Letter to the Editor

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Pre-analytical variability of the Lumipulse immunoassay for plasma biomarkers of Alzheimer's disease

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To the Editor,

Phosphorylated tau at threonine 181 (pTau), amyloid-β_{1-42} (AB 1–42), AB 1–40 and AB 1–42/AB 1–40 ratio are established cerebrospinal fluid (CSF) biomarkers for Alzheimer's disease (AD) that mirror amyloid and tau pathology as identified by gold standard imaging techniques [1]. The need for less invasive and costly procedures for diagnosis and follow-up led to recent improvements in the analytical sensitivity of different assays, that have undertaken the path for technical and clinical validation of blood testing [2].

As long established in clinical laboratory medicine, preanalytical phase is a crucial source of errors in the testing cycle continuum [3], thus sampling and storage procedures should be carefully monitored and standardized to assure accuracy in studies evaluating the use of blood neurodegeneration biomarkers in specific clinical context [4]. The development of standardized operating procedures (SOPs) for CSF handling, supported the implementation in routine testing of AD biomarkers after their clinical validation [5]. Similarly, the evidence on the efficacy of plasma pTau and AB 1–42/AB 1–40 ratio measurements for the diagnosis of AD and its early disease [6] should nowadays be supported by blood SOPs.

Initial studies on sample handling for AD biomarkers in plasma measured with an automated immunoassay describe tube anticoagulants, time between sample collection and centrifugation and time between centrifugation and testing as relevant factors of variability [7]. Recommendations were then extended to ELISA, single molecule array (Simoa) and mass spectrometry-based techniques by the Standardization of Alzheimer's Blood Biomarkers (SABB) workgroup of the Alzheimer's Association, that included processing and storage temperatures in its recommendations [8].

In this preliminary study we aimed to estimate the effect of pre-analytical variables (storage temperature, time to centrifugation and hemolysis) on the stability of biomarkers of AD in plasma measured on the electrochemiluminescence automated platform Lumipulse by Fujirebio, Japan.

pTau, AB 1–42 and AB 1–40 were tested in plasma with a research use only (RUO) method on Lumipulse G1200 having as measuring ranges: 0.05–60 ng/L pTau, 0.10–1,000 ng/L for AB 1–42, 0.10–5,000 ng/L AB 1–40. Blood from a healthy volunteer (female, 46 years old) that gave informed consent for blood use for research was collected in 5 K_2-EDTA tubes (Becton Dickinson, US), and then centrifuged at 2,150 *g* for 5 min: 4 tubes within 3 h and 1 tube after 4 h from collection and at room temperature. Immediately after centrifugation plasma obtained within 3 h from collection was aliquoted in 14 polypropylene vials, which were stored at different temperatures: +4 °C

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(n=4), −20 °C (n=5) and −80 °C (n=5). Plasma stored at +4 °C were tested every day for 3 consecutive days (day 1–day 3) not exceeding the maximum storage (72 h) recommended by the manufacturer. Plasma stored at −20 °C and −80 °C were tested for 4 consecutive days (day 2–day 5) and at day 8. Frozen samples were centrifuged again after thawing. Additionally, we tested 8 fresh plasma aliquots mixed with a serially diluted sample frozen at −80 °C after collection to obtain different hemolysis levels, as measured with HIL index (Cobas 8000, Roche Diagnostics, Italy); manufacturer declares that HIL index values correspond approximately with hemoglobin (Hb) concentration in mg/dL.

One-way ANOVA, Student's t-test and Spearman correlation coefficient were used to estimate the effect of storage temperature and hemolysis. Statistical analyses were performed with GraphPad Prism ver 5.01.

Comprehensive results are reported in Table 1; from day 2 samples showed time-dependent variations in concentrations.

In particular, for pTau, all temperatures considered showed a significant effect on mean concentrations (p=0.0217), especially when comparing the concentrations measured in samples stored at +4 °C (mean 0.93 ng/L) with those of frozen samples at −20 °C (mean 1.73 ng/L, p=0.0366) or −80 °C (1.60 ng/L, p=0.0008) showing in both conditions higher levels. The difference in mean values observed between samples stored at −20 °C and −80 °C, instead, was not statistically significant (p=0.5961). Finally, about AB 1–42, AB 1–40 proteins and AB 1–42/1–40 ratio, the storage temperatures did not significantly affect the mean levels (p=0.0785, p=0.2280 and p=0.3887, respectively),

Therefore, the biomarkers variability at different storage temperatures as described by the difference between single day-by-day value and baseline value at Day 1 (bias %) was not equal (Figure 1). At temperature −20 °C all biomarkers showed overall the worst performances, particularly for pTau (+130.4% at day 4). Samples stored at +4 °C showed similar variations; at −80 °C pTau showed still not acceptable values (>40%) in comparison to the fresh sample (Table 2).

The delay in centrifugation (>3 h) had only a minor impact on concentrations compared to those of sample centrifugated within 3 h and tested after the storage for few hours at +4 °C as recommended by manufacturer (pTau=1.07 vs. 1.02 ng/L, AB 1–42=23.955 vs. 26.51 ng/L, AB 1–40=285.19 vs. 299.23 ng/L).

Evaluating the analytical performance, inter-day CV% calculated on internal quality controls (QC) provided by manufacturer were: 4.7% for level 1 (mean 5.03 ± 1.01 ng/L) and 3.3% level 2 (44.16 ± 8.83 ng/L) for pTau (lot U2B3011); 2.2 and 2.3% for AB 1–42 level 1 and 2 (mean 21.28 ± 4.26 ng/L and 206.96 ± 41.39 ng/L, lot T8B2091); 3.3 and 4.2% for AB 1–40 level 1 and 2 (204.94 ± 40.99 ng/L and 2050.84 ± 410.17 ng/L, lot T8B2091).

Our results highlight that storage conditions might be critical to obtain results accurate and consistent with the clinical status, due to a possible temperature-dependent biological instability of pTau and amyloid proteins in blood. For samples stored at −20 °C the variations in concentrations (bias%) in comparison to the baseline values was in fact greater than those observed for other storage temperatures; this effect of −20 °C freezing was indeed not observed in the SABB blood SOPs [8], that suggested a storage up to two weeks at this temperature.

Table 1: Time and temperature storage effects on plasma biomarkers.

Day	Sample	Storage temperature	pTau ng/L	AB 1–42 ng/L	AB 1–40 ng/L	AB 1–42/1–40
1	1	4 °C	0.99	27.02	303.52	0.089
	2	4 °C	1.05	26.01	294.94	0.088
2	3	4 °C	0.84	23.52	262.3	0.090
	4	−20 °C	1.78	21.73	288.31	0.075
	5	−80 °C	1.82	25.51	295.63	0.086
3	6	4 °C	0.93	22.43	254.25	0.088
	7	−20 °C	1.99	22.83	290.08	0.079
	8	−80 °C	1.7	25.11	296.42	0.085
4	9	−20 °C	2.35	19.17	189.55	0.101
	10	−80 °C	1.43	24.8	287.82	0.086
5	11	−20 °C	1.03	13.41	218.17	0.061
	12	−80 °C	1.63	24.25	300.74	0.081
8	13	−20 °C	1.51	25.31	307.34	0.082
	14	−80 °C	1.43	27.58	314.97	0.088

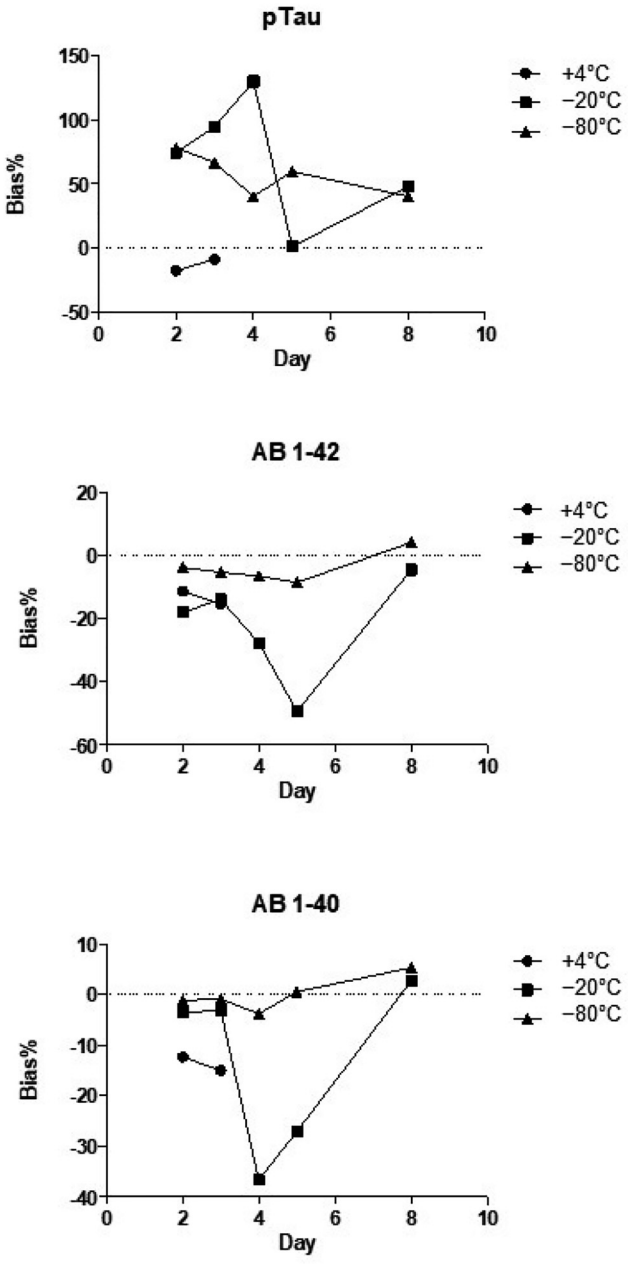

Figure 1: Effect of storage temperatures on day-by-day values of plasma biomarkers in different aliquots of sample from 1 healthy volunteer as expressed by difference between single measures and value at baseline (bias%).

Instead, the bias% observed for amyloids had the lowest values at −80 °C (≤8.5%), confirming the recommendation for long-term storage [8].

The described results of inter-day CV% on QC for plasma measurement fully confirm performances of CSF stored at −80 °C as reported in different papers evaluating the AD biomarkers testing on the same assay platforms (intra-laboratory repeatability <5.6%)

Table 2: Biomarkers variability on plasma. Bias% of samples at different storage temperature compared to baseline value (+4 of Day 1).

		Day 2	Day 3	Day 4	Day 5	Day 8
pTau	4 °C	−17.6%	−8.8%			
	−20 °C	74.5%	95.1%	130.4%	1%	48%
	−80 °C	78.4%	66.7%	40.2%	59.8%	40.2%
AB 1–42	4 °C	−11.3%	−15.4%			
	−20 °C	−18%	−13.9%	−27.7%	−49.4%	−4.5%
	−80 °C	−3.8%	−5.3%	−6.5%	−8.5%	4%
AB 1–40	4 °C	−12.3%	−15%			
	−20 °C	−3.6%	−3.1%	−36.7%	−27.1%	2.7%
	−80 °C	−1.2%	−0.9%	−3.8%	0.5%	5.3%
AB 1–42/1–40	4 °C	1.2%	−0.4%			
	−20 °C	−14.9%	−11.2%	14.2%	−30.6%	−7%
	−80 °C	−2.6%	−4.4%	−2.7%	−9%	−1.2%

[9].Therefore, further studies should clarify whether the suggested temperature-related instability might be ascribed to matrix-dependent factors or to sub-optimal analytical sensitivity of the assays at lower concentrations of the biomarkers in blood.

Based on our preliminary data, measurements performed in fresh and frozen samples may not be comparable. While optimum testing conditions might involve testing at the same day of collection or within 3 days when stored at +4 °C, or the storage at −80 °C if deferral is mandatory, it might be crucial to further assess if different cutoffs should be established for fresh and thawed samples.

We then tested samples with HIL: 5,676 (i.e. Hb=57 g/L), 4,898 (Hb=49 g/L), 3,544 (Hb=35 g/L), 2,810 (Hb=28 g/L), 910 (Hb=9 g/L), 291 (Hb=3 g/L), 21 (Hb=0.2 g/L) and 10 (Hb=0.1 g/L). Spearman coefficients showed a strong correlation between protein levels and hemolysis: r=0.9524, p=0.0011 for pTau; r= −0.9762, p=0.0004 for AB 1–42; r= −0.9762, p=0.0004 for AB 1–40 (Figure 2). Moreover, also AB 1–42/1–40 ratio had a significant variation based on hemolysis index (r=0.7857, p=0.0279).

We recommend checking for hemolysis before testing, as hemolysis may cause a direct and predictable effects on the results for the three biomarkers concentrations. In fact, HIL or other plasma hemolysis biomarkers are usually available in fully automated clinical chemistry instrumentations that are used in clinical laboratories.

The recent approval of the first beta-amyloid targeted immunotherapy [10] seems to allow the clinical application of blood-based biomarkers measurement as screening tools for at-risk individual or for therapeutic drug management. Therefore, laboratory professionals

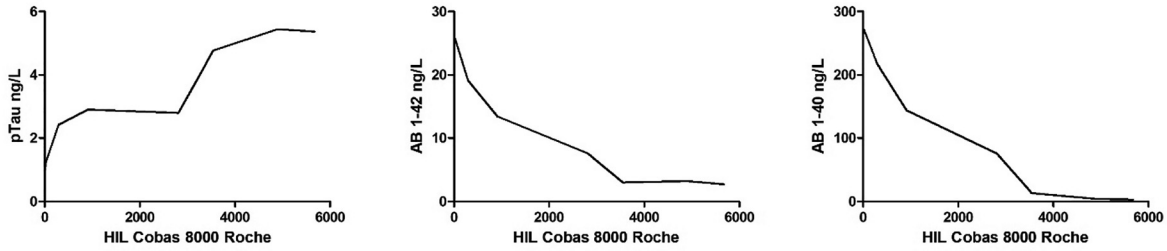

Figure 2: Effect of hemolysis on pTau, AB 1–42 and AB 1–40 concentrations; HIL index values=Hb concentration mg/dL.

should address comprehensive preanalytical and post-analytical issues in addition to analytical performances in view of a forthcoming implementation of these biomarkers in clinical practice. The main limitation of the study is the use of samples only from a healthy subject; as biomarkers might behave differently in pathological conditions and at different concentrations, further studies are needed to provide evidence in samples from AD patients.

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