elsewhere: (11; 16). Data for subjects included in the ADNI study (N=292) were accessed from the ADNI website (See Supplementary Methods and Table SI in Supplement 1) (8). Processing, aliquoting and storage were performed according to the ADNI Biomarker Core Laboratory Standard Operating Procedures (http://adni.loni.ucla.edu). Additional details for these two studies are provided elsewhere (10; 17)

Genotyping of rs7412 and rs429358 which define the *APOE* ε2/ε3/ε4 isoforms were previously described (17; 18).

Expression studies were carried out using cDNA obtained from the parietal lobe from 82 AD cases (86±7years; 45%male; and 41% APOE ε4 carriers) and 39 cognitively normal individuals (85±9; 41% male; and 23% *APOE* ε4 carriers) obtained through the Knight-ADRC Neuropathology Core. Real-time data were analyzed using the comparative Ct method (19) (See Supplementary Methods in Supplement 1).

## Analyte Measurement

The samples from both the Knight-ADRC and ADNI were evaluated by Rules Based Medicine, Inc. (RBM) (Austin, TX) for levels of 190 analytes using the Human Discovery Multi-Analyte Profile (MAP) 1.0 panel and a Luminex 100 platform. Only analytes with <10% missing values in each study were analyzed. The protocol used to quantify plasma analytes is described elsewhere (20; 21).

CSF $A\beta_{42}$, and phospho-tau181 ($ptau_{181}$) levels for the Knight-ADRC participants were obtained in duplicate by the WU-ADRC Biomarker Core by quantitative ELISA (Innotest; Innogenetics, Ghent, Belgium)(16). Quantification of CSF VILIP-1 and YKL-40 is described elsewhere (9; 10). For the ADNI participants the CSF $A\beta_{42}$, and $ptau_{181}$ levels were measured using the multiplex xMAP Luminex platform as described (8).

## Statistical analysis

The analyses were performed in R (The R Foundation for Statistical Computing v2.14.1). Analytes were log-transformed to approximate a normal distribution, and outliers were removed. The associations with the CSF $ptau_{181}$-$A\beta_{42}$ ratio were tested using linear regression models, adjusting for age at LP; sex and *APOE* genotype, encoded as five levels based on the genetic risk (*APOE* ε22=0; ε23=l; ε33=2; ε24=3; ε34=4; and ε44=5). For analyses using the combined datasets we included study as a covariate. We used two approaches to correct for the multiple testing associated with examining 64 analytes: Bonferroni correction and SimpleM. For 64 tests the Bonferroni corrected p-value corresponding to p=0.05 is p= 7.81E-04. Because this method is most likely too conservative given the correlation structure of the analyte measurements, we also extended and applied the method termed SimpleM(22) (see Supplementary Methods in Supplement 1), and obtained corrected *p-values* =1.39E-03 and 1.43E-03 for the Knight-ADRC and ADNI studies respectively. Logistic regression was used to test for association with CDR (CDR=0 vs. CDR>0), adjusting for the covariates mentioned above. Kaplan-Meier survival curves were calculated employing the function *survfit,* and the Cox proportional hazard regression models were tested using the function *coxph* (package *survival;* v2.36–10). The Cox proportional hazard tests were adjusted by age, sex, *APOE* genotype, and study. Hierarchical clustering was calculated using the complete linkage method provided (function *hclust),* and heatmaps were plotted using the function *heatmap.2* (package *gplots* v2.10.1). Multivariate stepwise model selection (Table 3AB) was performed using the function *stepAIC* (package *MASS,* v7.3–16), optimizing for the Bayesian Information Criterion; and included age, sex and *APOE* genotype as fixed covariates. The minimal

multivariate model shown in Table 3C was obtained by selecting the subset of analytes identified as significant in each study (Table 3AB). Moreover, we constrained this list to the analytes that were also significant (*p-value*<5.0E-2) when the identified models were applied to the other study. The principal component analysis was performed in R running the method *prcomp* (package stats v 2.14.1) The random forest results were obtained executing the function *randomForest* (implemented in the package *randomForest*, v4.6–6) to grow 1000 trees, and the importance measure was calculated by the mean decrease in accuracy.

## Results

### CSF $ptau_{181}/A\beta_{42}$ confers more statistical power to identify CSF analytes associated to AD

We first evaluated the statistical power of the CSF $ptau_{181}/A\beta_{42}$ measure compared to the Clinical Dementia Rating (CDR) at lumbar puncture (LP), using CSF levels of VILIP-1(9) and YKL-40(10), that had previously been measured and shown to be associated with CDR and CDR–sum of boxes in the Knight-ADRC study (11; 20; 23). The CSF $ptau_{181}$-$A\beta_{42}$ ratio has previously been shown to be a strong predictor of both the conversion of cognitively normal subjects to very mild or mild dementia (11), and the rate of decline across time in individuals with very mild dementia (24). The CSF $ptau_{181}$-$A\beta_{42}$ ratio is strongly associated with CDR at LP (*p-value*=5.05E-16).

We employed linear regression models to analyze the association of CSF VILIP-1 with the log-transformed levels of CSF $ptau_{181}$-$A\beta_{42}$ ratio, and observed a much stronger association than with CDR (*p-value*=3.18E-17 vs. 3.80E-05). The CSF $ptau_{181}$-$A\beta_{42}$ ratio remained more powerful than CDR even when converted to a dichotomous variable using two partitioning criteria. First, we split the subjects by the median value of CSF $ptau_{181}$-$A\beta_{42}$ ratio and second we compared the subjects from the upper tercile to the lower two terciles (intended to resemble the CDR distribution of the Knight-ADRC series). Both logistic models that included the CSF ratio showed much stronger associations (*p-values*=7.11E-08 and 4.00E-09 respectively) than the CDR model (*p-value*=3.80E-05).

Similarly, the association of YKL-40 with CSF $ptau_{181}$-$A\beta_{42}$ ratio was stronger than CDR (p-value =8.99E-09 vs. 7.76E-04 respectively). We also tested the normal quantile transformed CSF ratio and the same two dichotomous representations for YKL-40. In every case, the test that included the CSF ratio was more statistically significant than the CDR model (*p-value*=9.42E-06 and 3.04E-05 partitioning by the median and by the upper tercile respectively). Together these analyses provide a strong rationale for our subsequent use of the CSF $ptau_{181}$-$A\beta_{42}$ ratio for novel biomarker discovery.

### Correlation structure of the CSF analytes

We applied stringent quality criteria to the CSF analyte measurements, selecting only those that were measurable in >90% of the subjects in each series (n=311 and 293 for the Knight-ADRC and the ADNI series). A total of 64 CSF analytes met this criterion.

Analysis of the correlation matrix for the combined measurements of both datasets showed clear patterns in the analyte levels (Table S2 in Supplement 2). The hierarchical clustering method identified 10 clusters containing 48 of the CSF analytes, with a within-cluster

correlation $r^2$>0.50. Six clusters included 2 analytes, and the other 4 clusters included 3, 4, 9 and 20 analytes (Figure S1 in Supplement 1). The remaining 16 CSF analytes showed only weak correlations to other analytes in the dataset (mean $r^2$=0.16, and maximum $r^2$=0.35).

## CSF Analytes associated with ptau$_{181}$-A$\beta_{42}$ ratio

Next we tested for association between each of the CSF analytes and the ptau$_{181}$-A$\beta_{42}$ ratio. Thirteen analytes were significantly associated with CSF ptau$_{181}$-A$\beta_{42}$ ratio (*p-value*<1.0E-3) and exhibited the same direction of effect in the Knight-ADRC and the ADNI series (Table 1; and see Table S3 in Supplement 1 for the exhaustive list of the analytes evaluated); Eleven of these CSF analytes showed a mean $r^2$=0.58 with each other (Figure 1), while the other two, Sortilin (S0RT1) and Tumor necrosis factor–Related Apoptosis-Inducing Ligand R3 (TRAIL-R3), exhibited a slightly lower correlation ($r^2$=0.49 and 0.48, respectively) (Figure 1).

CSF Heart Fatty acid binding protein (H-FABP) was the most significantly associated analyte in both series (*p-value*= 4.77E-18 and 7.40E-18 for the Knight-ADRC and the ADNI studies, respectively), and was associated with higher levels of H-FABP in individuals with CSF ptau$_{181}$/A$\beta_{42}$ ratios indicative of AD (Figure 2a): the regression coefficients for the two studies (Table 1) did not differ significantly (F-Test *p-value*=0.24).

To investigate whether these associations were apparent in non-demented and demented individuals we combined the subjects from both studies and stratified them by the presence or absence of cognitive impairment. A total of 236 subjects had a CDR>0 and low CSF A$\beta_{42}$ levels, which we employed as a proxy for brain A$\beta_{42}$ deposition (16) (cutoff=500 pg/ml and 192 pg/ml for the Knight-ADRC and the ADNI studies, respectively). To evaluate the impact of A$\beta_{42}$ deposition we further stratified the 300 cognitively normal subjects (CDR=0) by their CSF A$\beta_{42}$ levels, distinguishing the 192 “clean” controls from the 105 preclinical subjects with lower CSF A$\beta_{42}$ levels. Each of the 13 CSF analytes was significantly associated (*p-value*<5.0E-4) in every stratum (Table 2). The ANCOVA analysis revealed that the effect size for CSF H-FABP was significantly lower in controls, compared to the inferred preclinical and clinically assessed cognitively impaired individuals (*p-value*=4.44E-04 and 5.87E-03 respectively); and that there was no significant difference in the effect observed in the preclinical and symptomatic cases (Figure 3). This is reflected in the different levels of CSF H-FABP among the strata. While H-FABP levels discriminate cognitively impaired individuals from preclinical AD (*p-value*= 5.05-03), the levels of H-FABP are not significantly different in preclinical individuals from the clean controls, indicating that early amyloid-β plaque deposition does not affect the association of CSF H-FABP with the CSF ptau$_{181}$-A$\beta_{42}$ ratio. Importantly, the association of these analytes with the CSF ptau$_{181}$-A$\beta_{42}$ ratio is robust to the presence of clinically assessed cognitively impaired individuals with uncharacteristic high levels of CSF A$\beta_{42}$. (Table S4 in Supplement 1).

In contrast, the plasma levels of these analytes were not significantly associated with CSF ptau$_{181}$-A$\beta_{42}$ ratio (*p-values*>0.05) in either the Knight-ADRC or the ADNI series (consistent with the null correlation between the CSF and plasma levels of each analyte) (Table S5 in Supplement 1).

### CSF heart fatty acid binding protein is associated with ptau$_{181}$-A$\beta_{42}$ ratio

To determine whether each of the novel analytes provided independent information to predict the CSF ptaui$_{181}$-A$\beta_{42}$ ratio, we combined the subjects from both studies and evaluated joint models that included CSF H-FABP and other CSF analytes from the candidate set, one at a time. Only Hepatocyte Growth Factor (HGF) remained significant after inclusion of CSF H-FABP in the model (p-value=8.56E-06) (Table S6 in Supplement 1).

CSF APOE, which is also associated with the CSF ptaui$_{181}$-A$\beta_{42}$ ratio (Table 1) has previously been reported to be associated with AD (17). To determine whether the previously reported association of CSF APOE levels with CSF ptau$_{181}$-A$\beta_{42}$ ratio is independent of the other analytes, we extended the models to include CSF APOE. Only CSF Angiopoietin 2 (ANGPT2) and CSF Angiotensin-Converting Enzyme (ACE) show a decrease in the significance of their association (*p-values*=3.53E-03 and 3.34E-02), while the other CSF analytes remained significant (p-value<1.0E-3) (Table S6 in Supplement 1), indicating they are capturing additional information. Similarly, the joint analysis of CSF H-FABP and VILIP-1(9) or YKL-40(10) demonstrated that CSF H-FABP is providing non-redundant information to the predictive model for CSF ptau$_{181}$-A$\beta_{42}$ ratio (See text in Supplement 1).

To determine whether CSF H-FABP levels were a strong predictor of future conversion to CDR>0, we compared the association of CSF H-FABP levels with CDR at LP and CDR after a mean of 4 yrs following LP. We tested the association of CSF H-FABP with CDR at LP and found that it is significantly associated in the ADNI study but marginally associated in the Knight-ADRC study (*p-values*= 1.55E-04 and 8.66E-02 respectively) (Figure 2b). We also evaluated the association with the latest available evaluation of CDR (mean elapsed years=4.29; and SD=2.30 years) and found it significant (*p-value*=3.72E-03) for the Knight-ADRC study (36 converted from CDR=0 to CDR>0 and for the ADNI study (*p-value*=6.17E-05; mean elapsed years=3.90; and SD=1.92 years). We observed a similar trend when we analyzed the CDR sum of boxes (CDR-SB), (25) and the Mini-mental state examination (MMSE) (26). H-FABP levels in the ADNI study were strongly associated with both endophenotypes at LP and the latest available evaluations, while in the Knight-ADRC study H-FABPH levels were significantly associated (p<0.05) only for the latest evaluations (Table S7 in Supplement 1).

To test whether the mRNA expression of H-FABP is also associated with CDR, we measured the mRNA levels in parietal lobe tissue from 112 subjects (73 AD cases and 39 controls) (27). mRNA levels of H-FABP in parietal lobe were not associated with expiration CDR (p-value=0.76) or Braak and Braak staging of the pathology (*p-value*=0.46) (28).

### CSF H-FABP as a predictor of developing cognitive impairment

To determine whether higher levels of CSF H-FABP predict conversion from CDR=0 to CDR>0) we performed a survival analysis. The Cox regression analysis showed similar hazard ratios for CSF H-FABP (Hazard ratio = 1.58, 95% CI = 1.02–2.44; *p-value* = 3.70-02), CSF ptau$_{181}$ (2.04; 95% CI=1.11–3.71; *p-value*=1.91E-02) and CSF A$\beta_{42}$ (2.93;

95% CI = 1.15–7.42; *p-value*= 2.74E-02). In addition, we compared the survival curves for CSF $ptau_{181}$-$A\beta_{42}$ ratio (11) and CSF H-FABP-$A\beta_{42}$ ratio (29). Both ratios showed similar results (Figure 4) (Cox proportional hazard: ratio=2.05 and 1.85; p-value=2.14E-03 and 3.93E-03; 95% CI= 1.29–3.27 and 1.21–2.79 for CSF $ptau_{181}/A\beta_{42}$ and CSF H-FABP/$A\beta_{42}$ respectively). Importantly, only 62% of the subjects were assigned to the same tercile when stratified by these ratios (i.e. P ($Q_{CSF\ ptau/A\beta42}$ = $Q_{CSF\ H\text{-}FABP/A\beta42}$)=0.65). Additionally, we observed that the CSF H-FABP-$A\beta_{42}$ ratio is associated with CDR-SB (Cox proportional hazard: ratio=1.76; p-value=2.83e-05; 95% CI= 1.35–2.29); and it is also associated with the MMSE (Cox proportional hazard: ratio=1.74; p-value=1.47e-04; 95% CI= 1.30–2.32)

## CSF Macrophage Migration Inhibitory Factor and Vascular Endothelial Growth Factor are also associated with the CSF $ptau_{181}$-$A\beta_{42}$ ratio

We investigated whether multivariate models would detect analytes that did not show strong evidence of association in single analyte models. Our approach included a discovery phase in which we made use of stepwise regression analysis to optimize a multivariate model for the CSF $ptau_{181}$-$A\beta_{42}$ ratio in one of the datasets, which was followed by a replication phase, in which the model was applied in the second dataset.

The multivariate model optimized for the Knight-ADRC dataset (Table 3a) showed an increased $R^2$ (0.14 for the discovery set and 0.12 for the ADNI study). It included H-FABP and HGF as well as CSF Vascular Endothelial Growth Factor (VEGF), Macrophage Migration Inhibitory Factor (MIF), Thrombomodulin and Adiponectin (Table 3a). The model optimized for the ADNI dataset (Table 3b) also increased the $R^2$ for both datasets (0.20 and 0.08 for the ADNI and Knight-ADRC dataset respectively). This model also selected H-FABP, HGF, VEGF and MIF. We constructed a multivariate model with these four analytes and observed that all of them remained significantly associated with CSF $ptau_{181}$-$A\beta_{42}$ ratio in the two datasets (Table 3c), reflecting an increased goodness of fit for the model (increment of $R^2$=0.08 and 0.09 for the Knight-ADRC and the ADNI datasets, respectively; Table 3c). To evaluate whether the correlation among the analytes produced spurious results, we applying principal component analysis to these selected analytes and evaluated a multivariate model with the values rotated. We found that that all of them remained significant (*p-value*<0.05), indicating that regardless of their correlation these analytes provide additional information.

CSF MIF levels show a trend toward association with the CSF $ptau_{181}$-Aβ42 ratio in the single analyte analysis (Table S3 in Supplement 1). Although CSF VEGF is mildly positively associated in the single analyte analysis (Table S3 in Supplement 1), it has a negative effect in the multivariate model. The stratified analysis revealed that the change of direction occurs only in the cases (Figure S2 in Supplement 1).

The random forest method was also used to analyze these datasets and also highlighted the importance of H-FABP, MIF, HGF and VEGF. These analytes are among the top 6 for the Knight-ADRC dataset; and H-FABP, HGF and MIF are among the top 8 analytes for the ADNI dataset (Table S8 in Supplement 1).

## Discussion

The goal of this study was to identify novel CSF biomarkers for AD using a discovery dataset collected by the Knight ADRC and a replication cohort ascertained by ADNI. Although the ascertainment and structure of the datasets is quite different, we were able to identify several biomarkers that showed consistent effects across the two datasets. The RBM discovery MAP 1.0 panel includes 64 analytes that are not totally independent of one another. We made use of the correlation structure of these analytes to approximate the number of independent tests, and consequently used this number to adjust our *p-values* for multiple testing. Moreover, we employed this information to understand the apparent excess of analytes associated with the CSF $ptau_{181}$-$A\beta_{42}$ ratio.

A novel feature of our study is the use of the CSF $ptau_{181}$-$A\beta_{42}$ ratio as the outcome variable in our analyses rather than comparing the levels of the test analytes in cases versus controls. The CSF $ptau_{181}$-$A\beta_{42}$ ratio provides several key advantages over the more traditional approach. It captures the progression of the disease before the onset of clinical symptoms by correcting the misclassification of control subjects with amyloid-β plaques. In addition, this method also correctly assigns clinically demented individuals who do not have Alzheimer's disease. In a traditional analysis these individuals would be included as cases, but when using the $ptau_{181}$-$A\beta_{42}$, ratio would be analyzed as non-AD dementias. We believe that each of these factors contribute to the gain in statistical power of the CSF $ptau_{181}$-$A\beta_{42}$ ratio, that we observed comparing its performance to the usual case-control model in the evaluation of the CSF levels of VILIP-1 and YKL-40.

The analytes associated with CSF $ptau_{181}$-$A\beta_{42}$ ratio were correlated with CSF $ptau_{181}$ but not $A\beta_{42}$ (Figure 1), indicating that they do not reflect the very early Aβ – related events in the development of the disease, but they do discriminate between preclinical and symptomatic AD. Query of the Database for Annotation, Visualization and Integrated Discovery (DAVID) (30) failed to identify any pathway that characterized the candidate set of analytes.

CSF levels of H-FABP, the most significant analyte, were consistently associated with the $ptau_{181}$-$A\beta_{42}$ ratio in both the Knight-ADRC and ADNI datasets, even when the model included other reported biomarkers such as CSF VILIP-1 and YKL-40. Two previous studies have reported the association of CSF H-FABP with AD in other smaller cohorts (31; 32). In the current study we show that this association is present even at very early stages of the disease, as demonstrated by the analysis of cognitively normal subjects with evidence of amyloid-β plaques. One limitation of our analysis is that although both studies are longitudinal, the novel biomarkers have only been measured in cross sectional data. We are therefore unable to address the role of FABP as a novel AD biomarker across the entire course of disease at the current time. Despite this limitation of our study design, we used the available longitudinal data to show how CSF H-FABP can be employed to predict the conversion from cognitive normality to cognitive impairment. It still remains to be determined whether addition of H-FABP in a model including both CSF $ptau_{181}$ and $A\beta_{42}$ levels improves the accuracy of predicting conversion to symptomatic AD. Subjects in different terciles for the CSF H-FABP-$A\beta_{42}$ and the CSF $ptau_{181}$-$A\beta_{42}$ ratios suggest this

trend, but the studies included in our analysis did not provide statistical power to test this hypothesis.

In contrast, H-FABP levels do not predict progression from CDR=0.5 to CDR>0.5, but it is noteworthy that the number of subjects in this analysis is small (N=81) compared to the analysis of conversion from CDR=0 to CDR>0 (N=219). Based on these results we believe that H_FABP may be a very useful biomarker in staging preclinical AD

H-FABP is a member of a family of proteins characterized as lipid chaperones (33) that transport lipids to specific compartments in the cell or even outside the cell (33). It has been demonstrated that *in vitro* fatty acids induce Aβ assembly and modulate the rate of tau polymer assembly (34). Furthermore, genetic studies have implicated several genes involved in lipid metabolism as risk factors for AD, including *APOE* (35) and *ABCA7* (36).

The multivariate analysis of the MAP 1.0 panel of analytes identified distinct complex models for the Knight-ADRC and the ADNI datasets. Nevertheless, these models included a common subset of analytes. CSF H-FABP is part of this subset of selected analytes, as well as HGF, which was reported previously to discriminate AD subjects from other neurodegenerative disorders (32). Similarly VEGF was reported to discriminate controls from moderately-severe AD cases, but not from mild-AD subjects (31). We confirmed the role of these analytes in our dataset by demonstrating an association with CDR at LP.

In summary, the employment of CSF $ptau_{181}$-$A\beta_{42}$ ratio as an endophenotype of AD led to the identification of a set of 13 correlated analytes associated with the disease in two independent cohorts. This set of candidate analytes is extended by 2 additional analytes, which are significantly associated only in the context of multivariate models. Despite the obvious differences in our two datasets (the ADNI study has many symptomatic individuals and fewer nondemented controls while the Knight-ADRC study is largely composed of controls and inferred preclinical AD samples), these analytes were consistently associated with the CSF $ptau_{181}$-$A\beta_{42}$ ratio and thus AD. Our analysis suggests that these analytes are capturing distinct information from that identified by the traditional analytes (CSF $A\beta_{42}$ and tau/$ptau_{181}$) for the disease, and thus describe novel facets of disease pathogenesis. Analysis of the role of these analytes as novel biomarkers of AD pathogenesis in additional datasets will help to determine the specificity of these changes to AD. Longitudinal analyses will also enable characterization of the temporal sequence of analyte changes in the pathological cascade of AD.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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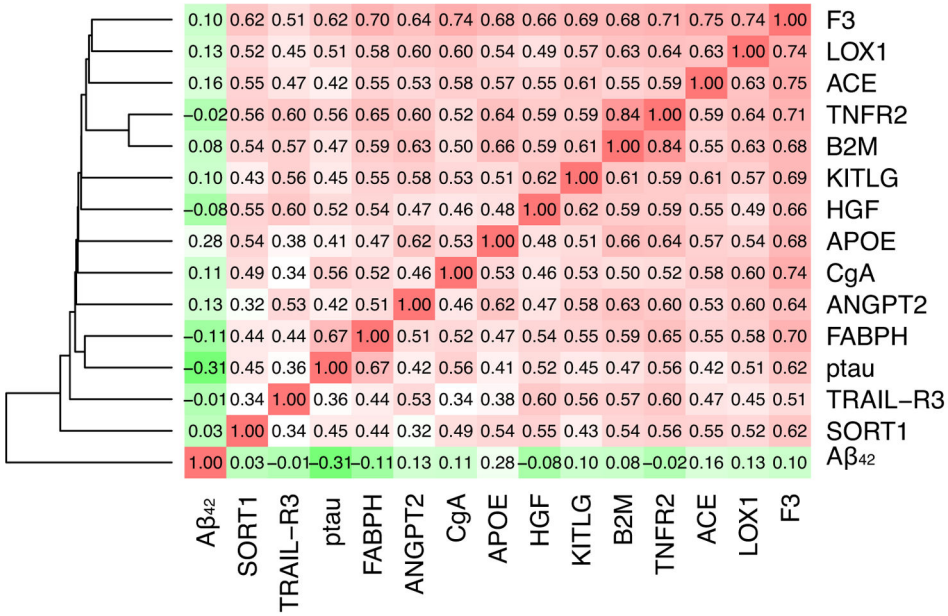


**Figure 1.**

Correlation among the analytes associated, CSF $A\beta_{42}$ and $ptau_{181}$ in the combined Knight-ADRC and the ADNI studies.

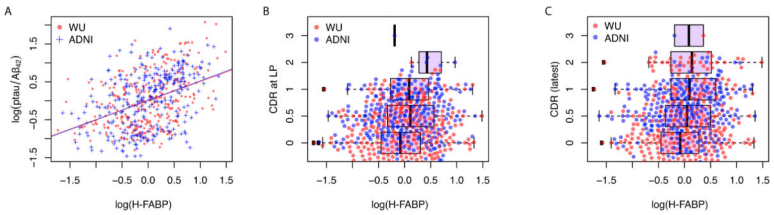


**Figure 2.**

Distribution of CSF H-FABP in the combined Knight-ADRC and the ADNI studies.

A) Linear model of CSF H-FABP compared to CSF ptau/$A\beta_{42}$; Boxplots for CSF H-FABP stratified by B) CDR at lumbar puncture (LP) and C) latest ascertained CDR.

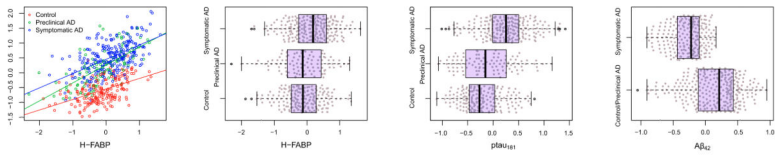


**Figure 3.**

Distribution of CSF H-FABP, $ptau_{181}$ and $A\beta_{42}$ stratified by the cognitive status of the subjects (cognitive normal; preclinical; and cognitive decline) in the combined Knight-ADRC and the ADNI studies..

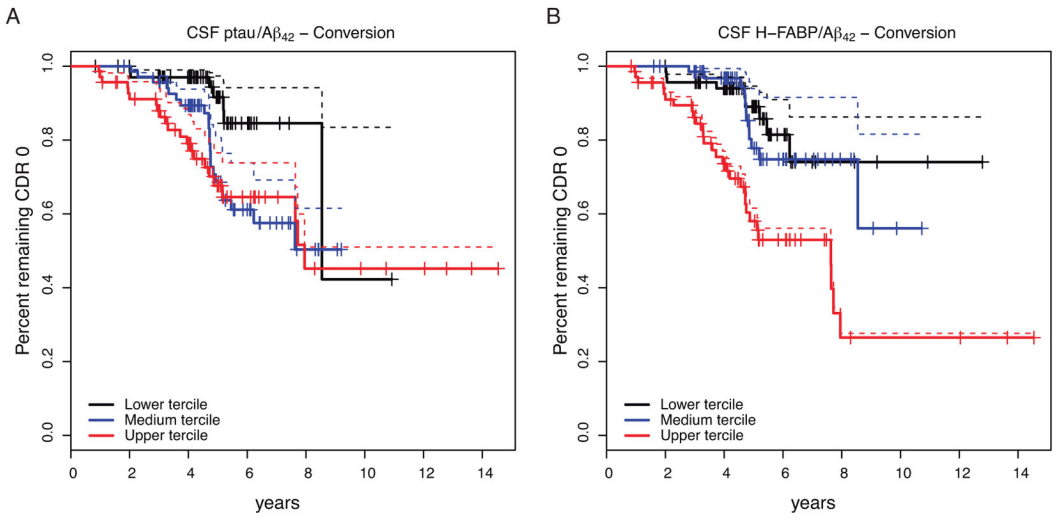


**Figure 4.**

Kaplan-Meyer (solid lines) and Cox survival (dashed lines) curves for the conversion (CDR=0 to CDR>0) for CSF $ptau_{181}/A\beta_{42}$ and CSF H-FABP/$A\beta_{42}$. Only 7.46% of the subjects within the lowest tercile for CSF ptau-$A\beta_{42}$ ratios converted after 5 years (Figure 4a), compared to 23.18% and 27.14% of the subjects in the intermediate and higher terciles. Similarly, 8.69, 10.92 and 34.78% of the subjects within the lower, intermediate and higher terciles of CSF H-FABP/$A\beta_{42}$ values had converted after 5 years.

**Table 1**

CSF analytes significantly associated with CSF ptau$_{181}$-A$\beta_{42}$ ratio (*p-value*<1.0E-03)*

| | Knight-ADRC | | | ADNI | | |
|---|---|---|---|---|---|---|
| **analyte** | ***p-value*** | **Effect** | **c.i.** | ***p-value*** | **Effect** | **ci** |
| Fatty Acid Binding Protein (Heart) - H-FABP | 4.77E-18 | 0.54 | (0.43–0.66) | 7.40E-18 | 0.50 | (0.39–0.61) |
| Tumor Necrosis Factor Receptor 2 - TNFR2 | 2.64E-13 | 0.91 | (0.68–1.15) | 6.11E-07 | 0.55 | (0.34–0.76) |
| Tissue Factor - F3 | 9.18E-13 | 0.54 | (0.40–0.69) | 4.20E-09 | 0.46 | (0.31–0.62) |
| Hepatocyte Growth Factor - HGF | 4.77E-11 | 0.88 | (0.62–1.13) | 5.13E-14 | 0.66 | (0.50–0.82) |
| Chromogranin A-CgA | 1.52E-10 | 1.11 | (0.78–1.44) | 1.27E-07 | 0.95 | (0.60–1.29) |
| Stem Cell Factor - KITLG | 1.08E-08 | 0.64 | (0.42–0.85) | 2.97E-05 | 0.43 | (0.23–0.63) |
| Sortilin - SORT1 | 3.22E-08 | 0.89 | (0.58–1.20) | 9.10E-04 | 0.50 | (0.21–0.79) |
| Lectin Like Oxidized LDL Receptor 1 - LOX1 | 1.54E-07 | 0.51 | (0.32–0.69) | 1.34E-05 | 0.47 | (0.26–0.68) |
| Beta$_2$Microglobulin (B2M) | 3.36E-07 | 0.70 | (0.44–0.97) | 5.07E-05 | 0.53 | (0.28–0.79) |
| TNF-Related Apoptosis-I.L. R3 - TRAIL-R3 | 3.67E-07 | 0.82 | (0.51–1.13) | 4.73E-04 | 0.38 | (0.17–0.58) |
| Angiopoietin 2-ANGPT2 | 2.01E-06 | 0.58 | (0.34–0.81) | 1.90E-04 | 0.36 | (0.17–0.55) |
| Apolipoprotein E - APOE | 4.83E-05 | 0.45 | (0.23–0.66) | 8.26E-04 | 0.40 | (0.17–0.63) |
| Angiotensin-Converting Enzyme - ACE | 6.18E-05 | 0.39 | (0.20–0.58) | 7.86E-04 | 0.34 | (0.14–0.54) |

*Only analytes showing similar effect

**Table 2**

Stratified analysis for cognitively normal, preclinical AD and symptomatic AD

| | Cognitive normal (N=195) | | | Preclinical AD (N=105) | | | Symptomatic AD (N=236) | | |
|---|---|---|---|---|---|---|---|---|---|
| **analyte** | ***p-value*** | **effect** | **c.i.** | ***p-value*** | **effect** | **c.i.** | ***p-value*** | **effect** | **c.i.** |
| Fatty Acid Binding Protein (Heart) - H-FABP | 1.67E-10 | 0.30 | (0.35–0.66) | 8.42E-14 | 0.60 | (0.46–0.74) | 1.59E-22 | 0.49 | (0.40–0.58) |
| Tumor Necrosis Factor Receptor 2 - TNFR2 | 1.41E-09 | 0.51 | (0.30–0.49) | 1.45E-09 | 1.07 | (0.75–1.39) | 1.64E-14 | 0.70 | (0.53–0.87) |
| Tissue Factor - F3 | 7.30E-14 | 0.40 | (0.25–0.59) | 1.49E-13 | 0.72 | (0.55–0.89) | 1.10E-23 | 0.63 | (0.52–0.74) |
| Hepatocyte Growth Factor - HGF | 1.71E-06 | 0.42 | (0.38–0.85) | 8.53E-09 | 1.04 | (0.71–1.37) | 1.86E-16 | 0.66 | (0.51–0.81) |
| Chromogranin A - CgA | 7.74E-07 | 0.61 | (0.32–0.59) | 3.13E-11 | 1.50 | (1.10–1.90) | 4.78E-25 | 1.45 | (1.21–1.70) |
| Stem Cell Factor - KITLG | 1.19E-10 | 0.46 | (0.13–0.57) | 4.59E-10 | 0.99 | (0.71–1.27) | 3.40E-16 | 0.71 | (0.55–0.87) |
| Sortilin - SORT1 | 2.30E-03 | 0.35 | (0.30–0.56) | 1.49E-06 | 1.05 | (0.64–1.45) | 1.88E-07 | 0.67 | (0.42–0.91) |
| Lectin Like Oxidized LDL Receptor 1 - LOX1 | 4.08E-10 | 0.43 | (0.46–0.80) | 1.30E-10 | 0.85 | (0.62–1.09) | 1.67E-11 | 0.57 | (0.41–0.73) |
| $Beta_2$Microglobulin (B2M) | 4.75E-12 | 0.63 | (0.22–0.59) | 5.38E-07 | 0.99 | (0.63–1.36) | 1.73E-09 | 0.64 | (0.44–0.85) |
| TNF-Related Apoptosis-I.L. R3 - TRAIL-R3 | 2.66E-05 | 0.41 | (0.42–0.69) | 1.12E-04 | 0.86 | (0.44–1.29) | 1.58E-03 | 0.32 | (0.12–0.52) |
| Angiopoietin 2 - ANGPT2 | 2.66E-14 | 0.55 | (0.22–0.56) | 2.86E-05 | 0.75 | (0.41–1.08) | 1.13E-08 | 0.49 | (0.33–0.65) |
| Apolipoprotein E - APOE | 6.82E-06 | 0.39 | (0.26–0.52) | 1.29E-06 | 0.76 | (0.47–1.05) | 1.68E-11 | 0.59 | (0.42–0.75) |
| Angiotensin-Converting Enzyme - ACE | 1.53E-08 | 0.39 | (0.46–0.74) | 2.32E-07 | 0.67 | (0.43–0.90) | 3.56E-12 | 0.59 | (0.43–0.75) |

## Table 3

Multivariate models. Stepwise optimization in A) the Knight-ADRC study, and B) the ADNI study. C) A minimal model that includes the analytes commonly selected in A and B.

| A) | Inferred Knight-ADRC Model | | Replication on ADNI | |
|---|---|---|---|---|
| | *p-value* | effect | *p-value* | effect |
| **H-FABP** | 3.18E-08 | 0.44 | **1.74E-11** | 0.47 |
| **VEGF** | 1.01E-05 | −0.63 | **1.85E-04** | −0.66 |
| TNF.RII | 8.56E-04 | 0.63 | 1.08E-01 | 0.22 |
| **Thrombomodulin** | 2.03E-03 | −0.37 | **1.01E-03** | −0.39 |
| ACE | 2.25E-03 | −0.36 | 8.66E-01 | −0.02 |
| **Adiponectin** | 2.29E-03 | 0.17 | **7.98E-03** | 0.23 |
| **HGF** | 2.69E-03 | 0.51 | **5.60E-08** | 0.60 |
| **MIF** | 3.30E-03 | 0.33 | **5.88E-04** | 0.16 |
| SAP | 4.26E-03 | −0.21 | 9.67E-01 | 0.00 |
| CXCL9 | 8.17E-03 | −0.14 | 9.23E-01 | −0.01 |
| FasL | 1.12E-02 | 0.21 | 2.95E-01 | −0.10 |
| age | 1.33E-01 | 0.01 | 3.17E-01 | −0.01 |
| sex | 4.84E-01 | −0.05 | 3.57E-01 | −0.07 |
| APOE Genotypes | 2.80E-07 | 0.14 | 3.64E-11 | 0.19 |
| R squared/BIC | 0.50/−323.91 | | 0.56/−222.42.68 | |

| B) | Replication on Knight-ADRC | | Inferred ADNI Model | |
|---|---|---|---|---|
| | *p-value* | effect | *p-value* | effect |
| **H-FABP** | **2.50E-09** | 0.44 | 5.06E-16 | 0.50 |
| **HGF** | **2.39E-02** | 0.37 | 2.19E-11 | 0.68 |
| **VEGF** | **1.65E-04** | −0.60 | 4.99E-06 | −0.68 |
| Complement 3 | 7.57E-01 | −0.03 | 2.62E-05 | −0.63 |
| ANGPT2 | 4.04E-01 | 0.12 | 2.10E-04 | 0.41 |
| **APOA** | **1.13E-02** | −0.26 | 2.58E-04 | 0.39 |
| **MIF** | **2.08E-02** | 0.33 | 6.71E-04 | 0.14 |
| Fibrinogen | 2.02E-01 | 0.09 | 1.73E-03 | −0.12 |
| Prolactin | 6.80E-01 | −0.04 | 9.88E-03 | −0.26 |
| TRAIL.R3 | 8.13E-01 | 0.04 | 1.70E-02 | −0.28 |
| sex | 4.96E-01 | 0.05 | 8.60E-01 | −0.01 |
| age | 7.24E-03 | 0.01 | 9.89E-01 | 0.00 |
| APOE Genotypes | 7.90E-08 | 0.16 | 6.16E-17 | 0.22 |
| R squared/BIC | 0.44/−296.91 | | 0.60/−370.60 | |

| C) | Knight-ADRC | | ADNI | |
|---|---|---|---|---|
| | *p-value* | effect | *p-value* | effect |
| H-FABP | 6.44E-10 | 0.45 | 1.87E-14 | 0.50 |
| HGF | 3.25E-02 | 0.33 | 5.93E-09 | 0.51 |
| MIF | 6.27E-04 | 0.40 | 3.11E-04 | 0.16 |
| VEGF | 3.47E-05 | −0.59 | 1.32E-06 | −0.66 |
| age | 6.78E-03 | 0.01 | 3.51E-01 | 0.00 |
| sex | 3.27E-01 | 0.06 | 9.73E-01 | 0.00 |
| APOE Genotypes | 9.46E-09 | 0.16 | 1.44E-14 | 0.21 |
| R squared/BIC | 0.42/−336.92 | | 0.53/−360.04 | |