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Characterization of pre-analytical sample handling effects on a panel of Alzheimer's disease–related blood-based biomarkers: Results from the Standardization of Alzheimer's Blood Biomarkers (SABB) working group

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Abstract

Introduction: Pre-analytical sample handling might affect the results of Alzheimer's disease blood-based biomarkers. We empirically tested variations of common blood collection and handling procedures.

Methods: We created sample sets that address the effect of blood collection tube type, and of ethylene diamine tetraacetic acid plasma delayed centrifugation, centrifugation temperature, aliquot volume, delayed storage, and freeze–thawing. We measured amyloid beta (Aβ)42 and 40 peptides with six assays, and Aβ oligomerization-tendency (OAβ), amyloid precursor protein (APP)$_{699-711}$, glial fibrillary acidic protein (GFAP), neurofilament light (NfL), total tau (t-tau), and phosphorylated tau181.

Results: Collection tube type resulted in different values of all assessed markers. Delayed plasma centrifugation and storage affected Aβ and t-tau; t-tau was additionally affected by centrifugation temperature. The other markers were resistant to handling variations.

Discussion: We constructed a standardized operating procedure for plasma handling, to facilitate introduction of blood-based biomarkers into the research and clinical settings.

KEYWORDS
Alzheimer's disease, amyloid beta, plasma biomarkers, pre-analytics, stability, tau

1 | BACKGROUND

Blood-based biomarkers for Alzheimer's disease (AD) have the potential to be implemented as non-invasive screening tools. Promising markers that reflect AD neuropathophysiological processes are amyloid beta (Aβ),[1–9] (phosphorylated) tau (p-tau),[10–13] glial fibrillary acidic protein (GFAP),[6,8,14–16] and neurofilament light (NfL).[8,15–17] Particularly GFAP and NfL are also implicated in other neurodegenerative diseases, such as non-AD dementias, multiple sclerosis, or traumatic brain injury.[14,18,19] Standardization of measurement across clinical trials and care settings will facilitate widespread adoption and implementation of blood-based biomarkers.

It took decades for cerebrospinal fluid (CSF) Aβ42 and p-tau to be widely accepted as fluid biomarkers for AD,[20,21] among other reasons due to large variability in levels between and within laboratories.[22,23] Variation has been partially mitigated by fully automated analytical instruments,[24,25] and development of Certified Reference Materials to which commercial assays were recalibrated.[26] Different procedures of sample handling in the pre-analytical phase, from body fluid collection until analysis, also caused variation.[27] This was standardized by development of standardized operating procedure (SOP)s.[28–31] It is conceivable that pre-analytical variations might also impact levels of the novel AD blood-based biomarkers.

A plasma sample handling SOP has been proposed,[32] but this was not based on empirical evidence. Initial pre-analytical experiments showed that use of different anticoagulants resulted in different Aβ42, Aβ40, GFAP, NfL, total tau (t-tau), and p-tau181 levels,[33–35] and that Aβ levels might be unstable in ethylene diamine tetraacetic acid (EDTA) blood pending centrifugation and after,[33,35–37] while t-tau and NfL appeared to be more stable but with some conflicting results.[33,37–40] A comprehensive study of a broad range of pre-analytical factors on the wide portfolio of novel AD blood-based biomarkers is lacking. We aimed to establish an analyte-independent *and* technology-independent blood sample handling SOP based on empirical evidence.

2 | METHODS

This study was a joint effort of the Standardization of Alzheimer's Blood Biomarkers (SABB) workgroup of the Global Biomarker Standardization Consortium (GBSC) of the Alzheimer's Association, which was launched in 2018 to bring together expertise from academia, government, and industry. In a stepwise approach, we (1) identified the breadth of variation in sample handling protocols applied by cohort studies and diagnostic and pharmaceutical companies, (2) investigated the effect of the most common and most relevant pre-analytical sample handling differences on the different blood-based biomarkers in systematic experiments, and (3) generated a unified SOP.

2.1 | Identification of pre-analytical sample handling variation between studies using inventories

We invited 42 GBSC members representing 30 organizations to fill out a survey detailing all steps of their pre-analytical sample handling protocols (19 variables; survey template in File S1 in supporting information). After reviewing, a next survey was sent to 48 GBSC members representing 32 organizations (template in File S2 in supporting information), inviting them to give priority rankings (scale 1 [= highest] to 5 [= lowest]) to short-list the most relevant pre-analytical factors.

2.2 | Generation of pre-analytical sample sets

At Amsterdam University Medical Center (UMC), sample sets were prepared to mimic the extremes of the reported variations of the short-listed pre-analytical factors. Fresh blood was obtained from adults who presented at the Clinical Chemistry Department for a diagnostic blood draw for any disease. As reference condition, whole blood was centrifuged for 10 min at 1800 × g at room temperature (RT), after standing 30 minutes at RT. Subsequently, separated plasma (or serum) was aliquoted in 250 μL portions in 0.5-mL polypropylene tubes, and stored at −80°C. For the experimental conditions, these handling steps were systematically varied (detailed experimental protocols in File S3 in sup-

porting information). Ninety-two volunteers participated, contributing to n = 10 to 12 inclusions per protocol, with ≤15 aliquots per condition.

The study was in accordance with the Declaration of Helsinki, and approved by the medical ethical committee of the Vrije Universiteit (VU) UMC (approval number: 2019.257–NL69123.029.19). All volunteers gave written informed consent to use their blood for research purposes. No demographic or clinical information was collected.

2.3 | Sample measurements

Samples were shipped to eight laboratories for quantitative analysis using different platforms (detailed below). All measurements were performed according to lab protocols and/or manufacturers' instructions. Sample handling from thawing until measurement is described in File S4 in supporting information.

Aβ42 and Aβ40 were measured at C$_2$N Diagnostics' Clinical Laboratory Improvement Amendments (CLIA)-certified lab (Precivity-AD) using immunoprecipitation liquid chromatography mass spectrometry (MS),[41] at the Shaw lab of University of Pennsylvania using Euroimmun enzyme-linked immunosorbent assays (ELISAs) and at Araclon Biotech using their ELISAs.[42,43] Aβ42, Aβ40, and amyloid precursor protein (APP)$_{669-711}$ were measured at the Hirtz lab with immunoprecipitation matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)-MS, using a protocol slightly modified from Nakamura

TABLE 1 Pre-analytical variation in sample handling protocols between studies

	Pre-analytical sample handling step	Summary of observed variation	Mean priority rating (range: 1-5; 1 = highest; 5 = lowest)	Included in empirical protocols?
Sampling factors	Time of day for collection	50% reported collection in the morning; 50% reported collection throughout the day.	2	No*
	Fasting status	57% reported non-fasted collection; 43% reported fasted collection.	2	No*
	Needle size and location of draw	Details reported by only a few. Only venous blood draws reported; needle size varied between 21- to 24-gauge needles.	4	No
	Vacutainer needle vs. butterfly needle vs. syringe	Details reported by only a few. Mostly, vacutainer needles were used. Sometimes butterfly needles for difficult blood draws. One study specifically reported syringe should be avoided due to risk of hemolysis.	3	No
	Plastic tubing or other tools used during the blood draw	None reported the use of tubings or other tools.	3	No
	EDTA type and concentration	Both 10 mL 18 mg K_2EDTA tubes and 6 mL 10.8 mg K_2EDTA tubes were used. One study reported Prostaglandin E1 addition. One study specifically reported spray-coated K_2EDTA is preferred, to not artificially dilute the plasma (i.e., with liquid EDTA).	2	No
	Tube types and additives, e.g., EDTA, citrate, heparin	Most studies collect K_2EDTA plasma. One study reported use of the additive-free S-Monovette Sarstedt tube. Some studies additionally collect serum, sodium-citrate plasma, or lithium-heparin plasma.	2	Yes
	Tube collection order	According to best practice: (1) sodium-citrate, (2) serum, (3) lithium-heparin, (4) EDTA.	3	No
	Tube filling volume	All reported complete tube filling.	3	No
Preparation factors	Tube inversion or manipulation before centrifugation	No variation. All studies invert the tube a few times immediately after collection; up to 10 times.	3	No
	Time from collection to centrifugation	A lot of variation observed: 30%: within 30 min; 30%: within 1 h; 20%: within 2 h; 10%: within 5 h; 10%: within 30 h.	2	Yes
	Temperature during period from collection to centrifugation	50% reported room temperature; 50% reported in the refrigerator or on wet ice	2	Yes
	Centrifugation parameters (specify speed, time, temp)	Most report centrifuging for 5–15 min at 1500-3000 x g. One study reports 4000 x g for 30 min. One study reports a stepwise approach to additionally collect platelets and buffy coat. 50% centrifuge at room temperature; 50% centrifuge at 4°C.	2	Yes: Centrifugation temperature. No: Centrifugation time and speed.
	Time from centrifugation to freezing	Details only reported by a few. 33% aliquot and store immediately; 33% within 30 min; 17% within 1.5 h; 17% within 4 h.	2	Yes
	Temperature during period from centrifugation to freezer	Details only reported by a few; 57% keep tubes at room temperature, 29% on ice/in the refrigerator, and 14% on dry ice.	2	Yes

(Continues)

TABLE 1 (Continued)

	Pre-analytical sample handling step	Summary of observed variation	Mean priority rating (range: 1-5; 1 = highest; 5 = lowest)	Included in empirical protocols?
Storage factors	Temperature of freezing	80% reported storage at −80°C; 6% reported liquid nitrogen freezing, 6% on dry ice, 6% at −20°C	2	Yes: intermittent storage at −20°C No: liquid nitrogen storage
	Freeze-thaw cycles	Most reported to use samples with no additional freeze–thaw cycles. One study reported that less than three cycles is possible.	2	Yes
	Aliquot size	Most report storage in 250-500 μL aliquots: 54%; 27%: > 500 < 1000 μL; 18%: > 1000 < 1500 μL. Storage tube sizes was not asked, thus no details on tube filling.	2	Yes
	Length of storage at low temperature	Length of storage depends on starting date of the cohort. Not formally investigated. One study reported intermediate storage at −20°C when immediate storage at −80°C is not possible.	2	Yes: intermediate storage in the refrigerator or at −20°C. No: long-term storage at −80°C.*

Notes: The reported percentages were calculated from valid answers only (i.e., if detail was not specified or answered with N/A it was not included in the counts). We were not able to investigate the variables indicated with an asterisk (*) within the current empirical protocols, although we do recognize these are relevant pre-analytical variables with high priority ranking.

Abbreviation: EDTA, ethylene diamine tetraacectic acid.

et al.[1] Aβ42, Aβ40, NfL, and GFAP were measured at the Quanterix lab , with the pre-commercial Simoa Neurology 4-Plex E kit, and Aβ42, Aβ40, and t-tau were measured at the Clinical Neurochemistry Laboratory of Gothenburg University with the Simoa Human Neurology 3-Plex A kit (Quanterix). Aβ oligomerization tendency (OAβ) was measured at PeopleBio (Republic of Korea), using multimer detection system-OAβ test.[7] Last, p-tau181 was measured at Amsterdam UMC in duplicate with 4-fold automated sample dilution on the Simoa HDx analyzer using a Simoa prototype two-step assay with AT270 (Thermo Fisher Scientific) as the capture and LRL (Eli Lilly and Company) as the detector antibody, which is according to the Meso Scale Discovery (MSD) set-up as published previously.[44] We analytically validated this novel assay prior to use and accepted it to be fit for purpose, according to validation consensus guidelines.[45]

2.4 | Data analysis

We applied Spearman correlation analysis to compare the raw blood biomarker values obtained in the reference conditions. Biomarker values obtained under experimental conditions were normalized against their reference condition (%recovery). We calculated group median %recovery per experimental condition. When the median changed > 10%, we considered this a relevant (clinically significant) change. This arbitrary value was chosen to balance intra-assay variability inherent to fluid biomarker measurements (generally < 10%), with the observation that plasma Aβ42/40 is decreased by only ≈20% in patients with AD compared to controls.[9] Data were visualized using R version 1.1.463.

3 | RESULTS

3.1 | Pre-analytical variations across studies identified with inventories

Fifteen investigators representing nine cohorts, five companies, and one experimental study filled out our pre-analytical sample handling survey. Results are summarized in Table 1. No relevant variation was reported in sampling factors needle size, location of blood draw, plastic tubing or other tools used, tube collection order, and tube filling volume. No variation was reported in preparation factor tube inversion or manipulation before centrifugation, and only little variation in storage factor freeze–thaw cycles. Much variation was reported in standing time from blood draw until centrifugation (longest: 30 hours), and from centrifugation to freezing (longest: 4 hours), during which 50% of the studies hold the tubes at RT and 50% hold the tubes cold.

The pre-analytical variables were ranked for priority for testing (average ratings in Table 1). In general, variables with little variation were ranked lowest, and variables with much variation were ranked highest. Exceptions were collection tube type and freeze–thawing.

3.2 | Effect of pre-analytical variation on the Aβ42 and Aβ40 peptides

Aβ42 and Aβ40 were measured with two MS assays, two Simoa assays, and two ELISAs. The absolute Aβ42, Aβ40, and Aβ42/40 values for each of the assays as measured in the non-mistreated reference conditions of the experimental sample sets (n = 80 for each assay) are presented in File S4 in supporting information. All values were measured well above the assay's lower limits of quantifications. In these reference samples, we observed moderate between-assay correlations of most of the Aβ42 values (range Spearman ρ: 0.3–0.6, all: $P < .04$), only the Araclon ELISA results did not correlate with the MALDI MS, the Euroimmun ELISA, and the N4PE Simoa results (File S5 in supporting information). We also observed moderate between-assay correlations of most of the Aβ40 values (range Spearman ρ: 0.3–0.6, all: $P < .02$), only between the MALDI MS assay and the N4PE Simoa assay there was no correlation (File S5).

A detailed summary of the median recovery with interquartile range (IQR) per pre-analytical sample handling variation, for each of the markers with each of the assays, is presented in File S6 in supporting information. For most assays, Aβ42 and Aβ40 values were somewhat lower in sodium–citrate samples (range of median recovery Aβ42: 85–100%; Aβ40: 82–99%) and somewhat higher in lithium–heparin samples (Aβ42: 104–131%; Aβ40: 97–128%; Figures 1 and 2) compared to plasma EDTA samples. Aβ42 and Aβ40 values in serum samples were either lower, the same, or higher depending on the assay used (Aβ42: 36–108%; Aβ40: 69–115%; Figures 1 and 2). Sample type-mediated higher or lower Aβ values seemed largely independent of the technology and/or assay used. An exception might be lithium–heparin, as qualitatively, recoveries of both MS assays as well as both Simoa assays were similar for Aβ42, and of both MS assays and both ELISAs for Aβ40.

Independent of the assay used, if centrifugation was delayed for 24 hours, plasma Aβ42 and Aβ40 values declined when tubes were held at RT (range Aβ42: 59–81%; range Aβ40: 62–93%), whereas this decline was not yet visible at earlier time points (1 and 3 hours) nor when tubes were held in the refrigerator up to 24 hours (Figures 1 and 2; except outliers in MALDI MS Aβ40 and N3PA Aβ40 data: see File S6). We observed this same assay-independent effect upon delayed storage of separated plasma (i.e., post-centrifugation and aliquoting; Figures 1 and 2). Plasma Aβ42 and Aβ40 values were decreased in samples that were held at RT for 24 hours prior to –80°C storage (Aβ42: 70–85%; Aβ40: 68–90%), whereas this decrease was not yet visible at time point 4 hours nor when the samples were held in the refrigerator for up to 24 hours (except outliers in MALDI MS Aβ40 data: see File S6). Two-week intermittent storage in the refrigerator prior to storage at −80°C

also resulted in decreased Aβ42 (41–79%) and Aβ40 values (27–80%), independent of the assay used, while two-week intermittent storage at −20°C did not (Figures 1 and 2). Centrifugation temperature (RT or 4°C), aliquot volume (250, 500, or 1000 μL in 1.5 mL-tubes) and sample freeze–thawing (up to four cycles) did not impact Aβ42 and Aβ40 values (Figures 1 and 2).

The Aβ42/40 ratio mitigated the pre-analytical variation observed on Aβ42 and Aβ40 values for only some of the assays and conditions (Files S6 and S7 in supporting information). The observed pre-analytical effect at 24-hour delayed centrifugation (RT condition) could be mitigated by the Aβ42/40 ratio for only the Euroimmun ELISA assay, and not the other five assays. The observed effect of 24-hour delayed storage (RT condition) was mitigated by the Aβ42/40 ratio for four of six assays (C$_2$N MS, Simoa N3PA, Euroimmun ELISA, Araclon ELISA), and the observed effect of 2-week storage in the refrigerator was mitigated by this ratio for three of six assays (C$_2$N MS, Simoa N3PA, Araclon ELISA).

3.3 | Effect of pre-analytical variation on other blood-based biomarkers

We also measured levels of two other amyloid forms OAβ and APP$_{699-711}$ and the non-amyloid biomarkers GFAP, NfL, t-tau, and p-tau181 (absolute values in non-mistreated reference samples in File S4; median (IQR) recovery per pre-analytical condition per analyte in File S6).

Sample type influenced OAβ, GFAP, NfL, t-tau, and p-tau181, but not APP$_{699-711}$ (Figure 3), in a similar way as for the Aβ42 and Aβ40 analytes. Compared to EDTA plasma samples, values were lower in sodium–citrate samples (range of median recovery: 74–103%), higher in lithium–heparin samples (except OAβ: recovery 52%; other: 103–206%) and either comparable (APP$_{699-711}$, GFAP, NfL) or lower (OAβ, t-tau, p-tau181) in serum samples.

OAβ, APP$_{699-711}$, GFAP, NfL, and p-tau181 were stable over 24 hours delayed centrifugation conditions both when tubes were held at RT or in the refrigerator pending centrifugation (Figure 3; except one outlier for OAβ: see File S6). t-tau, however, was not stable in whole blood, with lower values when tubes were held at RT for 3 hours (83%) and for 24 hours (49%), and higher values when tubes were held in the refrigerator for 3 hours (115%) and for 24 hours (129%) pending centrifugation. Also, centrifugation temperature affected t-tau levels (4°C compared to RT condition: recovery 81%), while it did not affect the other markers. Aliquot tube filling did not affect any of the markers. Short-term delayed storage (i.e., max 24 hours) did not affect any of the markers (except outliers in p-tau181: see File S6), but APP$_{699-711}$ and t-tau levels were lower after 2-week storage in the refrigerator (APP$_{699-711}$: 72%; t-tau: 85%). The marker levels remained stable upon 2-week −20°C storage. Last, only GFAP levels were increased in the samples that underwent four additional freeze–thaw cycles (113%), while no change was observed at the second cycle. Levels of the other markers were not changed after up to four times repeated freeze–thawing.
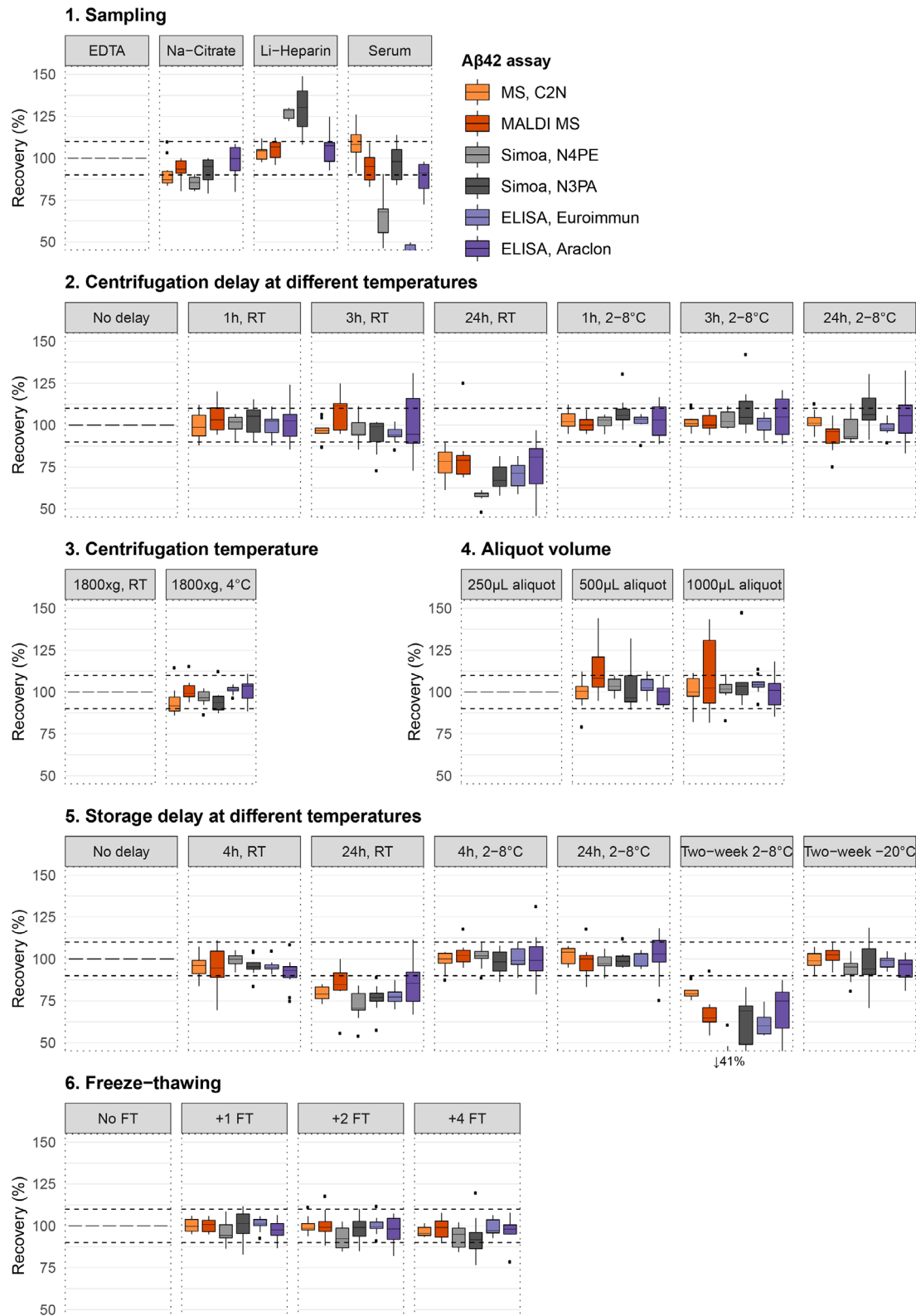
FIGURE 1 Effect of pre-analytical sample handling variations on Aβ42 levels, measured by different technologies. Values measured in the experimental conditions were normalized against the values measured in the reference conditions, which is EDTA plasma, centrifuged at 1800 × g at room temperature, after a standing time of 30 minutes at room temperature, immediately followed by aliquoting and −80°C storage. In 1. Sampling, levels in Na-citrate and Li-heparin were undetectable with the Euroimmun ELISA assay. In 5. Storage, 2-week 2–8°C condition, the recovery was 41% for Simoa N4PE, which is below the y-axis limit. Horizontal, dashed reference lines were set at 90% and 110%, to visualize a > 10% change of the median under an experimental condition compared to the reference condition. Aβ, amyloid beta; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FT, freeze–thaw cycle; Li-heparin, lithium–heparin; MS, mass spectrometry; Na-citrate, sodium–citrate; RT, room temperature
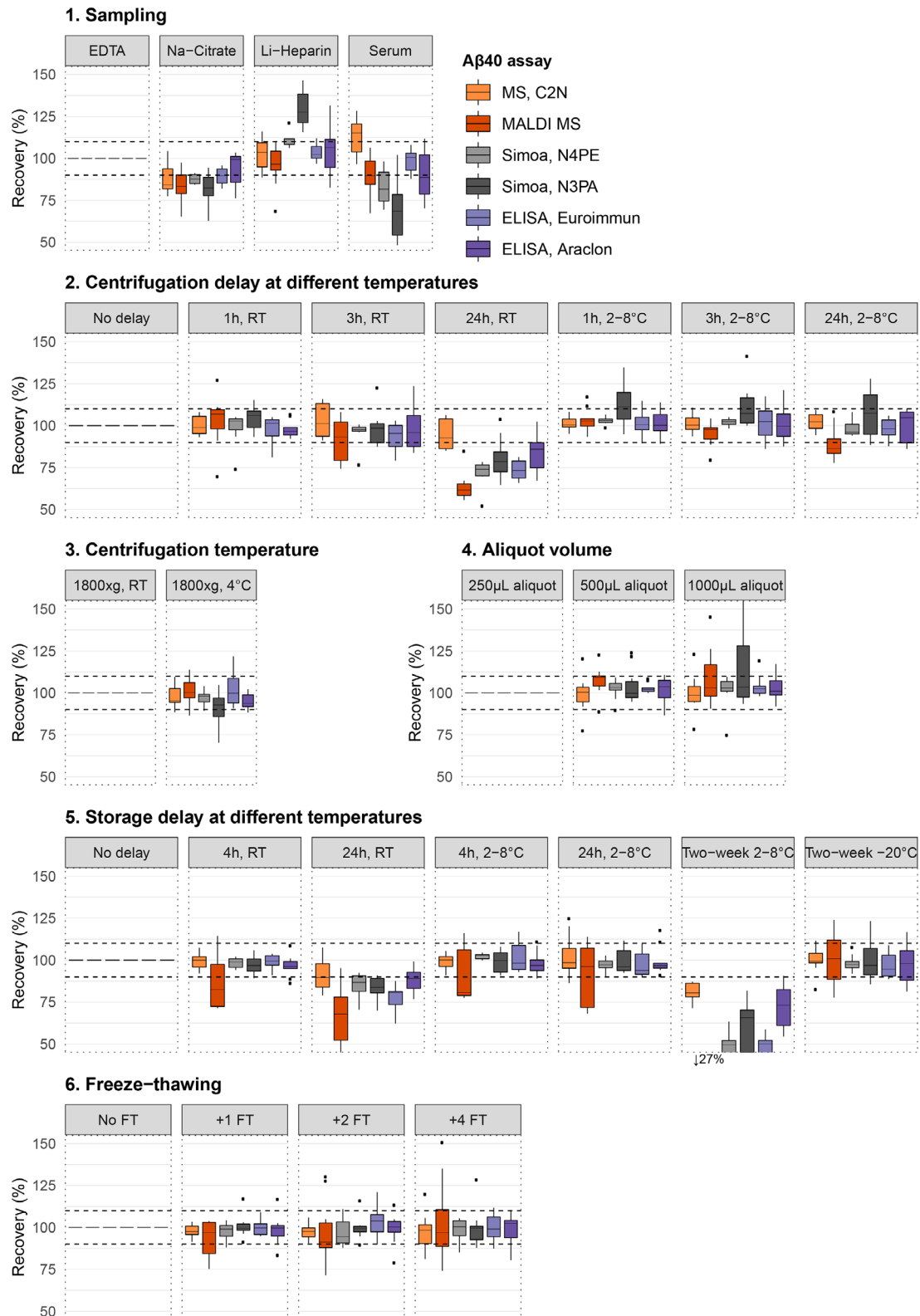
FIGURE 2 Effect of pre-analytical sample handling variations on Aβ40 values, measured by different technologies. Values measured in the experimental conditions were normalized against the values measured in the reference conditions, which is EDTA plasma, centrifuged at 1800 × g at room temperature, after a standing time of 30 minutes at room temperature, immediately followed by aliquoting and −80°C storage. In 5. Storage, 2-week 2–8°C condition, the recovery was 27% for the MALDI MS assay, which is below the y-axis limit. Horizontal, dashed reference lines were set at 90% and 110%, to visualize a > 10% change of the median under an experimental condition compared to the reference condition. Aβ, amyloid beta; EDTA, ethylene diamine tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FT, freeze–thaw cycle; Li-heparin, lithium–heparin; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; Na-citrate, sodium–citrate; RT, room temperature
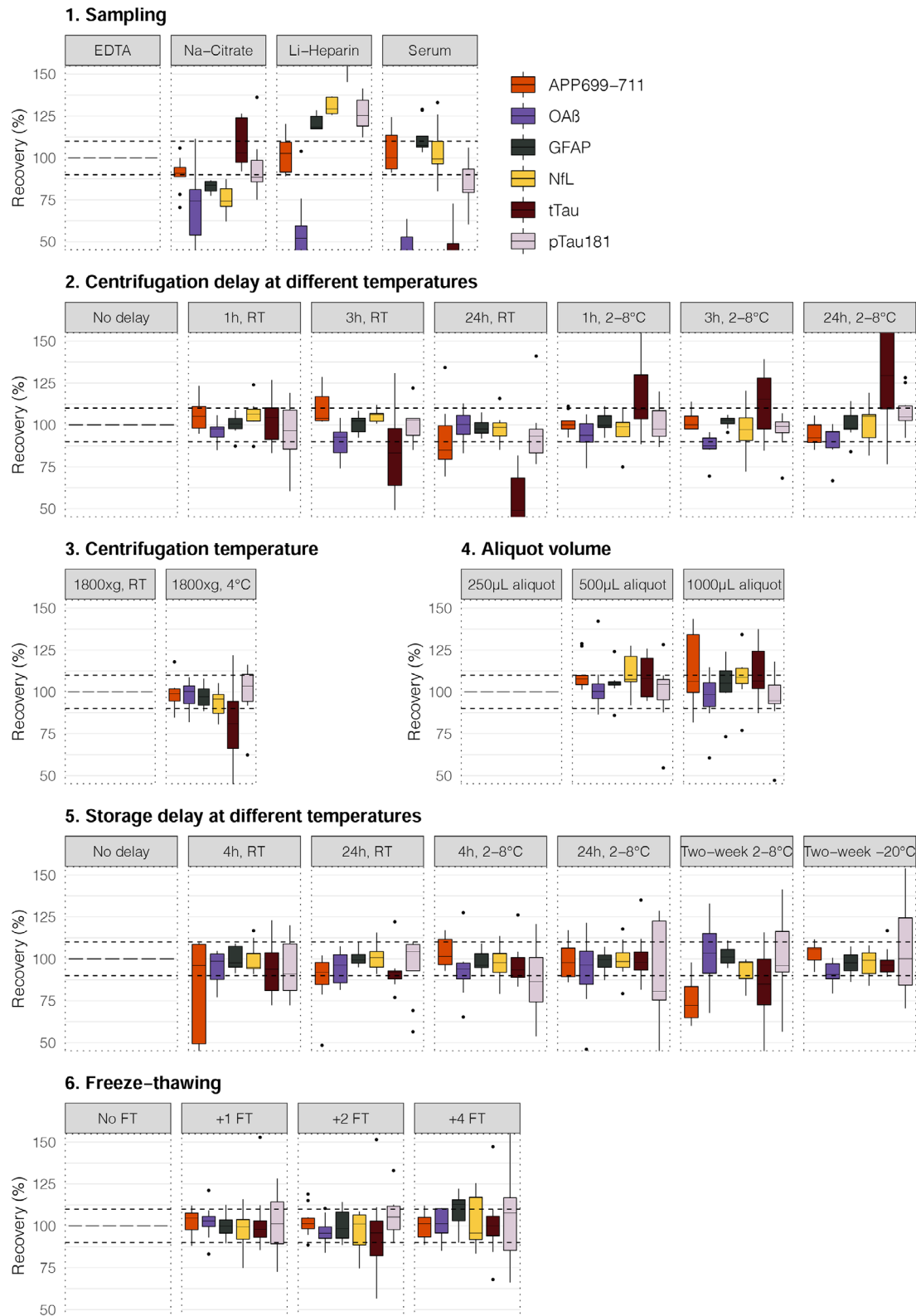
FIGURE 3 Effect of pre-analytical sample handling variations on biomarkers APP$_{699-711}$, OAβ, GFAP, NfL, t-tau, and p-tau181. Values measured in the experimental conditions were normalized against the values measured in the reference conditions, which is EDTA plasma, centrifuged at 1800 × g at room temperature, after a standing time of 30 minutes at room temperature, immediately followed by aliquoting and −80°C storage. Horizontal, dashed reference lines were set at 90% and 110%, to visualize a > 10% change of the median under an experimental condition compared to the reference condition. APP, amyloid precursor protein; FT, freeze-thaw cycle; GFAP, glial fibrillary acidic protein; Li-heparin, lithium–heparin; Na-citrate, sodium–citrate; NfL, neurofilament light; OAβ, amyloid beta oligomerization tendency; p-tau181, tau phosphorylated at threonine 18; RT, room temperature; t-tau, total tau
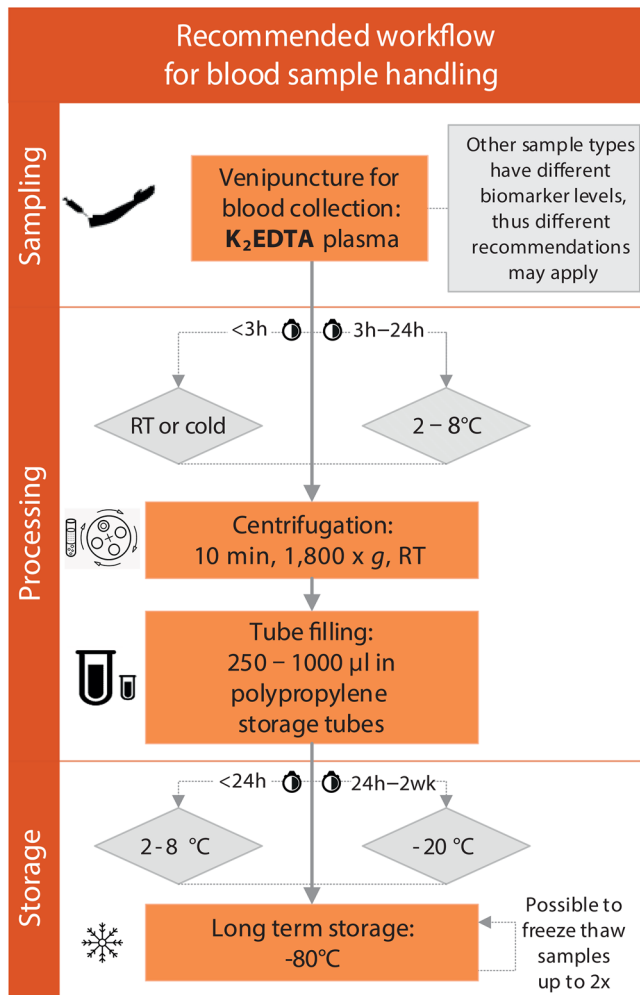
FIGURE 4 Recommended plasma handling standardized operating procedure (SOP). The SOP was constructed according to results obtained in systematic pre-analytical experiments. Deviating from this SOP might result in less reliable Aβ42 and Aβ40 measurements. For reliable total tau measurement, a maximum processing time of 1 hour might be necessary. Longer delays than 24 hours for centrifugation or 2 weeks for storage might be possible, but this was not investigated in this study. Aβ, amyloid beta; RT, room temperature; wk, week

4 | DISCUSSION

We showed that between studies, largest pre-analytical variation is in time to centrifugation and time from centrifugation to storage, during which tubes are kept at RT or cold. Our experiments showed that time and temperature to centrifugation and storage are critical pre-analytical variables, as was blood collection tube type. Levels for all investigated markers were unaffected by differences in aliquot tube filling, short-term −20°C storage, and up to three cycles of sample freeze–thawing. Based on our empirical observations, we recommend an SOP for the handling of EDTA plasma from collection to measurement (Figure 4). Understanding pre-analytical sample handling effects on blood-based biomarkers and implementing SOPs to limit variation is key to reach consistency across studies, technologies, and laboratories. This will contribute to validating and interpreting blood-based biomarker results across studies, and facilitates implementation of blood-based biomarkers into diagnostic and trial settings.

Aβ values were decreased in whole blood and in separated plasma at 24 hours when held at RT but not when stored in the refrigerator, and after 2 weeks in the refrigerator but not at −20°C. Therefore, we recommend in our SOP to store whole blood or separated plasma in the refrigerator when centrifugation and aliquot freezing is not possible within a short time window. Biomarkers OAβ, APP[699-711], GFAP, NfL, and p-tau181 were stable under delayed centrifugation and storage conditions. It is noted that t-tau was not resistant to delayed centrifugation, with lower t-tau levels observed when whole blood was held at RT for ≥3 hours, and higher levels when whole blood was held in the refrigerator for ≥3 hours. t-tau findings in our study were the exception among the biomarkers investigated, and are not in line with earlier publications using other t-tau assays, that showed that t-tau was stable for up to 6[33] or 8 hours[37] prior to centrifugation. Additionally, clinical studies on plasma t-tau suggest that when measured in its current format, this is not an accurate biomarker for AD.[13] Therefore, we decided not to adapt the SOP to the t-tau findings. Instead, we recommend t-tau biomarker studies to be conducted in biobanks with minimal centrifugation delays. Further, only t-tau levels were influenced by centrifugation temperature, with lower levels in samples that were centrifuged at 4°C. We recommend adhering to RT centrifugation.

At this time, few studies have been published on pre-analytical stability of blood-based Aβ42, Aβ40, t-tau, GFAP, NfL, and p-tau181.[33,34,36,37,40,46,47] None studied the variables included in our study comprehensively and only one analytical platform was used within each study. Results of previous studies corroborated our observed resistance of Aβ42 and Aβ40 to sample freeze–thawing (up to 3x)[33,34,40,46] and to delayed centrifugation or storage when kept cold.[36] Results on the effect of delayed centrifugation and storage at RT on Aβ42 and Aβ40 levels were conflicting.[33,36,37] With different assays than used in our study, Aβ42 and Aβ40 levels in whole blood were found stable at RT over 8 hours,[37] started declining from 1 hour onward,[33] or from 6 hours (Aβ42) and 48 hours (Aβ40) onward.[36] In separated plasma, levels were shown to decline at RT from 6 hours (Aβ42) and 8 hours (Aβ40) onward.[36] We did not include intermediate standing times between 3 or 4 and 24 hours, but in our hands, Aβ42 and Aβ40 values started declining somewhere within this time window. Using the Aβ42/40 ratio instead of the single peptides was not a solution to mitigate all pre-analytical effects in the current study. The observed declines were somewhat more pronounced for Aβ42 compared to Aβ40, suggesting that plasma Aβ42 is more unstable. Because we did not see an effect of tube filling on Aβ levels, adsorption of Aβ to plastics likely does not explain the observed decreases in Aβ levels, in contrast to earlier observations for the CSF for which adsorption to plastics was an important pre-analytical variable.[48] Ex vivo aggregation or proteolytic cleavage could be mechanisms at play.

Prior NfL studies showed, in agreement with our findings, that levels are stable in the refrigerator for up to 24 hours prior to processing and storage,[38] and for 48 hours prior to processing at RT.[47] It was also shown that NfL levels increase when samples are stored either at RT

or in the refrigerator for 5 days,[39] but we did not see such an increase after 2 weeks in the refrigerator. There is some conflicting literature on the effect of freeze–thawing on NfL levels, with some studies reporting no effect of sample freeze–thawing,[34,38] while another study did report an effect.[40] Furthermore, it was reported that plasma p-tau181 levels were decreased at freeze–thaw cycle 4, while GFAP remained stable up to freeze–thaw cycle 4.[34] Thus, if possible, using fresh aliquots might be preferred, although our findings show it is possible to freeze–thaw several times after initial freezing. So far, none studied the effects of delayed storage and processing on p-tau and GFAP.

A strength of the current study is that we had a standardized, systematic study design to investigate variations in the pre-analytical phase on a broad panel of blood-based biomarkers. With all biomarkers measured in the same samples, we could make well-founded conclusions on the relevance of pre-analytical variables and establish a biomarker-independent SOP, even though there was some measurement variation for some assays. Further, we included six different assays with different technologies for Aβ, resulting in a technology-independent SOP. Interestingly, the observed pre-analytical effects on Aβ42 and Aβ40 values were largely technology-independent, although Spearman correlations between the assay results were only moderate. Possible causes for these moderate correlations are that different assays target different epitopes of the Aβ peptides and there are differences in assay sensitivity and selectivity,[49] and inherent to the assay used, there are differences in how samples are handled from thawing until measurements.

Our study has some limitations as well. We empirically tested effects of pre-analytical variations on plasma EDTA samples only, because this is the matrix most commonly stored in dementia biobanks and used for AD blood-based biomarker testing. Different collection tubes resulted in different absolute biomarker values, which was also confirmed in an independent study,[34] but we do not know yet if pre-analytical effects are similar for different blood sample types, which is the subject of follow-up studies. Further, we did not investigate differences between K_2EDTA and K_3EDTA, which are both used commonly in dementia biobanks. Also, it might be that pre-analytical variations are dependent on the presence of a disease (e.g., healthy controls vs. patients with AD) or on the biomarker starting concentration, which we did not investigate here. Also, we must note that RT is not a standardized term. In this study, RT was by estimation in the range of 17–22°C, and we did not test higher ambient RTs. Further, we only investigated single pre-analytical parameters per experiment, while there might be interactions between parameters, which should be addressed in follow-up studies. Also, it is noted that pre-analytics might affect both measurement precision and analyte stability, which both affect the measured biomarker concentrations, and which affects clinical applicability of the biomarkers. Last, it is important to note that only one p-tau181 assay was included in this study, which is a prototype Simoa assay applying the antibodies used in the Eli Lilly MSD set-up. This assay is not yet published elsewhere. How the findings obtained with this assay relate to other assays, such as the commercially available p-tau181 Quanterix assay, remains to be established.

We recommend follow-up studies to confirm, and expand, our current findings, for example, including the multiple plasma p-tau assays that are currently becoming available. For a comparative p-tau pre-analytics study, we recommend the inclusion of AD patients, to include samples with higher starting concentrations. Especially for p-tau217, many control samples were reported to be measured below the detection limit in previous research,[50] which impacts measurement precision, therewith hinders interpretation of possible variation in levels due to pre-analytical conditions. Also, we recommend to extend our findings. Time of day of collection and fasting status, as well as long-term stability at –80°C, were not investigated in the current study but were deemed relevant by expert opinion. It is noted that the SOP we designed might be tailored most to observational studies that store samples to measure multiple biomarkers now and in the future. If one has a specific marker defined in the protocol or a specific context of use in mind, different sample handling protocols may be preferred. Also, with novel blood-based biomarkers and assays entering the field and follow-up experiments to come, we realize the SOP presented here may need fine-tuning, which will only increase adoption of the SOP in a wide range of settings.

To conclude, we established a technology-independent and biomarker-independent SOP for plasma EDTA sample handling, from collection to storage to measurement. We recommend biobanks and clinical trials adopt this SOP, to limit pre-analytical variability on blood biomarker results. Also, once the AD blood-based biomarkers reach clinical implementation, this SOP can be applied to reach optimal results in routine AD diagnostic blood biomarker measurements.

CONFLICTS OF INTEREST

Inge Verberk, Els Misdorp, Jannet Koelewijn, Jana Kindermans, and Teresa Waligorska report no financial disclosures nor conflicts of interest related to the present work or outside the submitted work. Andrew Ball and Dandan Shan are employees of Quanterix Corporation, and hold Quanterix Corporation stock and stock options. Simoa assay development work associated with this study was performed at and funded by Quanterix Corporation. Andrew Ball received a National Institutes of Health (NIH) Rapid Acceleration of Diagnostics grant (RADx 5534) for development/scaling of a SARS-CoV-2 assay, which was paid to the institution and was not associated with this study, and resulted in two provisional patent applications. Outside the submitted work, Kaj Blennow has served as a consultant or at advisory boards for Abcam, Axon, Biogen, JOMDD/Shimadzu, Lilly, MagQu, Prothena, Roche Diagnostics, and Siemens Healthineers, and as data monitoring committee for Julius Clinical and Novartis. All payments were made to him personally. Kaj Blennow is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program. Jeffrey L. Dage is an employee and stockholder of Eli Lilly and Company, which also supports his costs for travel and attending of meetings. Subject matter relating to the p-tau181 assay, methods, reagents, and/or compositions of matter set forth herein are subject to patents and/or patent applications of Eli Lilly and Company. Noelia Fandos is an employee of Araclon Biotech. Oskar Hansson has acquired research support (for the institution) from AVID Radiopharmaceuticals, Biogen, Eli Lilly, Eisai, GE Healthcare, Pfizer, and Roche. In the past 2 years, he has received consultancy/speaker fees from AC Immune, Alzpath, Biogen, Cerveau, and Roche. Christophe Hirtz has a collaboration contract with Shimadzu European Innovation Center including a PhD thesis. Shorena Janelidze is supported by research grants from Alzheimerfonden Kockska stiftelsen Stiftelsen för Gamla Tjänarinnor, and travel grants from the Strategic Research Area MultiPark (Multidisciplinary Research in Parkinson's disease) at Lund University. All payments were made to Lund University. Sungmin Kang and Ryan Lee are employees and stockholders of PeopleBio. Kristopher Kirmess, Matthew Meyer, and Tim West are employees of C2N Diagnostics. C2N has received funding in support of their assay from the following grant sources: NIH, BrightFocus Foundation, GHR Foundation. Leslie M. Shaw has grant support from NIH: NIA ADNI U19 AG024904; UPenn ADRC P30 AG010124; IIS grant support from Roche; serves on Advisory Boards for Biogen, Roche, FujiRebio, Siemens Healthineers. 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These payments were made to him personally. He has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure, and Biogen. Further, he is a chair of the Alzheimer's Association Global Biomarker Standardization Consortium and a chair of the Alzheimer's Association Biofluid-Based Biomarker PIA (no payments), and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (payments made to him personally). Rebecca M. Edelmayer is a full-time employee of the Alzheimer's Association. She serves on the external advisory board of the TREAT-AD Consortium, NIH (2020-Present; no payments made), and the MODEL-AD Consortium, NIH (2019-Present; no payments made). 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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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