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Original Article

The impact of pre-analytical factors on plasma biomarkers for Alzheimer's disease: The ASPREE Healthy Ageing Biobank☆

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ABSTRACT

Background: The conditions under which samples were collected, processed, and stored in biobanks may influence Alzheimer's disease (AD) biomarker levels.
Objectives: This study aims to investigate whether a range of pre-analytical factors influence plasma levels of AD biomarkers.
Methods: Data were obtained from the ASPREE Healthy Ageing Biobank, a cohort of healthy community-dwelling older individuals aged 70+ years in Australia. Five biomarkers were measured using plasma from 11,868 individuals: phosphorylated-tau181 (p-tau181), neurofilament light chain (NfL), glial fibrillary acidic protein (GFAP), and amyloid-beta 42 and 40 (Aβ42/Aβ40). Linear regression examined the association between pre-analytical factors and biomarker levels.
Results: Participants were aged 70–96 years, and 54 % were female. The mean storage time for samples was 10.6 years (range: 7.7–13.5). Some significant associations were identified between pre-analytical factors and biomarkers, in particular for p-tau181, but the effect sizes were small. Weak negative associations were found between p-tau181 and the time from venepuncture to laboratory (transport) (β: −0.82, p = 0.03), laboratory processing to frozen storage (β:−1.56, p < 0.001), and total years of storage (β: −0.45, p = 0.007), while a positive association was found with intermediate storage at −20 °C/−30 °C compared to −80 °C (β: 2.24, p = 0.004). Longer fasting time was associated with higher levels of both NfL (β: 0.15, p < 0.001) and GFAP (β: 1.75, p < 0.001).
Conclusion: Following standard operating procedures, AD biomarkers can be measured in plasma from biobanks stored for up to 13 years, with minimal impact from long-term storage or other pre-analytical factors.

1. Introduction

Alzheimer's disease (AD) is a growing public health concern worldwide, with a significant impact on the healthcare system and society. As the most common form of dementia, AD places a considerable burden on the healthcare system and society [1], highlighting the need for effective tools that can detect the disease and monitor its progression accurately. Blood-based biomarkers, including amyloid-beta (Aβ), phosphorylated-tau181 protein (p-tau181), neurofilament light (NfL) and glial fibrillary acidic protein (GFAP), have been increasingly shown as promising tools for AD diagnosis and screening [2,3]. Compared to the use of neuroimaging (e.g., amyloid PET), or a lumbar puncture for the collection of cerebrospinal fluid (CSF), blood tests are minimally invasive, inexpensive and more accessible, making them ideal for large-scale use.

Biobanks are important resources for the understanding of AD pathophysiology and the discovery of biomarkers. They enable the collection and storage of biological samples collected from large prospective studies, which facilitates population-based investigations of blood biomarkers years before the onset of dementia symptoms. However, to ensure their accuracy and reliability, it is important to understand whether long-term storage in biobanks, as well as other factors in the pre-analytical phase, from collection to storage, could affect biomarker levels. There have been studies suggesting that blood concentrations of AD biomarkers may change after several years of storage [4,5]. The

☆ Pre-analytical effects on AD biomarkers.

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number of freeze-thaw cycles the blood samples undergo before the assay may also affect biomarker levels [6,7]. However, these studies have generally been small (n < 100) [7,8], limiting the statistical power to detect differences. Meanwhile, previous studies have mostly focused on storage conditions [4,6,9], but less is known about the earlier phases before storage, such as length of fasting, and time spent in transport and processing.

Using laboratory data obtained from a large biobank involving over 11,000 older community-dwelling individuals, this study aimed to determine whether the length of storage and a range of other important pre-analytical factors, including processing conditions and duration, were associated with plasma levels of p-tau181, NfL, GFAP, as well as Aβ42 and Aβ40.

2. Methods

2.1. ASPREE healthy ageing biobank

The sample population consisted of the Australian participants of the ASPREE (ASPirin in Reducing Events in the Elderly) clinical trial who agreed to provide blood samples within the first year of the study, mostly close to recruitment. ASPREE was a randomised double-blind placebo-controlled trial, examining the effects of daily low-dose aspirin versus placebo on disability-free and dementia-free survival in community-dwelling older individuals. A total of 19,114 participants, aged predominantly 70 years and above were recruited from Australia and the United States. According to the inclusion criteria at enrolment, participants were without major cognitive impairments (with a score of modified Mini-Mental State Examination >78 and no dementia diagnosis), independence-limiting physical disabilities, and cardiovascular diseases or any health condition that could be life-threatening within the five years after enrolment. Full details of the clinical trial have been published previously [10].

All Australian participants (n = 16,703) of the clinical trial were invited to provide blood samples for the ASPREE Healthy Ageing Biobank. Blood collections were prioritised to be within the first four weeks after enrolment and before starting trial medication, but baseline collections could be conducted within the first 12 months of the trial. The baseline biobank samples were collected between 2010 and 2015, largely in parallel with the recruitment period of the ASPREE clinical trial, and occurred across metropolitan and regional areas of four Australian States (Victoria, Tasmania, South Australia, New South Wales) and the Australian Capital Territory. For detailed information regarding the biobank recruitment procedure, please refer to the publication by Parker and colleagues [11].

2.2. Sample collection, processing and storage

The entire procedure, from blood collection to initial (intermediate, short-term) freezer storage, was instructed to be completed within a four-hour time window. Blood collection was conducted in or near the original ASPREE recruitment sites. Most blood collections were conducted by trained biobank staff during pre-booked biobank visits, with processing conducted in the nearby laboratory. In some instances, a local pathology service centre was contracted to collect the samples and our courier transferred the biospecimens on ice to a processing laboratory. Processing laboratories included the ASPREE central laboratory (Monash, Melbourne), regional/metropolitan hospital laboratories with staff instructed following rigorous standard operating procedures or biobank staff who travelled to process in the local laboratory, or a mobile laboratory called the ASPREE biobus (11). The biobus services were necessary for participants living in rural, regional or remote areas. Around 40 mL of blood was collected from each participant, with fasting status determined based on the recorded time of the last meal before blood collection. The blood sample was drawn into six tubes, including three EDTA tubes, two sodium citrate tubes and one serum separator. All samples were kept on ice or < 4 ˚C throughout all phases of processing, including the transport process to local laboratories for samples collected at contracted pathology service centres.

Upon arrival at the processing sites, EDTA samples were centrifuged at 4 ˚C (800 g, 15 minutes) with plasma, buffy coat and red blood cells separated. The upper layer of plasma was then pipetted from the tubes and then pooled into a single-use collection tube. The pooled plasma sample of each participant was then aliquoted into a maximum of 28 cryovials, with a maximum volume of 0.5 mL for each cryovial. All tubes during processing were kept in an ice slurry at 4 ˚C. Subsequently, the cryovials were stored at −80 ˚C freezer after aliquoting. If -80 ˚C freezers were not immediately available because of location, the cryovials were temporarily placed in intermediate storage including −80 ˚C shuttle biobus, −80 ˚C dry ice, 170 ˚C cryo-shipper (resin embedded with liquid nitrogen) or −20 ˚C /−30 ˚C freezer. Aliquots were then transferred to long-term storage in either a −80 ˚C freezer or nitrogen vapour phase tank. The mean intermediate storage time under the different conditions was as follows: −80 ˚C biobus–mean 1.12 days; −80 ˚C dry ice–mean 1.52 days; −170 ˚C cryo-shipper–mean 1.14 days; -20 ˚C/−30 ˚C freezer–mean 4.92 days.

2.3. Assays

All AD biomarker assays were conducted at the Advanced Research and Diagnostic Laboratory (ARDL) at the University of Minnesota, USA, in 2023. Samples were shipped to the laboratory from Melbourne (Australia) on dry ice. The Quanterix HD-X platform was used to measure plasma samples for the levels of AD biomarkers. Specifically, the Simoa® Human Neurology 4-Plex E (N4PE) platform was used to measure the levels of Aβ40, Aβ42, NfL and GFAP [12]. P-tau181 was measured using the Simoa® p-Tau181 v2 assay [13]. Samples were assayed in singlets with the inclusion of 9.5 % duplicates for p-tau181 and 9.6 % for N4PE. Laboratory personnel were blinded to the inclusion of replicate samples. The average intra-assay coefficients of variation (CV) from 148 samples were 3.2 % for Aβ40, 3.8 % for Aβ42, 4.6 % for GFAP, 4.6 % for NfL and 5 % for p-tau181. The average inter-assay CV from 145 samples was 4.8 % for Aβ40, 4.8 % for Aβ42, 6.1 % for GFAP, 6.3 % for NfL and 6 % for p-tau181.

2.4. Pre-analytical factors

Detailed information for each step of sample handling was documented on a Biospecimen Information Sheet. We analysed total storage time along with three additional sets of pre-analytical factors in this study, including

- Total storage time (time between sample collection and assay);
- Time of sample handling: transport time (time between blood collection and the first centrifugation), processing time (time between the first centrifugation and final sample storage);
- Pre-collection conditions: the fasting time before blood collection (0–4 hours, 4–8 hours, 8+ hours), history of blood transfusion (no, yes, unsure);
- Sample handling conditions: haemolysis (at least one aliquot in the whole sample was haemolysed: no, yes), thawing (at least one aliquot in the whole sample has been thawed unintentionally: no, yes), intermediate storage temperature (−80 ˚C, −170 ˚C, −20 ˚C/−30 ˚C).

2.5. Statistical analysis

Multivariable linear regression was used to analyse the associations between pre-analytical factors (independent variables) and the level of each plasma biomarker (dependent variable), including p-tau181, GFAP, NfL, Aβ40, Aβ42, as well as the Aβ42/40 ratio (log-transformed

for the analysis). Each pre-analytical factor was analysed in separate models, with key demographic characteristics of the participants (age at collection (numerical), sex (male, female)) and the location of sample collection (Monash-Central, biobus, other) adjusted. In each case, the reference group for the pre-analytical factor represented best practices to minimise biomarker variation [14,15]. For example, eight or more hours of fasting time, no history of blood transfusion, no thawing and storage at −80 ˚C. Time of sample handling and storage were analysed as continuous variables, with their associations assessed based on incremental increases. This approach allowed us to assess the independent associations of each pre-analytical factor with biomarker levels, while controlling for other covariates. We also used η^2 to assess the effect size of independent variables, with threshold values of 0.01 indicating a small effect, 0.06 a medium effect, and 0.14 a large effect [16]. To examine the potential confounding of long-term storage, we conducted sensitivity analyses by further adjusting for total storage time in the regression models of each pre-analytical factor. A $p < 0.05$ was considered statistically significant, indicating rejection of the null hypothesis of no difference. All analyses were performed using Stata version 17.0 (Stata Corp., College Station, Texas, USA) on the biobank dataset version 2.

2.6. Ethics

The ASPREE Healthy Ageing Biobank was approved by the ethics review board at each participating institution. All participants provided written informed consent.

3. Results

Among the 12,219 participants from Australia who contributed baseline biospecimens to the ASPREE biobank, 11,868 were eligible to be included in this study, after excluding those with no available blood samples, or with incomplete data for pre-analytical factors or biomarkers (Fig. 1).

The characteristics of the samples are presented in Table 1. The majority of participants were aged between 70–74 years (n = 6996, 59 %), were female (n = 6361, 53.6 %) and had their biospecimens collected at Monash-Central laboratory (n = 4226, 35.6 %) or on the biobus (n = 4601, 38.8 %). Most participants fasted for 0–4 hours (n = 9856, 83.1 %) and had no history of blood transfusion (n = 9854, 83.0 %). Most samples had no haemolysis (n = 11,530, 97.1 %) or unintentional thawing (11,729, 98.8 %). Over half of the samples were stored in −80 ˚C freezers for their initial short-term storage (n = 7009, 59.1 %), while others were mostly stored in −170 ˚C cryo-shippers temporarily (n = 3046, 26.7 %) before being transferred to long-term ultra-cold freezers at either −80 ˚C or −170 ˚C. The average time of transport (from collection to initiation of processing) and processing were 0.74 hours (44.37 minutes) and 1.74 hours (104.68 minutes), respectively. The average time of storage before the assay was 10.56 years.

The associations of time of transport, processing and long-term storage with levels of AD biomarkers are shown in Table 2. The β-coefficients refer to the change in biomarker levels for each unit of increase in numerical variables, or in comparison to the reference group for categorical variables. Specifically, the plasma level of p-tau181 decreased with increasing hours of transport ($\beta = -0.82$, $p = 0.03$), hours of processing ($\beta = -1.56$, $p < 0.001$), and years of storage ($\beta = -0.45$, $p = 0.007$). Meanwhile, Aβ42 levels were negatively associated with processing hours ($\beta = -0.09$, $p = 0.006$), while Aβ40 levels were positively associated with both transport hours ($\beta = 0.76$, $p = 0.04$) and storage years ($\beta = 0.36$, $p = 0.03$). However, the ratio of Aβ42 relative to Aβ40 only showed a negative association with total storage years ($\beta = -0.01$, $p < 0.001$). There was no association between any of the biomarkers and length of time at intermediate storage (data not shown, $p > 0.1$ for all comparisons).

The relationships between the remaining pre-analytical factors and plasma levels of AD biomarkers are detailed in Table 3 (p-tau181, NfL and GFAP) and Table 4 (Aβ42, Aβ40, Aβ42/40 ratio). Compared to those who fasted for 8+ hours before sample collection, participants with fewer fasting hours showed a higher level of p-tau181, and lower levels of NfL, GFAP, Aβ42 and Aβ40 (all $p < 0.05$).

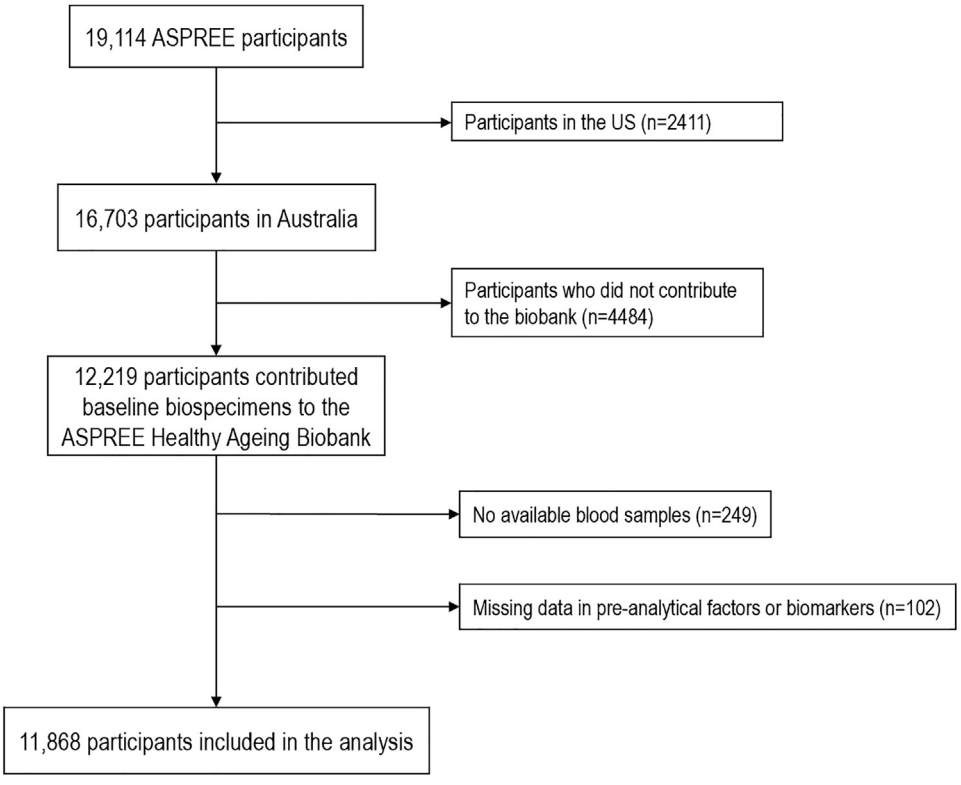

Fig. 1. Flowchart of participant selection.

Table 1
Basic characteristics of pre-analytical factors (n=11,868).

Pre-analytical factors Demographics/laboratories	Number (percentage)
Age at collection, years	
70–74	6996 (59.0)
75–79	3122 (26.3)
80+	1750 (14.7)
Sex	
Male	5507 (46.4)
Female	6361 (53.6)
Location of processing	
Monash-Central	4226 (35.6)
Biobus	4601 (38.8)
Other laboratories*	3041 (25.6)
Sample handling conditions	**Number (percentage)**
Fasting time, hours†	
0–4	9856 (83.1)
4–8	1664 (14.0)
8+	348 (2.9)
History of blood transfusion	
No	9854 (83.0)
Yes	1750 (14.8)
Unsure	264 (2.2)
Haemolysis in blood sample	
No	11,530 (97.1)
Yes‡	338 (2.9)
Thawed blood sample	
No	11,729 (98.8)
Yes‡	139 (1.2)
Intermediate storage type	
−80 °C freezer	7009 (59.1)
−80 °C shuttle biobus	387 (3.3)
−80 °C dry ice	541 (4.6)
−170 °C cryo-shipper§	3046 (26.7)
−20 °C/−30 °C freezer	885 (7.5)
Sample handling and storage time	**Mean (standard deviation)**
Transport time, hours‖	0.74 (0.56)
Processing time, hours{	1.74 (0.44)
Total storage time (p-tau181), years#	10.56 (1.18)
Total storage time (N4PE), years#	10.56 (1.18)
AD biomarker levels	**Mean (standard deviation)**
P-tau181 (pg/mL)	36.7 (20.9)
NfL (pg/mL)	22.1 (10.9)
GFAP (pg/mL)	134.6 (68.5)
Aβ42 (pg/mL)	5.3 (1.6)
Aβ40 (pg/mL)	87.0 (21.3)
Log-transformed Aβ42/40 ratio	−2.81 (0.24)

* Includes laboratories with trained ASPREE staff in Canberra, Adelaide, Bendigo, Geelong, Gippsland, Tasmania, Warrnambool, Wodonga, Wollongong and Shepparton.
† Time between the last meal and sample collection.
‡ At least one aliquot from the total blood aliquots collected for this participant.
§ Cryo-shippers were resin embedded with liquid nitrogen.
‖ Time between sample collection and the first blood centrifugation.
{ Time between the first blood spin and sample storage.
Time between sample storage and assay.

Higher NfL level was observed in samples with previously thawed aliquots (β = 1.89, p = 0.03). Moreover, intermediate storage temperature was also linked to AD biomarker levels. Compared to samples initially stored at −80 °C, those stored in −20 °C or -30 °C freezers showed higher p-tau181 (β = 2.24, p = 0.004) and GFAP levels (β = 9.93, p < 0.001), and lower Aβ42 (β = −0.36, p < 0.001) and Aβ40 levels (β = -5.09, p < 0.001). In addition, samples initially stored at −170 °C had higher levels of GFAP (β = 7.08, p < 0.001) and Aβ40 (β = 1.87, p = 0.005). Box plots illustrating biomarker levels across categories of these pre-analytical factors showing the median, interquartile range and overall range (outliers excluded) are provided in Supplementary Figures 1–5. The η^2 values indicated small effects for total storage time and all other pre-analytical factors, ranging from < 0.001 to 0.007. The sensitivity analysis did not show materially different results from those of the main analysis (Supplementary Tables 1–3).

4. Discussion

This study used plasma samples collected from nearly 12,000 older individuals aged 70+ years stored in the ASPREE Healthy Ageing Biobank–a large-scale, community-based biobank that includes sample collection and processing across multiple sites and regions in Australia. Using data from samples stored for an average of 10.56 years, we examined the associations between a range of pre-analytical factors and the levels of five AD biomarkers–p-tau181, NfL, GFAP, Aβ42 and Aβ40, as well as the Aβ42/40 ratio. Our results indicate that with strict adherence to the standard operating procedures, the pre-analytical factors, from sample collection to long-term storage, had minimal impact on the plasma levels of these biomarkers. The protocols applied in the ASPREE biobank, particularly the transport and processing of samples at 4 °C and freezing within 4 hours after blood collection, resulted in a remarkably consistent range of AD biomarker levels. However, there are small but statistically significant changes in the biomarker levels influenced by several factors throughout the sample-handling process. Samples stored for a longer time showed lower levels of p-tau181 and Aβ42/40 ratio, while longer transport time and processing time were associated with lower levels of p-tau181.

The impact of prolonged storage on biomarkers is important, particularly for dementia research with a longitudinal design. Due to the long pre-clinical and prodromal phases of dementia, samples need to be stored for decades to identify potential associations with incident dementia at an asymptomatic stage. Our study adds to the current evidence that long-term storage may indeed have some minor impacts on the plasma levels of certain AD biomarkers, such as p-tau181 and Aβ42/40 ratio. Similarly, one study observed that plasma total tau showed slight degradation after 1.5 years of storage at −80 °C, with the concentrations significantly reduced after two years [4]. Another study suggested slightly greater variability in the levels of Aβ42, Aβ40 and NfL in samples stored for more than 14 years [5]. The reasons for these changes remain unknown, but several biochemical mechanisms seem plausible, such as protein degradation, enzyme activities and self-consumption

Table 2
Adjusted associations between sample transport/processing/total storage time and plasma levels of p-tau181, NfL, GFAP, Aβ42, Aβ40 and Aβ42/40 ratio (n = 11,868).

	β (95 % CI)					
Biomarkers (pg/mL)	Transport time (hours)	P-value	Processing time (hours)	P-value	Total storage time (years)	P-value
P-tau181	−0.82 (−1.53, −0.10)	0.03	−1.56 (−2.42, −0.70)	<0.001	−0.45 (−0.77, −0.12)	0.007
NfL	0.16 (−0.19, 0.50)	0.38	−0.13 (−0.55, 0.29)	0.55	−0.07 (−0.22, 0.09)	0.42
GFAP	−0.17 (−2.35, 2.02)	0.88	−0.80 (−3.45, 1.85)	0.55	0.90 (−0.09, 1.89)	0.08
Aβ42	0.04 (−0.01, 0.10)	0.13	−0.09 (−0.16, −0.03)	0.006	−0.00 (−0.03, 0.02)	0.76
Aβ40	0.76 (0.05, 1.48)	0.04	−0.77 (−1.64, 0.10)	0.08	0.36 (0.03, 0.68)	0.03
Aβ42/40 ratio	−0.00 (−0.01, 0.01)	0.86	−0.01 (−0.02, 0.00)	0.27	−0.01 (−0.01, −0.00)	<0.001

Note: a) Age at collection (years, numerical), sex (male, female) and location of collection (Monash Central-Melbourne, Biobus and others). b) All time variables were analysed as numerical variables. c) The Aβ42/40 ratio was log-transformed.

Table 3
Adjusted associations between pre-analytical factors and plasma levels of p-tau181, NfL and GFAP (n = 11,868).

Pre-analytical factors	P-tau181 (pg/mL)		NfL (pg/mL)		GFAP (pg/mL)	
	β (95 % CI)	P-value	*β* (95 % CI)	P-value	*β* (95 % CI)	P-value
Fasting time, hours*						
Continuous (per hour increase)	−0.11 (−0.26, 0.04)	0.14	0.15 (0.07, 0.22)	<0.001	1.75 (1.30, 2.20)	<0.001
8+	Ref		Ref		Ref	
4–8	2.62 (0.22, 5.01)	0.03	−0.56 (−1.73, 0.62)	0.35	4.80 (−2.54, 12.15)	0.20
0–4	1.98 (−0.24, 4.19)	0.08	−1.23 (−2.32, −0.15)	0.03	−9.89 (−16.68, −3.10)	0.004
History of blood transfusion						
No	Ref		Ref		Ref	
Yes	−0.09 (−1.16, 0.97)	0.86	0.25 (−0.27, 0.78)	0.34	−2.39 (−5.66, 0.88)	0.15
Unsure	−0.04 (−2.56, 2.49)	0.98	0.14 (−1.10, 1.38)	0.82	−4.51 (−12.29, 3.26)	0.26
Haemolysis of blood sample						
No	Ref		Ref		Ref	
Yes†	−0.56 (−2.80, 1.68)	0.62	0.83 (−0.27, 1.92)	0.14	1.78 (−5.11, 8.67)	0.61
Thaw of blood sample						
No	Ref		Ref		Ref	
Yes†	−1.23 (−4.69, 2.23)	0.49	1.89 (0.19, 3.59)	0.03	1.93 (−8.74, 12.59)	0.72
Intermediate storage temperature						
−80 °C‡	Ref		Ref		Ref	
−170 °C§	1.32 (0.02, 2.61)	0.05	0.06 (−0.57, 0.70)	0.85	7.08 (3.10, 11.05)	<0.001
−20 °C/−30 °C‖	2.24 (0.73, 3.75)	0.004	0.55 (−0.19, 1.29)	0.15	9.93 (5.30, 14.57)	<0.001

Note: a) Age at collection (years, numerical), sex (male, female) and location of collection (Monash Central-Melbourne, Biobus and others). b) The A*β*42/40 ratio was log-transformed.
* Time between the last meal and sample collection.
† At least one aliquot from the total blood aliquots collected for this participant.
‡ Include -80 °C freezers, shuttle biobus and dry ice.
§ Include -170 °C cryo-shippers resin embedded with liquid nitrogen.
‖ Include -20 °C/-30 °C freezers.

Table 4
Adjusted associations between pre-analytical factors and plasma levels of A*β*42, A*β*40 and A*β*42/40 ratio (n = 11,868).

Pre-analytical factors	A*β*42 (pg/mL)		A*β*40 (pg/mL)		A*β*42/40 ratio	
	β (95 % CI)	P-value	*β* (95 % CI)	P-value	*β* (95 % CI)	P-value
Fasting time, hours*						
Continuous (per hour increase)	0.02 (0.01, 0.03)	0.003	0.28 (0.13, 0.42)	<0.001	−0.00 (−0.00, 0.00)	0.95
8+	Ref		Ref		Ref	
4–8	0.06 (−0.12, 0.25)	0.50	0.80 (−1.61, 3.21)	0.52	−0.00 (−0.03, 0.03)	0.97
0–4	−0.07 (−0.24, 0.10)	0.42	−1.95 (−4.18, 0.29)	0.09	0.01 (−0.02, 0.03)	0.50
History of blood transfusion						
No	Ref		Ref		Ref	
Yes	0.04 (−0.05, 0.12)	0.40	0.98 (−0.09, 2.05)	0.07	−0.01 (−0.02, 0.01)	0.31
Unsure	0.19 (−0.01, 0.38)	0.06	2.43 (−0.12, 4.98)	0.06	0.00 (−0.03, 0.03)	0.90
Haemolysis of blood sample						
No	Ref		Ref		Ref	
Yes†	−0.02 (−0.19, 0.16)	0.84	1.43 (−0.83, 3.69)	0.22	−0.02 (−0.05, 0.00)	0.06
Thaw of blood sample						
No	Ref		Ref		Ref	
Yes†	0.05 (−0.22, 0.32)	0.71	2.29 (−1.21, 5.78)	0.20	−0.02 (−0.06, 0.02)	0.27
Intermediate storage temperature						
−80 °C‡	Ref		Ref		Ref	
−170 °C§	0.09 (−0.01, 0.19)	0.09	1.87 (0.57, 3.17)	0.005	−0.01 (−0.03, 0.00)	0.09
−20 °C/−30 °C‖	−0.36 (−0.47, −0.24)	<0.001	−5.09 (−6.61, −3.57)	<0.001	−0.00 (−0.02, 0.02)	0.95

Note: a) Age at collection (years, numerical), sex (male, female) and location of collection (Monash Central-Melbourne, biobus and others). b) The A*β*42/40 ratio was log-transformed.
* Time between the last meal and sample collection.
† At least one aliquot from the total blood aliquots collected for this participant.
‡ Include -80 °C freezers, shuttle biobus and dry ice.
§ Include -170 °C cryo-shippers resin embedded with liquid nitrogen.
‖ Include -20 °C/-30 °C freezers.

[17]. However, it is worth noting that the size of time effects shown in these and the current studies is small, especially when compared to the standard deviations of biomarker levels. This suggests that changes in AD biomarkers after long-term storage in appropriate conditions, even up to 13 years, are unlikely to substantially impact their validity for research.

Time spent on the sample handling procedure is another important factor, which has been rarely investigated in previous studies. All samples in our biobank had been kept at low temperatures since collection, strictly adhering to the best practices recommended [18]. However, the durations of the two phases, transport and processing of biospecimen, still appeared to influence the blood samples. An empirical survey led by Verberk et al., involving 42 AD researchers from academia, government and industry, showed a large variation among the responses regarding transport time and processing time [15], suggesting that more evidence is needed to standardise these times. However, evidence re-

garding the extent to which the time of these intermediate phases can impact biomarker levels remains scarce. One study with 30 samples found that serum levels of NfL remained stable after delayed freezing after centrifugation [6]. Our results complement this with the findings that some other biomarkers, particularly p-tau181, can also be influenced, highlighting the importance of rapid transport and immediate processing. However, as mentioned above, given the small effect sizes relative to the natural variability of biomarker levels, these times may not substantially impact the reliability of AD biomarkers when rigorously adhering to the pre-specified time window.

The present findings suggest that fasting status may affect AD biomarker levels, in particular for p-tau181 and to a lesser extent (a meal within 4 hours versus fasting) for NfL and GFAP. This aligns with previous findings of changes in these biomarkers after food intake [19,20]. A few biological mechanisms have been proposed, including the increase in blood glucose and triglycerides, as well as changes in gut microbiota following food intake, which may affect the release and clearance of AD biomarkers in the blood [21–23]. It is worth noting that our findings indicate that while Aβ42 and Aβ40 were affected by fasting time, the Aβ42/40 ratio remained stable. This suggests that the Aβ42/40 ratio may be a more reliable measure of amyloid pathology, highlighting its importance over Aβ42 and Aβ40 alone. Together these findings suggest the importance of considering fasting status for the testing of blood-based AD biomarkers in clinical practice and research. However, the survey led by Verberk et al. indicated that only 43 % reported using fasted blood samples in biomarker testing [15].

Another important finding is the variations of biomarker levels across different intermediate storage temperatures. Although -80 °C is the optimal temperature for long-term storage of blood samples, it is necessary to temporarily store samples at higher temperatures when −80 °C freezers are not immediately available. The potential impact of such intermediate storage remains inconclusive in previous studies. For example, one study observed no difference in the recovery rate of any AD biomarkers between fresh and previously frozen samples (at −20 °C) [24]. Another study observed lower levels of Aβ42 and Aβ40 in samples initially stored at room temperature or in the refrigerator, but not the samples at −20 °C before long-term storage [15]. Our findings indicate that intermediate storage at −20/−30 °C may have influenced biomarker levels, compared to storage at −80 °C This highlights the importance of ultra-low freezer storage to minimise variability in biomarker measurements. Laboratories using the higher temperature for intermediate storage may have had different operational practices, which could have also contributed to the biomarker differences.

With almost 12,000 plasma samples, this is the largest study so far investigating the pre-analytical factors of AD biomarkers. The sample size allows for the detection of even subtle pre-analytical effects, which is not feasible in prior studies that are far smaller. In addition, the multi-site design and detailed monitoring of sample handling facilitate an investigation into several highly critical factors that have rarely or never been examined before. This includes intermediate storage temperature, transport time and processing time. These strengths, together with the findings, contribute to a more comprehensive understanding of the standardised sample handling procedure. However, there are also limitations. First, the rigorous inclusion criteria of this biobank ensured that all participants were generally healthy at enrolment, without any cognitive deficits or other major health conditions. This homogeneity in sample characteristics may reduce the variations in the biomarker levels and limit the generalisability of the current findings. Meanwhile, biomarker levels observed in this study may be different from those of patient populations (e.g., AD patients), which may limit the applicability of our findings to clinical diagnostic contexts. However, these biomarkers also have promise for screening among the asymptomatic populations, making our findings important for the early detection of at-risk individuals. Second, with such a large sample size and high statistical power, some effects that are small and practically unimportant in clinical chemistry may appear to be statistically significant, warranting careful interpretation of the results. Third, due to the application of rigorous standard operating procedures, this study is limited to the pre-determined biobank protocols, such as strict temperature and time window set for the process. Therefore, this study cannot examine the pre-analytical effects beyond the established thresholds of these conditions.

In conclusion, in this large study leveraging the ASPREE Healthy Ageing biobank where standardised procedures were maintained from sample collection through to storage, we observed minimal pre-analytical effects on plasma levels of AD biomarkers. However, some weak but statistically significant associations were found. Long-term storage time was associated with plasma levels of p-tau181 and Aβ42/40 ratio, whereas a longer duration of transport and processing seemed to influence p-tau181 and GFAP. A shorter fasting time was associated with lower plasma levels of NfL and GFAP, and a higher level of p-tau181. Samples initially stored at −20/−30 °C before long-term storage at −80 °C showed different levels of p-tau181, NfL and GFAP, compared to those directly stored at −80 °C after aliquoting. These findings suggest that long-term storage lasting up to 13 years may not substantially impact blood-based AD biomarkers under standardised conditions. However, there are still minor pre-analytical effects arising from sample collection and processing.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Anne M. Murray reports grants from the National Institute of Health. Michelle Mielke reports grants from the National Institute of Health, DOD, Alzheimer's Association and Davos Alzheimer's Collaborative, payments from Athira, Biogen, Eisai, Lilly, Merck, Roche, Siemens Healthineers, Novo Nordisk and Alzheimer's Drug Discovery Foundation. Danni Li reports funding from the U.S. Food and Drug Administration, the National Institute of Health and the Minnesota Office of Higher Education. Joanne Ryan reports funding from the National Institute of Health, the National Health and Medical Research Council, the Medical Research Future Fund and the Australian Research Council, honoraria from the Victorian Coronial Council–Department of Justice and Community Service, as well as serving as a non-paid committee member for Dementia Australia and as a member of the scientific board for its research foundation. Zimu Wu, James Phung, Alice Owen, Robyn L. Woods and Jo Wrigglesworth report no conflict of interest.

CRediT authorship contribution statement

Zimu Wu: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Michelle M. Mielke:** Writing – review & editing, Investigation, Funding acquisition. **Anne M. Murray:** Writing – review & editing, Investigation, Funding acquisition. **James Phung:** Writing – review & editing, Project

administration, Methodology, Investigation. **Alice Owen:** Writing – review & editing, Resources, Project administration, Investigation, Funding acquisition. **Robyn L. Woods:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition. **Danni Li:** Writing – review & editing, Resources, Project administration. **Jo Wrigglesworth:** Writing – review & editing, Data curation. **Joanne Ryan:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.tjpad.2025.100058.

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