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ORIGINAL ARTICLE

Impact of pre-analytical sample handling factors on plasma biomarkers of Alzheimer's disease

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Abstract

An unmet need exists for reliable plasma biomarkers of amyloid pathology, in the clinical laboratory setting, to streamline diagnosis of Alzheimer's disease (AD). For routine clinical use, a biomarker must provide robust and reliable results under pre-analytical sample handling conditions. We investigated the impact of different pre-analytical sample handling procedures on the levels of seven plasma biomarkers in development for potential routine use in AD. Using (1) fresh (never frozen) and (2) previously frozen plasma, we evaluated the effects of (A) storage time and temperature, (B) freeze/thaw (F/T) cycles, (C) anticoagulants, (D) tube transfer, and (E) plastic tube types. Blood samples were prospectively collected from patients with cognitive impairment undergoing investigation in a memory clinic. β-amyloid 1–40 (Aβ40), β-amyloid 1–42 (Aβ42), apolipoprotein E4, glial fibrillary acidic protein, neurofilament light chain, phosphorylated-tau (phospho-tau) 181, and phospho-tau-217 were measured using Elecsys® plasma prototype immunoassays. Recovery signals for each plasma biomarker and sample handling parameter were calculated. For all plasma biomarkers measured, pre-analytical effects were comparable between fresh (never frozen) and previously frozen samples. All plasma biomarkers tested were stable for ≤24 h at 4°C when stored as whole blood and ethylenediaminetetraacetic acid (EDTA) plasma. Recovery signals were acceptable for up to five tube transfers, or two F/T cycles, and in both polypropylene and low-density polyethylene tubes. For all plasma biomarkers except Aβ42 and Aβ40, analyte levels were largely comparable between EDTA, lithium heparin, and sodium citrate tubes. Aβ42 and Aβ40 were most sensitive to pre-analytical handling, and the effects could only be partially compensated by the Aβ42/Aβ40 ratio. We provide recommendations for an optimal sample handling protocol for analysis of plasma biomarkers for amyloid pathology AD, to improve the reproducibility of future studies on plasma biomarkers assays and for potential use in routine clinical practice.

Abbreviations: 9NC, trisodium citrate; AD, Alzheimer's disease; Aβ, β-amyloid; CSF, cerebrospinal fluid; CV, coefficient of variation; DMT, disease-modifying therapy; EDTA, ethylenediaminetetraacetic acid; F/T, freeze/thaw; FNF, fresh (never frozen); GFAP, glial fibrillary acidic protein; IQR, interquartile range; K3, tripotassium; K3E, tripotassium ethylenediaminetetraacetic acid; LiHep, lithium heparin; NA, not available; NaCit, sodium citrate; NFL, neurofilament light chain; PE-LD, low-density polyethylene; PET, positron emission tomography; PF, previously frozen; phospho-tau, phosphorylated-tau; PP, polypropylene; Q, quartile; RT, room temperature; WB, whole blood.

KEYWORDS
Alzheimer's disease, beta-amyloid (Aβ), phosphorylated-tau (phospho-tau), pre-analytical stability, glial fibrillary acidic protein (GFAP), neurofilament light chain (NFL)

1 | INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia in the elderly. Recent forecasts predict that by 2050, there will be more than 152 million people living with dementia worldwide, with up to 70% of cases being attributed to AD (Nichols et al., 2022; World Health Organisation, 2022). AD is commonly characterized by β-amyloid (Aβ) deposition (amyloid pathology) and tau pathology in the brain (DeTure & Dickson, 2019).

Until recently, there were no disease-modifying therapies (DMTs) available that could attenuate the cognitive and functional decline associated with AD and improve the patient's quality of life (Rasmussen & Langerman, 2019). However, in June 2021, the United States Food and Drug Administration granted accelerated approval for aducanumab, an Aβ-directed monoclonal antibody for the treatment of patients with mild symptomatic AD (Esang & Gupta, 2021) and in September 2022, phase three clinical trial results for lecanemab for the treatment of mild cognitive impairment due to AD and mild AD with confirmed presence of amyloid pathology in the brain were announced (Eisai, 2022). Such novel DMTs will likely be most effective during the early stages of AD; as such, routine clinical use of plasma biomarkers that correlate with cerebral amyloid and tau pathologies are required to enable early identification of patients requiring further evaluation and initiation of DMTs (Rasmussen & Langerman, 2019; Rózga et al., 2019). Given that there are many underlying causes of dementia, and co-pathology is common in individuals with AD, it is also important to be able to differentiate the clinical syndrome from the underlying pathological process to arrive at a diagnosis of AD (Rabinovici et al., 2017; Staffaroni et al., 2017).

Validated cerebrospinal fluid (CSF) biomarkers (including Aβ, total-tau and phosphorylated-tau [phospho-tau]) and positron emission tomography (PET) biomarker testing are routinely used for the diagnosis of AD; however, these procedures are expensive, invasive, and have limited availability in the primary care setting (Blennow et al., 2015; Grimmer et al., 2009; Janelidze et al., 2020). There exists an unmet need for reliable plasma biomarkers of amyloid pathology and AD that are accessible, minimally invasive and easy to use, to aid identification of patients who would benefit from confirmatory diagnostic evaluation using CSF and PET, and rule out patients with a low likelihood of amyloid pathology and AD (Rózga et al., 2019). Collection of plasma via blood draw is associated with less risks, is less invasive, and inexpensive compared with CSF collection via lumbar puncture and PET imaging. Furthermore, a strong correlation has been observed between several of the biomarkers found in CSF and plasma (Barthélemy et al., 2020; Blennow et al., 2015; Karikari et al., 2020; Khoury & Ghossoub, 2019; Ovod et al., 2017; Tatebe et al., 2017; Zetterberg, 2019). In particular, the plasma β-amyloid 1–42/β-amyloid 1–40 (Aβ42/Aβ40) and CSF phospho-tau/Aβ42 ratios are reflective of amyloid pathology in the brain, with a diagnostic accuracy of approximately 90% versus PET, dependent on the analytical platform used (Doecke et al., 2020; Hansson et al., 2018; Nakamura et al., 2018; Ovod et al., 2017; Zetterberg, 2019).

Variability exists between institutions in the procedures used to process plasma and CSF samples prior to analysis, and differences in these procedures may contribute to the significant inter-laboratory and batch-to-batch variability observed in previous studies on CSF biomarkers (Hansson et al., 2021; Snyder et al., 2014; Watt et al., 2012). Recent studies have evaluated the impact of common pre-analytical parameters on the measurement of plasma biomarkers for amyloid pathology and AD, and provide recommendations to standardize sample handling procedures and improve the reliability of analyses on these biomarkers (Rózga et al., 2019; Verberk et al., 2021). Further investigation into appropriate pre-analytical sample handling procedures across a wide range of plasma biomarkers, in fresh (never frozen) samples, using fully automated platforms is required to aid the reliable analysis of plasma biomarkers for amyloid pathology and AD in routine laboratory practice and in clinical trials.

This exploratory study aimed to evaluate the effects of storage time and temperature in fresh (never frozen) and previously frozen plasma; these evaluations were conducted using plasma separated from stressed whole blood (WB), and plasma stressed following separation from WB. In addition, the effects of freeze/thaw (F/T) cycles were studied in fresh (never frozen) plasma, the effects of anticoagulants and tube transfer in previously frozen plasma, and the effects of plastic tube type in both in fresh (never frozen) and previously frozen plasma (Table 1; Figure S1).

2 | METHODS

2.1 | Study design

This prospective, non-interventional study was conducted between December 2020 and October 2021 in Germany (sample collection at University Hospital, LMU Munich, Munich, Germany; measurement at Roche Diagnostics GmbH, Penzberg, Germany). The study was conducted in accordance with the principles of the Declaration of Helsinki and the International Council for Harmonisation Good Clinical Practice guidelines. All participants provided written informed consent to participate in the *PsyCourse* biobank project, including the collection, storage, analysis, scientific utilization, and distribution of samples. Blood samples were anonymized, and no clinical data was shared. *PsyCourse* was conducted by the Department of Psychiatry and Psychotherapy, LMU Munich, and received approval from the ethics committee of LMU Munich (project number 18-716).

TABLE 1 Summary of pre-analytical sample handling parameters and assays evaluated in this study and their results

	Sample handling parameter														
	Storage time and temperature									Anticoagulant				Tube type	
	RT				4°C										
	WB (h)		EDTA plasma (h)		WB (h)		EDTA plasma (h)								
Assay	FNF	PF	FNF	PF	FNF	PF	FNF	PF	Number of F/T cycles (–20°C or –80°C)	EDTA plasma (reference)	LiHep	NaCit	Number of tube transfers	PP (reference)	PE-LD
Aβ40	2	6	2	2	24	24	24	24	2	✓	X	✓	5	✓	✓
Aβ42	2	2	2	2	24	24	24	24	2	✓	X	✓	5	✓	✓
Aβ42/ Aβ40	2	6	6	2	24	24	24	24	4	✓	✓	✓	5	✓	✓
Apolipoprotein E4	–	–	6	2	–	–	24	24	4	✓	–	–	5	✓	✓
GFAP	24	24	24	24	24	24	24	24	4	✓	✓	✓	5	✓	✓
NFL	24	24	24	24	24	24	24	24	4	✓	✓	✓	5	✓	✓
phospho-tau-181	24	–	24	–	24	–	24	–	4	✓	–	–	–	✓	✓
phospho-tau-217	24	24	6	24	24	24	24	24	4	✓	✓	✓	5	✓	✓

Note: Hours indicate the time for which the plasma biomarker levels were within pre-defined acceptance criteria. Tick marks (✓) indicate parameters for which plasma biomarker levels were within pre-defined acceptance criteria; crosses (X) indicate parameters for which plasma biomarker levels did not meet pre-defined acceptance criteria. En dashes (–) indicate parameters for which no data were collected.

Abbreviations: Aβ40, β-amyloid(1–40); Aβ42, β-amyloid(1–42); EDTA, ethylenediaminetetraacetic acid; FNF, fresh (never frozen); F/T, freeze/thaw; GFAP, glial fibrillary acidic protein; h, hours; LiHep, lithium heparin; NaCit, sodium citrate; NFL, neurofilament light chain; PE-LD, low-density polyethylene; phospho-tau-181, phosphorylated-tau 181; phospho-tau-217, phosphorylated-tau 217; PF, previously frozen; PP, polypropylene; RT, room temperature; WB, whole blood.

The plasma levels of Aβ40, Aβ42, apolipoprotein E4, glial fibrillary acidic protein (GFAP), neurofilament light chain (NFL), phospho-tau-181, and phospho-tau-217 were measured using fully automated Elecsys® plasma prototype immunoassays on the cobas® e 601 analyzer (all Roche Diagnostics International Ltd). The Elecsys Aβ40, Aβ42, apolipoprotein E4, GFAP, NFL, phospho-tau-181, and phospho-tau-217 plasma prototype immunoassays are intended for investigational/research purposes and are not currently approved for clinical use or commercially available. The prototype immunoassays were validated according to internal procedures adapted from the bioanalytical method validation guideline EMEA/CHMP/EWP/192217/2009 Rev.1 Corr.2 from the European Medicines Agency. The coefficients of variation for intra-assay, inter-assay and inter-instrument precision are summarized in Table S1. The Aβ42/Aβ40 ratio was calculated from the Aβ40 and Aβ42 measurements obtained in each analytical assessment procedure.

2.2 | Participants and sample collection/handling

Eligible participants were patients with cognitive impairment due to possible or probable AD according to the National Institute on Aging-Alzheimer's Association criteria (McKhann et al., 2011), presenting for routine clinical visits at the University Hospital, LMU Munich. The laboratory site that received the samples for plasma biomarker measurement had no further patient information. Thus, no randomization was performed to allocate individuals in this study, no blinding was necessary, and no pre-determined sample size calculations were employed. We did not conduct power calculations for this analysis, as the results were based on signal recovery rates versus the reference sample, and no p-values were produced. Despite the small sample size, the effects were consistent across patients, which gives us confidence that the changes seen are robust effects. No samples were excluded from the analyses.

All samples were collected at three independent blood donation events and processed as previously described (Rózga et al., 2019), unless otherwise specified in Section 2.3. Venepuncture was performed according to a standard operating procedure and free flow of blood with mild aspiration was ensured to avoid hemolysis. WB samples were collected in tripotassium (K3) ethylenediaminetetraacetic acid (EDTA) S-Monovette tubes (Sarstedt Inc.) and the tubes were immediately inverted five times after sample collection to ensure sufficient mixing of the WB with the anticoagulant present in the tube. To achieve the correct WB to anticoagulant ratio, each tube type was filled to a nominal volume according to the manufacturer's instructions. Unless otherwise stated, WB samples were stored in the collection tube at room temperature (RT) and centrifuged within 1 h of collection for 10 min at 2000x*g* at RT in a swing bucket centrifuge. Separated plasma was then transferred within 1 h of centrifugation into a polypropylene (PP) tube, inverted several times to ensure sufficient mixing of the sample, and immediately aliquoted into PP tubes. Further details of the tubes used are provided in Table S2.

2.3 | Pre-analytical sample handling parameter assessments

The effects of the following pre-analytical sample handling parameters on plasma biomarker levels were assessed: (A) storage time and temperature, (B) F/T cycles, (C) anticoagulant type, (D) tube transfer, and (E) plastic tube type.

2.3.1 | Storage time and temperature assessment

To assess the effects of storage time and temperature on the stability of WB prior to plasma separation, one 'baseline' WB sample set was kept at 4°C for <6 h prior to centrifugation and plasma collection. This delay of up to 6 h was unavoidable due to logistical circumstances. At the time of this experiment, sufficient information had already been collected on the stability of the analytes at 4°C and storage of <6 h at 4°C was considered acceptable. Thus, fresh (never frozen) baseline plasma samples were stored for <6 h at 4°C prior to preparation, and previously frozen baseline plasma samples were stored for 30 min at RT prior to preparation and frozen at −20°C prior to analysis. Plasma samples were assessed using the Elecsys Aβ40, Aβ42, apolipoprotein E4, GFAP, NFL, phospho-tau-181, and phospho-tau-217 plasma prototype immunoassays to determine a baseline measurement for each of the biomarkers tested.

Additional WB sample sets were 'stressed' by keeping them at RT or 4°C for an additional 2, 6, or 24 h before centrifugation and plasma separation. These 'stress' procedures were intended to simulate laboratory situations in which samples may be left unattended prior to, or after, processing, causing a delay in handling. The samples where then either measured immediately (hereafter referred to as 'fresh (never frozen) plasma from stressed WB') or frozen at −20°C prior to analysis (hereafter referred to as 'previously frozen plasma from stressed WB').

To evaluate the effects of storage time and temperature on the stability of plasma, WB samples were processed as described above. Separated plasma was then pooled and split into aliquots. Aliquoted plasma samples were assayed or frozen immediately to establish a baseline measurement, or 'stressed' by keeping them at RT or 4°C for an additional 2, 6, or 24 h, and either assayed immediately after the delay ('fresh (never frozen) stressed plasma' samples) or frozen at −20°C and assayed immediately after thawing ('previously frozen stressed plasma' samples).

For previously frozen plasma samples, three possible handling scenarios were investigated, whereby 'stress' simulation occurred at different times after plasma separation. In these scenarios, plasma was (1) transferred to a measuring tube and stressed prior to freezing, (2) stressed in the original blood collection tube and then transferred to measuring tubes for freezing, or (3) transferred to a measuring tube, frozen and stressed after thawing. Note, there are no data available for phospho-tau-181 measured in previously frozen samples.

2.3.2 | F/T cycle assessment

Samples undergoing a F/T cycle analysis were kept at 4°C for <6 h prior to centrifugation. To evaluate the effects of F/T cycles on plasma biomarker levels, aliquoted plasma was assayed immediately to determine the baseline level of each plasma biomarker in fresh (never frozen) plasma, or after one, two, three, or four F/T cycles. During each F/T cycle, samples were frozen at either −20°C or −80°C for ≥8 h before thawing at RT.

2.3.3 | Anticoagulant assessment

In addition to K3 EDTA tubes, WB was also collected in S-Monovette lithium heparin (LiHep) and S-Monovette sodium citrate (NaCit) tubes (both Sarstedt Inc.) to assess the effects of anticoagulants on plasma biomarker levels. After collection, WB samples were processed as described in Section 2.2 and frozen at −20°C. After thawing, the level of plasma biomarkers were assessed as described above. Note, there are no data available for phospho-tau-181 measured in anticoagulant samples.

2.3.4 | Tube transfer assessment

To determine the effects of tube transfer on the stability of plasma biomarkers, previously frozen plasma samples (processed as described in section 2.2; tube 0 [baseline level], PP tube) were thawed at RT, before being transferred into the next PP tube (tube 1). This process was completed one, three, and five times. For every transfer, a new pipette tip was used. Plasma biomarker levels were then determined for each plasma sample in tubes 0, 1, 3, and 5.

2.3.5 | Plastic tube type assessment

To assess the effect of plastic tube type on plasma biomarker levels, WB samples were collected and then processed either as described in Section 2.2, or with prolonged storage of WB for <6 h at 4°C prior to centrifugation. Separated plasma was transferred into a PP or low-density polyethylene (PE-LD) measuring tube (Sarstedt Inc. and Roche Diagnostics International Ltd, respectively), and assayed immediately after transfer ('fresh [never frozen]') or frozen before measurement ('previously frozen').

2.4 | Statistical analysis

Most of the plasma biomarkers included in the present analysis were measured in single determination. Where duplicate measurements were available, mean values were calculated. For all sample handling parameters tested, recovery signals for Aβ40, Aβ42, apolipoprotein E4, GFAP, NFL, phospho-tau-181, and phospho-tau-217 compared with the reference sample were reported. For each participant and sample, and for all of the plasma biomarkers measured, the acceptance criteria were ±10% of the signal recovery rate compared with the reference sample. The Aβ40/Aβ42 ratio was calculated based on analyte concentrations, and the recovery signal of the ratio compared with the reference sample was reported. A summary of the reference values used to compare the signal recovery for each sample handling parameter tested is provided in Table S3. The detection of plasma apolipoprotein E4 is intended to determine carrier *APOE ε4* status; however, it does not establish homozygosity versus heterozygosity. For apolipoprotein E4 analysis, analyte results were separated into apolipoprotein E4 positive (i.e., presence of at least one *APOE ε4* allele) and negative (i.e., no *APOE ε4* alleles). Box plots of the analyte levels were generated using JMP version 15.2.0 (SAS Institute Inc.). Assessment of the normality of the data was omitted due to the small sample size. In addition, no parametric tests for outliers were included in the analyses as an abnormal distribution of the data was assumed.

3 | RESULTS

WB samples were collected from a total of *N = 16* patients across three independent blood donation events. An overview of the analyses performed in this study and results obtained is provided in Table 1.

3.1 | Effect of storage time and temperature on WB and plasma

All of the plasma biomarkers tested were stable for up to 24 h at 4°C when stored as WB and EDTA plasma (Figure 1). Aβ42, Aβ40, and the Aβ42/Aβ40 ratio were unstable if stored for more than 2 h at RT in WB and EDTA plasma. Phospho-tau-181, GFAP, and NFL were stable for up to 24 h at RT in WB and EDTA plasma; phospho-tau-217 was stable at RT for up to 24 h in WB and 6 h in plasma. For all of the plasma biomarkers and pre-analytical effects measured, there was no marked difference in the median recovery signal between fresh (never frozen) and previously frozen plasma samples. Furthermore, there were no marked differences between the three handling scenarios employed for previously frozen plasma (Figure S2).

3.2 | Effect of F/T cycles on fresh (never frozen) plasma

Recovery signals for all plasma biomarkers except Aβ42 and Aβ40 were acceptable for up to four F/T cycles (Figure 2a). For Aβ42 and Aβ40, up to two F/T cycles were acceptable, whereas the Aβ42/Aβ40 ratio was acceptable for up to four F/T cycles. Recovery signals for all plasma biomarkers measured were comparable between samples frozen at −20°C and samples frozen at −80°C.

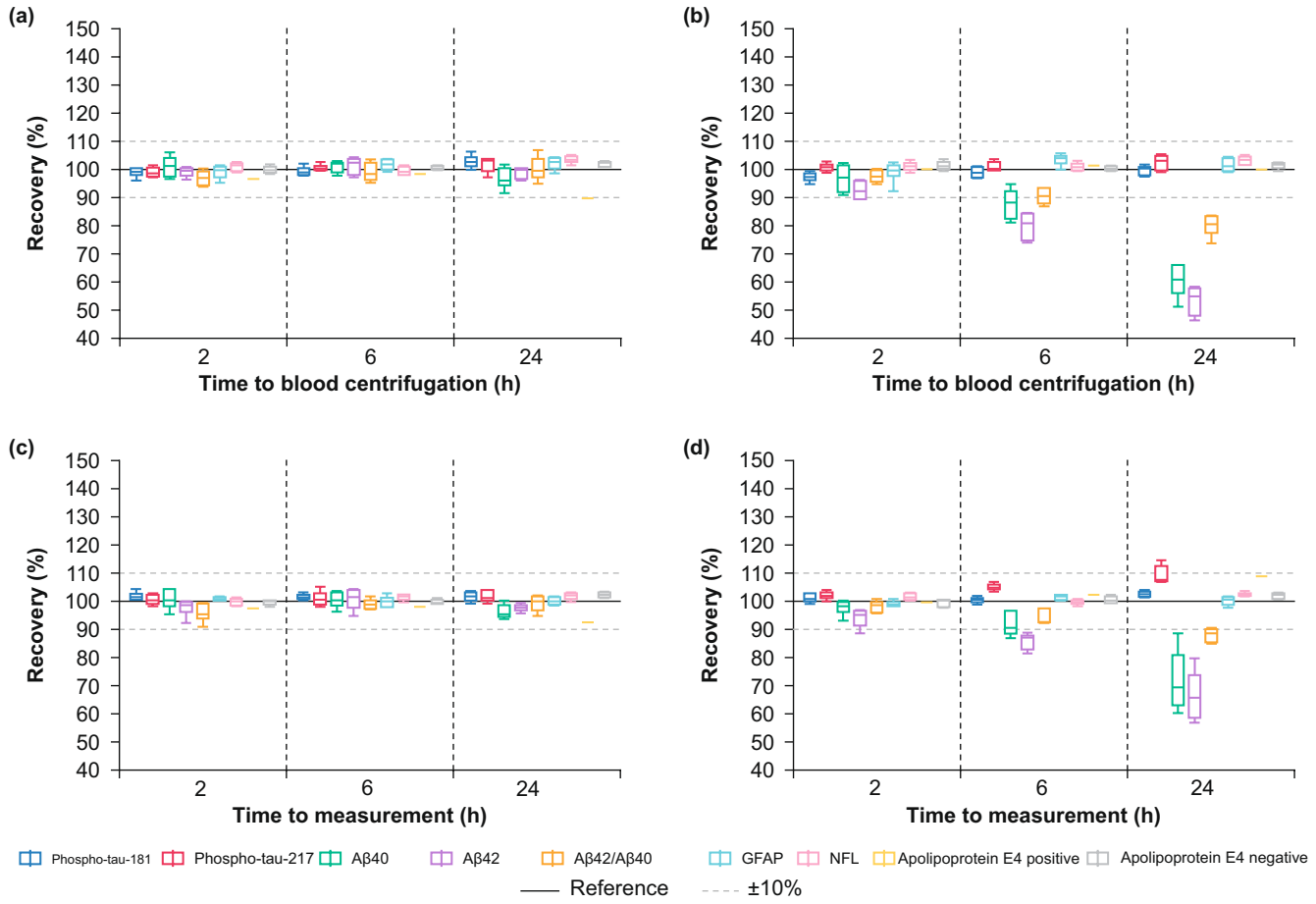

FIGURE 1 Effect of storage time and temperature on plasma biomarker levels in WB stored at (a) 4°C and (b) RT, and plasma stored at (c) 4°C and (d) RT (measured in fresh (never frozen) plasma). For all plasma biomarkers measured $n = 6$, except for apolipoprotein E4 positive and negative, where $n = 1$ and $n = 5$, respectively. Box plots shown comprise data from $n = x$ individual donors and one data point per donor. Boxes represent the median and interquartile range; the lower whisker represents the higher of the minimum values and the 25th percentile (Q1) to 1.5*IQR; the higher whisker represents the lower of the maximum values and the 75th percentile (Q3) to 1.5*IQR. Aβ40, β-amyloid 1–40; Aβ42, β-amyloid 1–42; GFAP, glial fibrillary acidic protein; IQR, interquartile range; NFL, neurofilament light chain; phospho-tau-181, phosphorylated-tau 181; phospho-tau-217, phosphorylated-tau 217; Q, quartile; RT, room temperature; WB, whole blood.

3.3 | Impact of anticoagulant type

For all plasma biomarkers, except Aβ42 and Aβ40, analyte levels were largely comparable between EDTA, LiHep, and NaCit tubes (EDTA was used as a reference; Figure 3a). Comparing LiHep tubes with EDTA tubes, the median recovery signals for Aβ42 and Aβ40 increased by 10% and 9%, respectively, with LiHep tubes. In NaCit tubes compared with EDTA tubes, the median recovery signals for Aβ42 and Aβ40 decreased by 6% and 4%, respectively, when using NaCit tubes. Furthermore, with NaCit tubes, a downward trend was observed for GFAP and NFL when compared with both EDTA and LiHep tubes. There were no samples from apolipoprotein E4 positive donors available for this analysis.

3.4 | Effect of tube transfer

All plasma biomarkers assessed were stable for up to five tube transfers in previously frozen EDTA plasma (Figure 3b). The median recovery signals for Aβ42 and Aβ40 decreased progressively between one, three, and five tube transfers; however, this decrease was within the predefined acceptance criteria of ±10% of the median recovery signal.

3.5 | Effect of plastic tube type

There was no marked change observed in the median recovery signal between PP and PE-LD tubes for any of the plasma biomarkers measured in fresh (never frozen) plasma (Figure 2b). Median recovery signals for all plasma biomarkers measured were comparable between fresh (never frozen) and previously frozen plasma samples (Figure 2b; Figure S3).

4 | DISCUSSION

The present study evaluated the impact of storage time and temperature on Aβ40, Aβ42, the Aβ42/Aβ40 ratio, apolipoprotein E4,

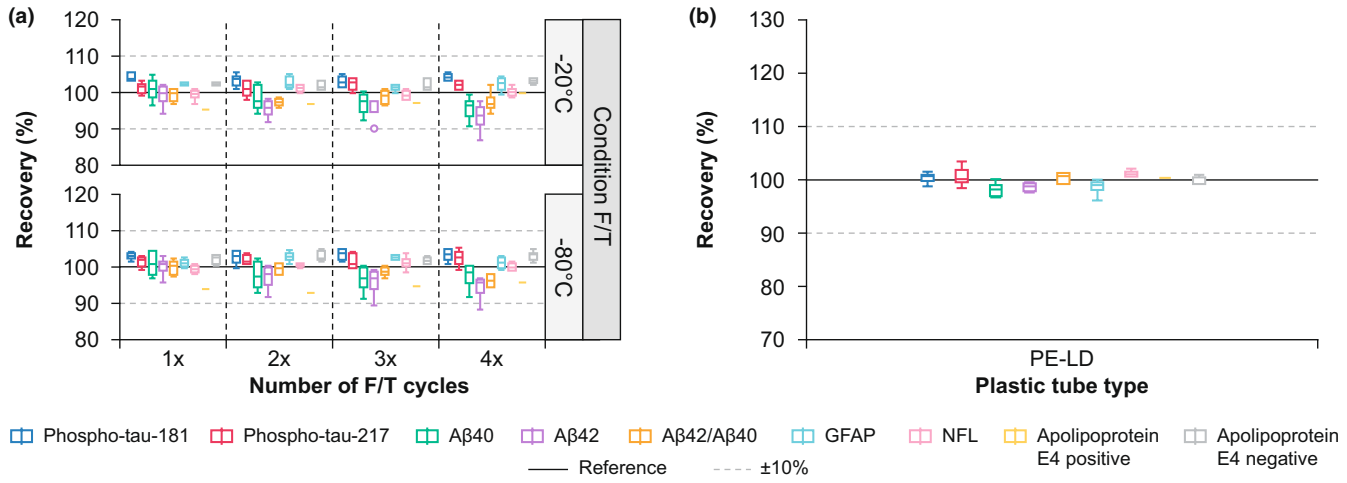

FIGURE 2 Effect of (a) F/T cycles and (b) plastic tube type on plasma biomarker levels in fresh (never frozen) plasma. For all plasma biomarkers measured $n = 6$, except for apolipoprotein E4 positive and negative, where $n = 1$ and $n = 5$, respectively. Box plots shown comprise data from $n = x$ individual donors and one data point per donor. For panel B, a PP tube was used as the reference. Boxes represent the median and interquartile range; the lower whisker represents the higher of the minimum values and the 25th percentile (Q1) to 1.5*IQR; the higher whisker represents the lower of the maximum values and the 75th percentile (Q3) to 1.5*IQR. Values above or below the whiskers are plotted as dots. Aβ40, β-amyloid 1–40; Aβ42, β-amyloid 1–42; F/T, freeze/thaw; GFAP, glial fibrillary acidic protein; IQR, interquartile range; NFL, neurofilament light chain; phospho-tau-181, phosphorylated-tau 181; phospho-tau-217, phosphorylated-tau 217; Q, quartile.

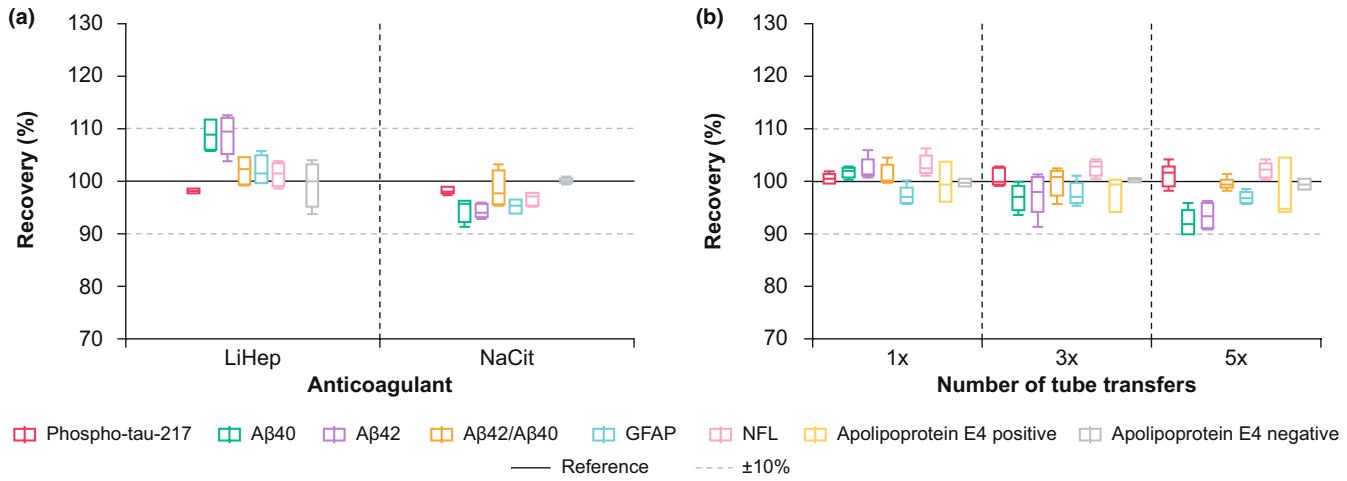

FIGURE 3 Effect of (a) anticoagulants and (b) tube transfer on plasma biomarker levels in previously frozen plasma. For panel A, for all plasma biomarkers measured $n = 4$, except for apolipoprotein E4 positive and negative, where $n = 0$ and $n = 4$, respectively. For panel B, for all plasma biomarkers measured $n = 5$, except for apolipoprotein E4 positive and negative, where $n = 3$ and $n = 2$, respectively. Box plots shown comprise data from $n = x$ individual donors and one data point per donor. Boxes represent the median and interquartile range; the lower whisker represents the higher of the minimum values and the 25th percentile (Q1) to 1.5*IQR; the higher whisker represents the lower of the maximum values and the 75th percentile (Q3) to 1.5*IQR. Aβ40, β-amyloid 1–40; Aβ42, β-amyloid 1–42; GFAP, glial fibrillary acidic protein; IQR, interquartile range; LiHep, lithium heparin; NaCit, sodium citrate; NFL, neurofilament light chain; phospho-tau-217, phosphorylated-tau 217; Q, quartile.

GFAP, NFL, phospho-tau-181, and phospho-tau-217 in fresh (never frozen) and previously frozen plasma, and the effects of storage time and temperature in plasma separated from stressed WB compared with plasma stressed following separation. In addition, the effects of F/T cycles in fresh (never frozen) plasma, the effects of anticoagulants and tube transfer in previously frozen plasma, and the effects of plastic tube types in both in fresh (never frozen) and previously frozen plasma were studied.

All plasma biomarkers tested were stable for up to 24 h at 4°C in WB and EDTA plasma. This means that WB can be stored directly in the collection tube in the fridge with no immediate processing needed. Moreover, EDTA plasma can be stored in the original blood collection tube at 4°C after centrifugation (mimicking a scenario when the sample is mistakenly left in the centrifuge). Measured levels of all plasma biomarkers except Aβ42 and Aβ40 were acceptable for up to four F/T cycles; for Aβ42 and Aβ40, a maximum of

two F/T cycles were acceptable. For all plasma biomarkers tested, pre-analytical effects were comparable between fresh (never frozen) and previously frozen samples, although the data for apolipoprotein E4-positive were more difficult to interpret due to having only a single sample available for fresh (never frozen) plasma analysis. We also demonstrated that the timing of plasma 'stressing' (i.e., storage at RT/4°C) can occur either before, or after, the freezing cycle. Again, this finding will make sample handling and processing more straightforward as samples will not need to be measured immediately and can instead be frozen. In combination, these findings allow for greater flexibility in pre-analytical blood sample handling and processing meaning that testing could take place at e.g., primary care centers, which in turn, would make large scale assessment for amyloid pathology and AD more feasible.

A previous study, conducted using earlier versions of the Elecsys Aβ42 and Aβ40 plasma prototype immunoassays at Roche Diagnostics GmbH (Penzberg, Germany), reported that Aβ42 and Aβ40 levels in previously frozen plasma samples were stable for up to 6 h when kept at 4°C prior to measurement; if the Aβ42/Aβ40 ratio was reported, these analytes remained stable for up to 24 h at RT and 4°C (Rózga et al., 2019). Conversely, the present findings indicate that Aβ42 and Aβ40 remain stable for up to 24 h at 4°C thawed EDTA plasma but were only stable for up to 2 h at RT if reporting the Aβ42/Aβ40 ratio. Our results show that Aβ42 levels decreased faster over time compared with Aβ40, reducing the ability of the Aβ42/Aβ40 ratio to compensate for this effect.

Our data are broadly in line with a previous report (Liu et al., 2020), in which a downwards trend in the levels of Aβ42 and Aβ40 with increasing number of F/T cycles was noted, similar to that observed here. Although the prior report did not find a statistically significant difference in the levels of Aβ42 and Aβ40 in fresh (never frozen) plasma compared with plasma experiencing five F/T cycles (whereas we found maximum of two F/T cycles were acceptable for Aβ42 and Aβ40 in fresh [never frozen] plasma), we consider that this discrepancy is likely due to differences in the rigidity of data interpretation.

Present findings indicate that apolipoprotein E4, GFAP, NFL, phospho-tau-181, and phospho-tau-217 levels were comparable between EDTA, LiHep, and NaCit tubes. This is encouraging given that phospho-tau is one of the most promising plasma biomarkers currently being investigated; the comparable stability of this analyte between EDTA, LiHep and NaCit tubes is beneficial for future large-scale studies. However, we did not test stability in these sample matrices, and so our results should be interpreted in that context. In contrast with present findings, previous studies found that levels of phospho-tau-181, GFAP, and NFL were lower in NaCit tubes (range of median recovery: 74%–103%) and higher in LiHep tubes (103%–206%) compared with EDTA tubes (Ashton et al., 2021; Verberk et al., 2021). The increase and decrease in recovery signal observed herein for Aβ42 and Aβ40 in LiHep and NaCit tubes, respectively, is in accordance with findings from previous studies (Ashton et al., 2021; Rózga et al., 2019; Verberk et al., 2021). One explanation for the decrease in analyte levels observed when using NaCit tubes may be that the citrate solution in the tubes dilutes the sample. Our study provides further evidence that use of LiHep and NaCit tubes can impact the measurement of Aβ42 and Aβ40 in plasma and indicate that EDTA tubes may be preferable if Aβ42 and Aβ40 are the analytes of interest.

A previous study found that consecutive transfer of CSF samples between tubes significantly affected measured levels of Aβ42 and Aβ40 (Toombs et al., 2014). Conversely, another report demonstrated that tube transfers had no effect on Aβ42 and Aβ40 in plasma, which supports the present findings (Rózga et al., 2019). Herein, a downwards trend in Aβ42 and Aβ40 levels was observed with increasing numbers of tube transfers; however, this was within the pre-defined acceptance criteria and can most likely be explained by the hydrophobicity of Aβ42, which leads to aggregation and adherence to tube surfaces (Willemse et al., 2017).

Previous studies have indicated a sensitivity in measured levels of AD biomarkers, especially Aβ42 and Aβ40, related to the use of different plastic tubes used to process CSF samples (Lehmann et al., 2014; Perret-Liaudet et al., 2012). In this study, we evaluated data using two tubes commonly utilized for Elecsys assays and found no noticeable change in median plasma biomarker recovery signals.

Based on the results of this study, we provide recommendations for an optimal handling protocol for blood collection and sample handling for analysis of plasma biomarkers for amyloid pathology and AD based on measurement in fresh (never frozen) samples (Figure 4). Our recommendations are largely in line with recent findings from the Standardization of Alzheimer's Blood Biomarkers working group and include the use of EDTA anticoagulant tubes for blood sampling, a maximum of five tube transfers, and a maximum of two F/T cycles at −20°C or −80°C to maintain biomarker stability in plasma samples (Verberk et al., 2021). Aβ42 and Aβ40 were the analytes most sensitive to pre-analytical sample handling and the effects could only be partially compensated by using the Aβ42/Aβ40 ratio.

One key finding from the study is that WB and EDTA plasma can be stored at 4°C for up to 24 h, while storage at RT should be avoided or limited to 2 h maximum. These findings could (1) improve the feasibility of conducting AD testing meaning that such assessments could be carried out at, for example, primary care facilities, and (2) simplify the handling of the samples in laboratories.

Strengths of this study include the measurement of a large set of plasma biomarkers in fresh (never frozen) plasma samples and the use of the fully automated and highly precise Elecsys plasma prototype immunoassays, which allow for global scalability; this demonstrates novelty in comparison to previous studies (Rózga et al., 2019; Verberk et al., 2021). Other novel aspects of this study include expanding the analysis of biomarkers beyond that of Aβ42 and Aβ40, evaluating samples stored at 4°C before further processing, and using clinical samples from patients with cognitive impairment due to possible or probable AD, as opposed to samples from healthy patients. Our results confirm findings from previous studies and are therefore likely to be reliable (Rózga et al., 2019; Verberk

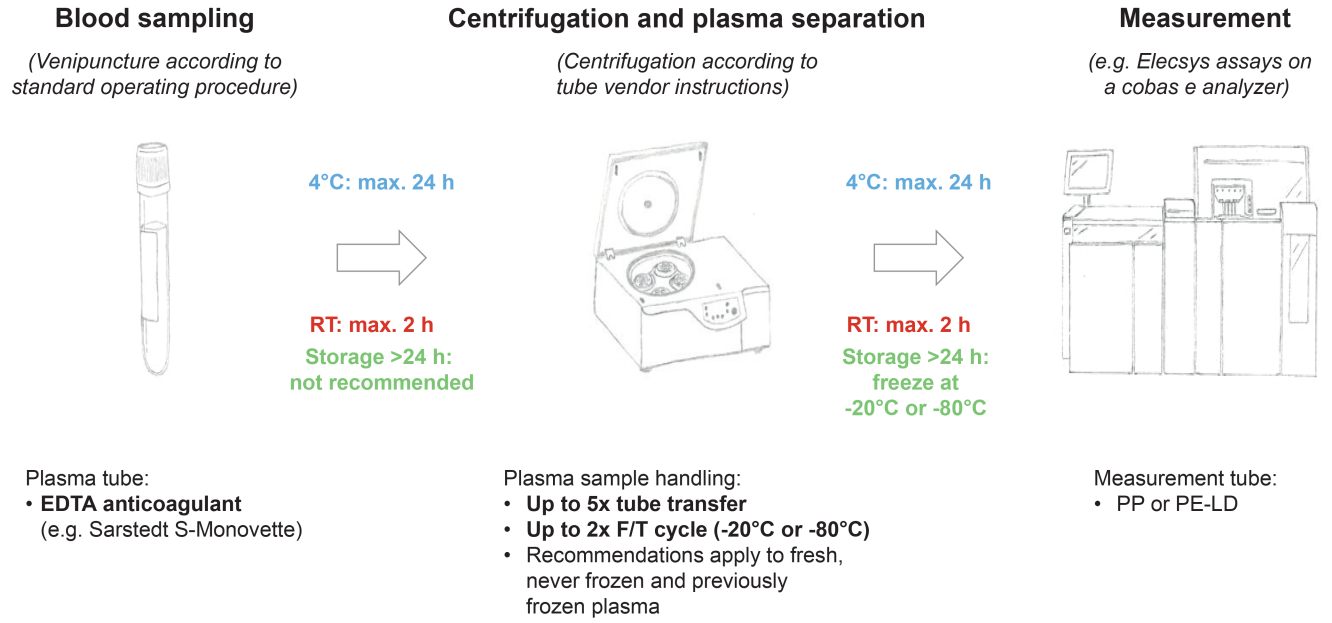

FIGURE 4 Summary of recommendations for blood collection and pre-analytical sample handling for the analysis of plasma biomarkers for amyloid pathology and AD. AD, Alzheimer's disease; EDTA, ethylenediaminetetraacetic acid; F/T, freeze/thaw; h, hours; max., maximum; PE-LD, low-density polyethylene; PP, polypropylene; RT, room temperature.

et al., 2021); however, future studies involving plasma biomarker immunoassays from other manufacturers are required to allow for comparison between platforms.

We acknowledge that this study also has some limitations. Samples were included from a relatively small number of patients ($n = 4–6$ per experiment, from a total of 16 donors), and very few were *APOE* $\varepsilon 4$ carriers. Despite the small sample size, the effects observed were consistent across patients, which gives us confidence that the results are robust. Additionally, the study population is reflective of real-world use of the Elecsys Aβ40, Aβ42, apolipoprotein E4, GFAP, NFL, phospho-tau-181, and phospho-tau-217 plasma prototype immunoassays (i.e., in patients with cognitive impairment).

The recommendations for an optimal handling protocol for blood collection and sample handling for analysis of plasma biomarkers for amyloid pathology and AD presented here will improve the reproducibility of future research into plasma biomarker assays and may support the adoption of these assays into routine clinical practice.

AUTHOR CONTRIBUTIONS

Carolin Kurz, Laura Stöckl, Tobias Bittner, Alexander Jethwa, and Robert Perneczky: study conception/design. Carolin Kurz, Laura Stöckl, Selim Üstün Gürsel, and Robert Perneczky: data acquisition. Carolin Kurz, Laura Stöckl, Isabelle Schrurs, Ivonne Suridjan, Tobias Bittner, Alexander Jethwa, and Robert Perneczky: data analysis/interpretation. All authors provided critical review of the manuscript and approved the final version for submission.

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CONFLICT OF INTEREST

CK and SUG declare no conflicts of interest. LS and AJ are full-time employees of Roche Diagnostics GmbH. ISc and ISu are full-time employees of Roche Diagnostics International Ltd. TB is a full-time employee of F. Hoffmann-La Roche and Genentech and a shareholder in F. Hoffmann-La Roche.

RP has received consultancy fees and speaker honoraria from Roche.

DATA AVAILABILITY STATEMENT

The anonymized data that support the findings of this study are available to qualified investigators on request from the corresponding author, Professor Robert Perneczky (email: robert.perneczky@med.uni-muenchen.de).

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

Not applicable.

CLINICAL TRIAL REGISTRATION

This study was not pre-registered as a clinical trial.

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