Biomarkers Consortium Project
BACE activity and sAPPβ measures as Novel Cerebrospinal Fluid (CSF) Biomarkers in Alzheimer's Disease (AD)

Data Primer

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Background

The data described within this document represents the work of the Biomarkers Consortium Project "Use of Targeted Multiplex Proteomic Strategies to Identify Novel CSF Biomarkers in AD." This overall project was submitted to the Biomarkers Consortium Neuroscience Steering Committee by a subgroup of the Alzheimer's Disease Neuroimaging Initiative (ADNI) Industry Private Partner Scientific Board (PPSB) for execution and was managed by a Biomarkers Consortium Project Team that includes members from academia, government and the pharmaceutical industry (see Appendix I). Funding for this project was provided by the Alzheimer's Drug Discovery Foundation, Eisai, Lilly, Merck, Pfizer, and Takeda. The data described within this document addresses the second aim within the overall project: To examine BACE1 enzymatic activity and secreted APP-beta (sAPPβ) in CSF from the ADNI baseline and one year CSF samples. This original aim was modified subsequent to project approval to examine only the baseline samples, based on a team decision.

Project rationale

Amyloid-beta (Aβ) levels increase in the brains of subjects with Alzheimer's Disease (AD). Increased activity of enzymes that cleave Aβ from the amyloid precursor protein (APP) may lead to this elevation. Beta secretase-1 (BACE1) liberates the free amino terminus of Aβ and increased BACE1 activity in brain and body fluids of AD patients has been reported in relatively small patient cohorts. Several groups reported increased BACE1 protein and enzymatic activity in AD brain (Fukumoto et al., 2002; Yang et al., 2003; Li et al., 2004; Johnston et al., 2005; Ahmed et al., 2010) or cerebrospinal fluid (CSF; Holsinger et al., 2004; Verheijen et al., 2006; Zhong et al., 2007; Zetterberg et al., 2008) compared with control subjects. However, others have observed either no change or reductions in BACE1 levels in brains or CSF from sporadic AD subjects (Preece et al., 2003; Wu et al., 2008). Overall, this data set supports the hypothesis that BACE1 activity is elevated in AD.

Several studies have also measured CSF levels of sAPPβ, the N-terminal secreted fragment of APP generated by BACE 1, and reported conflicting results ranging from an increase in these fragments in AD to no change or a slight decrease compared with control samples (Palmert et al., 1990; Prior et al., 1991; Van Nostrand et al., 1992; Sennvik et al., 2000; Olsson et al., 2003; Lewczuk et al., 2008; Zetterberg et al., 2008). Levels of sAPPβ product, coupled with a direct measure of secretase activity are two methods to assess BACE activity.

Therefore, the two primary aims of this study are to evaluate in CSF samples from ADNI-1

- BACE1 activity
- sAPPβ product of BACE1 activity

Significance

The primary causes of sporadic AD are unknown. Elevated BACE1 activity may be one of the causes and the ADNI sample set represents a large and well-characterized cohort in which to test this hypothesis. Although BACE1 is primarily a trans-membrane, cell-localized enzyme, a cleaved, active form of BACE1 is present in CSF. This form lacks the C-terminus that anchors it to the membrane (Wu, et al., 2008). Whether this cleaved form correlates with ongoing BACE1 activity in the brain is not known, but may be surmised if levels of CSF BACE activity and sAPPβ (thought to be generated primarily in brain) correlate. Several AD therapeutics are in the clinic, and overall treatment response could vary if patients have a wide range of BACE1 activities. For example, patients with elevated BACE1 activity could have a more aggressive disease, analogous to patients with the apoE4 genotype. In a clinical trial of passive anti-amyloid immunotherapy, patients with apoE4 genotype had reduced positive cognitive signals and increased adverse events compared to patients without an apoE4 allele (Salloway et al., 2009). It may be possible to stratify patients on the basis of BACE1 activity to better understand and monitor both disease progression as well as response to treatment with therapeutic agents. The ADNI-1 CSF samples were evaluated for both BACE1 activity and sAPPβ levels to determine whether there is a change in BACE1 activity in AD patient CSF compared to age-matched controls.

Experimental Design

The data contained herein were generated using analytically-validated assays that were the subject of two prior publications:

- Wu, et al., (2011) Decrease in brain soluble amyloid precursor protein β (sAPPβ) in Alzheimer's disease cortex. J Neurosci Res. 89:822-32
- Wu, et al., (2008) Decrease in age-adjusted cerebrospinal fluid beta-secretase activity in Alzheimer's subjects. Clin Biochem. 41:986-96

A total of 402 samples were obtained in blinded fashion from ADNI sample stocks held at the University of Pennsylvania site (U Penn) and thawed on ice. BACE1 activity and sAPPβ were measured concurrently, using aliquots obtained from the same vial at the same thaw according to the protocols found in the two manuscripts cited above (with methods and assay characteristics detailed further below). Standard curves were generated with recombinant BACE1 or sAPPβ (as described in the manuscripts)

and used to calculate absolute values within the patient samples. The blinded data was subjected to a statistical quality control review at Merck and Company and forwarded, along with the raw data to U Penn for unblinding and preparation for posting to the ADNI website.

NOTE: Figures 1-7 and Tables 1-2 are derived from the papers listed above (Wu, et al., 2008 and Wu, et al., 2011) and support analytical and pharmacological validation of BACE activity and sAPPβ assays. Some assay metrics are slightly different compared to the recent data accompanying the ADNI sample analysis, but still within acceptable ranges. For additional details of reagents produced at Merck, refer to the Wu, et al., manuscripts.

BACE activity assay

Diagram of two step assay: 1) Enzyme + substrate; 2) ELISA to measure product

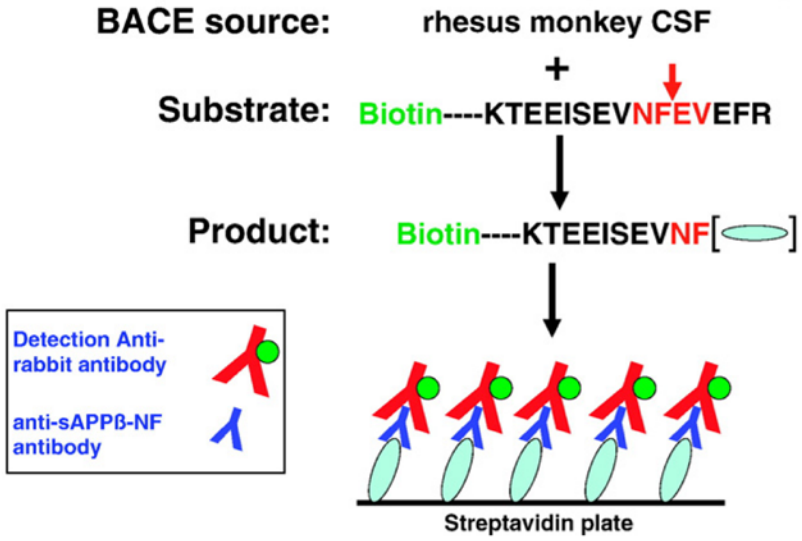

Fig. 1. BACE activity assay format. A source of BACE using either purified recombinant truncated BACE, human or rhesus monkey CSF was co-incubated with a BACE substrate. A biotinylated substrate "KTEEISEVNFEVEFR" was used where the wild type KMDA–BACE cleavage sequence in APP was replaced with an artificial optimized NFEV sequence. The BACE cleavage product was then detected using an "anti-NF" neo-epitope specific antibody and an indirect horseradish peroxidase (HRP) or alkaline phosphatase (AP) development of the reaction.

Reagents

1. **BACE running buffer, pH 4.5:** 50 mM NaOAc (Sigma, S2889), 0.01% BSA (Sigma, A3294), 15 mM EDTA (Promega, V4231), 0.2% CHAPS (Pierce, 28300), 1 mM Deferoxamine Mesylate

(Sigma, D9533), 1 µM pepstatin A (Calbiochem, 516481) pH to 4.5 with glacial acetic acid (Sigma, 320099).

2. **BBACE-1 (purified recombinant Baculo virus expressed BACE) enzyme:** Produced at Merck. Stock = 37 µM.
3. **Substrate (Biotin-KTEEISEVNFEVEFR):** Provided by Merck & Co., Inc. Chemistry Department. Stock = 1 mM in DMSO.
4. **Quench Solution:** 1 M TRIS-HCl, pH 8.0 (Invitrogen, 15568-025).
5. **PBS-T**: PBS + 0.05% Tween-20, pH 7.4.
6. **SuperBlock buffer:** Thermo Scientific, 37515.
7. **CDP-Star**: Applied Biosystems, T-2214.

Part A: BACE Enzyme/Substrate Assay

1. 25 µL of either BBACE-1 standard or ADNI CSF samples was added into a 96-well assay plate (Costar, # 3365). Note: BACE standard curve (200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0) was prepared in BACE running buffer; ADNI CSF samples were thawed on ice and added undiluted.
2. 25 µL of BACE running buffer was then added into each well and incubated at RT with shaking at 40 rpm for 15 min.
3. Then 100 µL of 200 nM substrate prepared in BACE running buffer was added, plate was sealed and incubated at 37C with shaking (40 rpm) for 2.5 hrs.
4. Enzymatic reaction was stopped by adding 50 µL of 1M Tris-HCl, pH 8.0.

Part B: ELISA Assay

5. Transfer the quenched product from above to a HBC streptavidin coated plate (Thermo Scientific, 15503), that was blocked with 0.1% Tween/SuperBlock, and incubate overnight at 4C.
6. Wash plates 2 times with PBS-T.
7. Add 100 µL of substrate absorbed NF-epitope polyclonal antibody (produced at Merck) at 1:2500 (in 0.1% Tween/SuperBlock). Incubate 1hr, RT.
8. Wash plates 3 times with PBS-T.
9. Add 100 µL of goat anti-rabbit IgG-AP at 1:30,000 (in 0.1% Tween/SuperBlock). Incubate 1hr at RT.
10. Wash 5 times with PBS-T.
11. Add 100 µL of CDP-Star. Incubate 30 min at RT.
12. Read luminescence on EnVision (PerkinElmer, model 2104).
13. The conc. of BACE in samples was calculated according to a quadratic fit to the std.

Dynamic range and Least Detectable Dose

The threshold for the lowest limit of reliable quantification (LLRQ) was set as the lowest standard with CV less than 20%. Based on this criterion, the LLRQ of this assay is less than 0.8 pM of recombinant BACE1 standard. This sensitivity limit is typically more than 25-fold lower than the endogenous human CSF BACE1 activity level (**Figure 2**). BACE1 activity within human and rhesus CSF dilutes linearly, without apparent matrix effects (**Figure 3**).

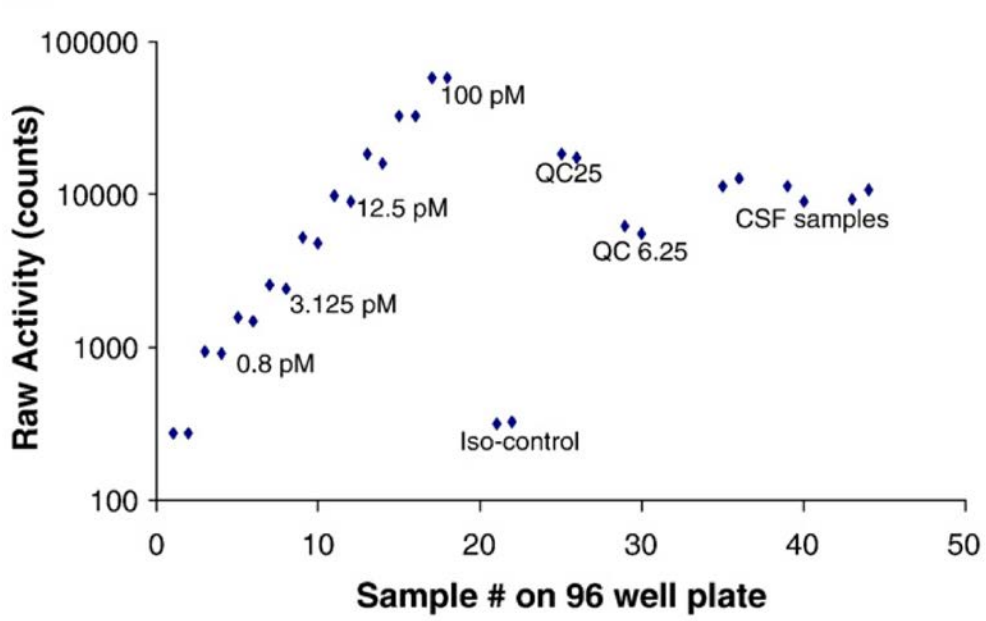

Figure 2

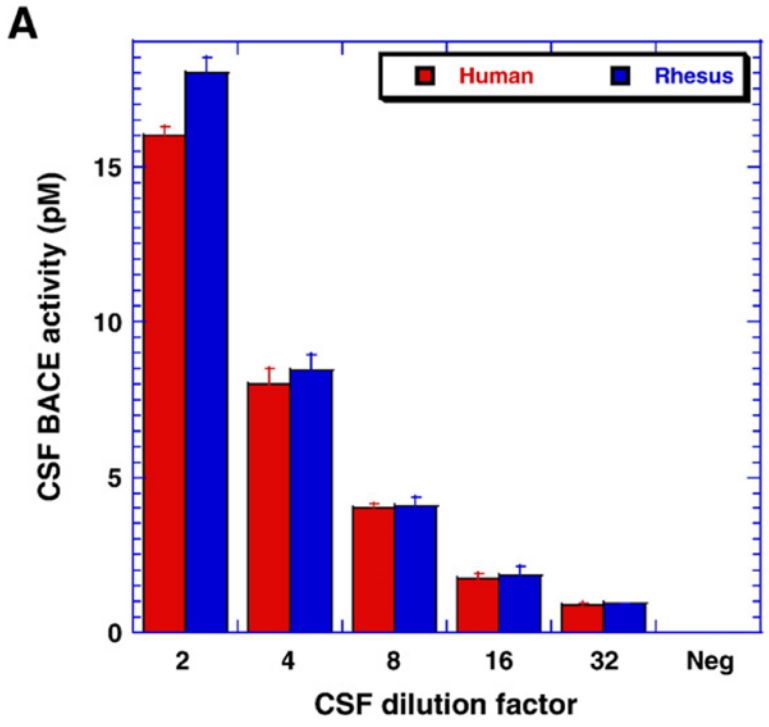

Figure 3

Precision

The reproducibility and precision of the assay was determined by evaluating the coefficient of variation (CV) for the validation samples and rhesus CSF samples with or without spike-in of BACE1. The intra-day CV's for the 6.25 and 25 pM validation samples were < 15%, while the inter-day CV's were < 10%. (Table 1). The CVs for the rhesus CSF were comparable.

Reproducibility of samples and QC

	Intraday		Interday		CSF
	QC-25	QC-6.25	QC-25	QC-6.25	
Average BACE (pM)	27.7	7.3	25	7.3	32.3
SD	3.4	0.4	1.8	0.3	1.8
CV	12.2	5.3	7.2	4.5	5.4
Min	25	6.9	23.2	15	30.6
Max	31.5	7.6	26.8	16.2	34

Table 1

Cross-reactivity

When measured in CSF in the presence of Pepstatin A (**Figure 4**, lane 3), the remaining enzyme activity is due to BACE1 since the BACE1 inhibitor Merck 3 (**Figure 4**, Lane 6) blocked all remaining activity (about 40%). In addition, CSF BACE1 activity was dose-dependently inhibited with Merck-3 (**Figure 5**), and Statin-Val- (not shown). Specificity and window of the solution-based CSF BACE activity assay. Rhesus monkey CSF was incubated with BACE substrate in the presence or absence of protease inhibitor (PI) cocktail, Pepstatin A (10 μM), BACE-specific inhibitor Merck-3 (10 μM) or various combinations thereof. Lane 1 indicates total CSF "BACE" activity, lanes 2, 3, and 4 indicate CSF BACE activity in the presence of PI, Pepstatin A, and Merck-3, respectively. Lane 5 indicates activity in the presence of all 3 inhibitors. Lanes 6, 7 and 8 indicate activity in different combinations of two inhibitors, while lane 10 indicates the activity in CSF following heat inactivation. The assay has a signal to noise ratio of ~20 and is indicated by the signal ratio between lane 8 (activity in the presence of PI and Pepstatin A) and lane 9 or 10, which represents the non-specific signal in the assay. The combination of Pepstatin A and Merck-3 (lanes 5 and 6) can completely inhibit all BACE-specific activity in the CSF samples (n=3 replicates per condition).

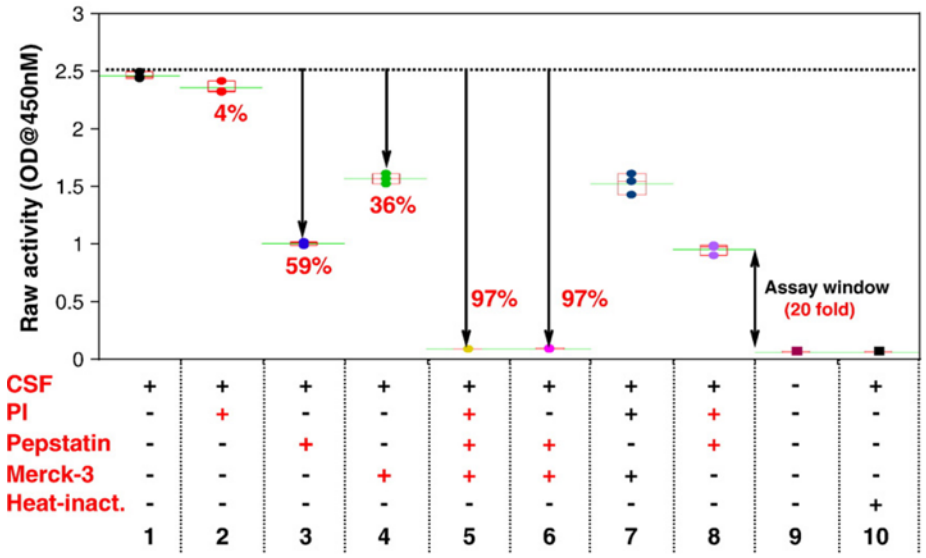

Figure 4

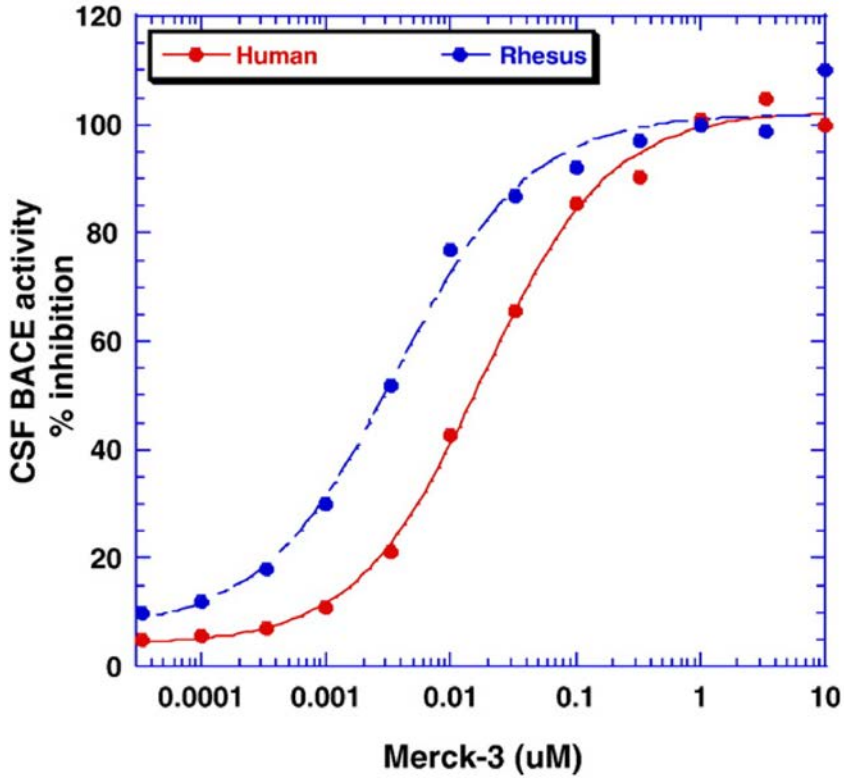

Figure 5

Spike Recovery

The CV's for the rhesus CSF samples (diluted 1:2) following spike-in at 6.25 and 25 pM were < 10% in both the intra-day and inter-day runs The recovery following spike-in in either buffer (not shown) or in normal rhesus CSF was between 99-112% (**Table 2**).

Spike-in recovery

	Intraday		Interday	
	Diluted CSF		Diluted CSF	
	25 pM	6.25 pM	25 pM	6.25 pM
Average BACE (pM)	45	25	41	22
SD	4.1	1.1	1.5	2.1
CV	9.2	4.3	3.7	9.2
% Recovery	110	112	99	100

BACE-1 concentration in neat CSF was 32 pM in this experiment.

Table 2

sAPPβ Assay

Reagents

1. **50 mM Carbonate/Bicarbonate buffer, pH 9.6:** Sigma, C3041.
2. **MRK-3-61 antibody:** Produced at Merck.
3. **PBS-T**: PBS + 0.05% Tween-20, pH 7.4
4. **CDP-Star**: Applied Biosystems, T-2214.
5. **sAPPβ std:**, Stock = 100 nM (Generated at Merck).
6. P2-1-AP: antibody P2-1 purchased from Invitrogen (CA) and coupled with alkaline phosphatase at Merck.

Procedure

1. Costar # 3925 plates were coated with 2.8 µg/mL MRK-3-61 in 50 mM Carbonate/Bicarbonate buffer pH 9.6 (100 µL/well). Plates were incubated overnight at 4C with shaking. The next day, plates were washed 1x with PBS-T (200ul/well) and incubated 5min/RT/shaking. The plates were blocked with 200 µL/well of 3% BSA/PBS. Plates were stored in cold room until use.
2. The ADNI CSF samples were thawed on ice and diluted 1:50 in 3% BSA/PBS.

3. The sAPPβ std. curve started at 200 pM with 2-fold serial dilutions, a 0 pM was included. Std curve (pM): 200, 100, 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78, 0.

Plate Set-up

4. Remove the blocking buffer and pat plates dry.
5. Add 0.1 mL of std. or sample (1:50) per well.
6. Add 0.05 mL of detection antibody P2-1-AP (1:1000 in 3% BSA/PBS/0.3% Tween-20) to each well except the controls. For IgG controls, use IgG-AP (1:8000) in 3% BSA/PBS/0.3% Tween-20.
7. Cover plates with foil seal and incubate overnight at 4C with shaking.
8. The next day, wash the plates 5x with 200 µL/well of PBS-T at RT and allow ~ 10 min between washes.
9. At last wash pat plates dry and add 0.1 mL of CDP-Star. Allow plates to incubate for 30 min at RT and read luminescence using EnVision (PerkinElmer, model 2104).
10. Data was collected and processed.

Least Detectable Dose

The LLRQ of sAPPβ was 1.6 pM, determined as the lowest point above background on the standard curve with %CV less than 20%.

Precision

The coefficient of variation (%CV) was determined for purified proteins (standards run at 40 pM and 10 pM in buffer) and human CSF samples with or without addition of exogenous sAPPβ. The intra-day CVs for the 40 and 10 pM validation samples were <10%, whereas the inter-day CVs were <12%. The CVs for the human CSF samples were 5.2% without addition of exogenous sAPPβ, whereas, after addition of either 40 or 10 pM, CVs were <11%.

Cross-reactivity and Dynamic Range

This assay was highly specific for sAPPβ compared with sAPPα, with a linear signal between 1 and 50 pM of protein (**Figure 6**).

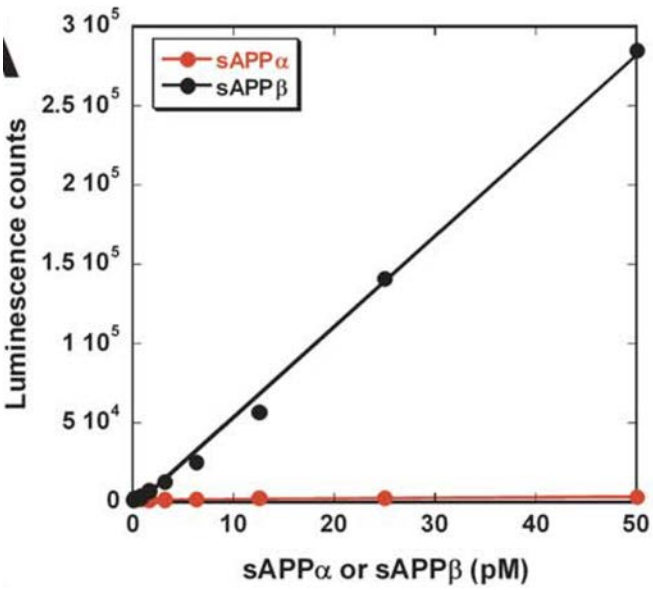

Figure 6

The potential for nonspecific signals was evaluated by performing immunodepletion experiments. Compared with non-immunodepleted samples, a complete loss of sAPPβ signal was observed following immunodepletion with the KM neo-epitope monoclonal antibody (equivalent to that of the negative control), whereas 6E10 and rabbit IgG showed no loss of signal (**Figure7**).

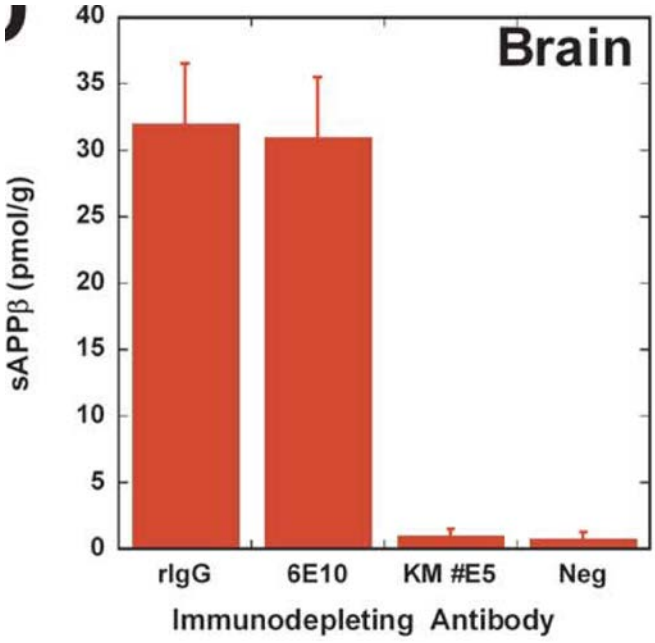

Figure 7

Spike Recovery

The recovery after addition of exogenous sAPPβ to buffer or normal human CSF was 85–100% in both the intra- and the inter-day runs.

BACE Activity and sAPPβ in CSF: Analysis of Quality Control Samples

The quality control samples (QCs) were analyzed to judge the quality and precision of the assay results. QCs for BACE activity and sAPPβ followed the same format. Quality was explored graphically and using a repeated measures model. For each analyte, the following graphs were created. These graphs are contained within the accompanying **Appendix II**.

1. Analyte vs. Plate number
2. Standardized residuals vs. fitted values
3. Coefficient of Variation (CV) vs. mean and Standard Deviation (SD) vs. mean
4. Mean, SD, and CV vs. plate number
5. Counts vs. analyte concentration
6A. Distribution of values for the unknowns
6B. Distribution of log(values) for the unknowns

Results for BACE activity are presented first, followed by results for sAPPβ.

Statistical Model

All estimates were based on modeling log(concentration) where "log" indicates the natural log. For each QC a mixed effects model was fit

$\log(conc_{ij}) = mean + plate_i + e_{ij}$
where
i indexes plate, and j indexes replicates within a plate
$plate_i \sim N(0, \sigma_p^2)$, eij ~ $N(0, \sigma^2)$, $cor(plate_i, e_{ij}) = 0$

REML estimates were obtained for σ_p^2 ($=s_p^2$) and σ^2 ($=s^2$)
Coefficients of variation were estimated as follows,
CVtotal = 100* sqrt(exp($s_p^2 + s^2$)-1)
CVplate = 100* sqrt(exp(s_p^2)-1)
CVresi = 100* sqrt(exp(s^2)-1)

Results for BACE Activity

Table 3 gives estimates of the geometrics mean, and CVs for 4 QCs for BACE activity. The QCs for BACE activity were derived from a pool of 1mL CSF samples acquired from 5 rhesus monkeys. The material was diluted as indicated with assay buffer and run in the assay at 2 wells per sample. Note the QC1 was neat rhesus CSF, analyzed as were the unknown ADNI CSF samples, while QC2, QC3, and QC4 were rhesus CSF diluted 1:2, 1:4, and 1:8, respectively. The geo-means of the signal in the rhesus CSF demonstrates linearity across the entire dilution range. The CV performance for both the neat and 1:8 dilution (7-18%), is within range of the previous publication for both QCs spiked standards and neat rhesus CSF (7-12%, Table 1). The 1:2 and 1:4 dilution demonstrated CV outside the range, driven by higher values across 3/13 plates for the 1:2 dilution and 6/13 plates for the 1:4 dilution, where the values were approximately 2 fold higher in each case. .The estimate of the between plate variance to the total variance, using all 4 QCs was 0.18. Geometric mean BACE activity for the unknown neat CSF samples was 43.0 pM with CV=7.2%, and the neat rhesus CSF had values closest to the unknown ADNI samples

As the neat rhesus CV in both this experiment (**Table 3**) and the validation set (**Table 1**) in the original publication, as well as the ADNI human sample CV are acceptable, we consider the assay overall as having passed quality control evaluation. The QC samples 2 and 3 may have been compromised on some plates due to a handling effect.

	BACE (pM)		
names	Geomean	CV within (%)	CV.total (%)
QC1	**23.6**	**18.0**	**18.0**
QC2	11.0	35.9	41.1
QC3	4.9	44.8	48.8
QC4	2.1	15.5	15.5
QC Btwn Plate/Total Var		0.18	
	Geomean	CV within (%)	
Unknowns	43.0	7.2	

Table 3

Figures described below are found in **APPENDIX II**.

Figure 1.1a shows BACE activity vs. plate number, with the geometric mean for each QC given as a horizontal reference line. There are a few of the diluted QCs with higher than expected values, shown in **Figure 1.1b**.

Figure 1.2 gives the standardized residuals vs. fitted values for each of the QCs.
Figure 1.3 shows that the SD for BACE activity tends to increase with the mean.
Figure 1.4 shows that the mean and precision of BACE was relatively consistent across plates.
Figure 1.5 gives an indication of the counts observed for the standards, unknowns, and QC1.
Figure 1.6A shows that the distribution of the unknown samples is skewed to the right.
Figure 1.6B shows that after a log transformation, distribution of the unknowns is fairly symmetric.

Results for sAPPβ

Table 4 gives estimates of the geometrics mean, and CVs for 4 QCs for sAPPβ. The QCs for sAPPβ were derived from a pool of 1mL CSF samples acquired from 5 rhesus monkeys. The material was diluted as indicated with assay buffer and run in the assays at 2 wells per sample. Note the QC1 was rhesus CSF diluted 1:50, while QC2, QC3, and QC4 were rhesus CSF diluted 1:100, 1:200, and 1:400, respectively. The estimate of the between plate variance to the total variance, using all 4 QCs was 0.17. Geometric mean sAPPβ for the unknown samples, diluted 1:84, was 3760.8 pM with CV=8.2%.

Table 4

	sAPPb (pM)		
names	Geomean	CV within (%)	CV.total (%)
QC1	**1227.6**	**5.9**	**6.4**
QC2	573.6	6.2	7.5
QC3	238.7	8.7	9.5
QC4	109.7	5.2	6.7
QC Btwn Plate/Total Var		0.17	
	Geomean	CV within (%)	
Unknowns	3760.8	8.2	

Figures described below are found in the accompanying **APPENDIX II.**

Figure 2.1 shows sAPPβ vs. plate number, with the geometric mean for each QC given as a horizontal reference line.
Figure 2.2 gives the standardized residuals vs. fitted values for each of the QCs.
Figure 2.3 shows that the SD for sAPPβ tends to increase with the mean.
Figure 2.4 shows that the mean and precision of sAPPβ was relatively consistent across plates.
Figure 2.5 gives and indication of the counts observed for the standards, unknowns, and QC1.
Figure 2.6A shows that the distribution of the unknown samples is fairly symmetric and.
Figure 2.6B shows that a log transformation causes the distribution to be skewed slightly to the left.

Methodology for obtaining the CSF samples:

Table 5 summarizes the demographics of the population selected. A total of 402 CSF samples from the baseline ADNI sample set were assessed (N= 107 Controls, 99 AD, 196 for amnestic mild

cognitive impairment (MCI) 20 technical replicates. Approximately 75% of these baseline CSF samples have matching aliquots from year 1 CSF so that possible future studies on longitudinal change would be possible if funding becomes available for such a follow-up investigation. Of the 196 MCI subjects, 87 subjects had progressed to dementia as of March 29, 2011. In addition the selected samples have additional biomarker data sets available. For example, all samples from controls, MCI and AD subjects have associated CSF Aβ42/tau measures and/or Pittsburgh Compound B (PIB) one year data were included in the AD subset.

The CSF samples were obtained in the morning following an overnight fast at the baseline visit in the ADNI 1 study. For the majority of samples, the time from collection to freezing was within 60 minutes. Processing, aliquoting and storage at -80^{0}C were performed according to the ADNI Biomarker Core Laboratory Standard Operating Procedures.

	Control	MCI	AD
N baseline	106	184	92
Age	76 (64-87)	75 (57-87)	76 (58-88)
Gender M/F (baseline)	53/53	123/61	51/41
ApoE4% (baseline)	24%	52%	69%
MMSE (range)	29.1 (27-30)	26.9 (24-30)	23.6 (20-26)

Table 5

What is posted on the ADNI Website and cautionary notes to data analysis

There is one data set posted on the ADNI website relating to the CSF BACE Study from the Biomarkers Consortium Project. The raw dataset is entitled ***Biomarkers Consortium ADNI CSF BACE activity and sAPPbeta.XLS***. Additional information can be found in the original manuscripts describing the methods: **Wu, et al., 2008 and Wu, et al., 2011**.

The statistical analysis plan is exploratory and not comprehensive. Results from this study will be compared with those from other studies on CSF proteins in AD, and will need confirmation in subsequent studies using other, independent data sets.

The CSF Proteomics Project Team will conduct the analysis described in the attached Statistical Analysis Plan at the time that the unblinded assay results are posted to the ADNI website. Results from this statistical analysis will be made available via scientific meeting presentations and will be submitted for publication in a peer-reviewed journal in the near future.

APPENDIX I

Acknowledgements

In addition to the Project Team Members cited below, the following individuals employed at Merck and Company are acknowledged for their efforts in this study:

Guoxin Wu
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Wan, Hong (Pfizer)
Wildsmith, Kristin (Genentech)

APPENDIX II
Figures representing statistical analysis on blinded data

Figure 1.1a

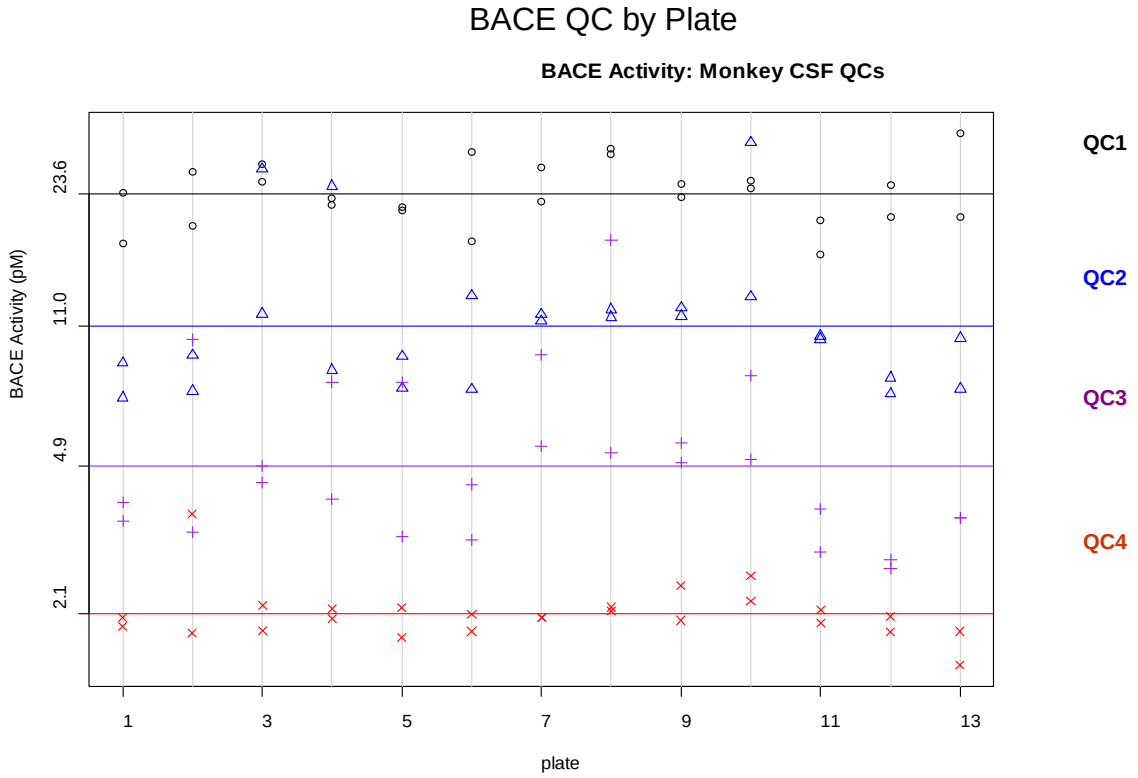


Figure 1.1b

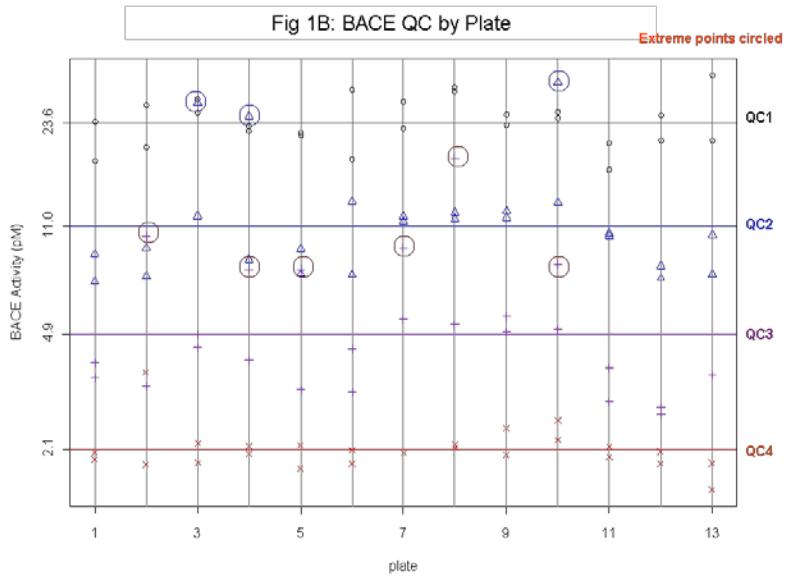


Figure 1.2

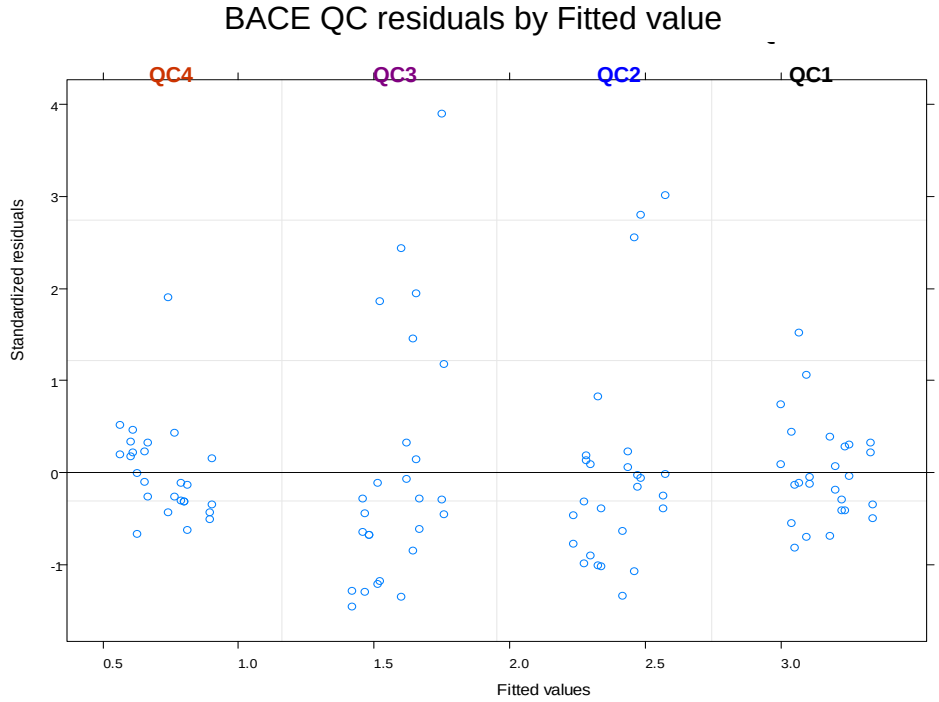

BACE QC residuals by Fitted value
QC4
QC3
QC2
QC1
Standardized residuals
4
3
2
1
0
-1
0.5
1.0
1.5
2.0
2.5
3.0
Fitted values

Figure 1.3

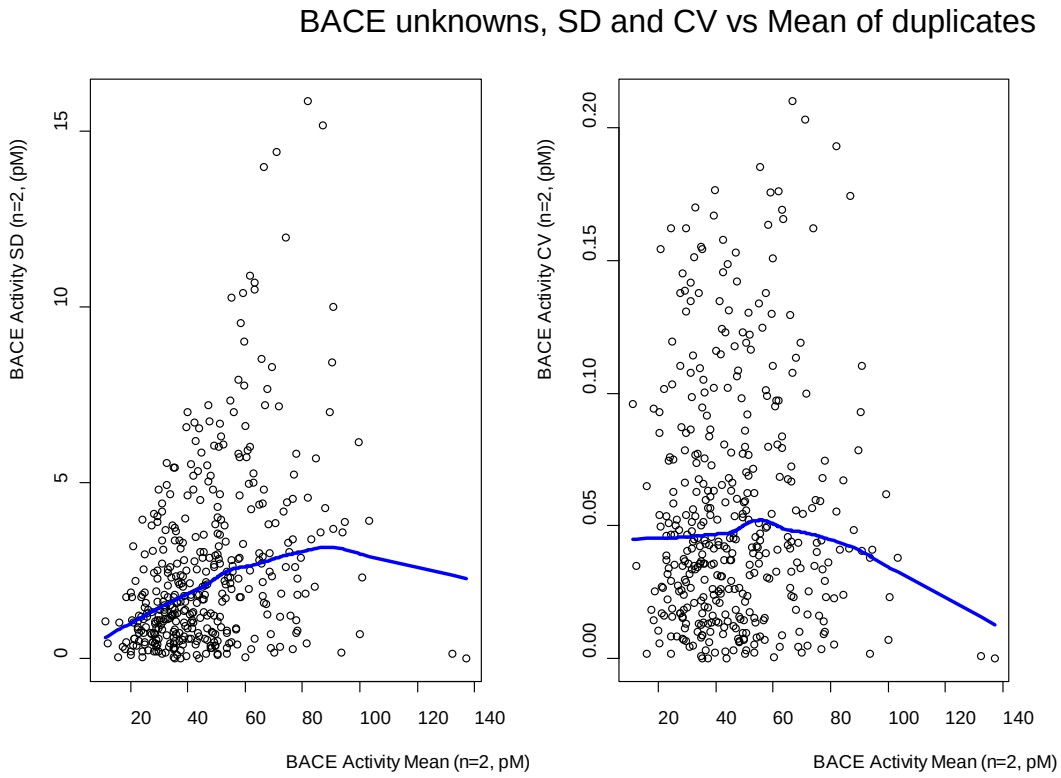


Figure 1.4

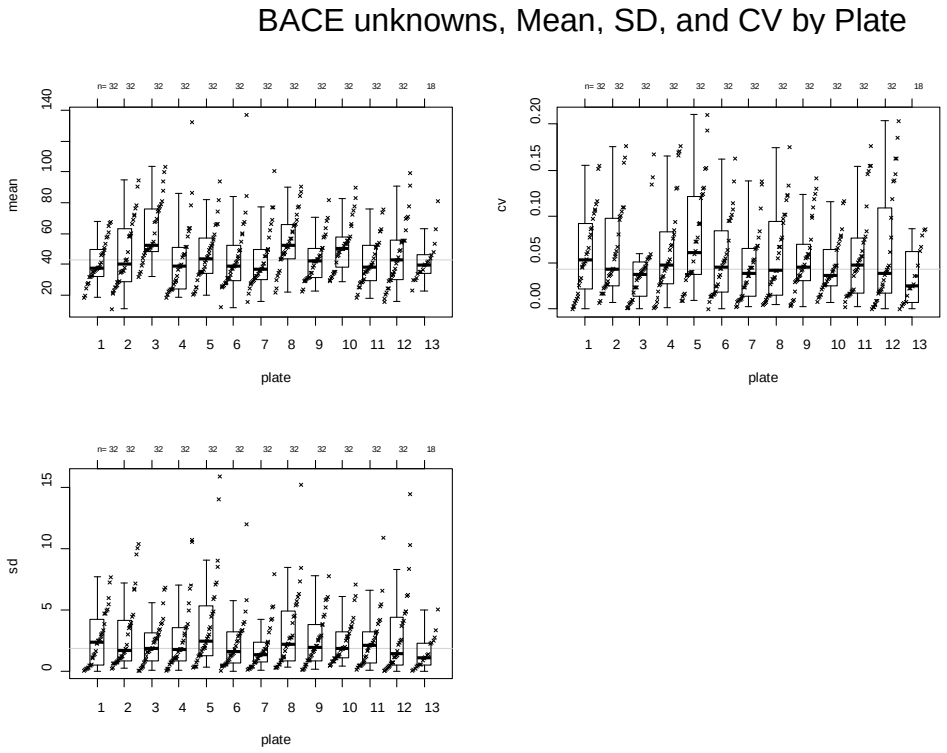


Figure 1.5

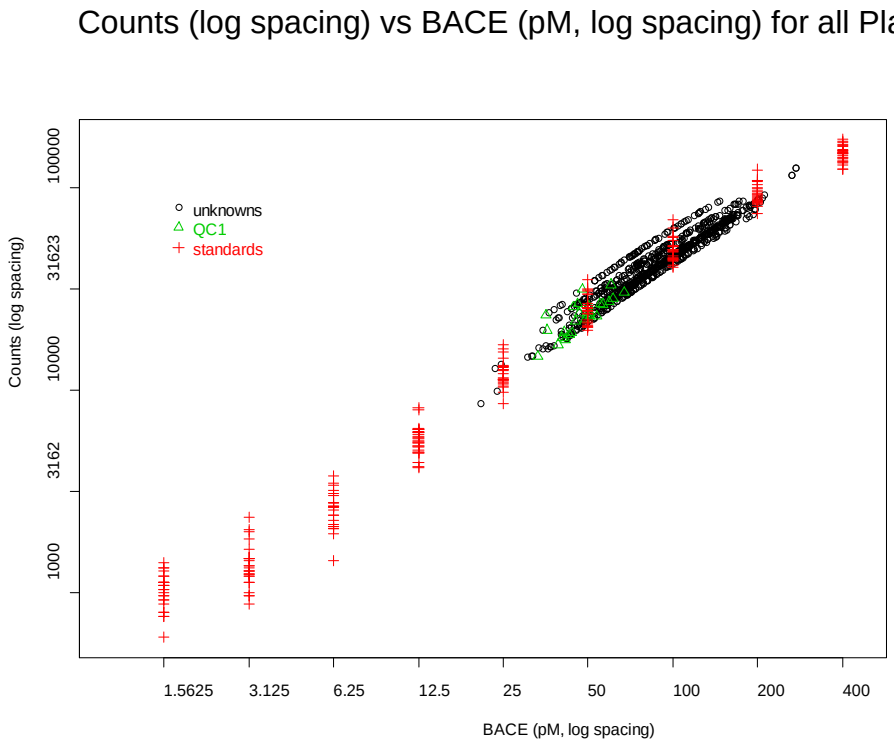


Figure 1.6A

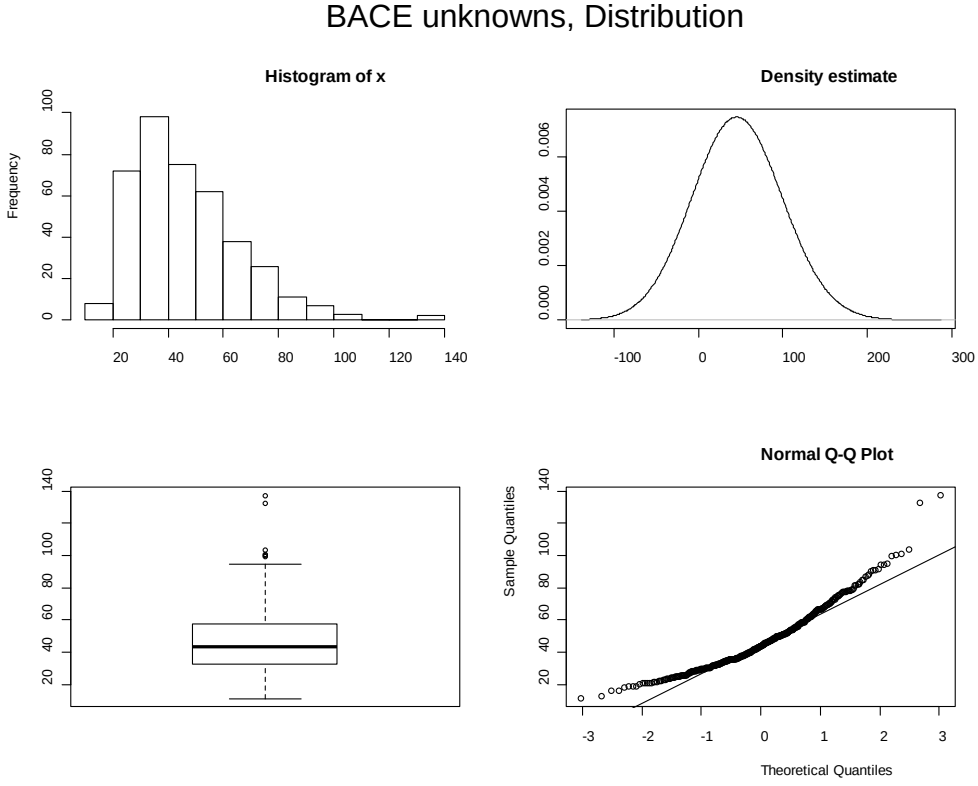


Figure 1.6B

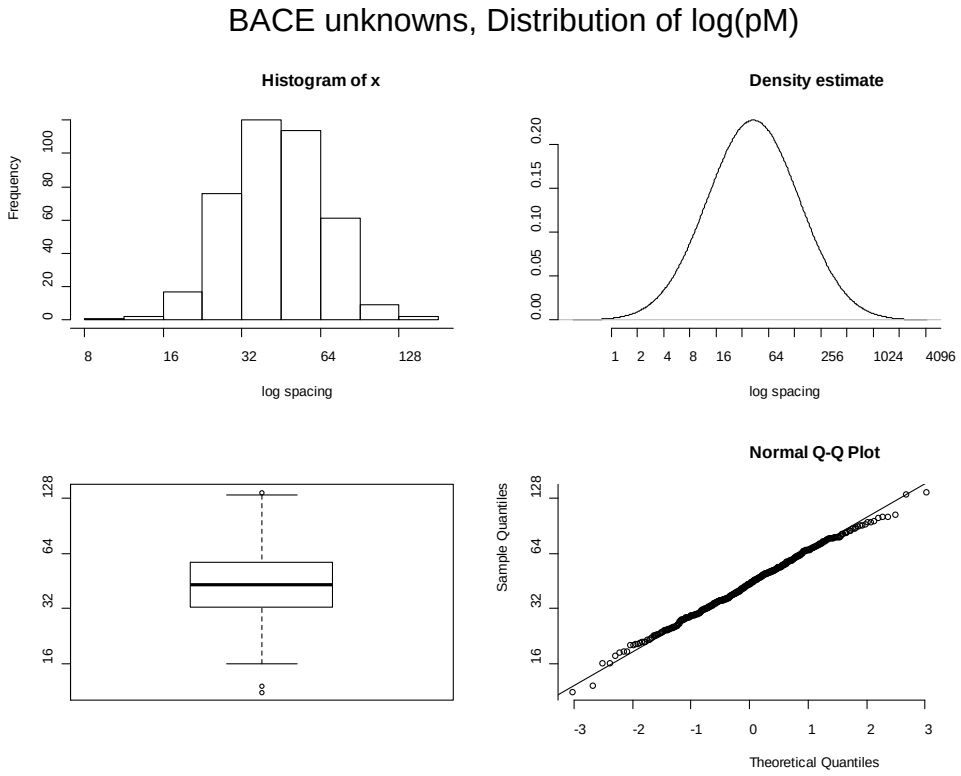


Figure 2.1

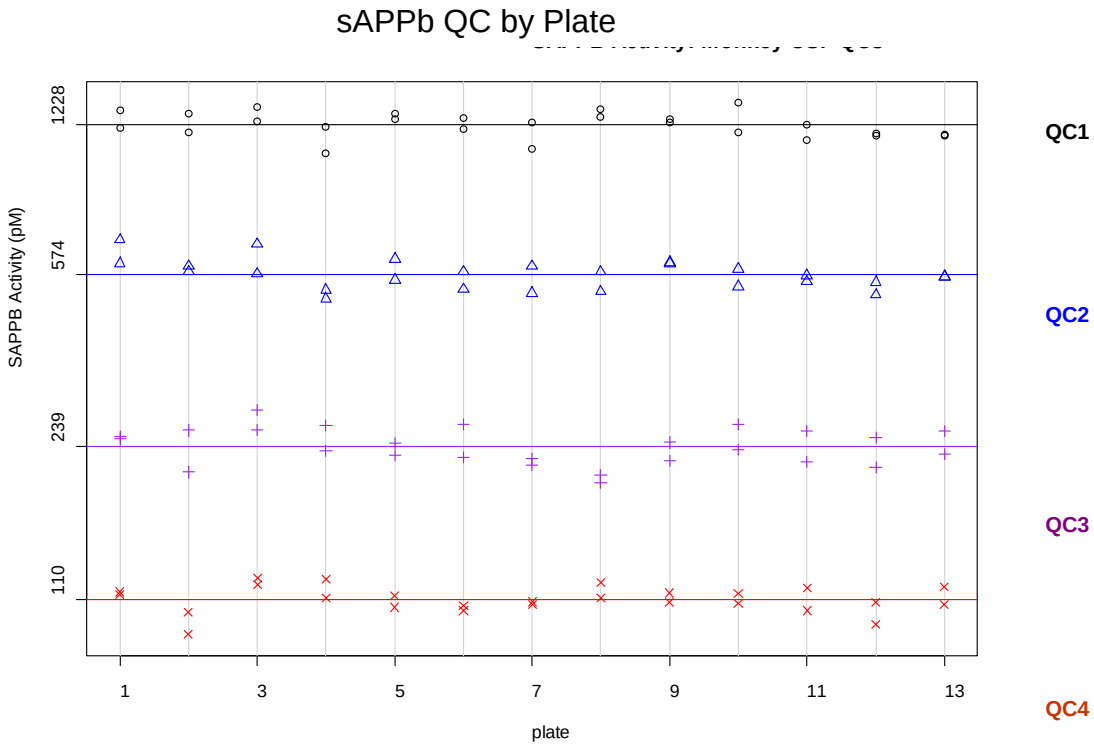


Figure 2.2

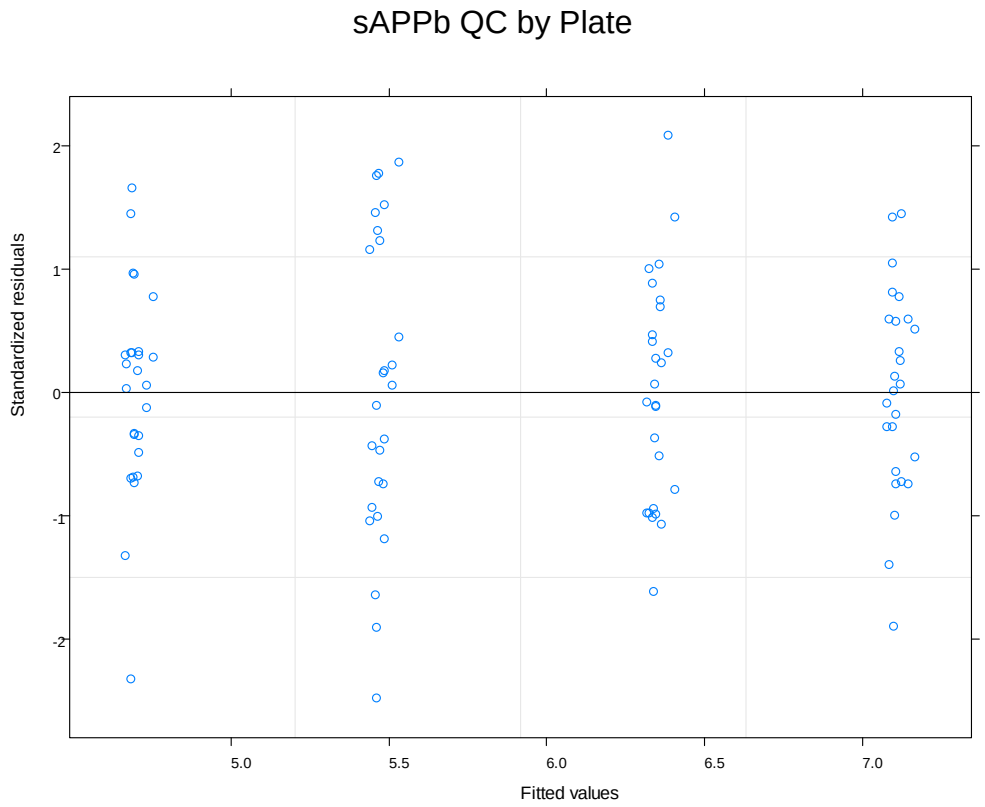


Figure 2.3

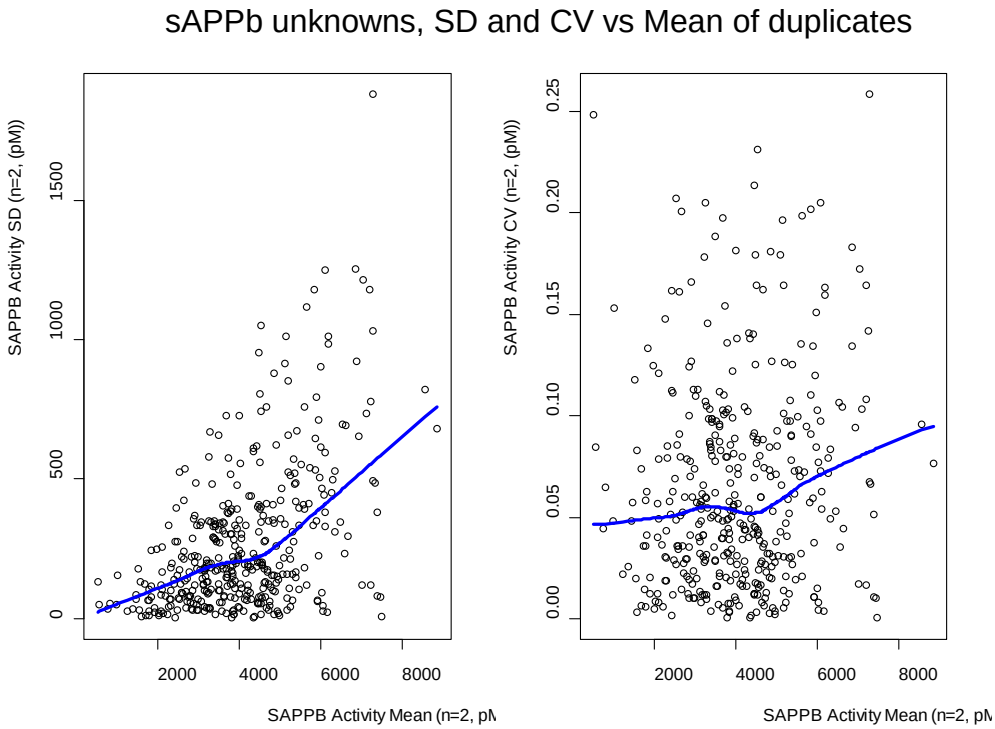


Figure 2.4

sAPPb unknowns, Mean, SD, and CV by Plate

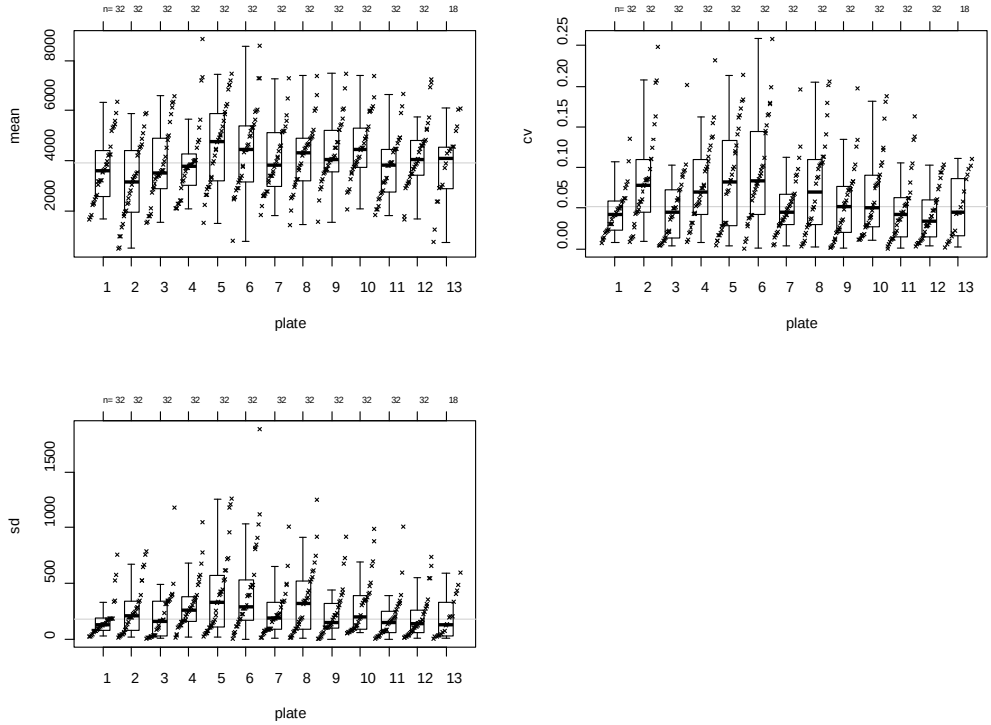


Figure 2.5

Counts (log spacing) vs sAPPb (pM, log spacing) for all Plates

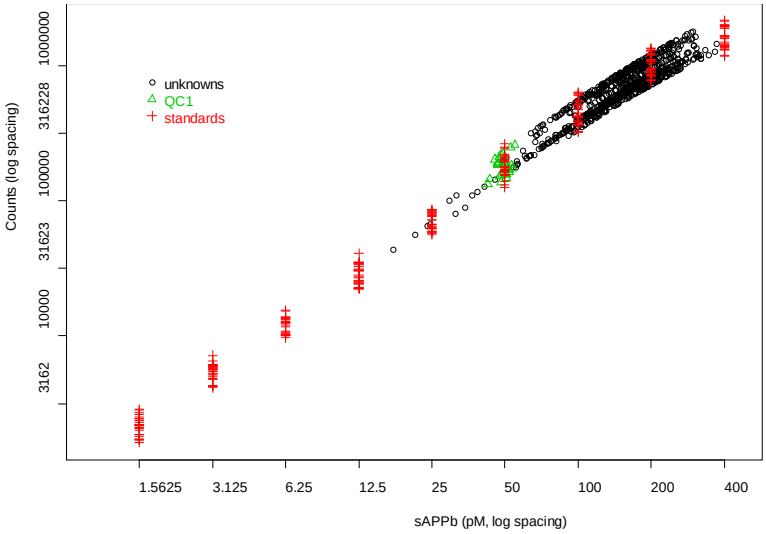


Figure 2.6A

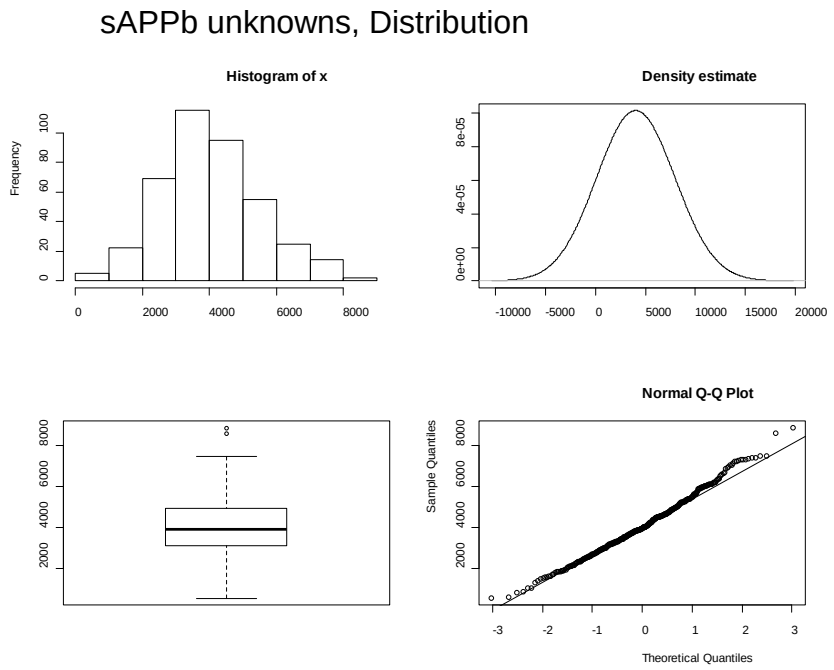
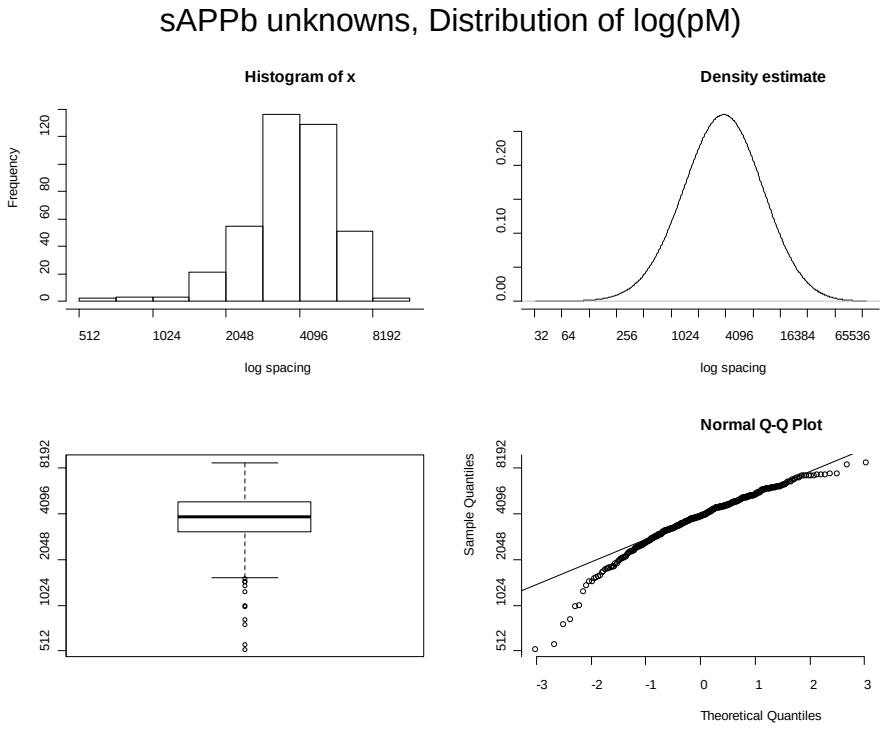


Figure 2.6B



APPENDIX III

Biomarkers Consortium Project
Use of Targeted Multiplex Proteomic Strategies to Identify CSF-Based Biomarkers in Alzheimer's Disease
BACE Activity and sAPPβ in CSF Statistical Analysis Plan

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Introduction

Biomarker tools for early diagnosis and disease progression in Alzheimer's disease (AD) remain a critical gap in AD drug development. Identification and validation of cost-effective methods to identify early AD and to monitor treatment effects in mild-moderate AD patients would revolutionize current clinical trial practice. Treatment prior to the onset of dementia may also ensure intervention occurs before irreversible neuropathology.

The aim of this project is to determine concentration levels of BACE activity and sAPPβ in CSF from baseline samples collected by ADNI. Comparisons will be made between diagnosis groups (Controls, AD, stable and progressing MCI). Comparisons will also be made between the CSF proteins measured in this project (BACE activity and sAPPβ) and characteristics previously measured (age, gender, ApoE genotype, and CSF concentrations of tau, ptau, Aβ42 and others).

The analyses described in this statistical analysis plan should be regarded as exploratory and meant for hypothesis and model generation, rather than for hypothesis confirmation and model validation. Results from this study will be compared with those from other studies on CSF proteins in AD, and findings will need to be confirmed and expanded upon in subsequent studies using other, independent data sets. Also, consultancy with a trained statistician is highly recommended prior to reporting results based upon multiple comparisons.

Study Design and Objectives

A total of 402 CSF samples from the baseline ADNI sample set were assessed (N = 106 Controls, 92 AD, 184 amnestic mild cognitive impairment (MCI), and 20 technical replicates). Approximately 75% of these baseline CSF samples have matching aliquots from year 1 CSF so that possible future studies on longitudinal change would be possible if funding becomes available for such a follow-up investigation. Of the 184 MCI subjects, 87 subjects had progressed to dementia as of March 29th, 2011. In addition the selected samples have additional biomarker data sets available. For example, all samples from controls, MCI and AD subjects have associated CSF Aβ42/tau measures and/or Pittsburgh

Compound B (PIB) one year data were included in the AD subset. This statistical analysis plan addresses the analysis of data from these samples. The study objectives are

- To determine whether baseline levels for CSF BACE activity and sAPPβ are associated with patient demographics (age, gender) or disease status.
- To determine whether baseline levels for CSF BACE activity and sAPPβ are associated with baseline levels of other CSF or plasma proteins or combinations of proteins, such as Tau, pTau, and Aβ42.

Classification Endpoints

Clinical diagnosis at time of enrollment/collection will be used to classify AD, MCI and control groups. Clinical diagnosis of amnestic MCI followed by diagnosis of AD will be used to classify pre-demented progressors. Explicit comparisons will be made between the AD group and the baseline MCI group and the control group. Also the progressing MCI group will be compared with the non-progressing MCI group.

General approach

Receiver-Operator Characteristic (ROC, see for example Pepe 2003) curves will be used to describe the ability of each analyte to discriminate between diagnosis groups. Differences in location (e.g., mean) of each population will be tested using a Mann-Whitney-Wilcoxon rank sums test (Mann & Whitney, 1947).

Linear models (ANOVA/ANCOVA) will be used to compare mean analyte levels among groups of interest. These models will include the diagnosis/disease status group and other covariates including age, gender and apoE4 genotype/status, as well as possible interactions among these factors. The interactive effect between diagnosis group and other covariates will be tested. Depending on the outcome of these tests, the differences between groups will be tested either by the main effect of diagnosis or the effect of diagnosis at a fixed level of other covariates (i.e., apoE4 status).

Analytes will be modeled on the log scale unless the data suggest that another scale is more appropriate. Correlation between CSF BACE activity, sAPPβ, and other CSF or plasma proteins will be measured by the Spearman (rank) correlation coefficient.

Hypotheses to Be Tested

The following hypotheses will be addressed for each analyte:

HO1i: Analyte i is not associated with age [age treated as a continuous variable]

HO2i: Analyte i is not associated with gender

HO3i: Analyte i is not associated with ApoE status

HO4i: Analyte i is not associated with disease status or change in disease status (adjusted for age, gender, and/or ApoE status as necessary)

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