

## **Supplementary Information**

**A $\beta$ 34 is a BACE1-derived degradation intermediate associated with amyloid clearance and Alzheimer's disease progression**

**Liebsch et al.**

## Supplementary Methods

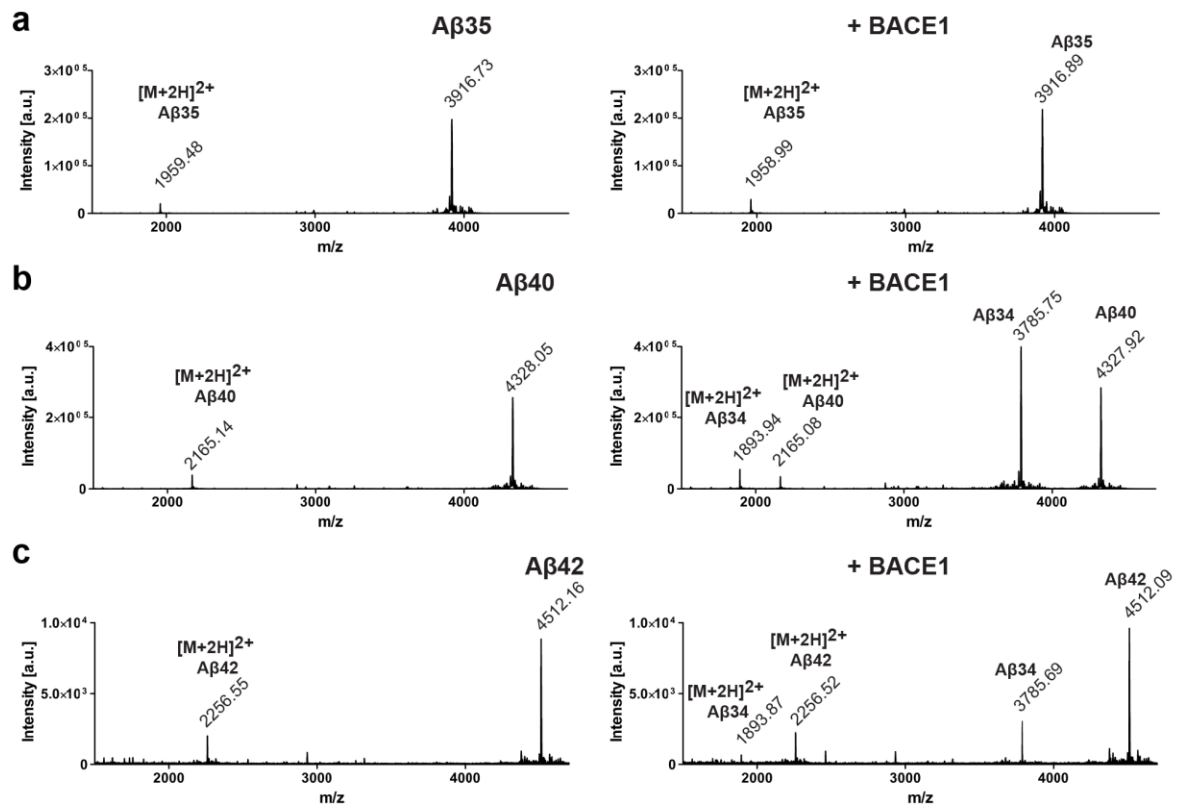
**Anti-A $\beta$ 34 antibody.** To quantify A $\beta$ 34 without cross-reactivity from other A $\beta$  species, we generated a high-specificity and high-affinity anti-A $\beta$ 34 monoclonal antibody (mab34) directed against A $\beta$ 27-34. We complied with all relevant ethical regulations for animal testing and research. Balb/cJ mice were immunized with synthetic A $\beta$ 27-34 peptide (from PSL, Germany) conjugated to KLH (Thermo Scientific). Splenocytes were fused to P3x63Ag8.653 myeloma cells (DSMZ No.: ACC 88; DSMZ, Braunschweig/ Germany). Hybridoma cells were cultivated in RPMI 1640, 10% fetal bovine serum (FBS w/o bovine Ig), 50  $\mu$ M  $\beta$ -Mercaptoethanol, 50 U/ml mu rec.IL-6, 1 x Penicillin-Streptomycin, 1 x L glutamine, and 25 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES) in a humidified incubator at 37°C with 5% CO<sub>2</sub>. Mab34 was purified using protein Protein G Sepharose <sup>1</sup>.

**Surface Plasmon Resonance (SPR).** Using a BIACORE T200 system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden; v2.0 Control and v1.0 Evaluation software), SPR experiments were performed at 25°C using filtered (0.2  $\mu$ m) and degassed HBS-EP+ buffer (10 mM HEPES pH 7.4; 150 mM NaCl; 3 mM EDTA; 0.05% (v/v) Tween-20). Using Biacore's Amine Coupling Kit, Mouse Antibody Capture Kit, and CM5 sensors, an anti-mouse Fc antibody (14  $\mu$ g/mL in 10 mM sodium acetate pH 5.0) was immobilized to the flow cells (~8000 RU each) as recommended by GE. To assess binding specificity, a variety of A $\beta$  peptides (0 – 2  $\mu$ M; 4-fold dilution series) were titrated over reference (anti-mouse Fc antibody only) and active (~1000 RU mab34 captured to anti-mouse Fc antibody) surfaces in single-cycle kinetic mode (25  $\mu$ L/min. x 5 min. association + 10 min. dissociation). Between each sample series, surfaces were regenerated at 50  $\mu$ L/min. using two 30 sec pulses of Pierce Gentle Ag/Ab Elution Buffer pH 6.6 (Thermo Scientific #21027) as well as 10 mM glycine pH 1.7. To assess binding affinity, A $\beta$ 34 peptide titrations (0 – 1.6  $\mu$ M; 3-fold dilution series) were performed in the similar manner using multi-cycle kinetic mode (25  $\mu$ L/min. x 10 min. association + 20 min. dissociation). All of the double-referenced data <sup>2</sup> presented are representative of duplicate injections acquired from at least three independent trials. To predict an apparent equilibrium dissociation constant ( $K_D$ ) for mab34, steady-state binding responses ( $R_{eq}$ ) in the multi-cycle titrations were averaged near the end of each association phase, plotted as a function of analyte concentration (C), and then subjected to non-linear regression analysis (Steady state affinity model in Biacore evaluation software). Based upon its unique neo-epitope design, mab34 specifically recognizes A $\beta$ 34 but not A $\beta$ 35, A $\beta$ 38, A $\beta$ 40, and A $\beta$ 42, as analyzed by single-cycle surface plasmon resonance (SPR; Supplementary Figure 2a). Additionally, we tested whether A $\beta$ 34 variants could bind to mab34. While the A $\beta$ 34 K16N - mab34 interaction demonstrated similar binding kinetics compared to the wild type peptide, A $\beta$ 34 G33A failed to interact with the antibody

(Supplementary Figure 2b). Complementary multi-cycle SPR titrations (Supplementary Figure 2c) further demonstrated that the dose-dependent, saturable binding between mab34 and the A $\beta$ 34 peptide is a high-affinity interaction (apparent equilibrium dissociation constant,  $K_D \sim 40$  nM; Supplementary Figure 2d). Overall, these results demonstrate that mab34 binds specifically with nanomolar affinity to the C-terminus of A $\beta$ 34 (Supplementary Figure 2e).

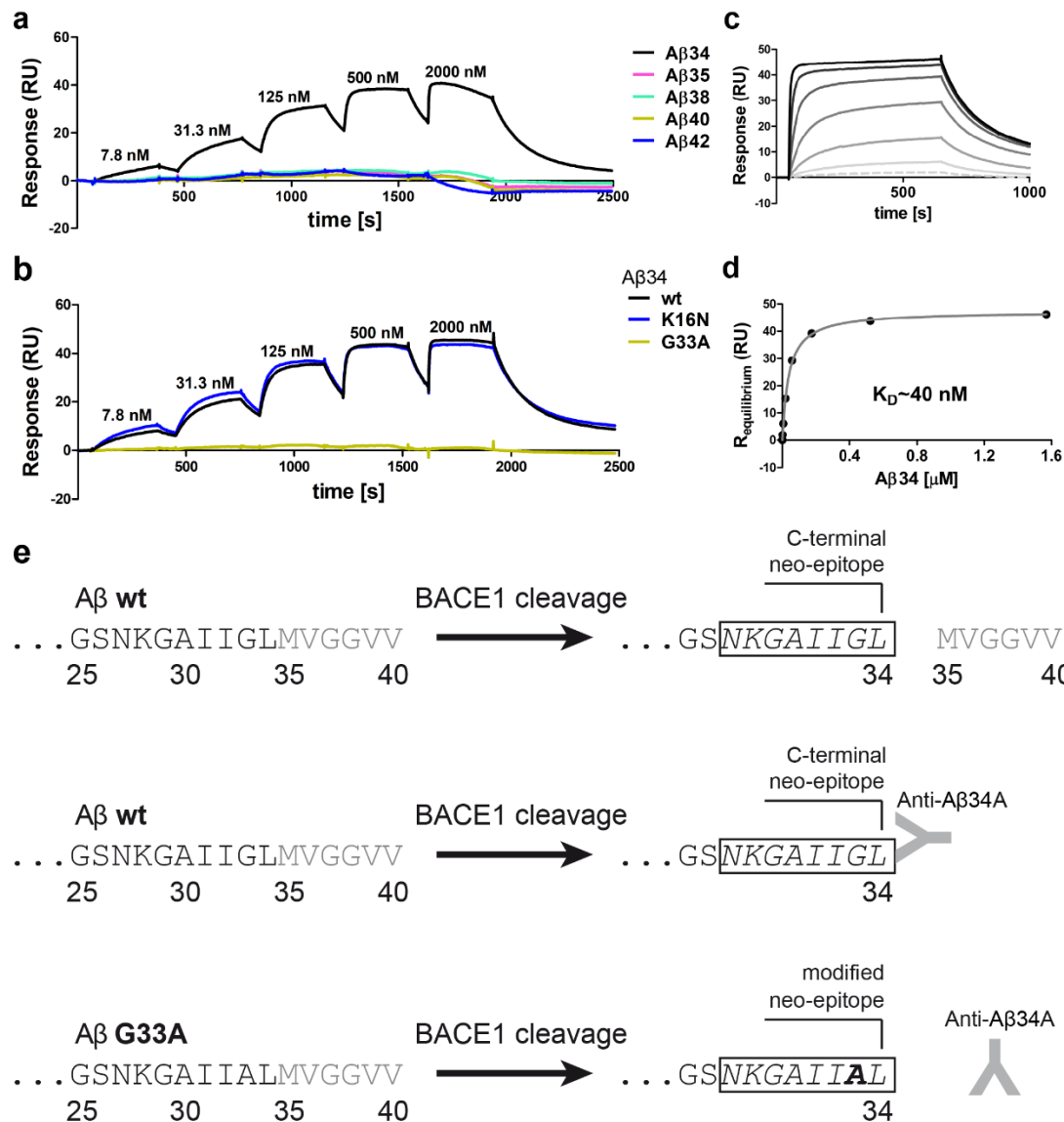
***Development and characterization of an ultrasensitive A $\beta$ 34 assay.*** We developed an ultra-sensitive multiplexing assay (Meso Scale Discovery (MSD)) for the simultaneous quantification of A $\beta$ 34, A $\beta$ 38, A $\beta$ 40, and A $\beta$ 42. Our mab34 and MSD's validated mouse monoclonal anti-A $\beta$ 38, anti-A $\beta$ 40, as well as anti-A $\beta$ 42 antibodies (see MSD A $\beta$  peptide V-PLEX) were spotted as the capture antibodies and we used the SULFO-tagged anti-A $\beta$  N-terminal 6E10 antibody (provided by the manufacturer) for detection. The MSD-based assay yielded excellent intra- and inter-assay variances (CVs below 5%), a lower limit of detection (LLOD) <2.5 pg/mL, a high dynamic range (2.5 – 10,000 pg/mL), and no detectable cross-reactivity with A $\beta$ 35, A $\beta$ 38, A $\beta$ 40, or A $\beta$ 42 (Supplementary Figure 3a). Furthermore, we tested whether the different biophysical properties of the A $\beta$  peptides tested had an effect on the response in an electrochemiluminescence-based assay. In a pan-A $\beta$  assay, based on the mouse monoclonal 4G8 capture antibody (recognizing A $\beta$  residues 17-24) all peptides, A $\beta$ 34, A $\beta$ 35, A $\beta$ 38, A $\beta$ 40, and A $\beta$ 42 yielded similar responses that were statistically not different from one another (Supplementary Figure 3b). For CSF measurements, repeated freeze-thaw cycles differentially affected the measurement of the four different A $\beta$ -species, and the measured concentration of all four A $\beta$  species was reduced after a second thawing cycle (Supplementary Figure 3c-f). These results demonstrate that the effect is less severe for shorter A $\beta$ -species and most prominent for A $\beta$ 42. However, we conclude that for a precise assessment of the absolute concentration, A $\beta$ -species are best quantified in CSF samples that were only thawed once. Since CSF samples are thawed at room temperature (see procedures from the BIOMARK-APD consortium that we followed here), these results further support that A $\beta$ 34 is neither generated nor degraded directly in CSF <sup>3</sup>.

## Supplementary Figures



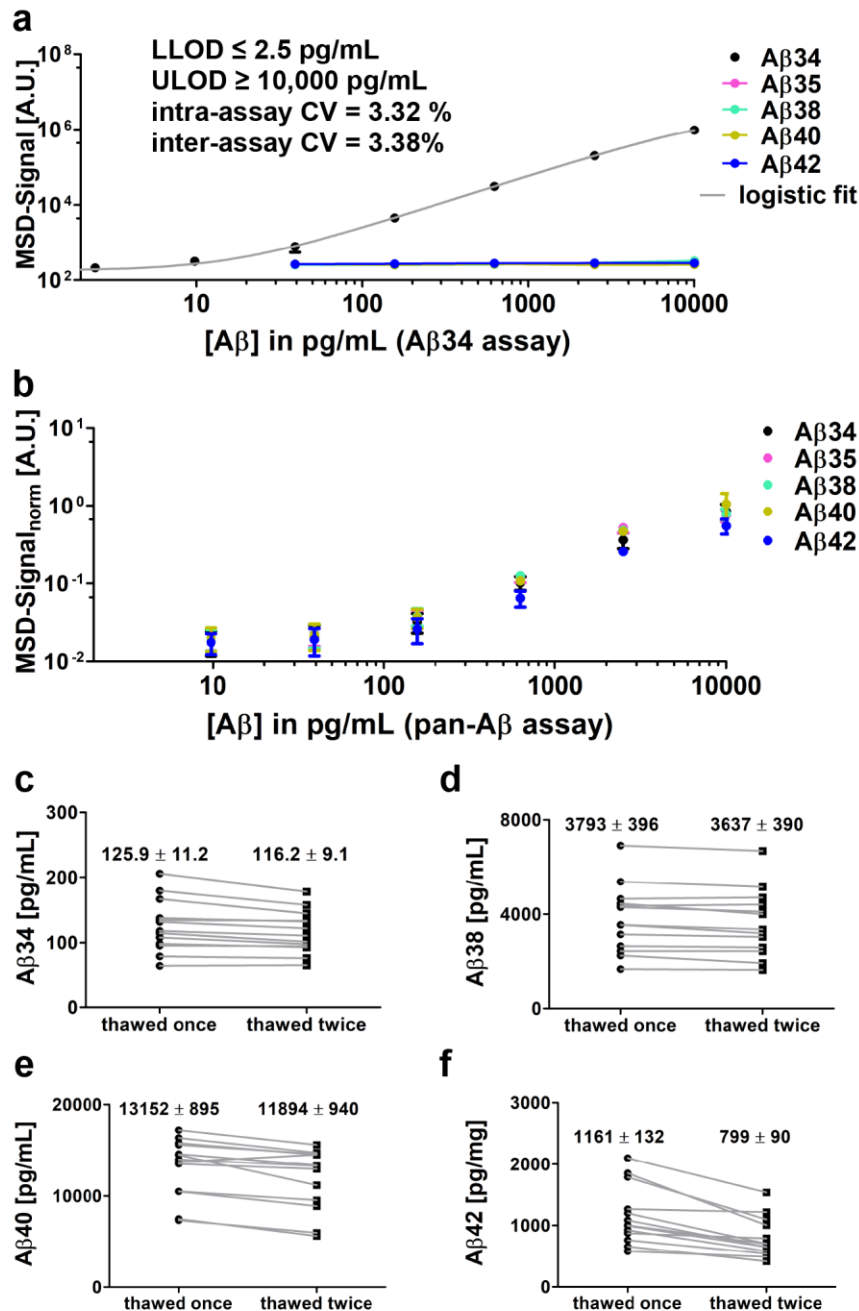
### Supplementary Figure 1: BACE1-mediated Aβ degradation *in vitro*

Cleavage of Aβ40 and Aβ42 by BACE1 *in vitro* was analyzed by MALDI-MS. Data were collected from 3 independent experiments. Representative MALDI-MS spectra for Aβ35 (**a**), Aβ40 (**b**), and Aβ42 (**c**), in the absence (left) or presence (right) of BACE1.



## Supplementary Figure 2: High-affinity, neo-epitope antibody against Aβ34

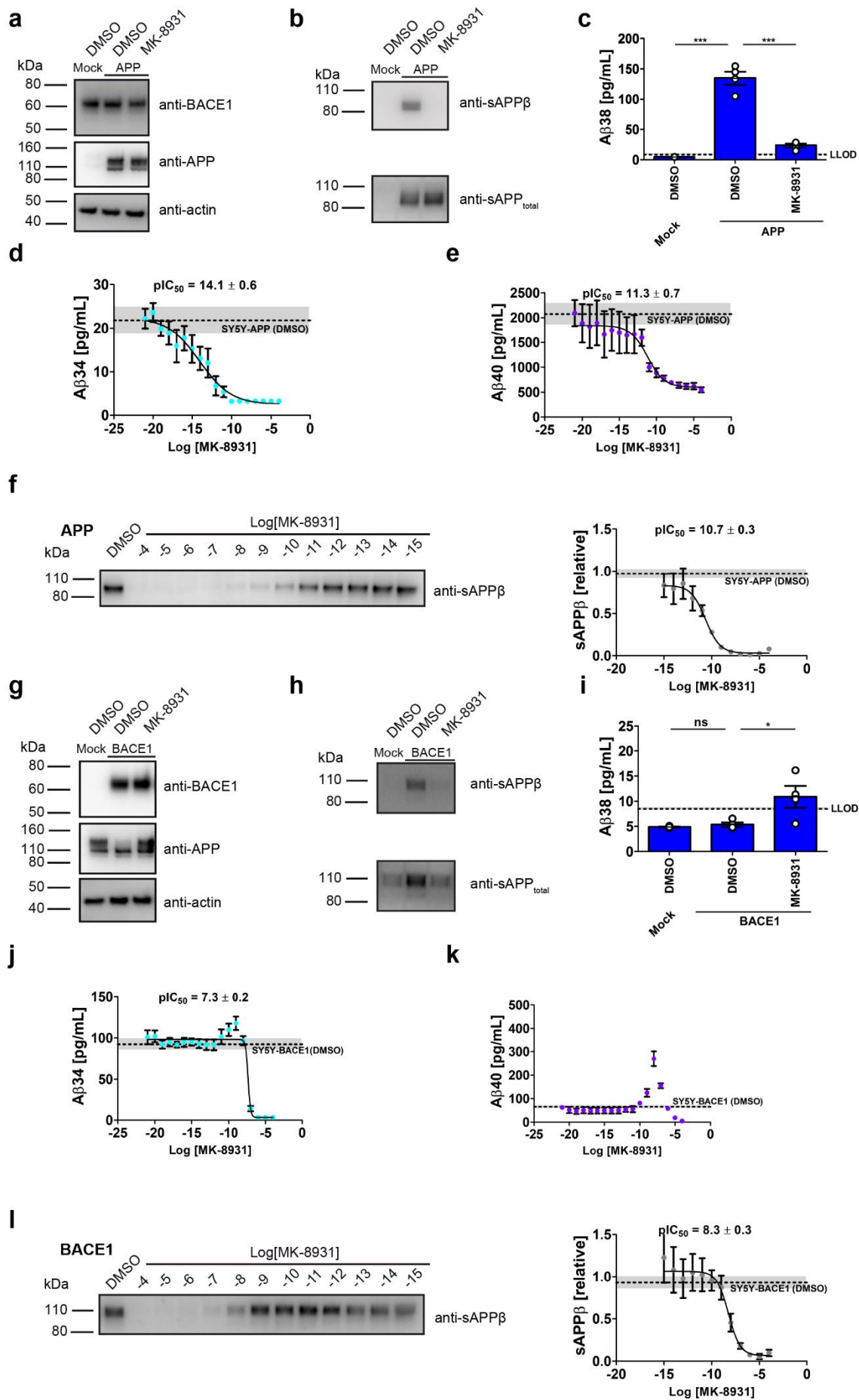
(a) Representative single-cycle SPR to demonstrate the specificity and kinetics of synthetic Aβ peptides binding to immobilized anti-Aβ34 antibody (i.e. mab34 captured to anti-Fc sensor). (b) Representative single-cycle SPR to demonstrate the specificity and kinetics of synthetic Aβ34 peptide variants binding to mab34 captured to anti-Fc sensor. (c,d) Representative multi-cycle SPR demonstrates saturable, dose-dependent binding of Aβ34 peptide (0 – 1.6  $\mu\text{M}$ ; 3-fold dilution series, lightest to darkest) to immobilized anti-Aβ34 antibody; steady-state amounts of Aβ34 bound ( $R_{\text{equilibrium}}$ ) were plotted as a function of concentration (symbols, raw data) and then subjected to non-linear regression analysis (line, curve fitting) to determine the apparent equilibrium dissociation constant ( $K_D$ ). (e) Cleavage between L34 and M35, e.g. by BACE1, generates the C-terminal neo-epitope, which is specifically recognized by mab34. The G33A modified neo-epitope is not recognized by mab34.



### Supplementary Figure 3: Ultrasensitive assay for the quantification of A $\beta$ 34

(a) Based on the monoclonal neo-epitope specific mab34, an ultra-sensitive, electrochemiluminescence-based immunoassay was developed using MSD technology. The assay was specific for A $\beta$ 34 without any significant cross-reactivity against A $\beta$ 35, A $\beta$ 38, A $\beta$ 40, or A $\beta$ 42 (n=3 independent experiments, data points for A $\beta$ 35, A $\beta$ 38, and A $\beta$ 40 are overlaid by A $\beta$ 42). Measures of assay performance are summarized in the top left corner (n=7 independent experiments). (b) Based on the monoclonal anti-A $\beta$  mid-domain (residues 17-24) antibody 4G8, we tested the response of A $\beta$ 34, A $\beta$ 35, A $\beta$ 38, A $\beta$ 40, or A $\beta$ 42 (n=4 independent experiments) in a pan- A $\beta$  electrochemiluminescence-based assay. Data were analyzed with a 2-WAY ANOVA. While A $\beta$  concentration showed a significant effect  $F(5,91) = 57.98$   $p < 0.0001$ , there was neither a significant effect for A $\beta$  species  $F(4,91) = 1.57$   $p = 0.19$  nor a

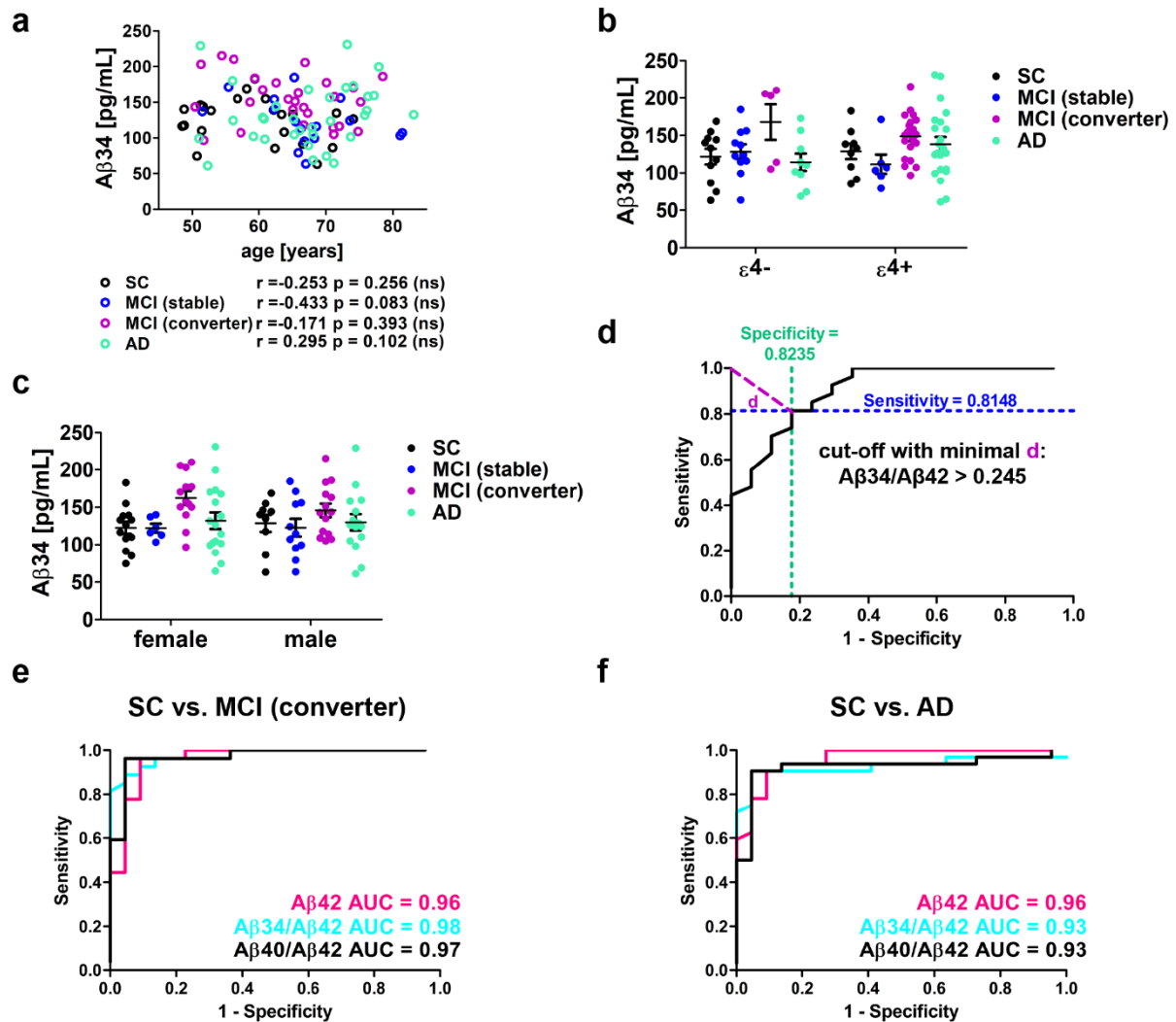
significant interaction  $F(20,91) = 0.73$ ,  $p = 0.78$ . **(c-f)** Using custom-printed MSD 4-plex plates (for A $\beta$ 34, A $\beta$ 38, A $\beta$ 40, and A $\beta$ 42), the effect of thawing cycles on the A $\beta$  quantification was tested. Measured concentrations of A $\beta$ 34 (**c**), A $\beta$ 38 (**d**), A $\beta$ 40 (**e**), and A $\beta$ 42 (**f**) from CSF samples that were thawed only once or thawed twice (n=15 CSF samples). The mean concentration  $\pm$  s.e.m. is displayed above the data points. Horizontal lines indicate mean  $\pm$  s.e.m.





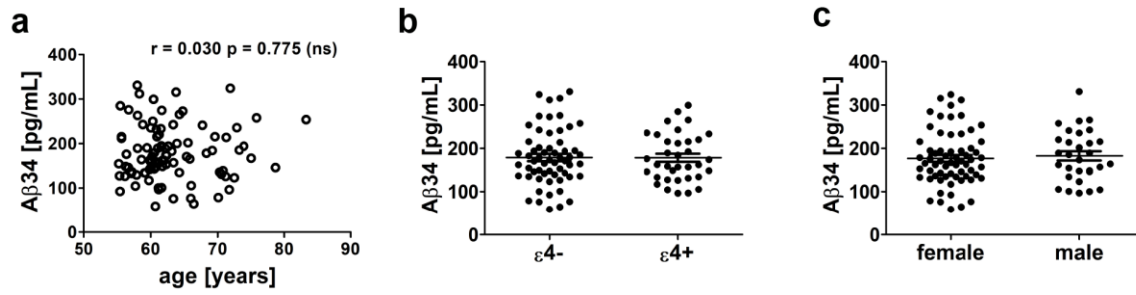
#### Supplementary Figure 4: BACE1 inhibition at high substrate or enzyme levels

Representative Western blots for the examination of APP, BACE1, sAPP $\beta$ , and sAPP $_{total}$  in APP (**a-f**) or BACE1 (**g-l**) overexpressing SH-SY5Y cells treated with vehicle (DMSO) or BACE1 inhibitor (MK-8931). (**c** and **i**) MSD multiplexing to quantify the absolute amounts of A $\beta$ 38. Data were collected from 4 independent experiments. Bars and error bars indicate mean  $\pm$  s.e.m. (**c,i**) Data were analyzed with 1-WAY ANOVAs and Tukey's post-hoc tests were performed for pairwise comparisons. (**c**) A $\beta$ 38,  $F(2,9)=118.4$ ,  $p < 0.0001$ , (**i**) A $\beta$ 38,  $F(2,9)=6.8$ ,  $p < 0.05$ . (**d-f,j-l**) Quantification of relative amounts of sAPP $\beta$ , including representative Western blots (**f,l**), and absolute amounts of A $\beta$ 34 (**d,j**) and A $\beta$ 40 (**e,k**). Data were collected from 4 independent experiments. Circles and error bars or dashed line with grey shaded area indicate mean  $\pm$  s.e.m. Dose-inhibition curves were fit with variable slope equations (four parameter model). Above graph  $-\log_{10}(IC_{50}) = pIC_{50} \pm$  s.e.m are displayed.



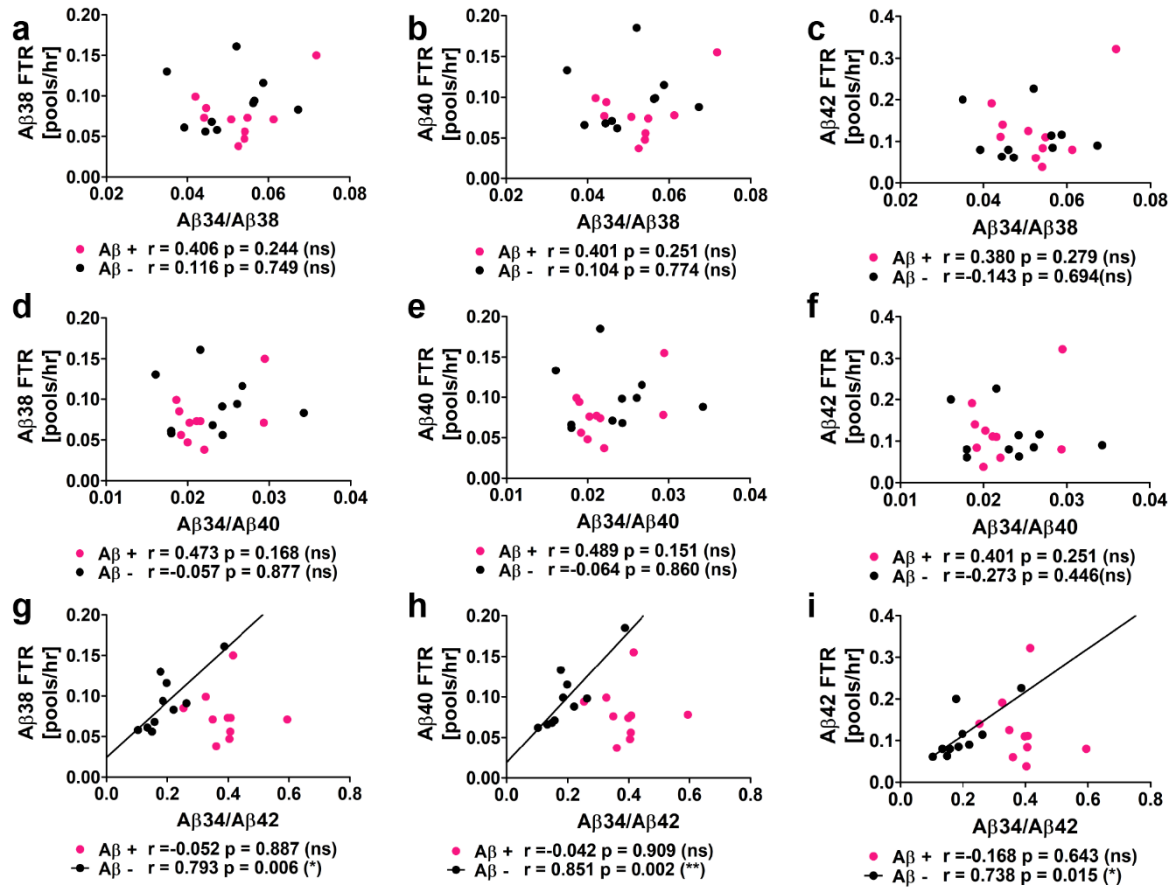
**Supplementary Figure 5: CSF-Aβ34 in the Amsterdam Dementia Cohort**

(a) Scatterplot of Aβ34 with age. Spearman correlation coefficients ( $r$ ) were computed to assess the relationship between the variables. (b,c) The effect of  $\epsilon 4$  (b) and gender (c) on CSF- Aβ34. The data were analyzed with 2-WAY ANOVAs, (b)  $\epsilon 4$   $F(1,86) = 0.02$ ,  $p=0.89$ ; diagnosis  $F(3,86) = 3.70$ ,  $p=0.02$ ; interaction  $F(3,86) = 1.54$ ,  $p=0.21$  (c) gender  $F(1,90) = 0.15$ ,  $p=0.70$ ; diagnosis  $F(3,90) = 3.58$ ,  $p=0.02$ ; interaction  $F(3,90) = 0.41$ ,  $p=0.74$ . Horizontal lines indicate mean  $\pm$  s.e.m. (d) The optimal cut-off (point on the ROC curve with the minimum distance (d) to sensitivity = 1 and specificity = 1) was determined using Pythagoras' theorem and yielded the best cut-off for a ratio > 0.245, with a sensitivity of 0.8148 and a specificity of 0.8235. (e,f) ROC curves were computed on CSF levels of Aβ42, Aβ34/Aβ42, Aβ40, and Aβ40/Aβ42 in samples from  $n=27$  MCI (converter) and  $n=22$  SC (e) and  $n=32$  AD and  $n=22$  SC (f).



### Supplementary Figure 6: CSF-Aβ34 in PREVENT-AD

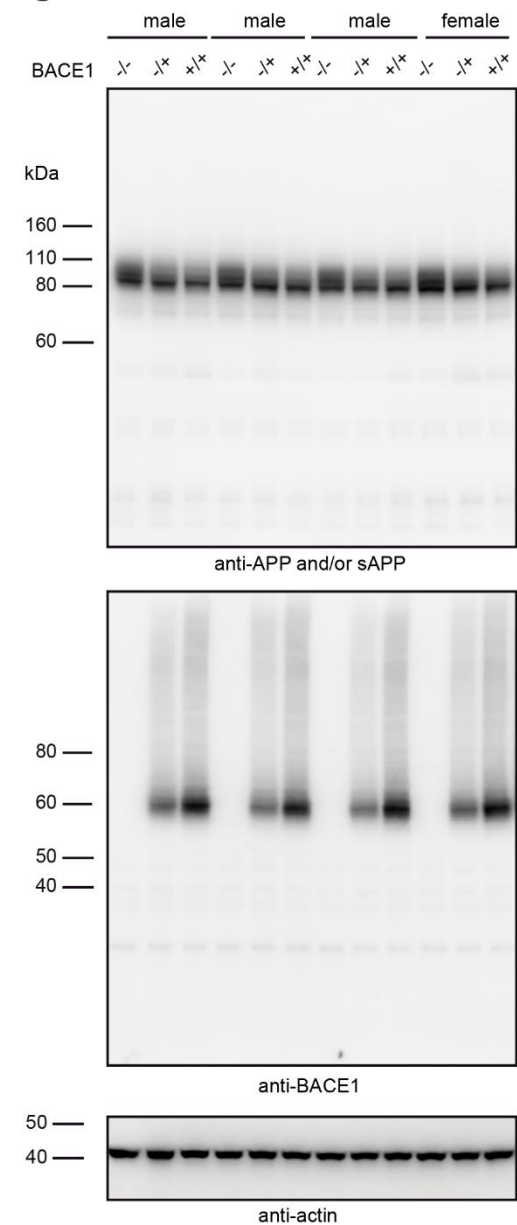
(a) Scatterplot of Aβ34 with age.  $n=94$  CSF samples. Spearman correlation coefficient ( $r$ ) was computed to assess the relationship between the variables (two-tailed  $p$ -value). (b,c) The effect of  $\epsilon 4$  (b) and gender (c) on CSF- Aβ34. The data were analyzed with unpaired two-tailed  $t$ -tests, (c)  $\epsilon 4$   $t(92) = 0.02$ ,  $p = 0.98$ ; (d) gender  $t(92) = 0.43$ ,  $p = 0.67$ . Horizontal lines indicate mean  $\pm$  s.e.m.



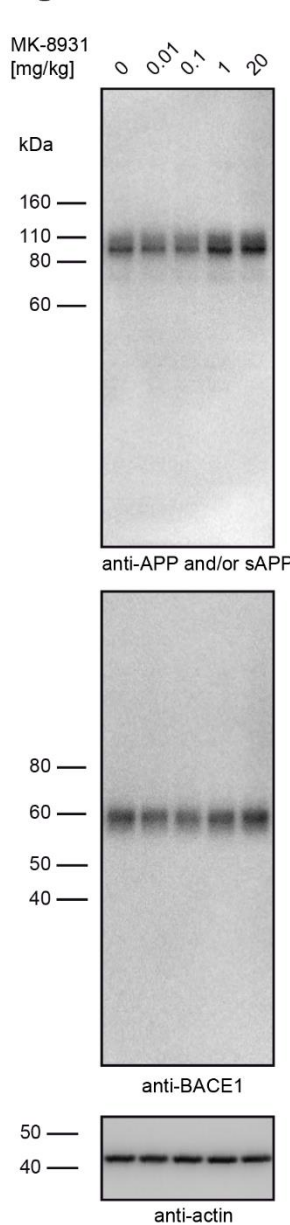
### Supplementary Figure 7: Association between CSF-A $\beta$ 34/A $\beta$ 42 and A $\beta$ clearance

Analysis of A $\beta$ 34, A $\beta$ 38, A $\beta$ 40, and A $\beta$ 42 in human CSF with ultra-sensitive assays (Meso Scale Discovery (MSD)). A $\beta$ 38, A $\beta$ 40, and A $\beta$ 42 clearance (fractional turnover rate, FTR) was previously measured using stable isotope labeling kinetic (SILK)<sup>39</sup>. Samples were from  $n=10$  A $\beta^+$  and  $n=10$  A $\beta^-$  individuals. Scatterplots of CSF-A $\beta$ 34/A $\beta$ 38 (**a-c**), A $\beta$ 34/A $\beta$ 40 (**d-f**), or A $\beta$ 34/A $\beta$ 42 (**g-i**) with A $\beta$ 38 FTR (**a,d,g**), A $\beta$ 40 FTR (**b,e,h**), or A $\beta$ 42 FTR (**c,f,i**). Pearson correlation coefficients ( $r$ ) were computed to assess the relationship between the variables. The Bonferroni adjusted  $p$ -values are: \*\* $p < 0.003$ , \* $p < 0.016$ , ns = non-significant  $p > 0.0125$ .

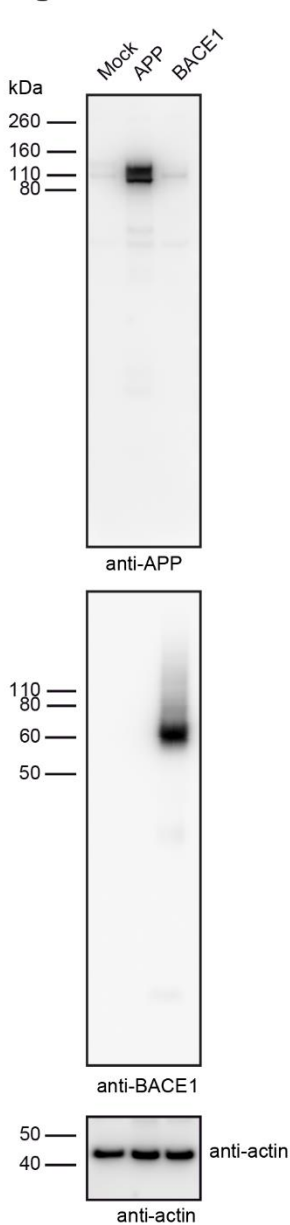
**Figure 1a**



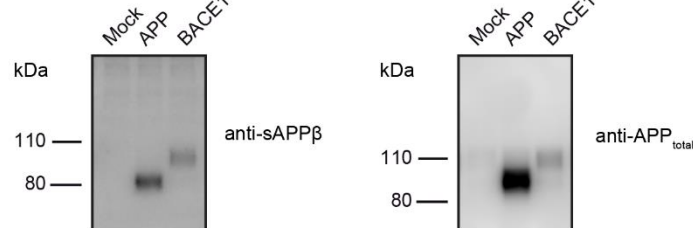
**Figure 2a**



**Figure 3a**



**Figure 3b**



**Supplementary Figure 8: Full-size Western blots**

Full-size images of Western blots shown in Figures 1, 2, and 3.

## Supplementary Tables

**Supplementary Table 1.** Spike-and-recovery and linearity-of-dilution assessments for human CSF-A $\beta$ 34 with MSD

	n	mean Recovery [%] (SD)
neat	4	100
1:2	4	115 (2)
1:4	4	114 (1)
1:8	4	105 (2)
Spike low (1 ng/mL)	4	81 (3)
Spike high (3 ng/mL)	4	85 (3)

**Supplementary Table 2.** Number and age of Individuals in the Separate Studies

	cognitively normal	SC	MCI	AD
Amsterdam Dementia Cohort (n=98)		n=22	n=44	n=32
female		13/22	19/44	17/32
ε4-carrier (%)		45 (two missing values)	63 (one missing value)	71 (one missing value)
Cognition, Mini-Mental State Examination score (SD)		27.7 (1.4)	26.5 (2.1)	23.3 (4.9)
Age in years (SD)		59.5 (8.1)	67 (8.1)	65.3 (7.7)
PREVENT-AD (n=94)	n=94			
female	64/94			
ε4-carrier (%)	37			
Cognition, Montreal Cognitive Assessment score (SD)	27.8 (1.6)			
Age in years (SD)	63.1 (5.8)			

## Supplementary References

- 1 Ida, N. *et al.* Analysis of heterogeneous A4 peptides in human cerebrospinal fluid and blood by a newly developed sensitive Western blot assay. *The Journal of biological chemistry* **271**, 22908-22914 (1996).
- 2 Myszka, D. G. Improving biosensor analysis. *J Mol Recognit* **12**, 279-284, doi:10.1002/(SICI)1099-1352(199909/10)12:5<279::AID-JMR473>3.0.CO;2-3 (1999).
- 3 Portelius, E. *et al.* Ex vivo (18)O-labeling mass spectrometry identifies a peripheral amyloid beta clearance pathway. *Mol Neurodegener* **12**, 18, doi:10.1186/s13024-017-0152-5 (2017).