Bioanalysis

Neuro biomarker levels measured with high-sensitivity digital ELISA differ between serum and plasma

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Aim: Digital ELISA-based assays for blood biomarkers of neurological disease are on the verge of clinical use. Here, we aimed to determine whether different preanalytical blood processing techniques influence results. **Materials & methods:** Concentrations of neurofilament light chain (NfL), Tau and amyloid beta (Aβ) were measured in human plasma and serum specimens using digital ELISA and compared between blood products. Measured levels of NfL were highly equivalant between serum and plasma in all analyses, however, measured levels of Tau and Aβ were consistently lower in serum relative to plasma. **Conclusion:** Tau and Aβ are likely lost during clotting in serum preparations, and should be assayed in plasma to get an accurate measure of circulating levels.

Due to recent technological advances in proteomic detection, blood-based biomarker assays with utility for diagnosis and monitoring of neurological conditions are on the verge of clinical use. The proteomic composition of the brain is highly unique relative to other organs, and damage to neural tissue results in the release of brain-specific proteins into peripheral circulation. Thus, the detection of these proteins in the blood can serve as biomarkers of acute and chronic neural pathology. For example, circulating levels of brain-specific proteins have been shown to be elevated in a wide variety of acute and chronic neural conditions including traumatic brain injury [1], stroke [2], multiple sclerosis [3], HIV-related neurodegeneration [4], dementia [5] and Alzheimer's disease [6]. Unfortunately, these proteins are typically only present in circulation at low-picogram to subpicogram concentrations, and the lower limits of detection associated with conventional ELISA techniques traditionally used to assay blood are not robust enough to detect them at early enough time-points in most pathologies to make them clinically informative.

However, recent advances in proteomic techniques are allowing for detection of these markers at low enough concentrations to allow for the development of assays with true clinical utility. Digital ELISA is a recently developed immunoassay methodology which allows for femtogram-level detection of protein analytes in biofluids. First, antibody conjugated paramagnetic beads are used to capture single molecules of target protein, and protein–bead complexes are labeled with fluorophore-conjugated detection antibody. Beads are then assessed for the presence or absence of target protein using a precision-fabricated microwell array capable of capturing one bead per well. This technique has been shown to be as much as 1000-times more sensitive than traditional ELISA methods [7], and is now being rapidly adopted for measurement of neuro biomarkers blood. For example, digital ELISA has now been successfully implemented to detect blood biomarkers of neural damage in individuals with subclinical concussion [8], Alzheimer's disease in individuals prior to onset of symptoms [9], and in relapsing-remitting multiple sclerosis patients prior to development of new lesions [10]. As a result, there has been a rapid push for the development of digital ELISA-based clinical assays targeting brain biomarkers, and there now tests which are close to consideration by regulatory bodies for *in vitro* diagnostic use.

As these tests become closer to clinical use, it is important to understand the preanalytical factors which need to be considered for constant and reliable measurements. In particular, blood preparation methods can affect the results of a wide range of clinical tests; most notably, assayed levels of many biomarkers can differ between serum

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and plasma, simply as a result of true differences in analyte concentration between the two blood products, or due to interference from the anticoagulants used to prepare plasma [11]. It is currently unknown whether blood processing methods affect the levels of neuro biomarkers measured with digital ELISA. Here, we measured the levels of three commonly tested neuro biomarkers in both serum and plasma using digital ELISA to determine if different blood processing techniques influence results.

Materials & methods

Experimental design
Parallelly-drawn paired plasma and serum specimens were obtained from a small group of heathy human donors (n = 8), and the concentrations of neurofilament light chain (NfL), Tau and amyloid beta (Aβ) were measured using digital ELISA; measured concentrations were subsequently compared between the two different blood products. In order to confirm our results, digital ELISA was used to measure the levels of the same biomarkers in a larger but unpaired pool of serum (n = 85) and plasma (n = 47) samples collected from donors with a variety of health statuses.

Subjects
Blood donors of varying health statuses were recruited as part of various different clinical investigations at Case Western Reserve University (OH, USA), University Hospitals Cleveland Medical Center (OH, USA), and West Virginia University (WV, USA). Demographic information was collected from either the subject or significant other by a trained clinician. All procedures were approved by the institutional review boards of University Hospitals (IRB protocol #20181112) and West Virginia University (IRB protocol #1410450461R001). Written informed consent was obtained from all subjects or their authorized representatives prior to any study procedures.

Blood collection & processing
Blood for serum specimens was drawn via venipuncture and collected in clot-activating serum separator tubes (Becton Dickinson, NJ, USA). Blood was allowed to clot for 30 min at room temperature, and spun at $2500 \times g$ for 10 min to separate serum. Resultant serum was aliquoted and stored at -80 °C until analysis. Blood for plasma specimens was drawn via venipuncture and collected in either spray coated K_2ETDA or lithium heparin tubes (Becton Dickinson). Blood was spun at $2500 \times g$ for 10 min to separate plasma. Resultant plasma was aliquoted and stored at -80 °C until analysis.

Digital ELISA
Levels of NfL, Tau and Aβ in serum and plasma specimens were measured via the Quanterix HD-1 Single Molecule Array (SiMoA) platform at the Quanterix Accelerator Laboratory (Quanterix Corporation, MA, USA) by a technician blinded to specimen information. NfL was measured via a single-plex assay kit targeting total NfL (Quanterix catalog# 103186), while Tau and Aβ were measured via a multiplex assay kit (Quanterix catalog #101995) targeting total Tau and the 40 amino acid isoform of Aβ specifically. All assays were performed using manufacture recommended protocols. Standard curves were generated via triplicate measurements of recombinant proteins (Quanterix catalog #102255, #103221) and all specimens were assayed in duplicate. Intra-assay coefficient of variation (CV) values were calculated from technical replicate measures of specimens assayed within a single run. Interassay CV values were calculated from technical replicate measures of high- and low-range control specimens (Quanterix catalog #102256, #102257, #103222, #103223) assayed across multiple runs.

Statistics
Statistics were performed via R version 2.14. T-test or analysis of covariance (ANCOVA) was used for comparison of continuous variables where appropriate. Strength of correlational relationships were assessed via Spearman's ρ. The null hypothesis was rejected when $p < 0.05$. The parameters of all statistical tests performed are outlined in detail within the figure legends.

Results

Levels of neuro biomarkers in paired serum & plasma specimens
Demographic characteristics of healthy donors are indicated in Table 1. Healthy donors included three males and five females, with an average age of 50.0 ± 4.0. All plasma specimens were anticoagulated with K_2EDTA.

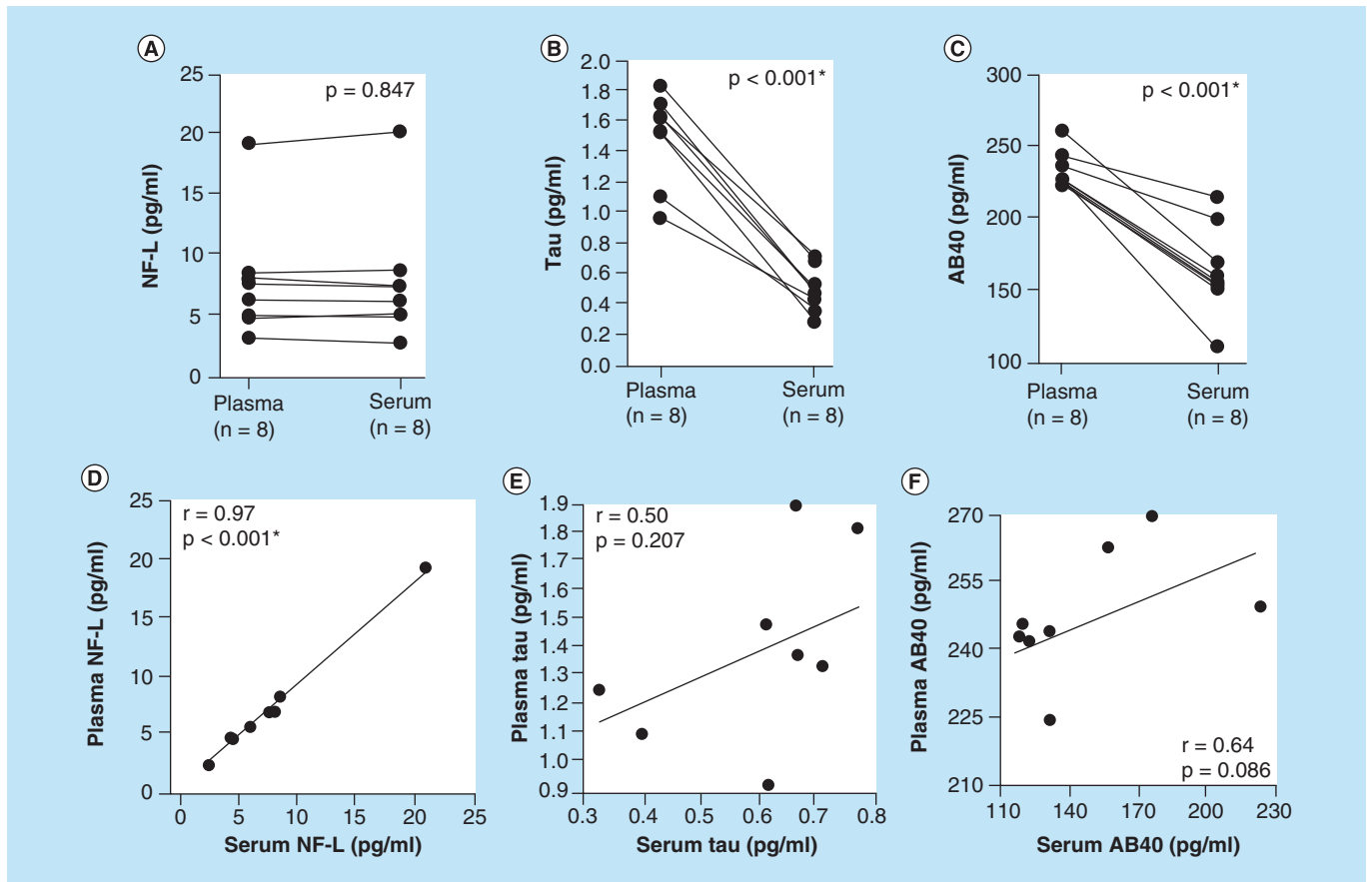| Table 1. Demographic characteristics of paired serum and plasma specimens donors.	
Demographic	Healthy donors (n = 8)
Age; mean ± standard deviation	50 ± 4.0
Female; n (%)	5 (62.5)
Male; n (%)	3 (37.5)
African–American; n (%)	8 (100)
Caucasian; n (%)	0 (0.0)

Figure 1.   Measured levels of neuro biomarkers in paired serum and plasma specimens. (A–C) Measured levels of NfL, Tau and Aβ in parallelly-drawn paired serum and plasma specimens. Lines connect paired specimens. Means were compared via two-tailed paired t-test. **(D–F)** Correlation of measured levels of NfL, Tau and Aβ between serum and plasma. Strength of correlations were tested via Spearman's ρ. NfL: Neurofilament light chain.

Measured biomarker levels for all technical replicates for all specimens used in the paired analysis fell within the quantitative ranges for each assay. Intra-assay CVs for NfL, Tau and Aβ measurements in plasma were 3.5, 9.3 and 2.6% respectively, while intra-assay CVs for NfL, Tau and Aβ measurements in serum were 4.3, 9.8 and 2.7%, respectively.

Measured levels of NfL were highly consistent between serum and plasma; we observed a negligible 5.4 ± 4.1% average percent difference in measured levels between paired specimens, which was not statistically significant (p = 0.847). Furthermore, measured NfL levels in serum and plasma exhibited a linear relationship and were highly correlated (r = 0.97; Figure 1A & E). Conversely, measured levels of Tau were on average 65.7 ± 8.7% lower in serum specimens compared with paired plasma specimens. This difference was highly statistically significant (p < 0.001), and levels were positively correlated (r = 0.50; Figure 1B & F). Similar to Tau, measured Aβ levels were on average 29.5 ± 12.1% lower in serum specimens versus paired plasma specimens. This difference was also highly statistically significant (p < 0.001), and levels were again positively correlated (r = 0.64; Figure 1C & G).

| Table 2. Clinical and demographic characteristics of unpaired serum and plasma specimens donors.		
Characteristic	Plasma donors (n = 47)	Serum donors (n = 85)
Age; mean ± standard deviation	54.9 ± 13.3	50.8 ± 15.3
Female; n (%)	23 (48.9)	57 (67.1)
Male; n (%)	24 (51.1)	28 (32.9)
African–American; n (%)	32 (68.1)	52 (61.2)
Caucasian; n (%)	15 (31.9)	33 (38.8)
Healthy; n (%)	0 (0.0)	26 (30.6)
Cardiovascular disease; n (%)	22 (46.8)	49 (57.6)
HIV positive; n (%)	25 (53.2)	3 (3.5)
Acute brain injury; n (%)	14 (29.8)	13 (15.3)
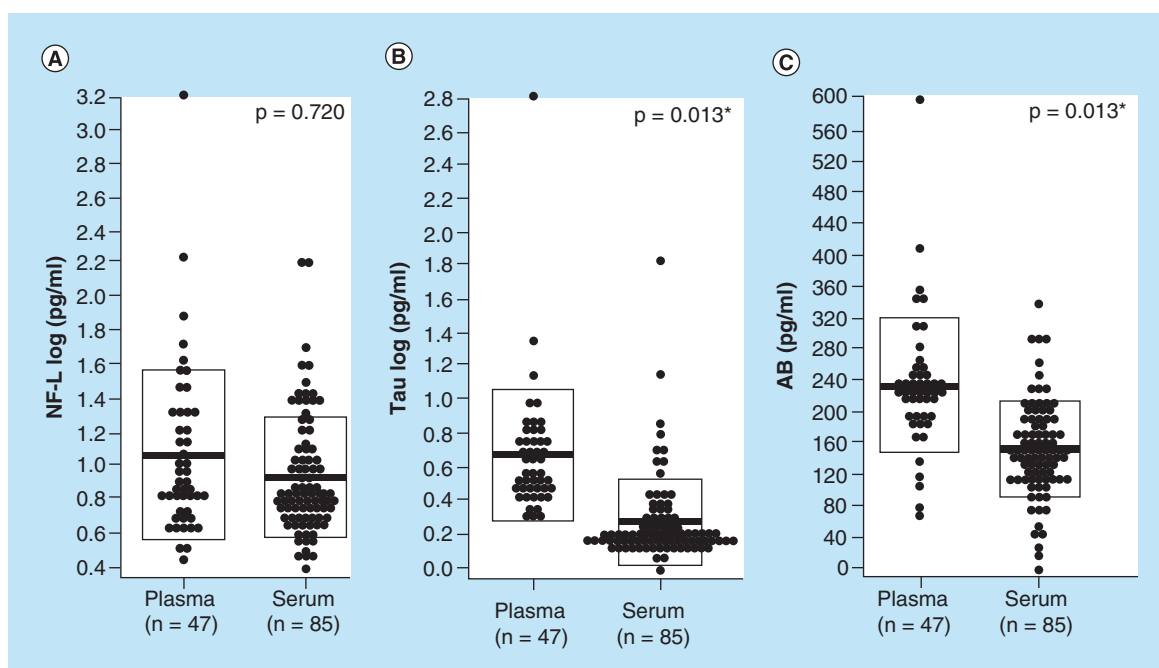
Figure 2. Measured levels of neuro biomarkers in unpaired serum and plasma specimens. (A–C) Measured levels of NfL, Tau and Aβ in unpaired serum and plasma specimens. Means were compared via one-way ANCOVA controlling for donor health status and type of anticoagulant used in plasma preparation. NfL and Tau values were log transformed prior to comparison.
ANCOVA: Analysis of covariance; NfL: Neurofilament light chain; Aβ: Amyloid beta.

Levels of neuro biomarkers in unpaired serum & plasma specimens

Clinical and demographic characteristics of donors associated with serum and plasma specimens are indicated in Table 2. Donors associated with serum specimens were of similar age to those associated with plasma specimens, and exhibited similar gender and ethnic demographics. A total of 47% of plasma specimens were anticoagulated with K_2EDTA, and the remaining 53% with lithium heparin.

Measured neuro biomarker levels for all technical replicates for all specimens in the unpaired analysis fell within the quantitative ranges for each assay, and interassay CVs were all less than 15%. Intra-assay CVs for NfL, Tau and Aβ measurements in plasma were 4.6, 9.1 and 9.5%, respectively, while intra-assay CVs for NfL, Tau and Aβ measurements in serum were 4.7, 11.4 and 2.7%, respectively.

Mean levels of NfL were 19.1% lower in serum relative to plasma, however, constant with our analysis of paired specimens, this difference was not statistically significant after controlling for donor health status and the type of anticoagulant used in plasma preparation (p = 0.720; Figure 2A). Mean levels of Tau and Aβ were respectively 54.1 and 33.9% lower in serum specimens relative to plasma specimens. These differences were both statistically
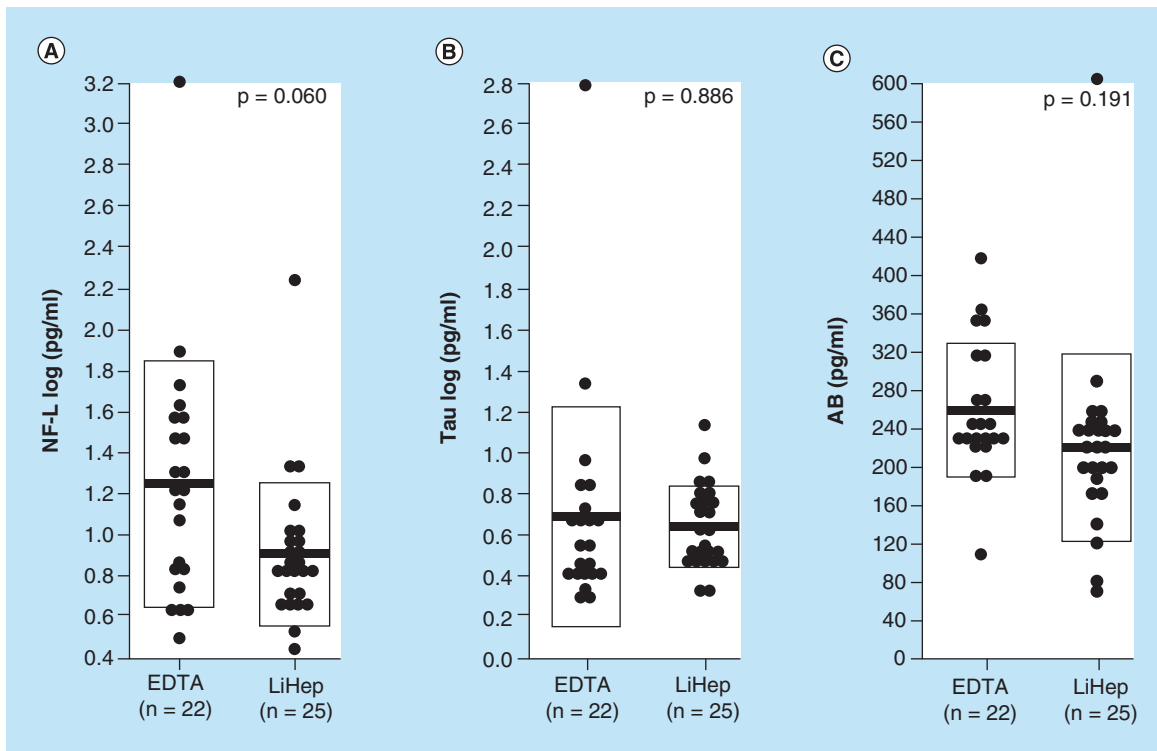
Figure 3.   Measured levels of neuro biomarkers in plasma specimens anticoagulated with EDTA and lithium heparin.
(A–C) Measured levels of NfL, Tau and Aβ in plasma specimens anticoagulated with either EDTA or lithium heparin.
Means were compared via one-way ANCOVA controlling for donor health status. NfL and Tau values were log
transformed prior to comparison.
Aβ: Amyloid beta; ANCOVA: Analysis of covariance; LiHep: Lithium heparin; NfL: Neurofilament light chain.

significant while controlling for donor health status and the type of anticoagulant used in plasma preparation
(p = 0.013, p = 0.013; Figure 2B & C).

Mean levels of NfL, Tau and Aβ were respectively 32.9, 2.2 and 14.6% lower in plasma prepared using lithium
heparin versus K_2EDTA (Figure 3B). However, none these differences were statistically significant after controlling
for donor health status (p = 0.060, p = 0.886, p = 0.191; Figure 3A–C).

Discussion

The aim of this work was to better understand what preanalytical factors may affect the levels of commonly-tested
neuro biomarkers measured via digital ELISA; specifically, we wanted to determine whether the use of serum or
plasma specimens influences results.

We observed negligible differences in measured NfL levels between serum and plasma in both our analyses
of paired and unpaired specimens, suggesting that the use of serum or plasma has little effect on levels of NfL
measured with digital ELISA. This is encouraging, as it suggests that different blood processing protocols can
produce consistent results when measuring NfL in the clinical setting, and that retrospective research studies using
archived specimens can likely do pooled analysis using a mix of different blood products without having to be
concerned about confounds. Conversely, we observed up to a 65% difference in measured Tau and Aβ levels
between blood products in some instances, suggesting that levels of these markers are not directly comparable
between serum and plasma, and that consistent blood processing protocols need to be implemented to produce
reliable results.

In terms of the mechanism which drove the observed differences in measured Tau and Aβ levels between serum
and plasma, we believe it is a result of true differences in their concentrations in the blood products, and not due
to a falsely-high signal caused by the presence of anticoagulants in plasma. It is unlikely that the difference was
driven by an anticoagulant interfering with assay chemistry for two reasons, first, we did not observe a similar effect
when measuring NfL using the same platform, and second, the effect was observed independently of the type of

anticoagulant used in plasma preparation. Due to their propensity to aggregate, it is most probable that Tau and Aβ become trapped in the fibrin-platelet matrix during clotting, which would account for the lower levels which we observed in serum. Interestingly, the degree of correlation between serum and plasma levels with respect to both markers was relatively weak, suggesting that there is a high degree of interindividual variability regarding the degree to which they are lost during serum preparation. Such variability could be due differences in the levels of various clotting components and overall clotting kinetics between individuals. Thus, our collective results suggest that measurement of Tau and Aβ in plasma is likely to give a better indication of true circulating levels.

It is important to note that we only analyzed plasma prepared using K_2EDTA and lithium heparin. While these are two of the most commonly used anticoagulants used to prepare plasma for immunoassays, there are other anticoagulants which are used clinically such as sodium citrate and potassium oxalate. It is well known that anticoagulants can cause interference in immunoassays, and that such interference is highly dependent on the type of anticoagulant, analyte and assay [12]. While we did not notice any dramatic or statistically significant differences in measured neuro biomarker levels between plasma prepared using K_2EDTA and lithium heparin, future work should more thoroughly examine whether the use of different anticoagulants affects results. Such work will be essential in developing the standardized blood processing protocols which are so clearly needed for reliable measurement of these markers.

It is also important to note that we only reported on three commonly measured neuro biomarkers, and that our results do not extend to others not directly assessed here. For example, we specifically assayed the 40 amino acid isoform of Aβ, as is the most biologically prevalent in humans [13]. However, there are other isoforms of Aβ, such as 38 and 42 amino acid variants, that are produced in some neurodegenerative conditions [14]; these other isoforms may not exhibit similar differences in concentration between serum and plasma as the 40 amino acid isoform. However, our results clearly indicate that preliminary experiments assessing the possible effects of different blood processing methods on measured levels of these other isoforms would have be completed in order to justify any type of combined analysis using a mix of different blood products.

Collectively, our results clearly indicate that different blood preparation methods can affect the levels of neuro biomarkers assayed by digital ELISA, and highlight the importance of considering what preanalytical factors need to be standardized in order to produce reliable measures. With respect to the specific markers which we assessed here, our findings suggest that serum and plasma levels of NfL are likely directly comparable, however, measurements of Tau and Aβ are not. Tau and Aβ are likely lost during clotting, and should be assayed in plasma to get an accurate measure of circulating levels.

Future perspective

Within the next decade, we fully expect digital ELISA-based blood assays measuring neuro biomarkers to be in clinical use for the diagnosis and monitoring of neurological damage. The findings reported here clearly indicate that the use of serum versus plasma can produce dramatically different measures, and that standardized blood processing methodologies need to be established in order to produce accurate and consistent results. Future studies should more thoroughly examine whether the type of anticoagulant used in plasma preparation influences the levels of these biomarkers. This work is essential if these assays are going to make the jump from the research environment to the clinic.

Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Summary points

Background
- Digital ELISA-based assays for blood biomarkers with utility for diagnosis and monitoring of neurological conditions are on the verge of clinical use.
- It is important to understand the preanalytical factors which need to be considered for reliable measurements.
- Here, we measured the levels of three commonly tested neuro biomarkers in both serum and plasma using digital ELISA to determine if different blood processing techniques influence results.

Methods
- Parallelly-drawn paired plasma and serum specimens were obtained from a small group of heathy human donors (= 8), and the concentrations of neurofilament light chain (NfL), Tau and amyloid beta (Aβ) were measured using digital ELISA and compared.
- Levels of the same biomarkers were also measured in a larger but unpaired pool of serum (= 85) and plasma specimens (= 47) collected from donors with a variety of health statuses to confirm results.

Results
- Measured levels of NfL were highly consistent between blood products in both analyses.
- Measured levels of Tau and Aβ were up to 65% lower in serum specimens relative to plasma specimens.

Conclusion
- Serum and plasma levels of NfL measured with digital ELISA are likely directly comparable, however, measurements of Tau and Aβ are not.
- Tau and Aβ are likely lost during clotting, and should be assayed in plasma to get an accurate measure of circulating levels.

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