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Abstract

Objectives: Glial fibrillary acidic protein (GFAP) is a promising biomarker that could potentially contribute to diagnosis and prognosis in neurological diseases. The biomarker is approaching clinical use but the reference interval for serum GFAP remains to be established, and knowledge about the effect of preanalytical factors is also limited.

Methods: Serum samples from 371 apparently healthy reference subjects, 21–90 years of age, were measured by a single-molecule array (Simoa) assay. Continuous reference intervals were modelled using non-parametric quantile regression and compared with traditional age-partitioned non-parametric reference intervals established according to the Clinical and Laboratory Standards Institute (CLSI) guideline C28-A3. The following preanalytical conditions were also examined: stability in whole blood at room temperature (RT), stability in serum at RT and –20 °C, repeated freeze-thaw cycles, and haemolysis.

Results: The continuous reference interval showed good overall agreement with the traditional age-partitioned reference intervals of 25–136 ng/L, 34–242 ng/L, and 5–438 ng/L for the age groups 20–39, 40–64, and 65–90 years, respectively. Both types of reference intervals showed increasing levels and variability of serum GFAP with age. In the preanalytical tests, the mean changes from baseline were 2.3% (95% CI: –2.4%, 6.9%) in whole blood after 9 h at RT, 3.1% (95% CI: –4.5%, 10.7%) in serum after 7 days at RT, 10.4% (95% CI: –6.0%, 26.8%) in serum after 133 days at –20 °C, and 10.4% (95% CI: 9.5%, 11.4%) after three freeze-thaw cycles.

Conclusions: The study establishes age-dependent reference ranges for serum GFAP in adults and demonstrates overall good stability of the biomarker.

Keywords: glial fibrillary acidic protein (GFAP); preanalytical; reference range; serum; stability.

Introduction

Glial fibrillary acidic protein (GFAP) is a type-III intermediate filament found primarily in astrocytes. It has been investigated as a promising biomarker of neurological disease, especially in the field of traumatic brain injury (TBI) [1]. Studies show that blood GFAP levels reflect the clinical severity of TBI [2] and may be superior to other serum biomarkers (e.g. S100B, UCH-L1, and NfL) in discriminating patients with and without abnormal head CT following a mild TBI [3–5]. Evidence also suggests a prognostic potential of blood GFAP in stroke [6–10], neurodegenerative diseases [11–14], and neuroinflammatory diseases such as multiple sclerosis [15–18].

Most of the initial studies on GFAP measured the biomarker in cerebrospinal fluid (CSF) where levels in healthy individuals are approximately 100 times higher than in blood and well within the detection range of conventional immunoassays [19]. However, the development of more advanced immuno assays in the last decade such as the ultrasensitive single molecule array (Simoa) has enabled the measurement of GFAP in blood samples even at the low levels found in some healthy individuals [3]. The use of blood instead of CSF has obvious advantages and makes the biomarker much more accessible for clinical use. To our knowledge, no age- and gender-specific reference ranges in healthy individuals have been defined, and only a few studies have examined the preanalytical properties of blood GFAP [14, 20–25].

Therefore, the aim of this study was to establish age- and gender-specific reference intervals for serum GFAP using both non-parametric quantile regression and the traditional non-parametric method described by the Clinical

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and Laboratory Standards Institute (CLSI) guideline C28-A3 [26], and to examine key pre-analytical properties of blood GFAP.

Materials and methods

The study was performed at our international accredited hospital laboratory at the Department of Clinical Biochemistry at Aarhus University Hospital, Denmark (DS/EN ISO/IEC 15189), between January and June 2022.

Sample collection

Reference interval study: The reference subjects were recruited among two groups: (1) blood donors aged 17–65 years from Aarhus University Hospital's blood bank, and (2) outpatients >65 years of age in the hospital's blood sampling units.

Danish blood donors are volunteers, between 17 and 65 years of age at enrolment, and have to fulfil strict health requirements. The blood samples were collected prior to blood donation by the laboratory technicians in the blood bank.

To recruit subjects >65 years, elderly outpatients referred to our blood sampling units for other biochemical tests were screened by a systematic interview after obtaining verbal consent for additional blood sampling. Subjects were excluded if self-reported to suffer from diabetes, dementia, current or previous stroke, brain disease or being referred from the departments of neurology, neurosurgery, or psychiatry. The blood samples were drawn by the laboratory technician doing the routine samples who also performed the systematic interview.

Preanalytical tests: Blood samples were collected from three age groups, approx. 30 years (n=3), 50–60 years (n=3), and >80 years (n=4), in the hospital's outpatient blood sampling units and selected inpatient wards after obtaining verbal consent for additional blood sampling.

Sample handling

All blood samples were drawn from the antecubital vein using Vacuette® SAFETY Blood Collection Set (Greiner Bio-One, Kremsmünster, Austria) and BD Vacutainer® serum clot activator tubes (Becton Dickinson, Lyngby, Denmark). All samples were anonymised and transported by hand to the laboratory, only accompanied by information on age and gender of the reference subjects. They were allowed to clot for at least 30 min before being centrifuged at 3,000 g for 5 min at 20 °C ± 1 °C (room temperature, RT) and subsequently frozen at –20 °C. For details about the timing of centrifugation and freezing, see below.

Reference interval study: All samples from reference subjects were kept at RT, centrifuged within 7 h of collection, and subsequently stored at –20 °C for a maximum of 2 months before analysis.

Preanalytical tests: The preanalytical stability of GFAP was assessed in (1) whole blood at RT, (2) serum at RT, (3) serum at –20 °C, and (4) serum after repeated freeze-thaw cycles, using an adaptation to The CRESS checklist for reporting stability studies by the EFLM [27].

(1) The stability in whole blood at RT was tested by collecting five serum tubes from 10 patients and storing them at RT for 1, 2, 5, 7, or 9 h before centrifugation and freezing.

(2) The stability in serum at RT was tested in samples from 10 patients that were centrifuged within 1 h, aliquoted and stored at RT for 0, 1, 2 3, 5, or 7 days before freezing.

(3) The stability in serum at –20 °C was tested by preparing three serum pools of different GFAP levels and aliquoting each into four tubes, of which one was analysed immediately and the rest after storage at –20 °C for 1, 2, or 4.5 months.

(4) Freeze-thaw stability was tested in four serum pools that were aliquoted and subjected to 1, 2, or 3 cycles of –20 °C/RT.

The effect of haemolysis was assessed in a dose-respond study as recommended by the CLSI approved Guideline EP07 [28]. Six serum pools with three different GFAP levels (low, medium, and high) were prepared. Haemolysate was prepared from whole blood using an adaptation to the Meites method [29]. Briefly, the erythrocytes from 5 mL of EDTA plasma was washed four times with 5 mL of isotonic saline. After centrifugation and discarding of the supernatant, the cells were diluted with demineralized water to a final volume of 5 mL, incubated at RT for 30 min and frozen at –20 °C overnight. After thawing, the sample was centrifuged again, and the H-index of the supernatant fluid determined using the Siemens Atellica Solution (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany). Dilution series with decreasing levels of haemoglobin (Hb) were prepared by spiking each serum pool with haemolysate to a concentration of 800 mg/dL and making 1:1 serial dilutions to obtain six samples with Hb in the range of 0–400 mg/dL.

GFAP assay

GFAP was measured using the Simoa GFAP Discovery Kit on the HD-1 Analyzer (Quanterix Corp., Billerica, MA) following the manufacturer's instructions. The assay is a 2-step digital immunoassay providing single molecule sensitivity by capturing and detecting immunocomplexes on singulated microbeads in arrays of femtoliter wells. According to the manufacturer, the lower limit of detection is 0.211 ng/L and the lower limit of quantification 0.686 ng/L. The calibration range is 0–1,000 ng/L and the dynamic range 0–4,000 ng/L. The stated within run coefficient of variation (CV) of three serum panels with mean concentrations 31.75 ng/L, 317.6 ng/L, and 2,282 ng/L is 6.3, 10.9, and 8.2%, respectively, and the between run CV 5.7, 13.0, and 13.6%. Two in-house made serum controls were used as internal controls. All samples were thawed at RT and analysed in singles by trained laboratory technicians blinded to data.

Statistical analysis

Reference interval study: Continuous reference intervals were modelled using non-parametric quantile regression as described by Holmes et al. [30] and compared to a traditional non-parametric method with age-partitioning as described in the CLSI guideline C28-A3 [26].

Quantile regression is robust to the shape of an empirical distribution and resistant to outliers. In this study, the package

quantregGrowth in R (version 4.1.3) was used with penalized splines and (n-1)-fold cross-validation. The 95%-confidence intervals were determined using the sandwich formula built into the quantregGrowth package [31, 32].

Figure 1 shows the effect of different smoothing values (λ) in the penalized spline function on the estimated continuous reference interval curves of the quantile regression. Small values of λ resulted in overfitting, whereas changing the value in the range of 1–40 had almost no visible effect on the reference interval. Using (n-1)-fold cross-validation, the optimum λ value was found to be 4.4.

To determine the traditional age-partitioned reference intervals, the data distribution was assessed by an QQ plot and Shapiro-Wilk test. Statistically significant outliers were detected by the Dixon D/R ratio. The decision to partition in age or gender groups was done according to the recommendations of Lahti et al. [33]. The age groups were predefined based on the demography of neurodegenerative disease patterns [34].

Apart from the quantile regression in R, the statistical analysis was carried out using Analyse-it 4.65.3 software for Microsoft Excel (Microsoft, Redmond, WA).

Preanalytical tests: The maximum permissible difference (MPD) for the preanalytical studies was established following the Milan hierarchy for analytical performance as recommended by the CRESS checklist [27]. The MPD could not be determined from outcome data from the literature due to lack of such available data. The MPD was therefore based on biological variation data [35]. The maximum allowable bias was calculated as $\pm 0.25 \times (CV_I^2 + CV_G^2)^{1/2} = \pm 10.2\%$ and the allowable total error as $\pm 1.65(0.5 \times CV_I) + bias = \pm 18.2\%$ [36].

Results

Analytical performance

Across the entire study period from February to June 2022, the intermediate precision of the low (average level 103.0 ng/L, n=28) and high (average level 1,398 ng/L, n=26) quality controls (QCs) was 13.1 and 14.2%, respectively. The reference samples were analysed in a total of seven runs with a between run CV for the low (n=14) and high (n=14) QCs of 10.6 and 14.1%, respectively. The preanalytical test samples were analysed in a single batch, except for the −20 °C condition, with a within run CV for the low (n=8) and high QCs (n=6) of 5.3 and 4.4%, respectively. The samples stored at −20 °C were analysed in four runs with a between run CV for the low (n=14) and high QCs (n=12) of 9.3 and 10.9%, respectively. There was one change of lot number during the study period, potentially affecting the −20 °C storage experiment at day 133.
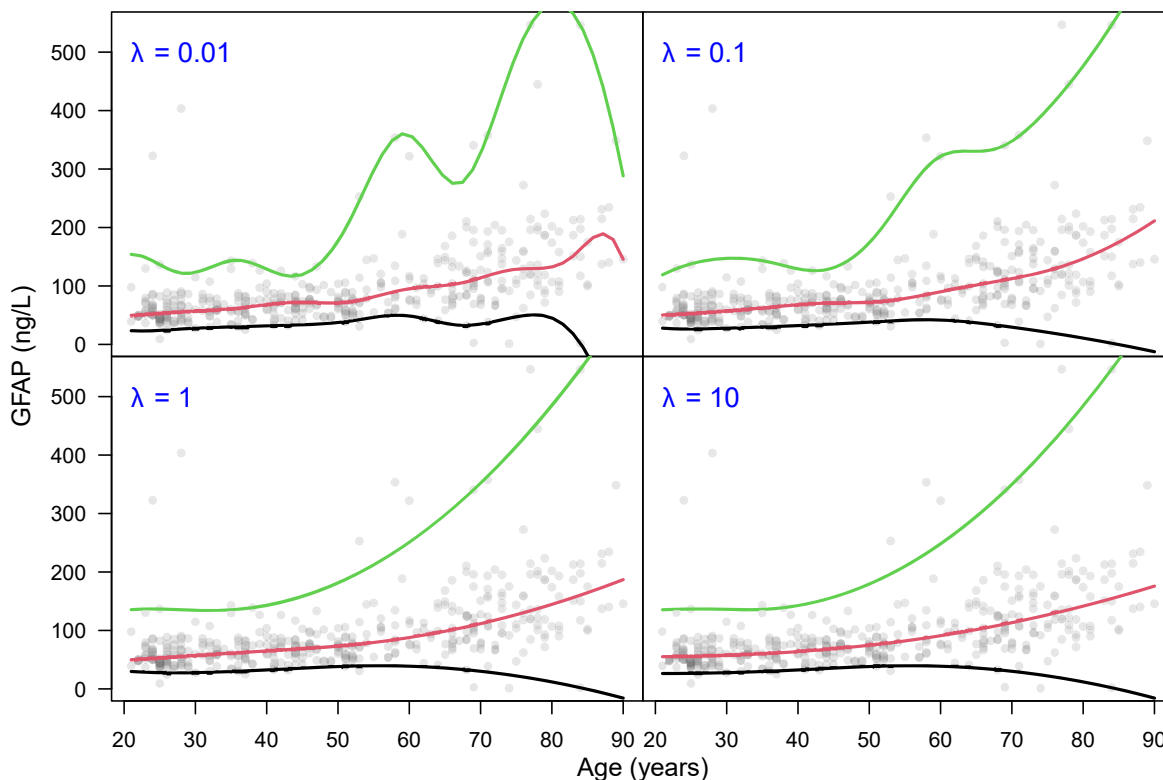
Figure 1: The effect of different smoothing values (λ) on the continuous reference interval curves.
The green, red, and black curves are the 97.5th, median, and 2.5th centiles, respectively.
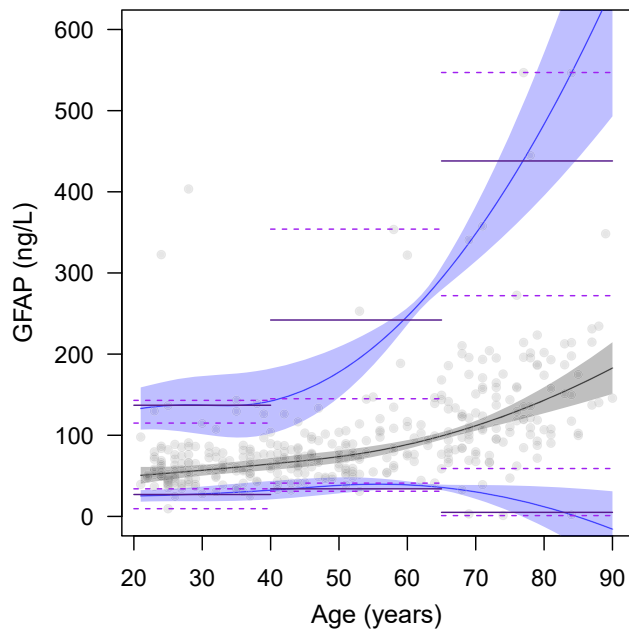
Figure 2: Continuous and age-partitioned reference intervals for serum GFAP.
Mean GFAP level (black line) and 95% reference limits (blue lines) from the continuous model with 95% confidence intervals calculated using the sandwich formula (shaded areas) are shown together with age-partitioned non-parametric reference intervals (solid purple lines) with 90% confidence intervals (dashed purple lines). The level of each individual is demonstrated by dots.

Reference interval study

A total of 371 reference subjects (165 males and 206 females) aged 21–90 years were included in the study. Of these, 254 were blood donors between 21 and 65 years of age and 117 were outpatients between 66 and 90 years of age. Figure 2 and Table 1 show the continuous reference intervals as well as the traditional age-partitioned non-parametric reference intervals.

In establishment of the age-partitioned non-parametric reference intervals, two measurements were identified as outliers by applying Dixons D/R on the predefined age groups. After removal of these, Lahti's recommendations applied on the age group 18–39 years vs. 40–64 years and 40–64 years vs. 65–90 years, respectively, led to partitioning of all three age groups. There was no statistically significant gender difference.

Preanalytical tests

Stability

Table 2 and Figure 3 show the results of the preanalytical stability studies. In all the tested conditions the individual samples remained within the allowable total error, except for one of the serum samples stored at RT which showed a 24.9% decrease after 1 day and a 19.2% increase after 7 days compared to the baseline value. The mean change from baseline remained within the maximum allowable bias of 10.2% for all tested conditions except for after 133 days at −20 °C and after 3 freeze-thaw cycles.

The mean change from baseline after storage of (1) whole blood at RT for 9 h before centrifugation was 2.3% (95% CI: −2.4%, 6.9%), (2) serum at RT for 7 days 3.1% (95%

Table 1: 5-year reference interval estimates from the continuous model and traditional age-partitioned reference intervals for serum GFAP.

Age, years	Reference interval quantile regression[a], ng/L			Reference interval age-partitioned, ng/L [90% CI]		
	n	2.5th	97.5th	n	2.5th	97.5th
20–24	26	26	135	123[b]	25 [9.5–34]	136 [115–143]
25–29	48	27	137			
30–34	22	29	137			
35–39	29	31	138			
40–44	34	34	148	124	34 [31–41]	242 [145–354]
45–49	23	37	166			
50–54	33	39	192			
55–59	17	40	226			
60–64	17	38	269			
65–69	35	33	320	122	5.3 [1.2–59]	438 [272–547]
70–74	29	27	379			
75–79	31	17	446			
80–84	17	6.0	522			
85–90	10	0	606			

[a]Based on point estimates determined at the mid-point of each respective age-bin. [b]Two outliers were removed for the age-partitioned reference interval.

Table 2: Stability of GFAP in whole blood and serum under different storage conditions.

Storage condition	Number of samples	Baseline median value, ng/L [range]	Results, PD%[a] [95% CI]	Instability equation
Whole blood at RT[b]	10	114 [52–299737]	2 h: 2.4 [−1.6, 6.4]; 5 h: −0.1 [−2.9, 2.7]; 7 h: 2.9 [−1.6, 7.5]; 9 h: 2.3 [−2.4, 6.9]	$y = 0.0028x$, $R^2 = 0.663$
Serum at RT[b]	10	102 [48–273928]	1 day: −4.3 [−12.2, 3.6]; 2 days: −0.6 [−5.7, 4.6]; 3 days: −1.4 [−7.1, 4.3]; 5 days: 1.4 [−4.3, 7.1]; 7 days: 3.1 [−4.5, 10.7]	$y = 0.0022x$, $R^2 = 0.126$
Serum at −20 °C	3 pools	155 [47–810]	42 days: −7.7 [−13.5, −2.0]; 57 days: −7.8 [−18.4, 2.7]; 133 days[c]: 10.4 [−6.0, 26.8]	$y = 0.0003x$, $R^2 = 0.044$
Freeze-thaw cycles	4 pools	140 [76–453]	1 cycle: 5.5 [−2.9, 13.8]; 2 cycles: 8.6 [−3.4, 20.5]; 3 cycles: 10.4 [9.5, 11.4]	$y = 0.0386x$, $R^2 = 0.978$

[a]PD%, percentage deviation compared to baseline value. [b]RT, room temperature. [c]Based on only two pools as analysis of the third failed.

CI: −4.5%, 10.7%), and (3) serum pools at −20 °C for 133 days 10.4% (95% CI: −6.0%, 26.8%). (4) Repeated freeze-thaw cycles led to a steady increase in GFAP up to a mean increase of 10.4% (95% CI: 9.5%, 11.4%) after 3 cycles.

Haemolysis

The GFAP concentration in the original six serum pools were between 53.4 and 215 ng/L. The mean concentrations of Hb in the serial dilutions were 11.6, 28.6, 53.2, 101.5, 200.0 and 386.9 mg/dL. There was no consistent trend in the effect of haemolysis except that most samples showed a slight positive change from baseline which did not increase with increasing Hb concentration. One sample showed a deviation from baseline of 27.5% at a Hb concentration of 103 mg/dL, but this measurement was rated as an outlier as the other samples in the serial dilution had a maximum deviation of 7.5%. The rest of the samples remained within the total allowable error. The mean change from baseline for the samples with highest Hb concentration (mean 386.9 mg/dL) was 4.3% (range −6.2%, 19.2%).

Discussion

In the present study we establish age-specific reference intervals for serum GFAP, both as continuous reference intervals and as traditional age-partitioned reference intervals, using the commercially available GFAP kit on the Simoa HD-1 platform. Moreover, we report on key pre-analytical properties of the biomarker.

One of the strengths of this study is the determination of a continuous reference interval using non-parametric quantile regression. The continuous interval shows good overall agreement with the traditional age-partitioned intervals but one of the main advantages is that it avoids large jumps between intervals of different age groups as often seen with age-partitioned intervals and also evident in our data. These jumps may result in misclassification of individuals close to age group partitions, a misclassification that is often difficult to realise for clinicians, especially as age partitions are not reported along with the lab results in many laboratory information systems. Other advantages of non-parametric continuous reference intervals include robustness to outliers and no requirement of any assumptions about the empirical distribution or arbitrary age partitions. A disadvantage of the method is less transparency in the way the reference ranges are determined compared to the traditional non-parametric approach and the fact that many laboratory information systems cannot yet handle continuous reference intervals. A solution to the latter could be calculating discrete e.g. 1-, 5-, or
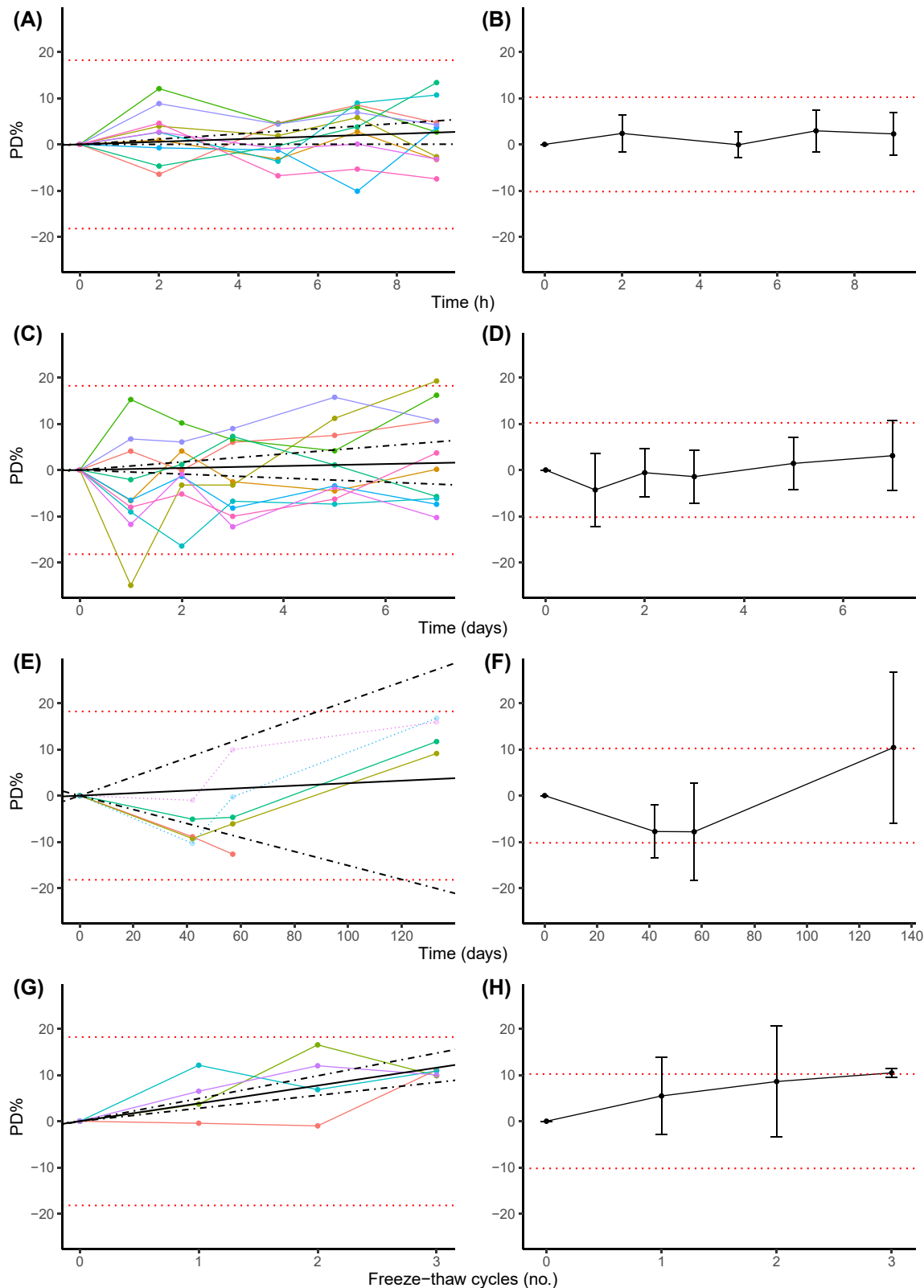
Figure 3: Percentage deviations (PD%) in GFAP compared to baseline level in different conditions.
(A and B) whole blood at RT, (C and D) serum at RT, (E and F) serum at −20 °C, and (G and H) repeated freeze-thaw cycles. (A), (C), (E) and (G) Individual sample data and instability equation calculation (black line) using the least squares method with confidence intervals for the slope (dashed lines). The change in the high and low QCs between runs is also displayed (dotted lines) for the −20 °C stability experiment. (B), (D), (F) and (H) Means with 95% confidence intervals. Red dotted lines represent the maximum permissible difference (MPD) for total error and bias based on biological variation.

10-year reference intervals based on point estimates determined at the mid-point of each respective age-bin as shown in Table 1.

To our knowledge, there are no previous reports on a reference interval for serum GFAP, but >20 studies so far have measured GFAP in groups of healthy control subjects using the Simoa technology: The reported median concentration among younger healthy controls <65 years in most studies is in the range of 55–71 ng/L [1, 17, 18, 37–44], whereas two larger studies looking at older healthy controls (n=61 with mean age 65.5 years and n=508 with mean age 82.2 years) found mean concentrations of 183.1 and 196 ng/L, respectively [12, 45]. This agrees well with the reference ranges established in the present study.

The preanalytical tests show excellent stability for all examined preanalytical variables, except for prolonged freezing (133 days at –20 °C) and three freeze-thaw cycles. In both cases the observed mean change form baseline was 10.4% and thus just outside the established MPD for bias of 10.2%. Inter-run variability may have affected the –20 °C stability experiment, though, as baseline and experimental conditions were measured in separate Simoa runs. In fact, the observed variation followed roughly the same pattern of increases/decreases as the level of our two QCs between runs (Figure 3E) and could thus have been caused by analytical imprecision across plates and change of lot number for the final measurement.

To our knowledge, only two previous studies have examined the effect of delayed storage and processing on serum GFAP. One study found GFAP levels to be stable in 4–5 °C refrigerated whole blood and serum samples for at least 72 h [24]. Another recent study reported good stability in whole blood for 24 h at RT and in serum for 24 h at RT and 2 weeks at 2–8 °C and –20 °C [21], in good agreement with another study by the same group on stability in EDTA plasma [22]. We have found no reports about prolonged storage at RT > 24 h or at –20 °C > 2 weeks. Regarding freeze-thaw stability, one study reported increased GFAP levels (13%) in EDTA plasma after four freeze-thaw cycles [22], whereas five others studies have reported stable levels in serum and/or plasma for three [23], four [21, 25] and five freeze/thaw cycles [14, 20], respectively. The accepted maximum deviation in the studies ranged from 10 to 20%, but most studies found mean deviations of <10%. Thus, although we found a mean change slightly higher than that, GFAP can probably be considered quite robust against freeze-thaw cycles. We are not aware of previous studies examining the effect of haemolysis on GFAP levels.

Our study has some limitations. GFAP was measured in serum only, but three studies (n=8 [25], n=10–12 [22], and n=20 [46]) using the Simoa technology have found comparable concentrations in paired serum and EDTA plasma samples, while one study (n=10) found slightly lower concentrations in EDTA plasma (87.1% [84.5–92.1]) [21]. Large differences between EDTA plasma and serum are therefore unlikely. Another limitation is that the included reference subjects comprise two distinct cohorts of younger blood donors ≤65 years and older outpatients >65 years, respectively. Danish blood donors must fulfil certain health requirements and may thus be healthier on average than the background population, whereas the older cohort consisting of outpatients may have a higher degree of morbidity than the background population. It cannot be ruled out that this combination has exaggerated the effect of age on GFAP. As there seems to be a slight increase in GFAP levels already from the age of 50 years, though, it most likely cannot account for the entire rise seen with age. Moreover, the observed age-dependent effect is similar to that seen in healthy controls in other studies [14, 17, 37, 38, 45, 47] and for other serum neurobiomarkers such as NfL [34].

The observed increase in GFAP levels with age is very important to consider in future studies looking at clinical cut-off values for different diseases. We also observed a larger variability with age which could have implications for the clinical use of the biomarker in diseases with relatively small differences between patients and healthy subjects such as psychiatric diseases [1] where it may be easier to establish cut-offs for younger compared to older individuals. A positive association between plasma GFAP and amyloid-β deposition on PET has been reported in several studies, which is present already well before clinical Alzheimer's symptoms develop [13, 47–49]. Since amyloid deposition increases with age [50], the observed higher GFAP level and variability with age in our study might be due to preclinical disease. The higher prevalence of preclinical disease in older compared to younger individuals is a general issue when establishing reference ranges.

In conclusion, the present study establishes age-dependent reference intervals for serum GFAP in adults, showing an age-dependent increase in levels, and demonstrates overall good stability of the biomarker.

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