Validation of soluble APP assays as diagnostic CSF biomarkers for neurodegenerative diseases

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Validation of soluble APP assays as diagnostic CSF biomarkers for neurodegenerative diseases

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Keywords

Method validation, standard operating procedures, soluble APP, CSF, Alzheimer's disease

Abbreviations

Aβ, amyloid-β peptide; AD, Alzheimer's disease; BIOMARKAPD, Biomarkers for Alzheimer's and Parkinson's Disease; FTD, frontotemporal dementia; IBL, IBL-international;

JPND, Joint Programme - Neurodegenerative Disease; LLOQ, lower limit of quantification;

MSD, Meso Scale Diagnostics; QC, quality control; sAPP, soluble amyloid-β precursor protein; sAPPf, full-length sAPP; SMC, subjective memory complaints; SOP, standard operating procedure.

Abstract

Background: Analytical validation of a biomarker assay is essential before implementation in clinical practice can occur. In this study, we analytically validated the performance of assays detecting soluble amyloid-β precursor protein (sAPP) α and β in CSF in two laboratories according to previously SOPs serving this goal.

Methods: sAPPα and sAPPβ ELISA assays from two vendors (IBL, MSD) were validated. The performance parameters included precision, sensitivity, dilutional linearity, recovery and parallelism. Inter-laboratory variation, biomarker comparison (sAPPα versus sAPPβ) and

clinical performance was determined in three laboratories using 60 samples of patients with subjective memory complaints, Alzheimer's disease or frontotemporal dementia.

Results: All performance parameters of the assays were similar between labs and within predefined acceptance criteria. The only exceptions were minor out-of-range results for recovery at low concentrations and, despite being within predefined acceptance criteria, non-comparability of the results for evaluation of the dilutional linearity and hook-effect. Based on the inter-laboratory correlation between Lab #1 and Lab #2, the IBL assays were more robust (sAPPα: r^2=0.92, sAPPβ: r^2=0.94) than the MSD assay (sAPPα: r^2=0.70, sAPPβ: r^2=0.80). Specificity of assays was confirmed using assay-specific peptide competitors. Clinical validation showed consistent results across the clinical groups in the different laboratories for all assays.

Conclusion: The validated sAPP assays appear to be of sufficient technical quality and perform well. Moreover, the study shows that the newly developed SOPs provide highly useful tools for the validation of new biomarker assays. A recommendation was made for renewed instructions to evaluate the dilutional linearity and hook-effect.

Introduction

The incidence of neurodegenerative diseases is a serious problem worldwide, in view of the annual 7.7 million new cases of dementia (Alzheimer's Association 2014). Currently, CSF biomarkers have been incorporated in the diagnostic criteria of Alzheimer's disease (AD) (McKhann *et al.* 2011), but additional biomarkers are needed for predicting disease progression or monitoring the success of disease-modifying therapies (Rosén *et al.* 2013).

Potential biomarkers for monitoring AD therapy targeting amyloid pathology may include soluble proteolytic fragments of the amyloid-β precursor protein (sAPP) apart from the amyloid-β (Aβ) peptide. APP can be processed in at least two ways. In the amyloidogenic pathway sAPPβ is formed when membrane-bound APP is cleaved by β-secretase. Subsequently, Aβ is released by cleavage of the remaining 99 amino acids long c-terminal C99 fragment by γ-secretase. In the non-amyloidogenic pathway APP is cleaved by α-secretase leading to the production of sAPPα (Jonsson *et al.* 2012, Cuchillo-Ibañez *et al.* 2015, Steiner *et al.* 2008). Inhibition of β-secretase and activation of α-secretase pathways may be promising approaches to treat AD (Kojro *et al.* 2001, Lichtenthaler & Haass 2004, Chasseigneaux & Allinquant 2012). Therefore, sAPPα and sAPPβ have been included in trials as potential CSF biomarkers for monitoring target engagement of treatments affecting APP metabolism (Lewczuk *et al.* 2008, Höglund & Salter 2013, Chasseigneaux & Allinquant 2012).

In order to implement established and new CSF biomarkers for routine use in clinical practice, clinical trials or research, there is a need for standardization at the pre-analytical, analytical and post-analytical level (Vanderstichele *et al.* 2013, del Campo *et al.* 2012). Standardization of pre-analytical aspects will improve the diagnostic value of CSF biomarkers. To achieve this, assays for specific CSF biomarkers need to be analytically validated, preferentially following clear and internationally accepted protocols. Within the Biomarkers for Alzheimer's and Parkinson's Disease (BIOMARKAPD) project, a large consortium of the European initiative Joint Programme - Neurodegenerative Disease (JPND) Research, two standard operation procedures (SOPs) for the evaluation of biomarker assays for neurodegenerative diseases were developed. The first SOP describes the purpose of

every analytical parameter and provides an outline of the procedures to be followed for thorough analytical validation in agreement with ISO 15189 guidelines. The second SOP describes a workflow for the practical implementation of the first SOP (Andreasson *et al.* 2015).

The aim of this study was to analytically validate the performance of the assays of two vendors detecting sAPPα and sAPPβ, using the novel SOPs developed in the BIOMARKAPD project. The resulting data give detailed insights into the performance of sAPP assays and provide proof of concept for the use of these SOPs.

Materials & Methods

Participants and assay kits

For this study informed consent of the participants and approval of the institutional review board was given. The following assays for the quantification of sAPPα and sAPPβ concentrations were validated: the duplex assay kit MSD® 96-Well MULTI-SPOT sAPPα / sAPPβ assay (Cat#: N45120B-1, Meso Scale Diagnostics, USA), and sAPPα high sensitive ELISA kit (Ref#: JP27734) and sAPPβ high sensitive ELISA kit (Ref#: JP27734) of IBL-international (Germany). These assays were distributed to three different experienced laboratories within the BIOMARKAPD consortium. The assays were performed according to the manufacturer protocols. Lab #1 and Lab #2 tested all performance parameters according to previously described SOPs (Andreasson et al. 2015). All three laboratories performed the inter-laboratory variation, biomarker comparison and clinical performance experiments. Lab

#3 only tested the IBL assays. Calculations of all parameters were done using a datasheet described previously (Andreasson et al. 2015).

Sensitivity

For the determination of the lower limit of quantifications (LLOQs), 16 blank samples were measured in one MSD or IBL plate. The calibration curves were calculated using a four parameter logistic (4PL) curve fit for MSD and a quadratic curve fit (second order polynomial) for IBL calibration curves.

Precision

Intra-assay variation (repeatability) was determined by measuring samples (MSD: n=14 and IBL: n=15) in four replicates within one plate. The mean coefficient of variation (%CV) was calculated by averaging the CVs of all tested samples. A %CV <20% was defined as acceptable.

Inter-assay variation (intermediate precision) was measured to determine the variation of analyses between different days. To quantify inter-assay variation, samples with low, medium and high concentrations were selected from the samples used for the intra-assay variation (quality control (QC) low, QC medium and QC high). These samples were measured in duplicate in four different plates at identical positions in the ELISA plates on four different days. The mean %CV was calculated for all samples. A %CV <20% was defined as acceptable.

Intra-plate variation was determined to explore the influence of different positions within a plate on the measured concentrations (stability of the plate). QC low, QC medium and QC high samples were measured in four replicates at different positions of the plate (columns 3/4 vs. columns 11/12). The mean %CV was calculated for all samples. A %CV <20% was defined as acceptable.

Dilutional linearity

Three different CSF samples, applied either undiluted (Lab #1, MSD and IBL), 40 times prediluted (Lab #2, MSD) or 80 times prediluted (Lab #2, IBL), were spiked with sAPPα or sAPPβ calibrator (Lab #1: MSD: 20 ng/mL and IBL: 50 ng/mL final concentration of calibrator in the spiked sample; Lab #2: MSD: 1000 ng/mL and IBL: 50 ng/mL final concentration of calibrator in the spiked sample). These samples were serially diluted to cover the entire measurement range and analyzed in duplicates. The dilutional linearity was expressed in:

$$\%Linearity = \frac{(observed\ C * dilution\ factor)}{(previous\ observed\ C * previous\ dilution\ factor)} * 100$$

C = concentration (ng/mL)

A linearity between 80% and 120% was defined as acceptable.

Recovery

Five different CSF samples, measured in duplicate, were diluted (MSD: 40 times, IBL: 80 times), and spiked with recombinant sAPPα or sAPPβ calibrator at three different levels. The final concentrations of recombinant sAPPα or sAPPβ in the samples were 100, 3.0 and 0.1

ng/mL for the MSD assay and 20, 8.0 and 2.0 ng/mL for the IBL assay. For neat samples, buffer without calibrator was added to the diluted CSF. Spike recoveries were calculated according to the formula:

$$\%Recovery = \frac{(C\ spiked\ sample - C\ neat\ sample)}{theoretical\ C\ spike} * 100$$

C = concentration (ng/mL)

A recovery between 80% and 120% was defined as acceptable.

Parallelism

Five different CSF samples, with high endogenous protein concentrations, were serially diluted. Both reciprocal relative dilution factor and OD450 absorbance signals of the samples and calibrator were log-transformed in order to use linear regression to calculate the slopes of the sample and calibrator curves. The OD of the lowest dilution of the samples was compared to a equivalent OD of the calibrator, calibrators with a higher OD were not included in the calibrator curves when calculating the slope of the calibration curve. The "in range %" was determined by the formula:

$$in\ range\ \% = \frac{slope\ of\ sample\ dilution\ series}{slope\ of\ calibration\ curve} * 100$$

A calculated in range % between 80% and 120% was defined as acceptable.

Clinical samples

Inter-laboratory variation, biomarker comparison (sAPPα versus sAPPβ) and clinical performance of the different assays were determined in three laboratories using CSF samples of patients with subjective memory complaints (SMC; n=20; male=10), Alzheimer's disease (AD; n=20; male=8) or frontotemporal dementia (FTD; n=20; male=4). All samples were age-matched. These clinical samples were collected in conformation with previously published guidelines (del Campo et al. 2012) and distributed in aliquots by one center. The clinical diagnosis was made according to internationally accepted criteria, for AD (McKhann et al. 2011) and for FTD (Neary *et al.* 1998). The laboratories tested the CSF samples blinded for the clinical status of the patients. The clinical samples were diluted 40 times for the MSD assay. For the IBL assays, Lab #1 and Lab #2 diluted their CSF samples 80 times and Lab #3, 16 times. Differences between the patient groups were tested for significance using a one-way ANOVA with Bonferroni as post-hoc test when data were normally distributed (D'Agostino and Pearson omnibus normality test) and using the non-parametric Kruskal-Wallis test with Dunns post-hoc test when the data were not normally distributed.

Specificity of the assays

To evaluate the specificity of the sAPPα and sAPPβ assays, we performed competitive blocking experiments with three different short synthetic peptides. The method of preparation of these peptides is described in Supplemental Data. PL416 is a synthetic peptide comprising the 10 amino acids sequence N-terminal to the Aβ region in APP. The peptide was designed in such a way that it was expected to compete with the detection of

sAPPβ only since it covers the antigen that was used for immunization (according to the product sheet by IBL). Two other synthetic peptides covering the first 10 ($Aβ_{1-10}$) or 16 ($Aβ_{1-16}$) amino acid residues of the Aβ peptide were tested for their expected ability to compete with sAPPα for binding to the capture antibodies employed in the MSD and IBL assays. The concentration added to the pooled CSF samples was 10 µg/mL of respectively PL416, $Aβ_{1-10}$ or $Aβ_{1-16}$.

Results

Sensitivity

The mean LLOQ's were comparable for both sAPPα assays (Supplemental Table 2), but differed by a factor 2.3 for the sAPPβ assays (i.e. 0.41 ng/mL for the MSD assay and 0.18 ng/mL for the IBL assay).

Precision

All intra-assay CVs (for both sAPPα and sAPPβ, using either the MSD or IBL assays, in both laboratories) ranged from 1% to 7%, which was well below the pre-defined value of 20% (Supplemental Figure 7A), and in range with the specifications provided by IBL (sAPPα: 6-7%, sAPPβ: 3-4%).

All inter-assay CVs were below the pre-defined value of 20% for both sAPPα and sAPPβ, using either the MSD or IBL assays, in both laboratories (Supplemental Figure 7B) and these were in range with the specifications provided by IBL (sAPPα: 7-13%, sAPPβ: 9-10%).

Obtained CVs were for both laboratories: MSD sAPPα, 3-8% and sAPPβ, 3-10%; for IBL sAPPα, 5-11% and sAPPβ, 4-12%.

The intra-plate CV for both MSD sAPPα and sAPPβ (2-7%) and for IBL sAPPα (5-10%) were comparable in the two laboratories and were all well below the pre-defined value of 20% (Supplemental Figure 7C). However, the intra-plate CV for IBL sAPPβ showed a three times higher mean CV for Lab #2 (15%) compared to Lab #1 (5%).

Dilutional linearity and hook-effect

The results of the linearity experiments for sAPPα and sAPPβ of both vendors are summarized in Supplemental Table 3. Results of all linearity determinations were well within the predefined ranges of 80-120%. However, it appeared that the original protocol to perform the experiments to determine linearity and a possible hook-effect allowed different interpretations and execution of the experiments. For MSD sAPPα, in Lab #1 linearity was achieved between 400 and 8,000 times dilution of the CSF and in Lab #2 between 480 and 69,120 times dilution of the CSF (note that Lab #2 started with prediluted CSF). For MSD sAPPβ, in Lab #1 linearity was achieved between 20 and 8,000 times dilution and in Lab #2 between 5,760 and 69,120 times dilution of the CSF. The linearity experiments for both IBL sAPPα and sAPPβ unfortunately failed in Lab #1. Linearity was achieved between 80 and 5,120 times dilution of the CSF for IBL sAPPα in Lab #2, and between 80 and 20,480 times dilution of the CSF for sAPPβ.

A hook effect, i.e. suppression of signal at concentrations above the upper limit of quantification, could only be evaluated in Lab #1, since Lab #2 diluted their samples to concentrations that were too low for the detection of such an effect. Lab #1 did not observe any hook effect for sAPPα and sAPPβ of both vendors (data not shown).

Recovery

The results of the recovery experiments for sAPPα and sAPPβ of both vendors are summarized in Supplemental Table 4. The mean recoveries of MSD sAPPα (Lab #1: 82%, Lab #2: 123%) and sAPPβ (Lab #1: 88%, Lab #2: 129%) assays did comply for Lab #1, but not for Lab #2, with the predefined range of 80-120%. However, for MSD sAPPα and sAPPβ in Lab #2, only the recovery obtained after spiking 3.0 ng/mL was out of the predefined range of 80-120%. At the highest spiked concentration, two out of five samples had to be excluded from analysis, due to analytical failures (for Lab #1 only).

The mean recoveries obtained for the IBL sAPPα (Lab #1: 96%, Lab #2: 96%) and sAPPβ (Lab #1: 91%, Lab #2: 75%) assays were within the predefined range of 80-120%, with the exception of sAPPβ in Lab #2. However, in Lab #2 for IBL sAPPβ, three out of five samples produced very low recoveries, in particular for low and medium spike levels. These were, however, in the range of the specifications provided by IBL (114-121% for sAPPα and 78-107% for sAPPβ).

Parallelism

The results for both the MSD and IBL sAPPα and sAPPβ assays by Lab #1 and Lab #2 showed that the recombinant standard paralleled native recognition (slope range: 91-107%), which was within the predefined range of 80-120% (Supplemental Table 5). The two laboratories obtained comparable results.

Inter-laboratory variation

The correlation between measured concentrations of Lab #1 and Lab #2 for MSD sAPPα (Figure 1A: r^2=0.70) in clinical samples was slightly weaker compared to MSD sAPPβ (Figure 1E: r^2=0.80). For the IBL assays, strong correlations of measured concentrations were observed between Lab #1 and Lab #2 for sAPPα (Figure 1B: r^2=0.92) and sAPPβ (Figure 1F: r^2=0.94). We observed a large discrepancy between the concentrations measured by Lab #3 versus either of the other two laboratories, for both the IBL sAPPα (Figure 1C: vs. Lab #1: r^2=0.53 and Figure 1D: vs. Lab #2: r^2=0.56) and for IBL sAPPβ (Figure 1G: vs. Lab #1: r^2=0.70 and Figure 1H: vs. Lab #2: r^2=0.64).

Biomarker comparison: sAPPα versus sAPPβ

We observed a strong correlation between MSD sAPPα and sAPPβ concentrations for Lab #1 (Figure 2A: r^2=0.95) and Lab #2 (Figure 2B: r^2=0.97). For the IBL assays, also a strong correlation between sAPPα and sAPPβ concentrations was shown for Lab #1 (Figure 2C:

r^2=0.94) and Lab #2 (Figure 2D: r^2=0.96). However, for Lab #3 the correlation between IBL sAPPα and sAPPβ concentrations was weak (Figure 2E: r^2=0.30).

Clinical performance

Both MSD and IBL sAPPα and sAPPβ concentrations in SMC, AD and FTD CSF samples showed no significant differences between clinical groups ($p>0.05$) as quantified by Lab #1 (sAPPα: Figure 3A, C; sAPPβ: Figure 3E, G) and Lab #2 (sAPPα: Figure 3B, D; sAPPβ: Figure 3F, H). In addition, a stronger correlation was observed for each clinical group (SMC, AD and FTD) using the IBL compared to the MSD assays (Supplemental Figure 8). In contrast, IBL sAPPα levels as quantified in Lab #3 were, however, significantly different between the clinical groups ($p<0.05$), and for IBL sAPPβ as quantified in Lab #3, decreased concentrations were observed in the FTD group compared to AD ($p<0.05$) and SMC ($p<0.01$). Because of the large discrepancy between the concentrations measured by Lab #3 versus the other two laboratories (Figure 1), clinical performance results of Lab #3 were not included in Figure 3.

In Lab #3 the IBL sAPPα concentrations in SMC, AD and FTD were lower compared to Lab #1 (all groups) and Lab #2 (SMC, AD) ($p<0.05$). Overall, the sAPPβ concentrations in SMC, AD and FTD were similar in all laboratories for MSD and IBL ($p>0.05$).

Specificity of the assays

In view of the strong correlation between sAPPα and sAPPβ, we defined the specificity of the epitopes detected by both assays, for both vendors. Supplementing the CSF-samples with Aβ 1-16, but not with PL416, an anti-sAPPβ blocker, reduced sAPPα signals in both the MSD and IBL assays to background level (Figure 4A, B). Aβ 1-10 strongly reduced the sAPPα signal in the MSD assay (Figure 4A), but did not affect the signal intensity in the IBL sAPPα assay (Figure 4B), indicating the epitope recognised by the IBL assay did not include the Aβ 1-10 sequence and thus recognition of different epitopes in the amino terminus of the Aβ sequence by the respective sAPPα capture antibodies. Conversely, addition of PL416, to a sample of pooled human CSF abolished sAPPβ signals in both MSD and IBL assays, while Aβ 1-10 and Aβ 1-16 had no effect (Figure 4C, D).

Comparison of calibrators from different vendors

The calculated sAPPα (MSD: 36-380 ng/mL, IBL: 40-635 ng/mL) and sAPPβ (MSD: 62-527 ng/mL, IBL: 200-1660 ng/mL) concentrations in biological samples were substantially different between the assays of the two vendors. We compared the respective calibrator proteins provided with the kits of both vendors side by side in both assay formats. In the MSD assay (Figure 5A, C), both the MSD sAPPα and sAPPβ calibrators yielded substantially higher signals than the corresponding IBL calibrators. In the IBL sAPPα assay (Figure 5B), the signal intensities obtained with the MSD calibrators were slightly higher than with the IBL calibrators. In the IBL sAPPβ assay (Figure 5D) the MSD calibrators produced substantially stronger signal intensities than the IBL calibrators.

Discussion

In this study, we validated the sAPP assays of MSD and IBL for the detection of sAPPα and sAPPβ. It should be noted that the MSD assay was only tested in two laboratories, since the MSD system was not available in Lab #3. Despite this limitation, this inter-laboratory comparison yielded interesting novel insights. Both sAPPα and sAPPβ assays passed the performance parameters and similar results were obtained by the two laboratories for both vendors. Thus, these assays can be used for the detection of sAPPα and sAPPβ in CSF. Furthermore, our study demonstrates that the use of a previously published SOP can be used to validate ELISAs in a multi-center setting, with the exception of the instructions provided to determine the dilutional linearity, which allowed for multiple interpretations. Because of these differences in protocol execution, although each lab reported acceptable levels for this dilutional linearity, comparison of the data was not possible. Therefore, we suggest that the instructions for the experiments to evaluate dilutional linearity and a possible hook-effect should be amended into: "Spike undiluted samples with calibrator stock solution to a final concentration of 100 times the highest standard of the calibration curve. Make serial dilutions of these spiked samples, without any predilution, until the theoretical concentration is below LLOQ. Use the concentrations above the highest standard to evaluate a possible hook-effect, and the concentrations between the LLOQ and the highest standard to calculate the dilutional linearity."

We observed a large discrepancy between the concentrations measured by Lab #3 versus either of the other two laboratories. This could be explained by a matrix effect in the samples of Lab #3, since Lab #3 diluted their clinical samples 16 times for the IBL assays, and

the other laboratories 80 times. For future and uniform analyses, we recommend to use 80x diluted CSF for the MSD duplex assay and 40x diluted CSF for the IBL assays to measure sAPPα and sAPPβ. The differences in the calibrators of the two assay formats may provide an explanation for substantial differences in reported CSF sAPPα and sAPPβ concentrations studied by MSD versus IBL assays (Perneczky *et al.* 2011, Lewczuk et al. 2008, Lewczuk *et al.* 2011).

Our results on the correlation between sAPPα and sAPPβ are in agreement with previous studies, which show a strong positive correlation between sAPPα and sAPPβ in cognitively healthy controls as well as patients with AD (Lewczuk et al. 2008, Gabelle *et al.* 2010, Mulugeta *et al.* 2011, Alexopoulos *et al.* 2012b, Pera *et al.* 2013). This is somewhat unexpected if we assume that the pathways leading to the production of either sAPPα or sAPPβ are mutually exclusive. However, at least in cell culture, this assumption was also not demonstrated (Dyrks *et al.* 1994). Although we confirmed the specificity of the assays using assay-specific inhibitors, it has recently been demonstrated that the sAPP assays detect not only sAPPα or sAPPβ but also homodimers, heterodimers and large heteromers of (full-length) APP (Cuchillo-Ibañez et al. 2015). Soluble full-length APP (sAPPf) co-exists in CSF with sAPPα and sAPPβ, and these proteins have overlapping antigenic epitopes, which may hinder the specific detection of the various proteins. All three forms of sAPP can assemble into heteromers, which affects the detection of sAPPα and sAPPβ. Therefore, the occurrence of sAPPf in CSF and its recognition in the sAPPα and sAPPβ assays may – at least partly – explain the strong correlation between sAPPα and sAPPβ. Additionally, detection of homodimers, heterodimers and large heteromers of (full-length) APP could be an

explanation for our poor recovery results, since the spiked synthetic proteins could interact with the endogenous proteins present in the CSF samples.

Our results showed that neither sAPPα nor sAPPβ in CSF consistently differentiated between AD, and FTD and SMC. These findings are in line with several previous studies that also reported no differences in either sAPPα or sAPPβ concentrations between AD or controls (Hock *et al.* 1998, Olsson *et al.* 2003, Zetterberg *et al.* 2008, Brinkmalm *et al.* 2013, Magdalinou *et al.* 2015, Rosén *et al.* 2012, Sennvik *et al.* 2000), FTD and controls (Kimura *et al.* 1999, Steinacker *et al.* 2009, Magdalinou et al. 2015) or AD and FTD (Gabelle *et al.* 2011), but they are in contrast to other studies reporting a decrease between groups (Lewczuk et al. 2008, Lewczuk et al. 2011, Taverna *et al.* 2012, Perneczky et al. 2011, Gabelle et al. 2010, Rosén et al. 2012, Sennvik et al. 2000, Palmert *et al.* 1989, Prior *et al.* 1991, Van Nostrand *et al.* 1992, Alcolea *et al.* 2015, Gabelle et al. 2011, Alcolea *et al.* 2014, Alexopoulos *et al.* 2012a). Differences between groups could be due to variations in the number of patients tested, in patients' characteristics, in methods used to detect sAPPα and sAPPβ, and even in the execution of the specific method.

sAPPα and sAPPβ have been used as CSF biomarkers for monitoring effects of treatments affecting APP metabolism (Lewczuk et al. 2008, Höglund & Salter 2013, Chasseigneaux & Allinquant 2012). Inhibition of β-secretase and activation of α-secretase pathways are under investigation for potential treatments of AD (Kojro et al. 2001, Lichtenthaler & Haass 2004, Chasseigneaux & Allinquant 2012). Whereas determination of Aβ concentrations in CSF

reflect the net treatment effects on Aβ production, aggregation and clearance mechanisms, quantification of sAPPα and sAPPβ levels may provide more specific information regarding target engagement and treatment effect of specific α-secretase and β-secretase inhibitors on the processing of APP.

In conclusion, the newly developed SOPs provide useful tools for the validation of new biomarker assays. However, in this manuscript we added an unequivocal instruction on how to execute uniform experiments to calculate the dilutional linearity, since the original SOPs allowed for multiple interpretations. The multicenter design of the validation study allowed identification of robustness and site-specific errors. All assays investigated here appear to be of sufficient technical quality and performed well. We recommend to use 80x diluted CSF for the MSD duplex assay and 40x diluted CSF for the IBL assays to measure sAPPα and sAPPβ. Both sAPPα and sAPPβ may have potential as CSF biomarkers for monitoring target engagement of treatments affecting APP metabolism.

ARRIVE guidelines have been followed:

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Conflicts of interest: This work was supported by the BIOMARKAPD research project within the EU Joint Programme – Neurodegenerative Disease (JPND). The project is supported through national funding organizations under the aegis of JPND (http://www.jpnd.eu). For the Netherlands, the Netherlands Organisation for Health Research and Development (ZonMw); for Germany, the Bundesministerium für Bildung und Forschung (BMBF grant 01ED1203A). The LVR-

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Figure 1: Inter-laboratory variation of sAPPα and sAPPβ assays.

(A-D) sAPPα; **(E-H)** sAPPβ. Laboratory numbers are indicated in the figure.

Figure 2: Comparison of sAPPα and sAPPβ per vendor.

(A) MSD Lab #1, samples were diluted 40 times. **(B)** MSD Lab #2, samples were diluted 40 times. **(C)** IBL Lab #1, samples were diluted 80 times. **(D)** IBL Lab #2, samples were diluted 80 times. **(E)** IBL Lab #3, samples were diluted 16 times.

Figure 3: Clinical performance of sAPPα and sAPPβ.

(A-D) sAPPα; **(E-H)** sAPPβ. Laboratory numbers are indicated in the figure. The dots in A and E in the FTD group indicate outliers.

Figure 4: Competitive blocking experiments with synthetic peptides.

Synthetic peptides PL146, Aβ 1-10, or Aβ 1-16 were spiked into a diluted sample of pooled human CSF. The electrochemiluminescence signals obtained for the spiked peptides in the MSD-duplex assay **(A, C)** and the absorptions at 450 nm (A450nm) in the IBL ELISAs **(B, D)**

were compared to those obtained with neat samples and blanks. The means and ranges of duplicate reads (technical repeats) are shown.

Figure 5: Comparison of calibrator proteins.

The sAPP calibrator proteins of the MSD-duplex and the IBL-monoplex assays were compared side by side within both assays. Electrochemiluminescence signals in the sAPPα and sAPPβ MSD-duplex assay **(A, C)** and absorption at 450 nm (A450nm) in the corresponding IBL ELISAs **(B, D)** are plotted against the nominal concentrations for both calibrators. The means ± ranges of two technical replicates within one assay plate are shown. Circles indicate the respective MSD-calibrators and squares represent the IBL-calibrator proteins.