Effects of storage conditions on the stability of blood-based markers for the
diagnosis of Alzheimer’s disease.

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Abstract

**Background**: Alzheimer’s disease (AD) is considered the most common cause of dementia in older people. Recently, blood-based markers (BBM) Aβ1-42, Aβ1-40, and phospho Tau181 (p-Tau181) have demonstrated the potential to transform the diagnosis and prognostic assessment of AD. Our aim was to investigate the effect of different storage conditions on the quantification of these BBM and to evaluate the interchangeability of plasma and serum samples. **Material and Methods**: Thirty-two individuals with some degree of cognitive impairment were studied. Thirty further patients were retrospectively selected. Aβ1-42, Aβ1-40 and p-Tau181 were quantified using the LUMIPULSE-G600II automated platform. **Results**: Storing samples at 4ºC for 8-9 days was associated with a decrease on Aβ fractions but not when stored for 1-2 days. Using the ratio partially attenuated the pre-analytical effects. For p-Tau181, samples stored at 4ºC presented lower concentrations, whereas frozen samples presented higher ones. To assess interchangeability between conditions, correction factors for magnitudes that showed strong correlations (p-Tau181) were calculated, followed by clinical agreement studies. **Conclusion**: Our findings provide relevant information for the standardization of sample collection and storage in the analysis of AD blood biomarkers in an automated platform. This knowledge is crucial to ensure their introduction into clinical settings.

**Keywords**: Alzheimer’s Disease, Automated platforms, Plasma, Serum, Blood-based Markers, Amyloid, Tau.

**Highlights**

1- Blood-based markers (BBM) for the diagnosis of Alzheimer disease (AD)
2- Impact of different storage conditions on the analysis of BBM
3- Interchangeability evaluation between plasma-EDTA and serum samples
4- Automated platforms
Abbreviations

Alzheimer's disease (AD)
Amyloid-beta (Aβ)
Blood-based markers (BBM)
Cerebrospinal fluid (CSF)
Ethylene diamine tetra acetic acid (EDTA)
Kappa (k)
Phospho Tau (p-Tau)
Room temperature (RT)
Confidence interval (CI)
Coefficient of variation (CV)
Standard deviation (SD)
Total Tau (t-Tau)
1. Introduction

The most frequent cause of dementia in older adults is Alzheimer’s disease (AD) (doi.org/10.1159/000197897), a chronic neurodegenerative condition with an increasing incidence expected to reach 150 million people by the year 2050 (DOI: 10.1016/S2468-2667(21)00249-8). Despite the fact that clinical symptoms still serve as the basis of the diagnosis, imaging and/or cerebrospinal fluid (CSF) biomarkers are required for the pathophysiological diagnosis of the disease (doi.org/10.1016/j.jalz.2018.02.018; doi.org/10.1016/S1474-4422(14)70090-0) (doi.org/10.1038/nrneurol.2014.232).

Amyloid-beta (Aβ) plaques and hyperphosphorylated tau protein aggregation in intracellular neurofibrillary tangles are two significant neuropathological hallmarks of AD (doi.org/10.1038/s41583-019-0240-3). These processes can be monitored by measuring amyloid peptides (Aβ1-42 and Aβ1-40), total Tau (t-Tau), and phospho Tau (p-Tau) proteins in CSF (doi.org/10.1038/nrneurol.2010.214; doi.org/10.1080/15622975.2017.1375556). As a result, AD patients present with a consistent and characteristic CSF profile with elevated levels of t-Tau and p-Tau together with lower concentrations of Aβ1-42 and a low Aβ1-42/Aβ1-40 ratio compared to cognitively normal controls (doi: 10.1002/alz.12545). More recently, blood-based markers (BBM) have demonstrated the potential to transform the diagnosis and prognostic assessment of AD as well as the planning of interventional trials (doi: 10.1001/jamaneurol.2021.3180; doi: 10.1002/alz.12756). However, and similar to what has been observed in CSF (doi.org/10.1016/j.jalz.2018.05.008), standardization of sample handling has shown to be crucial for preventing dementia misdiagnosis (doi: 10.1002/alz.12510). In that regard, understanding the effects of pre-analytical conditions on BBM and implementing standardized operation procedures to limit variation are critical for achieving consistency across studies, technologies, and laboratories. Several studies have explored pre-analytical stability of BBM including Aβ42, Aβ40 and phospho Tau181 (p-Tau181) (doi: 10.1016/j.dadm.2019.02.002; doi: 10.1002/dad2.12168; doi:
However, studies investigating such effects in more heterogeneous populations are still scarce. Another major question for the study of BBM is the type of sample to be used. The most common matrix in dementia biobanks is plasma, usually derived from blood collected in tubes containing ethylene diamine tetra acetic acid (EDTA) (doi: 10.1002/alz.12510). Nonetheless, other matrices such as serum are regularly used in clinical laboratories. Thus, comparing the effects of preanalytical conditions between matrices could facilitate the implementation of BBM in clinical practice.

The purpose of our investigation was to look into the effect of different routine storage conditions on the simultaneous quantification of the plasma biomarkers Aβ1-42, Aβ1-40, and p-Tau181 in the LUMIPULSE G600II fully-automated platform, as well as to evaluate the interchangeability of EDTA-plasma and serum samples.

2. Material and Methods

For the stability analysis, whole blood extraction was practiced by venepuncture in 32 consecutive individuals who were referred to the Sant Pau Memory Unit as consequence of some degree of cognitive impairment. Subjects underwent lumbar puncture for the analysis of AD CSF biomarkers and blood extraction during June and July 2022. Whole blood samples were obtained in EDTA-K2 tubes and transferred to our laboratory where they were fractioned by centrifugation at 2000 g for 10 minutes at 4°C within 2 hours after extraction. Plasma samples were aliquoted in 1.5 mL polypropylene tubes (Sarstedt, Ref. 72.690.001) and stored until analysis under the following conditions: A) Refrigerator 4°C (2-8°C) for 1-2 days; B) Refrigerator 4°C (2-8°C) for 8-9 days; C) Freezer -20°C (-15 - -25°C) for 8-9 days. Condition C was considered the reference condition, as it was expected to ensure higher stability of the samples. Cut-off values are currently under validation process by our group.
For the comparison of EDTA-plasma and serum analysis, 30 patients with both plasma and serum aliquots were retrospectively selected from the Sant Pau Initiative on Neurodegeneration (SPIN) cohort based on a balanced gender distribution. Pre-analytical protocol in the SPIN cohort for plasma and serum samples has been previously reported (doi: 10.1016/j.trci.2019.09.005). Briefly, plasma and serum samples were obtained from whole blood in EDTA-K2 and SST VACUTAINER tubes, respectively, and were subsequently centrifuged, aliquoted and stored at -80°C in 1.5 mL polypropylene Eppendorf tubs (Sarstedt, Ref. 72.690.001) until the day of the analysis.

All included subjects gave written informed consent for participation in research in accordance with the guidelines of the local ethics committee following the ethical principles set forth in the Declaration of Helsinki. The full protocol for sample processing is detailed in Figure 1.

The samples were brought to room temperature (RT) (21-23°C) on the day of the analysis, mixed thoroughly, centrifuged during 5 minutes at 2000 g and transferred to specific cuvettes for their quantification with the LUMIPULSE G600II automated platform using the same batch of kits and reagents. Because of the stability analysis' design, samples obtained from the same patient and stored for different periods of time had to be run in different days. Internal quality control samples were included in all runs to assess inter-assay variability.

The characteristics of the study population were summarized using descriptive statistics. Demographic and biochemical data are presented as mean and standard deviation (SD). Biomarker measurements were converted to percentages relative to the aliquot analysed under condition C (reference sample, 100%). Data are presented as mean (95% Confidence interval (CI)) for each condition. To compare continuous variables across the studied conditions, one-way ANOVA for repeated measures with Dunnett's Multiple Comparison test was used. Pearson's correlation coefficients were determined between
reference condition C vs. conditions A and B as well as between EDTA-plasma and serum samples. Regression analysis was performed for the comparisons that showed strong correlations. Cohen's kappa coefficient (k) was used to calculate diagnostic agreement. A k index of 0.41 to 0.6 was considered moderate agreement, 0.61 to 0.8 was considered substantial agreement, and 0.81 to 1 was considered nearly perfect agreement. For statistical analyses, the statistical software Graph Prism (version 5.01) was used. A p-value < 0.05 was considered statistically significant.

3. Results

The study included 32 patients for the plasma biomarker stability study and 30 independent patients for the plasma-serum comparison analysis. Tables 1 and 2 provide the demographics, mean, (SD), and range for each parameter that was evaluated under the reference condition, for both groups, respectively.

Regarding sample stability, Figure 2 depicts plasma biomarker measures in each storage condition. Results are expressed as percentage relative to the reference condition C (frozen 8-9 days). Our results show that the Aβ1-42 concentration was 30% lower (95% CI 25-35%, p<0.001) when samples were kept refrigerated for 8-9 days compared to the reference condition C. Aβ1-42 concentrations in condition A (refrigerated for 1-2 days) were not significantly different than those in the reference condition C (95% CI 98-112%, p=ns) (Fig. 2a). Similar results were seen for Aβ1-40 quantification, with concentrations being 39% lower in tubes stored for 8-9 days at 4°C (95% CI 35-43%, p<0.001) compared to those in the reference condition C. Aβ1-40 concentrations in condition A were not significantly different than those in the reference condition C (95% CI 92-102%, p=ns) (Fig. 2b). When using the Aβ1-42/Aβ1-40 ratio, significant differences were observed for both conditions involving storage at 4°C compared to the reference condition C. The ratios measured in conditions A and B were 8% (95% CI 4-12%, p<0.001) and 16% (95% CI 13-20%, p<0.001), respectively, higher
than those in the condition C (Fig. 2c). For the p-Tau181 concentrations, significantly lower values were observed for conditions A and B, with decreases of 21% for both conditions (condition A: 95% CI 15-26%, p<0.001; condition B: 95% CI 16-26%, p<0.001) compared to those observed in the reference condition C (Fig. 2d). No statistical differences were observed in p-Tau181 concentrations when samples were kept refrigerated for 1-2 days or for 8-9 days. Analysis using absolute values were repeated for all the studied magnitudes, obtaining identical results.

Correlation studies involving reference condition C vs. A and condition C vs. B for every measured magnitude were performed. All correlations were significant, with the following correlation coefficients for each comparison and each marker (from high to low): Reference condition C vs. A: p-Tau181 (0.93) > Aβ1-40 (0.75) > Aβ1-42 (0.72) > Aβ1-42/Aβ1-40 (0.55); Reference condition C vs. B: p-Tau181 (0.95) > Aβ1-42 (0.78) > Aβ1-40 (0.76) > Aβ1-42/Aβ1-40 (0.71) (Figure 3).

For the EDTA-plasma vs. serum analysis, we observed that concentrations for Aβ1-42, Aβ1-40 and p-Tau181 in serum were decreased 94.0%, 38.9% and 30.6% respectively when compared to the plasma ones. In the correlation analysis between both matrices, Pearson’s r was 0.43 (p=0.018) for Aβ1-42; 0.77 (p<0.0001) for Aβ1-40; 0.30 (p=0.101) for Aβ1-42/Aβ1-40; and 0.94 (p<0.0001) for p-Tau181 (Figure 4).

The inter-assay CV for Aβ1-42, Aβ1-40, and p-Tau181 were 4.9%, 6.1% and 1.9%, respectively. The inter-condition CV for Aβ1-42, Aβ1-40, Aβ1-42/Aβ1-40 ratio, and p-Tau181 were 23.3%, 27.1%, 9.3% and 16.3% respectively. Therefore, the observed differences between conditions were greater than expected by inter-assay variability.

To assess interchangeability between conditions, we performed regression analysis for those comparisons that showed strong correlations (Pearson’s r > 0.8), i.e. p-Tau181. For the amyloid peptides in plasma, the correlation coefficients between conditions were too low to perform regression analysis. Table 3 includes slope and intercept regression
parameters for p-Tau181 comparisons between condition C vs. A, condition C vs. B, and EDTA-plasma vs. serum. The regression parameters were used to calculate new p-Tau181 values which were used in diagnostic agreement studies (Figure 5). To assess the agreement between conditions, we calculated k indices for the classification of patients as positive or negative with cut-offs ranging from 1.6 to 4.2 pg/ml. The highest k values (>0.81) when comparing reference condition C vs. A were observed for cut-offs 2.9 to 3.0, 3.4 to 3.8 and 4.2 pg/mL (Fig. 5a), and when comparing reference condition C vs. B were observed for cut-offs 1.7 to 1.9, 2.1, 2.4, 2.9 to 3.1, 3.5 to 4 and 4.2 pg/mL (Fig. 5b). For the plasma vs. serum comparison analysis, the highest k values were observed for cut-offs 1.9, 2.5 to 3.4 and 4.1 to 4.2 pg/mL (Fig. 5c).

4. Discussion

As a consequence of the development of ultrasensitive immunoassays, a wide variety of easily measurable BBM of AD is now available. However, before these tests can be applied in clinical practice, a number of methodological and pre-analytical issues, as well as extended validation in matrices other than EDTA-plasma, need to be resolved. The current study explored the effects of different storage conditions, as well as the use of plasma or serum matrices, on the simultaneous quantification of the BBM Aβ1-42, Aβ1-40 and p-Tau181 on the LUMIPULSE G600II automated platform for the diagnosis of AD.

Amyloid peptides are known to be very sensitive to preanalytical conditions in CSF (doi: 10.1002/alz.12545). In our study and when compared to our reference condition C (sample storage at -20°C for 8-9 days), we found that sample storage at 4°C for 8-9 days was associated with a decrease on plasma Aβ fractions (both for Aβ1-42 and Aβ1-40), whereas storage at 4°C for 1-2 days had no significant impact on the results. Similar to our results, recent studies showed that storing plasma samples at 4°C leads to stable Aβ peptide concentrations up to 72 h (doi: 10.3233/JAD-200777), or that storage of plasma
samples in the refrigerator up to 24 hours does not affect Aβ42 and Aβ40 values (doi: 10.1002/alz.12510). Verberk et al. also reported that two-week storage in the refrigerator prior to storage at −80°C results in decreased Aβ42 and Aβ40 values while two-week storage at −20°C does not show effects (doi: 10.1002/alz.12510). On the contrary, a recent work found that after 24 hours, fresh plasma kept at 4°C presents with a reduction of 10% of Aβ1-40 but no effect on Aβ1-42 levels (doi: 10.1016/j.dadm.2019.02.002). In our study, applying the Aβ1-42/Aβ1-40 ratio partially compensated the decrease for plasma samples stored at 4°C for 8-9 days, but it aggravated the observed effect when storing at 4°C for 1-2 days. Thus, unlike what has been described for CSF (doi: 10.1515/cclm-2022-0134), using the Aβ1-42/ Aβ1-40 ratio is not as effective in attenuating the pre-analytical effects. Similar findings have been reported, as such, a recent work found that the Aβ ratio increases by 10% after 24 hours (doi: 10.1016/j.dadm.2019.02.002). Other authors with similar results hypothesized ex vivo aggregation or proteolytic cleavage to be mechanisms at play (doi: 10.1002/alz.12510). Comparably, but in another line of research, a work examining different blood-based assays to measure Aβ peptides and how they compare to each other and between centers, found no improvement in the correlations when using the ratio (doi: 10.1002/dad2.12242).

With respect to p-Tau181, all the conditions involving storage at 4°C for either 1-2 days or 8-9 days were associated with lower concentrations when compared to frozen samples. A recent study also reported lower concentrations of p-Tau181 in samples stored at 4°C when compared to frozen samples kept at −20°C (doi.org/10.1515/cclm-2022-0770). However, other works have found that p-Tau181 remains stable after delayed centrifugation and storage (doi: 10.1002/alz.12510). Plausible explanations to this observation could be that pTau-181 is degraded when stored at 4°C, or, based on preliminary findings, the detection of p-Tau181 increases after one freeze-thaw cycle. This could be attributed to the fact that during the freeze-thaw process, plasma proteins
that might interfere with the analysis in fresh samples are degraded or fragmented, allowing the immunoassay to detect more p-Tau181.

Other studies have previously explored different preanalytical conditions and their effect on the BBM levels. However, they focused on the impact of freeze-thaw cycles (but not fresh vs. frozen), storage at RT, type of anticoagulant used, and time until centrifugation (doi: 10.1159/000509358; doi: 10.1016/j.dadm.2018.06.001; doi: 10.1159/000506278; doi: 10.1002/alz.12510).

We found a strong correlation between plasma and serum for p-Tau181 but moderate to weak correlations for the amyloid peptides. In that sense, a recent study exploring the results from the Standardization of Alzheimer’s Blood Biomarkers group described that, compared to plasma measures, p-Tau181 concentrations in serum were lower, and Aβ42 and Aβ40 concentrations in serum samples were either lower, the same, or higher depending on the assay used (mass spectrometry, SIMOA, and ELISA) (doi: 10.1002/alz.12510). A recent work found strong correlations between serum and plasma p-Tau181, supporting the use of serum in research cohorts and hospital systems. However, and similar to what we found, the authors described that absolute biomarker concentrations might not be interchangeable, and suggest that they should be used separately (DOI:10.1186/s13195-022-01011-w). Another recent work directly recommended avoiding serum for Aβ42, Aβ40, and t-tau measures due to poor correlation (doi: 10.1002/dad2.12168).

Concerning clinical agreement studies in comparisons that revealed strong correlations, the k index was greater than 0.81 in a large number of the tested cut-offs, confirming the assay’s robustness. Nevertheless, and despite being highly correlated, serum and EDTA-plasma samples, as well as plasma samples stored under conditions different from the reference condition, should not be used interchangeably because they yield significantly different absolute concentrations.
A significant strength of our study is the standardization of the pre-analytical methodology except for the variable of interest. This included using a completely automated platform and the same batch of Lumipulse reagents throughout the study. To our knowledge this is the largest study to assess the effect of storage conditions in plasma measures on this automated analytical platform. The stability evaluation was also carried out on samples obtained from consecutive patients in their diagnostic evaluation in a memory clinic, thereby avoiding potential selection biases and covering the range of measurements commonly found in clinical settings. For the EDTA-plasma vs. serum comparison, patient selection was performed based on gender equity. Also, and to our knowledge, the EDTA-plasma vs. serum comparison represents the largest work addressing this issue. Some limitations should also be acknowledged, the most important of which is that the impact of the conditions tested may be dependent on brain pathology, and our study was not large enough to perform stratified analysis. Also, and due to the design of the study, samples obtained from the same patient had to be analysed in different days, however inter-assay CV was calculated to understand the impact on the current results.

5. Conclusion

The importance of early detection and treatment in limiting the impact of AD is critical. Our findings provide relevant information for the standardization of sample collection and storage in the analysis of AD blood-based markers in an automated platform. This knowledge is crucial to ensure their introduction into clinical settings.
Acknowledgements

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### Table 1

<table>
<thead>
<tr>
<th></th>
<th>Mean (SD)</th>
<th>Range</th>
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<tbody>
<tr>
<td>Age, years</td>
<td>73.4 (6.1)</td>
<td>62 - 84</td>
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<tr>
<td>Female/Male (%Female)</td>
<td>19/13 (59.4%)</td>
<td>-</td>
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<tr>
<td>MMSE score</td>
<td>23.7 (3.9)</td>
<td>17 - 30</td>
</tr>
<tr>
<td>Aβ1-40, pg/mL</td>
<td>21.9 (5.3)</td>
<td>11.7 – 31.0</td>
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<tr>
<td>Aβ1-42, pg/mL</td>
<td>295.8 (64.8)</td>
<td>180.5 – 435.1</td>
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<td>Aβ1-42/Aβ1-40</td>
<td>0.074 (0.009)</td>
<td>0.065 – 0.084</td>
</tr>
<tr>
<td>p-Tau181, pg/mL</td>
<td>2.8 (1.2)</td>
<td>1.1 – 6.7</td>
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Table 1. Mean (SD) and range for demographic and biochemical data under condition C for the stability analysis. MMSE: Mini-Mental Status Examination.

### Table 2

<table>
<thead>
<tr>
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<th>Mean (SD)</th>
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<td>Age, years</td>
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<tr>
<td>Female/Male (%Female)</td>
<td>15/15 (50%)</td>
<td>-</td>
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<tr>
<td>MMSE score</td>
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<td>14 - 30</td>
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<td>Aβ1-40, pg/mL Plasma</td>
<td>27.2 (9.1)</td>
<td>18.7 – 65.6</td>
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<td>Serum</td>
<td>1.6 (0.4)</td>
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<td>Aβ1-42, pg/mL Plasma</td>
<td>345.5 (110.5)</td>
<td>230.8 – 780.4</td>
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<td>Serum</td>
<td>211.2 (84.8)</td>
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<td>0.079 (0.009)</td>
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<td>Serum</td>
<td>0.009 (0.004)</td>
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<td>p-Tau181, pg/mL Plasma</td>
<td>2.9 (1.3)</td>
<td>1.2 – 6.1</td>
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<td></td>
<td>Serum</td>
<td>2.0 (1.3)</td>
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Table 2. Mean (SD) and range for demographic and biochemical data under condition C for EDTA-plasma vs. serum comparison analysis. MMSE: Mini-Mental Status Examination.
<table>
<thead>
<tr>
<th>Biomarker</th>
<th>Comparison</th>
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<th>Slope</th>
<th>Intercept</th>
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<tr>
<td>p-Tau181</td>
<td>C vs. A</td>
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<td>1.0233</td>
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<td></td>
<td>EDTA-Plasma vs. serum</td>
<td>30</td>
<td>0.9628</td>
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<td>0.94</td>
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</table>

**Table 3.** Slope and intercept regression parameters for comparisons between p-Tau181 conditions A and B, serum, and the respective reference system. R, Pearson's regression coefficient.