

## SHORT REPORT

# Differences between blood and cerebrospinal fluid glial fibrillary acidic protein levels: The effect of sample stability

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## Abstract

**Introduction:** Recent evidence has shown that the marker of reactive astrogliosis, glial fibrillary acidic protein (GFAP), has a stronger relationship with cerebral amyloid beta (A $\beta$ ) pathology in blood than in cerebrospinal fluid (CSF). This study investigates if pre-analytical treatment of blood and CSF contribute to these unexpected findings.

**Methods:** Paired CSF and serum samples from 49 individuals (A $\beta$ -negative = 28; A $\beta$ -positive = 21) underwent a series of seven freeze-thaw cycles (FTCs). All samples were analyzed for GFAP and neurofilament light (NfL) using single molecule array technology including a fresh unfrozen sample from each patient.

**Results:** FTC significantly affected CSF GFAP concentration (–188.12 pg/ml per FTC) but not serum GFAP. In the same samples, NfL remained stable. Serum GFAP had a higher discrimination of A $\beta$  burden than CSF GFAP, irrespective of FTC, which also included unfrozen samples.

**Discussion:** This study demonstrates large stability differences of GFAP in CSF and serum. However, this disparity does not seem to fully explain the stronger association of serum GFAP with A $\beta$  pathology. Further work should investigate mechanisms of GFAP release into the bloodstream under pathological conditions.

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## 1 | INTRODUCTION

The challenge of determining the underlying pathology of Alzheimer's disease (AD) in vivo is set to be simplified by a compendium of blood biomarkers reflecting AD pathophysiology. The latest addition to this blood biomarker toolbox, glial fibrillary acidic protein (GFAP), a marker of reactive astrogliosis, is elevated early in the AD continuum and further increased as disease symptomatology progresses.<sup>1-5</sup> This increase is seemingly closely related to the emergence of amyloid beta (A $\beta$ ) pathology and thus AD. Yet, blood GFAP levels are mildly increased in frontotemporal dementia and atypical parkinsonian disorders in comparison with healthy controls,<sup>6</sup> which predicts cognitive decline in these disorders.<sup>6,7</sup> However, unlike other blood biomarker candidates for AD (e.g., phosphorylated tau and A $\beta$ <sup>8</sup>), the blood component of GFAP is vastly superior in determining elevated cerebral A $\beta$  pathology, compared to GFAP levels in cerebrospinal fluid (CSF).<sup>1,2</sup> This goes against our current understanding of AD blood-based biomarkers, which are seen as proximal indications of cerebral pathologies and thus inferior to CSF and molecular imaging biomarkers, or at their very best, statistically comparable.<sup>9</sup> The reasoning behind this is currently unknown, but we, and others, have speculated different mechanisms that facilitate the drainage of GFAP, and its breakdown products, into blood under pathological conditions. A more practical reasoning, however, could be the differential pre-analytical stability of GFAP in blood and CSF, which has been reported separately.<sup>10-12</sup> If true, the capability to detect cerebral A $\beta$  by GFAP may be greatly affected by unavoidable pre-analytical considerations in research settings, and thus limits its use as a practical AD biomarker and general research tool in other areas of neurology. In this study, we sought to test the impact of sample stability by freeze-thaw cycles (FTCs) on the levels of GFAP in paired blood (serum) and CSF from 49 individuals. Serum<sup>10</sup> and CSF<sup>13</sup> levels of neurofilament light (NfL) are known to be stable in differing pre-analytical conditions and, thus, were used in this study as a comparative control measure. Furthermore, we assessed if GFAP stability differentially impacted the ability to identify abnormal cerebral A $\beta$  pathology determined by the CSF A $\beta$ 42/A $\beta$ 40 ratio.

## 2 | METHODS

Fresh and de-identified serum and CSF samples from 49 individuals were collected at the Sahlgrenska University Hospital Clinical Chemistry Laboratory in Mölndal, Sweden after routine analysis was secured, in accordance with a procedure approved by the ethics committee at University of Gothenburg (EPN140811). The time between CSF and serum collection was <1 h for all patients. CSF A $\beta$ 42 and A $\beta$ 40 concentrations were measured as a part of clinical routine using the commercial fully automated LUMIPULSE G1200 (Fujirebio). A $\beta$  positivity in these individuals was defined as CSF A $\beta$ 42/A $\beta$ 40 ratio <0.072.<sup>14</sup> After collection, polypropylene tubes containing 500  $\mu$ L of serum and CSF were snap frozen on dry ice (15 min) and thawed (30 min). This process was repeated a total of seven times (FTC 1 to 7).

### RESEARCH IN CONTEXT

- 1. Systematic review:** The authors reviewed the literature using traditional (e.g., PubMed) sources. The field of blood biomarkers of CNS-related pathophysiology has seen a great progress in recent years. A promising blood biomarker of astroglial activation, glial fibrillary acidic protein (GFAP), increases in blood early in the Alzheimer's disease (AD) continuum associating with amyloid- $\beta$  pathology. Interestingly, recent data has shown a superior performance of blood GFAP in detecting cerebral amyloid $\beta$  pathology than when measured in cerebrospinal fluid (CSF). However, the potential explanations to this unusual discrepancy are not yet known. We found no articles attempting to examine these differences.
- 2. Interpretation:** Our research highlights that freeze thaw cycles (FTCs) are important when interpreting CSF GFAP. CSF GFAP concentration clearly decreases with increasing number of FTCs, while serum GFAP remains stable. However, the inferior discriminative ability of amyloid burden by CSF GFAP is not solely due to FTCs, as serum GFAP was superior already in fresh and unfrozen samples. This highlights the need for further studies exploring these discrepancies focusing on release mechanism of CNS-related proteins into the bloodstream.
- 3. Future directions:** The manuscript proposes that CSF is an unreliable matrix when using GFAP in relation to AD pathophysiology, unless there is strict control and recording of the number FTCs which is equivalent in all patients. Further work could address: 1) are these differences unrelated to which assay is being used to quantify GFAP or, 2) are different drainage mechanisms contributing to these differences (e.g., blood brain barrier function or lymphatic clearance).

After each FTC, a fraction of the serum (50  $\mu$ L) and CSF (20  $\mu$ L) from each patient was collected in a new tube. These aliquots were stored at 4°C pending analyses the same day (maximum delay = 150 min), including an unfrozen sample (FTC 0). All samples were analyzed using commercially available single molecular array (Simoa) assays (serum: Neurology 4-plex E, CSF: GFAP Discovery and NfL advantage) on Simoa HD-X analyzers (Quanterix, Billerica, MA, USA). A Kruskal-Wallis test with a Dunn post hoc test compared biomarker % change from FTC 0 and all other FTC time points. *p*-values adjusted for multiple comparisons using false discovery rate (FDR) were considered significant at *p* < 0.05, two-tailed. Mann-Whitney *U* tests compared the % change FTC "X" to the previous FTC. Receiver-operating characteristic (ROC) curve analyses provided the area under the curve (AUC) for A $\beta$  positivity. A paired DeLong test was applied to statistically compare biomarker performance. In an alternative statistical approach, we used linear

**TABLE 1** Demographics and biochemical characteristics of the study participants

	A $\beta$ -negative (CSF A $\beta$ 42/40 > 0.072)	A $\beta$ -positive (CSF A $\beta$ 42/40 < 0.072)	p value
Participants, n	28	21	
Age, years	73.5 (62.8-76.3)	73 (67.0-76.0)	ns
Sex, M/F	17/11	9/12	ns
CSF A $\beta$ 42/40 ratio	0.095 (0.090-0.102)	0.045 (0.039-0.050)	< 0.0001
CSF p-tau181, pg/ml	34 (24.8-45.0)	80 (50.0-107)	< 0.0001
CSF t-tau, pg/ml	320 (211-471)	532 (358-671)	< 0.0001
CSF GFAP, pg/ml	1580 (1225-2073)	1829 (1230-3073)	< 0.01
Serum GFAP, pg/ml	160 (115-282)	231 (167-283)	< 0.001
CSF NfL, pg/ml	1672 (1102-3046)	2262 (1185-7344)	< 0.001
Serum NfL, pg/ml	33.5 (26.3-45.8)	32 (24-47.95)	ns

Abbreviations: A $\beta$ , amyloid beta; CSF, cerebrospinal fluid; GFAP, glial fibrillary acidic protein; NfL, neurofilament light; p-tau, phosphorylated tau. Data are median (interquartile range; Q1–Q3). CSF and serum biomarker measurement are at freeze-thaw cycle 0.

mixed-effects models to evaluate whether GFAP levels are affected by FTC. We fitted two models, one with CSF and one with serum GFAP, as the outcome variable, containing continuous-variable predictor terms for baseline GFAP levels (measurement with no FTC) and number of FTC. To better accommodate baseline inter-individual variability and model our parameter of interest, for example, trends of change in GFAP with FTC, we included random slopes on FTC and random intercepts for each participant. Confidence intervals were computed based on  $n = 500$  bootstrap permutations

### 3 | RESULTS

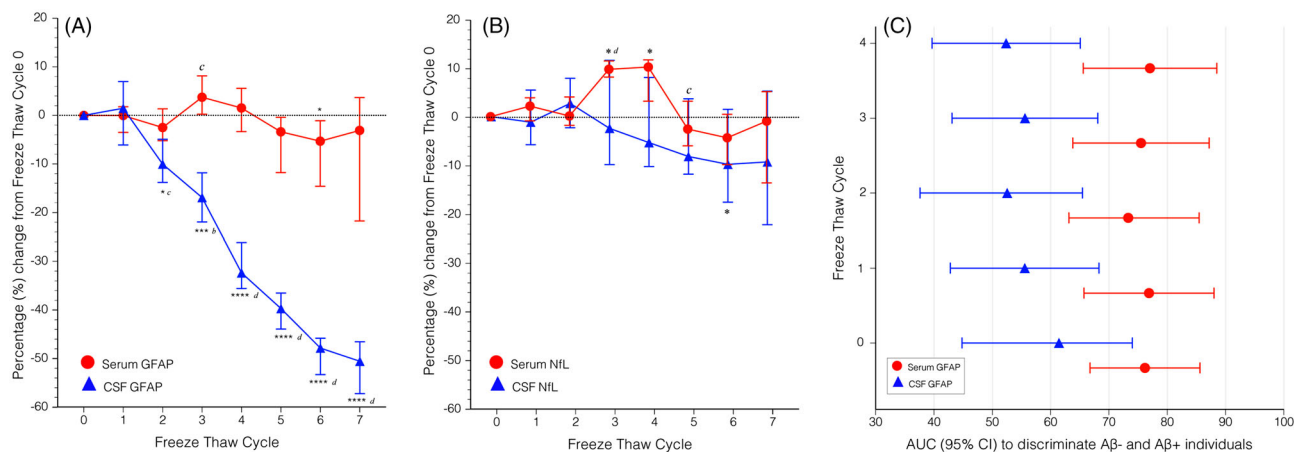
The basic demographics and biochemical characteristics of the study participants are shown in Table 1. In paired serum and CSF (A $\beta$ -negative = 28; A $\beta$ -positive = 21), CSF GFAP concentrations declined after each FTC, decreasing by a total of  $50.5\% \pm 10.2$  at FTC 7 (Figure 1A). In contrast, serum GFAP concentrations from the same individuals, treated in the same manner and analyzed at the same time, remained comparatively stable throughout this process (Figure 1A). Linear mixed-effects models indicated that FTC was associated significantly with a decline in CSF GFAP levels, with an average decrease of  $-188.12$  pg/ml (95% confidence interval [CI]  $-235.00, -141.27$ ;  $p < 0.0001$ ) per FTC. On the other hand, FTC were not associated with changes in serum GFAP ( $\beta$  estimate:  $-1.86$  pg/ml; 95% CI  $-4.35, 0.65$ ;  $p = 0.147$ ). CSF and serum NfL in the same samples were comparatively stable at all FTC (Figure 1B).

Serum GFAP could discriminate A $\beta$ -positive from A $\beta$ -negative individuals irrespective of FTC, with an average AUC of 75.8 (range, 73.3 to 77.1; Figure 1C) and was numerically higher than CSF GFAP but only significantly different at the fourth FTC ( $P_{DeLong} < 0.05$ ). The diagnostic performance of CSF GFAP, however, was only modest and was highest at FTC 0 (AUC = 61.4; 95% CI 41.8 to 74.1) but this was not significantly greater than subsequent FTCs in CSF GFAP (range, 52.4 to 61.4; Figure 1C).

### 4 | DISCUSSION

Recent studies have shown that blood GFAP is a robust biomarker for A $\beta$  plaque deposition across the AD continuum.<sup>1-5</sup> Two of these studies<sup>1,2</sup> also documented that blood GFAP significantly outperforms CSF GFAP in most analyses related to AD pathology. In this study, we hypothesized that stability differences between blood and CSF GFAP may have impacted on these results, given that bio-banked samples from research cohorts may undergo several FTCs before biochemical analysis. It was currently not known if fresh and unfrozen CSF yields a better relationship between GFAP and AD pathology than previously reported in cohort studies.<sup>1,2</sup> However, it has been shown previously that CSF GFAP levels decrease by 50% after only two FTCs in patients with primary progressive multiple sclerosis,<sup>11</sup> whereas GFAP in plasma or serum is robust in various pre-analytical conditions, including stability in more than four FTCs.<sup>10,12,15</sup> First, as anticipated, CSF GFAP is greatly impacted by increasing freeze-thawing, estimating an average decrease of  $\sim 200$  pg/ml per FTC, whereas serum GFAP from the same patients remained largely consistent. As a comparative control measured in the same samples, both CSF and serum NfL levels remained relatively stable. However, the instability of CSF GFAP upon freeze-thawing is not the sole reason for having an inferior relationship with AD pathology, as serum GFAP still exhibits a better relationship with cerebral A $\beta$ , indexed by the CSF A $\beta$ 42/A $\beta$ 40 ratio, than CSF GFAP in fresh, unfrozen samples but only significantly different at the fourth FTC.

This study demonstrates that CSF is an unreliable matrix for a robust GFAP measurement due to large decreases in measurable concentration after FTC. In contrast, serum GFAP has a stable association with AD pathology regardless of FTC stage. The reasons for these disparities need further investigation. It is possible that GFAP breakdown may already have taken place at the time of CSF sampling, given the  $\approx 4$  h delay for intracranial CSF to reach the lumbar sac where it is sampled. However, the superior stability of blood GFAP could be in part caused by a "hook effect" attributed to the formation of



**FIGURE 1** Effect of freeze-thaw cycling on GFAP and NfL in serum and CSF.

The % change of serum and CSF GFAP (A) and serum and CSF NfL (B) at multiple freeze-thaw cycles in comparison to fresh measurement. Data points are shown as median % change with 95% confidence intervals. The performance of serum and CSF GFAP at freeze-thaw cycle 0 to 5 to distinguish between amyloid-positive and amyloid-negative individuals (C).

Figure label: Kruskal-Wallis test with a Dunn multiple comparison test compared biomarker % change from freeze-thaw cycle 0 and all other time points; \* =  $p < 0.05$ , \* =  $p < 0.01$ , \*\* =  $p < 0.001$ , \*\*\* =  $p < 0.0001$ .

Mann-Whitney  $U$  tests compared the % change from freeze-thaw X to the previous freeze-thaw cycle;

<sup>a</sup> =  $p < 0.05$ , <sup>b</sup> =  $p < 0.01$ , <sup>c</sup> =  $p < 0.001$ , <sup>d</sup> =  $p < 0.0001$ .

Significance value above CI bars indicate serum, those below indicate CSF. GFAP, glial fibrillary acidic protein; NfL, neurofilament light; CSF, cerebrospinal flu

protein aggregates.<sup>16</sup> Our previous data shows that CSF GFAP exhibits a stronger relationship with measures of glial biomarkers (YKL40 and sTREM2), whereas plasma GFAP levels were not.<sup>1</sup> Thus it is possible that CSF GFAP better reflects reactive astrocytes in response to neuroinflammatory changes, whereas blood GFAP could be associated more closely with reactive astrogliosis due to A $\beta$  burden. Finally, we cannot exclude drainage mechanisms of GFAP, and fragments of, into the blood under pathological conditions as a principal reason for these differences. This could be via a bidirectional fluid exchange at astrocytic end-feet at the neurovascular unit, or through the glymphatic system and cervical lymph nodes, making it a better biomarker when measured in blood compared with CSF.

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#### CONFLICTS OF INTEREST

HZ has served at scientific advisory boards and/or as a consultant for Abbvie, Alector, Annexon, Artery Therapeutics, AZTherapies, CogRx, Denali, Eisai, Nervgen, Novo Nordisk, Pinteon Therapeutics, Red Abbey Labs, Passage Bio, Roche, Samumed, Siemens Healthineers, Triplet Therapeutics, and Wave; has given lectures in symposia sponsored by Cellectricon, Fujirebio, Alzecure, Biogen, and Roche; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program. KB has served as a consultant, at advisory boards, or at data monitoring committees for Abcam, Axon, BioArctic, Biogen, JOMDD/Shimadzu, Julius Clinical, Lilly, MagQu, Novartis, Ono Pharma, Pharmatrophix, Prothena, Roche Diagnostics, and Siemens Healthineers; and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program, outside the work presented in this paper. Author disclosures are available in the [supporting information](#).

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## SUPPORTING INFORMATION

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