Pre-analytical stability of serum biomarkers for neurological disease: neurofilament-light, glial fibrillary acidic protein and contactin-1

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Abstract

Objectives: Neurofilament-light (NfL), glial fibrillary acidic protein (GFAP) and contactin-1 (CNTN1) are blood-based biomarkers that could contribute to monitoring and prediction of disease and treatment outcomes in neurological diseases. Pre-analytical sample handling might affect results, which could be disease-dependent. We tested common handling variations in serum of volunteers as well as in a defined group of patients with multiple sclerosis (pwMS).

Methods: Sample sets from 5 pwMS and 5 volunteers at the outpatient clinic were collected per experiment. We investigated the effect of the following variables: collection tube type, delayed centrifugation, centrifugation temperature, delayed storage after centrifugation and freeze-thawing. NfL and GFAP were measured by Simoa and CNTN1 by Luminex. A median recovery of 90–110% was considered stable.

Results: For most pre-analytical variables, serum NfL and CNTN1 levels remained unaffected. In the total group, NfL levels increased (121%) after 6 h of delay at 2–8°C until centrifugation, while no significant changes were observed after 24 h delay at room temperature (RT). In pwMS specifically, CNTN1 levels increased from additional freeze-thaw cycles number 2 to 4 (111%–141%), whereas volunteer levels remained stable. GFAP showed good stability for all pre-analytical variables.

Conclusions: Overall, the serum biomarkers tested were relatively unaffected by variations in sample handling. For serum NfL, we recommend storage at RT before centrifugation at 2–8°C up to 6 h or at RT up to 24 h. For serum CNTN1, we advise a maximum of two freeze-thaw cycles. Our results confirm and expand on recently launched consensus standardized operating procedures.

Keywords: biomarkers; immunoassay; nervous system diseases; pre-analytical phase.

Introduction

Blood-based biomarkers are gaining increasing attention in the clinical and research field of neurological diseases, as they could contribute to accurate monitoring and prediction of disease and treatment outcomes. Neurofilament light (NfL), glial fibrillary acidic protein (GFAP) and contactin-1 (CNTN1) are biomarkers of interest in various neurological diseases, for example multiple sclerosis (MS) [1–4], dementia [5, 6], brain cancer [7] and chronic inflammatory demyelinating polyneuropathy [8]. These proteins have been shown to correlate with different mechanisms of neuro-axonal or axonal-glial dysfunction in CSF as well as in serum.

Pre-analytical variation might affect results of blood-based biomarkers and should be investigated to design standard operating procedures, an important step for reliable use of these biomarkers in both the clinical and research field of neurological diseases. Previous studies have reported that NfL remained stable after 3 freeze-thaw cycles in serum [9, 10] and plasma [9] and after delayed storage up to 7 days at both RT and in the refrigerator [11]. In previous work, we found that NfL and GFAP in EDTA plasma were unaffected by a comprehensive set of pre-analytical variables [12] and that serum CNTN1 could withstand up to 4 freeze-thaw cycles [4].
The effect of other common pre-analytical variables such as delayed centrifugation, centrifugation temperature and delayed storage up to 2 weeks for different temperatures on serum NfL, GFAP and CNTN1 levels however remains unknown. Furthermore, differences in pre-analytical effects might exist between diseased and non-diseased individuals. For example, if diseased individuals show lower biomarker levels compared to non-diseased individuals, the detection of variation between samples is more difficult and less reliable. Furthermore, in patients with MS (pwMS) specifically, proteins may be changed and thus have different stability in vitro due to chronic inflammatory processes or treatment with immunomodulatory compounds. The stability of NfL, GFAP and CNTN1 should therefore be confirmed specifically in the targeted patients to achieve swift and reliable clinical implication. In this study, we therefore aim to assess the effect of several prevalent sample handling variations on serum NfL, GFAP and CNTN1 in both pwMS and volunteers, to extend upon our recently launched consensus SOP for EDTA plasma [12].

Materials and methods

Subjects

Fresh blood was collected from a total of 70 subjects that visited the Clinical Chemistry Department, including a group of pwMS (n=35) and a group of volunteers (n=35). Inclusion criteria for the group of pwMS were: (1) age 18 years or older; (2) diagnosis of MS according to the 2017 McDonald criteria [13]. For the volunteer group, age of 18 years or older was the only inclusion criterion. Depending on the experiment (see Table 1), a volume of 12–40 mL blood was collected by standard venepuncture.

Sample handling

A total of 7 pre-analytical experiments were conducted, based on a previously described inventory of pre-analytical sample handling [12]. In each experiment, 5 pwMS and 5 volunteers were included. Tubes were inverted a few times for proper mixing immediately after tube filling. As reference condition, clot activator tubes (BD Vacutainer, ref 368815) were centrifuged for 10 min at 1,800 × g at room temperature (RT), after a standing time of 30 min at RT (except for experiment 3, see Table 1). Separated serum was aliquoted immediately in 250 µL portions in 0.5 mL polypropylene storage tubes (Sarstedt, Germany), and stored without any delay at −80°C until analysis. Sample handling steps were systematically varied for the experimental conditions. An overview of pre-analytical variables and experimental conditions is presented in Table 1.

NfL and GFAP analysis

Prior to the NfL and GFAP analyses, serum samples were shortly thawed, vortexed and centrifuged at 10,000×g for 10 min at room temperature. NfL and GFAP were simultaneously analyzed by a Single Molecule Array (Simoa) platform using the HDx analyzer (Quanterix, USA) according to manufacturer’s instructions (Neurology 2-Plex B multiplex assay, Quanterix, USA). All NfL and GFAP analyses were performed in duplicates. The measurement was repeated if the coefficient of variation (CV) was over 25%, which was found for 1 GFAP and 2 NfL samples. The median duplicate CV of the study samples (pwMS and volunteers) was 5.2% [interquartile range 2.4–8.1] for GFAP. For high, medium and low NfL QCs, the intra-assay CV’s (mean %CV within a run over 5 runs in total) were 6.7%, 3.9% and 11.2%, respectively, and the inter-assay CV’s (mean %CV of 5 runs in 3 days) were 8.2%, 6.5% and 9.3%, respectively. For high, medium and low GFAP controls, the intra-assay CV’s (mean %CV between duplicates within a run over 5 runs in total) were 6.4%, 6.5% and 18.8% and the inter-assay CV’s (mean %CV between runs over 5 runs in 3 days) were 3.6%, 3.6% and 16.6%, respectively.

<table>
<thead>
<tr>
<th>No.</th>
<th>Description</th>
<th>Conditions</th>
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<tr>
<td>2</td>
<td>Standing time at RT from collection to centrifugation</td>
<td>1. 30 min 2. 3 h 3. 6 h 4. 24 h</td>
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<tr>
<td>3</td>
<td>Standing time at 2−8°C from collection until centrifugation</td>
<td>1. 30 min 2. 3 h 3. 6 h 4. 24 h</td>
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<td>4</td>
<td>Centrifugation temperature</td>
<td>1. RT 2. 4°C</td>
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<tr>
<td>5</td>
<td>Standing time from centrifugation to freezer</td>
<td>1. Direct transfer 2. 4 h at RT 3. 4 h at 2−8°C 4. 24 h at RT 5. 24 h at −2−8°C</td>
</tr>
<tr>
<td>6</td>
<td>Short-term storage</td>
<td>1. −80°C directly 2. −20°C for 2 weeks, afterwards −80°C 3. 2−8°C for 2 weeks, afterwards −80°C</td>
</tr>
<tr>
<td>7</td>
<td>Freeze-thawing</td>
<td>1. 1 cycle 2. +1 cycle 3. +2 cycles 4. +4 cycles</td>
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Centrifugation settings were fixed at 1,800×g for 10 min at room temperature (RT). After centrifugation, serum was transferred in aliquots of 250 µl into 0.5 mL Sarstede polypropylene tubes, which were stored at −80°C until analysis. In experiment 3, temperatures during standing time and centrifugation 2−8°C and 4°C and, respectively.
Prior to CNTN1 analysis, in a second set of aliquots, serum samples were shortly thawed, vortexed and centrifuged at 10,000×g for 10 min at room temperature. The samples were handled according to the manufacturer’s instructions (Human contactin-1 Magnetic Luminex Assay, R&D systems, Minneapolis, USA) on a Bio-PlexTM 200 system (Bio-Rad Laboratories). All samples were analyzed in duplicate, for which a CV of <15% was accepted. The mean intra-plate CV (8 plates in total) for the high, medium and low QC samples was 1.5%, 2.2% and 2.9% and the intra-day CV (4 days in total) for high, medium and low QC samples was 4.8%, 10.3% and 8.6%, respectively. The inter-assay CV of the high, medium and low QC samples were 8.0%, 13.8% and 15.9%, respectively.

**Results of pre-analytical variable testing**

### Effect of pre-analytical variation on NfL levels

In the total sample set with pwMS and volunteers (Figure 1), levels of NfL measured in the serum gel separator, plasma EDTA, plasma EDTA gel separator and lithium-heparin samples were comparable (90.3–112.4%) to the serum reference samples clotted in clot activator tubes. Sodium-citrate samples showed substantially lower NfL level (63.3% [59.3–68.2%]).

Delay in centrifugation when tubes were kept at RT led to no relevant change in NfL levels after 3–24 h (103.2–103.5%), as compared to the reference condition where tubes were kept at RT for 30 min. Centrifugation delay when tubes were kept at 2–8°C resulted in no relevant change in NfL levels after 3–6 h (96.7–102.4%), but after 24 h, NfL levels were higher and showed increased variation among samples (120.9% [112.4–152.1]). Specifically, two samples per group showed a recovery above 110%: 122% and 144% in pwMS and 149% and 202% in volunteers (Supplementary Table 1C).

Compared to centrifugation at RT, centrifugation at 4°C did not result in a change in NfL level (97.3%).

Compared to immediate storage at −80°C after centrifugation, delayed storage when tubes were kept at RT did not show relevant changes in NfL levels after 4 and 24 h (94.1%–94.4%). Similar results were found for delayed storage when tubes were kept at 2–8°C up to 24 h (94.8%) or after storage for 2 weeks at 2–8°C or −20°C (98.2–110.7%).

NfL levels did not change significantly after up to 4 additional freeze-thaw cycles (96.0–100.0%).

When we investigated the pre-analytical effects on NfL levels in pwMS and volunteers separately, comparable stability was found for all experiments (data not shown).

### Effect of pre-analytical variation on GFAP levels

In the total sample set with pwMS and volunteers (Figure 2), the serum gel separator and lithium heparin samples showed comparable levels (100.6–105.2%) to the serum reference samples collected in clot activator tubes. Significantly lower levels were found in the plasma EDTA (87.1% [84.5–92.1]), plasma EDTA gel separator (86.5% [87.9–98.1]) and sodium-citrate samples (72.5% [66.4–75.7]).

Compared to 30 min standing time before centrifugation, no relevant change in GFAP levels was found after 3–24 h (101.6–101.9%). Standing time at 2–8°C resulted in comparable GFAP levels after 3–24 h (95–101.6%).

### Statistical analysis

Biomarker levels were compared between pwMS and volunteers using Mann-Whitney U test, for which a p-value of <0.05 was considered significant.

Biomarker levels were normalized against the reference condition, resulting in a recovery (%) for each condition. A median recovery of more than 10% above or below the reference condition was considered relevant (significant).

### Ethical considerations

The study protocol was approved by the Medical Ethics Committee (METc) of Amsterdam UMC (METc number 2019.257). All subjects gave written informed consent for the collection of their blood for the purpose of this study. The ethical principles as stated in the declaration of Helsinki were complied.

### Results

#### Biomarker values in pwMS compared to volunteers

In total, 280 serum samples were analyzed for NfL, GFAP and CNTN1. Median biomarker levels of the reference samples were compared between pwMS (n=35) and volunteers (n=35). Serum NfL levels were relatively higher in pwMS (median 6.9 pg/mL, interquartile range [IQR] 4.7–10.0) compared to volunteers (4.8 pg/mL [4.0–6.5], p=0.02). The same was found for serum GFAP levels (pwMS: 60.5 pg/mL [47.5–89.2]; volunteers: 52.1 pg/mL [45.6–62.1], p=0.03). Serum CNTN1 levels were relatively lower in pwMS (8,103 pg/mL [6,451–11,756]) compared to volunteers (10,671 pg/mL [8,611–13,539], p=0.03).
Centrifugation at 4 °C (102.1%) showed similar GFAP levels compared to centrifugation at RT.

Delayed storage at RT did not show any relevant changes in GFAP levels after 4–24 h (96.5–98.3%) compared to immediate storage at −80 °C after centrifugation. Similar results were found for delayed storage at 2–8 °C after 4–24 h (99.8–101.4%). GFAP levels did not change significantly after storage for 2 weeks at 2–8 °C (103.4%) and at −20 °C (103.8%).

GFAP levels remained stable after up to 4 additional freeze-thaw cycles (98.6–102.6%).

Figure 1: Overview of median recovery of NfL levels in the total group. Values measured in the experimental conditions were normalized against the values measured in the reference conditions (% recovery), which is serum from a clot activator tube, centrifuged at 1,800×g at room temperature (RT), after a standing time of 30 min at RT, immediately followed by aliquoting and −80 °C storage. RT=room temperature.
When pre-analytical effects in GFAP levels were investigated separately in pwMS and volunteers, comparable stability in both the pwMS and volunteer group for all experiments were found (Supplementary material, Tables 2A–F).

Effect of pre-analytical variation on CNTN1

In the total sample set of pwMS and volunteers, CNTN1 values in the serum reference samples clotted in clot activator tubes were similar to the serum gel separator, plasma
Figure 3: Overview of median recovery of CNTN1 levels.
Recovery results for the total group (first 7 graphs) and for pwMS and volunteers separately (bottom right graph). Values measured in the experimental conditions were normalized against the values measured in the reference conditions (% recovery), which is serum from a clot activator tube, centrifuged at 1,800×g at room temperature (RT), after a standing time of 30 min at RT, immediately followed by aliquoting and −80°C storage. RT=room temperature.
EDTA, plasma EDTA gel separator and lithium-heparin samples (93.6–100.6%) (Figure 3). Here, one heparin sample was excluded from data-analysis due to a CV of 19.3%, which did not result in significantly change in the results. Significantly lower CNTN1 levels were found in the sodium-citrate samples (78.9% [75.3–82.5]).

Standing time before centrifugation at RT showed no significant change in CNTN1 levels after 3–24 h (98.7–100.5%) compared to 30 min. Standing time at 2–8°C for 3–24 h resulted in comparable CNTN1 levels (96.9–102.0%). Centrifugation at 4°C did not show a significant change in CNTN1 level (99.7%) compared to RT.

Compared to immediate storage at −80°C after centrifugation, delayed storage at RT did not show any relevant changes in CNTN1 levels after 4–24 h (103.8–104.2%). Similar results were found for delayed storage at 2–8°C after 4–24 h (103–104.2%). CNTN1 did not change significantly after storage for 2 weeks at 2–8°C (94.7%) and at −20°C (100.4%).

CNTN1 levels remained stable after additional freeze-thaw cycle number 1 (94.2% [91.9–104.4]) and number 2 (101.2% [87.6–116.5]) but were slightly higher after number 4 (117.7% [97.4–141.4]).

Comparing recovery results between pwMS to volunteers, CNTN1 showed comparable stability in both the pwMS and volunteer group for all but the freeze-thawing experiment, in which pwMS showed slightly higher CNTN1 levels and increased variation among samples after additional freeze-thaw cycle number 2 (110.6% [92.2–117.5] to number 4 (140.9% [127.7–154.6]) (Figure 3).

**Recommendations for laboratory handling**

According to the obtained results of the systematic pre-analytical experiments, recommendations for laboratory handling of serum for NfL, GFAP and CNTN1 analysis are illustrated in the SOP (Figure 4), which is an extension of recently published SOP [12].

**Discussion**

In this study we assessed the effect of a comprehensive set of pre-analytical variables on serum NfL, GFAP and CNTN1 levels and compared results between pwMS and volunteers, aiming to further refine detailed recommendations for laboratory handling. The resulting SOP can be used to substantiate reliability of biomarker results, which is of great importance for example in (multicentre) medical trials, to monitor treatment response in pwMS and enable

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**Recommended SOP for serum NfL, GFAP and CNTN1**

**Sampling**

- Venipuncture for blood collection — Clot activator tube

**Processing**

- Centrifugation: 10 min, 1800 xg, RT or 4°C

- Tube filling: 250-1000μL in polypropylene storage tubes

**Storage**

- Long-term storage: −80°C

*Other sample types have different biomarker levels, thus different recommendations may apply.

**Figure 4:** Recommended serum handling standardized operating procedure (SOP). The SOP was constructed according to results obtained in systematic pre-analytical experiments. Deviating from this SOP might result in less reliable NfL, GFAP or CNTN1 measurements. RT=room temperature. *This SOP for serum NfL and GFAP analysis is an extension of our SOP for EDTA plasma analysis [12]. The effect of storage tube volume up to 1000 μL was previously investigated in EDTA plasma [12]. We do not expect a different effect on serum and therefore we here recommend the same volumes. †If this SOP is applied for measurement of NfL and GFAP only, up to 5 times freeze-thawing is possible.
blood sampling at local facilities closer to patients’ home before central processing.

Overall NfL, GFAP and CNTN1 levels showed good serum stability except for two variables. For NfL, we observed that delay prior to centrifugation between 6 and 24 h when tubes were kept at 2–8 °C resulted in increased variation among samples and significantly higher levels in both pwMS and volunteers, though not consistently in all samples. We did not see this effect on GFAP levels, which were analyzed from the same samples (thus exposed to exactly the same procedures), from the same subjects. In our SOP, we therefore chose to advise to store the samples at RT. However, given this good stability at RT, which is in line with previous studies [10, 11], our findings under fridge conditions are difficult to explain and should be interpreted with caution. It is also in contrast with a previous study that reported stable serum NfL levels at 4–8 °C up to 24 h in pwMS, Parkinson’s disease and healthy controls [10], as well as our previous EDTA study in a group of volunteers in which the same methods were applied [12]. We consider this to not be caused by our use of the NfL–GFAP duplex-kit compared to the NF-light kit in other experiments performed in this study. Since we also observed increased variation in NfL level among the samples exposed to this condition, we would not recommend to apply a correction factor. To our knowledge there are no previous reports on serum GFAP stability, although the current results in serum are comparable to our previous EDTA plasma study [12].

For CNTN1, pwMS but not volunteers showed increased variation among samples which were slightly higher after the second additional freeze-thaw cycle. This finding is novel compared to previously reported findings in another volunteer sample set, in which CNTN1 withstood freeze-thawing up to 4 cycles [4]. We hypothesize that the presence of pro-inflammatory components or second-line DMT levels in serum of pwMS could have amplified freeze-thawing instability of CNTN1 in pwMS. For reliable serum CNTN1 analysis in pwMS, we advise to apply a maximum of two freeze-thaw cycles and thus prepare small aliquots before freezing.

For all three markers, NfL, GFAP and CNTN1, the current study supports the novel recommendations for laboratory handling [12] and extends these to serum (i.e. centrifugation at 4 °C, storage delays at RT, short-term storage at 2–8 °C and −20 °C and up to 4 freeze-thaw cycles).

Strengths of this study include the performance of the same comprehensive set of pre-analytical experiments as in our previous EDTA plasma study, which was based on a systematic inventory of prevailing pre-analytical variations [12]. In addition, we have provided novel information compared to the previous studies that reported on serum stability by using a duplex assay for NfL and GFAP. Furthermore, the inclusion of samples from pwMS has contributed to our detailed recommendations for laboratory handling. Our study has some limitations as well. Choosing serum samples as a reference, we observed significantly different values in other sample types (i.e. heparin for NfL, EDTA, EDTA gel separator for GFAP and sodium-citrate for all markers NfL, GFAP and CNTN1), which limits the usefulness of our SOP if laboratories are restricted to the use of other sample types. We did not include additional timepoints between 6 and 24 h of delayed storage. Finally, intrinsic subject factors such as fasting time, use of co-medication and differences between specific DMT in pwMS were outside the scope of this study.

In conclusion, our results illustrate the value of pre-analytical variation testing for specific biomarkers, since effects differed per biomarker and upon disease. We propose an SOP to limit variation in biomarker levels across laboratories and take an important step towards implementation of novel blood-based biomarkers in the clinical and research field of neurological diseases.

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**Informed consent:** Informed consent was obtained from all individuals included in this study.

**Ethical approval:** Research involving human subjects complied with all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by the authors’ Institutional Review Board (Amsterdam UMC Medical Ethics Committee, approval number 2019.257).

**Data availability:** The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

**References**


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