Stability of progranulin under preanalytical conditions in serum and cerebrospinal fluid
Chapter 8

Abstract

Progranulin (PGRN) levels in blood and cerebrospinal fluid (CSF) are increasingly studied as potential markers for neurodegenerative disorders. We aimed to 1) characterize two commercially available PGRN ELISAs on several assay validation parameters, 2) assess the stability of PGRN in serum and CSF under pre-analytical conditions, and 3) compare stability in the two assays.

Intra-assay variation, inter-assay variation, inter-lot variation, linearity, lower limit of detection, and kit correlations were assessed for the Adipogen and R&D PGRN ELISA kits. Blood and serum samples were experimentally exposed to ≤9 freeze/thaw cycles, delayed processing for ≤24h at room temperature and 4°C, and to temperature stability tests for ≤3 weeks at –20°C, 4°C, room temperature, and 37°C. Both commercial PGRN ELISA kits showed acceptable ranges for intra- and inter-assay variation, with the R&D kit performing more accurate than the Adipogen kit, especially for inter-assay variation (intra-assay serum: 6.7 and 8.3%, respectively; inter-assay serum: 9.2 and 21.0%, intra-assay CSF: 3.6 and 12.0%, inter-assay CSF: 16.0 and 44.5%). Absolute serum PGRN concentrations were 1.9-fold higher in Adipogen than R&D (p<0.001) and strongly correlated between both kits (ρ =0.86, p<0.0001) and CSF PGRN levels were on the borderline of detection in both kits. PGRN was typically stable under all pre-analytical conditions addressed, although two weeks at 37°C resulted in decreased PGRN concentrations in CSF, only when using the Adipogen kit.

These results support further examination of PGRN as a potential marker in neurodegenerative diseases, since PGRN is stable in serum and CSF and can be measured using ELISA kits from several providers.
Introduction

Progranulin (PGRN) is a secreted 593-amino acid long glycoprotein. PGRN-gene mutations are causative of familial frontotemporal dementia (FTD) 1, a neurodegenerative disorder preferentially affecting the frontal and/or temporal lobes. PGRN has also been related to several other neurodegenerative diseases, such as Alzheimer’s disease (AD), multiple sclerosis, and other brain disorders such as bipolar disorder and central nervous system (CNS) HIV-infection 7,8. PGRN is abundantly expressed throughout the body, and, in the CNS in particular, it is mainly expressed in microglia and neurons where it functions in brain neuroinflammatory processes, neurite outgrowth, and synapse biology 19. PGRN-gene mutations probably have a complex interaction with other proteins such as TAR DNA binding protein-43 (TDP-43) and fused in sarcoma protein (FUS), for example illustrated by TDP-43 depositions that can be detected in FTD patients carrying a PGRN-gene mutation at autopsy 19. In another recent study, administration of PGRN was suggested as a therapy for AD, based on an inverse dose-dependent relation of PGRN with amyloid load mediated via a PGRN related loss of phagocytosis function of the microglial cells 10. Besides studies into PGRN’s pathological mechanism 11, blood and cerebrospinal fluid (CSF) levels of PGRN are extensively studied as markers of FTD, since low blood PGRN levels were found to predict mutations in the PGRN-gene with high sensitivity 12–16. Moreover, PGRN is studied as a blood marker for cancer 18 and metabolic disorder pathologies 22. All these advances have led to the availability of several commercial PGRN ELISA kits.

Levels of human PGRN vary widely among different studies in blood as well as in CSF 4,11,12. In fact, ranges of PGRN levels in group analyses often do not even overlap between different studies in clinically comparable groups. For example, serum PGRN concentrations in healthy individuals in one study are estimated at 49.3 ng/mL with a range of 35 to 70 ng/mL in 60 subjects 15, while in another study the median level is 226.5 ng/mL with a range of 101 to 387 ng/mL in 22 subjects 16, both using the same primary antibody from R&D but different secondary antibodies from R&D.

Hence, the PGRN enzyme-linked immunosorbent assay (ELISA) is not harmonized or standardized (bias between methods) and possible pre-analytical (e.g., stability issues) and analytical (precision) factors hamper international comparison of studies. This discrepancy between concentration ranges could be due to a) bias between methods due to a lack of standardization or harmonization, b) analytical performance (imprecision), and c) variation in preanalytical factors affecting the protein, a well-known issue in biomarker studies in body fluids comprising changes in patient-characteristics (e.g., age, dietary factors, physical exercise) as well as processing and storage of the sample, before the actual analysis has taken place 24. Sensitivity of PGRN to preanalytical conditions might explain the large variation in serum PGRN levels between studies, however, no studies have addressed the effect of differences in
preanalytical handling on PGRN levels in either blood or CSF. Since the kit robustness is another source of pre-analytical variation, it is essential that these kits are thoroughly validated. Therefore, we aimed to (1) test the analytical performance of two commonly used commercial ELISA kits by applying the newly developed BIOMARKAPD protocols for assay validation 25; (2) test the stability of PGRN under various preanalytical conditions, such as time delay of sample processing, temperature stability up to three weeks, and multiple freeze/thaw cycles in both serum and CSF; and (3) test whether this stability is kit dependent.

**Materials and methods**

**General processing and analysis procedures of human blood and CSF samples**

Centrifugation was performed at 1,800 g for 10 min at room temperature (RT) in all conditions. Aliquots were prepared in polypropylene (PP) tubes (Sarstedt, Numbrecht, Germany) and stored at −80°C until analysis.

Blood was donated by healthy volunteers and collected and processed according to the standard procedure described above after clotting at RT, and was used for the assay validations and the stability tests.

CSF surplus pools were prepared from leftovers from diagnostic routine stored at −20°C. All samples were collected and used conform the ‘Research Code for Proper Secondary Use of Human Material’ of the VU University Medical Center (VUmc) Amsterdam.

Serum samples of 17 FTD patients from the Amsterdam Dementia Cohort 26 were used for method comparison of PGRN concentrations. 2–6 ml clotted blood was processed and stored according to international consensus standard operation procedures 27.

**Commercial human PGRN ELISAs**

2 commonly used commercial human PGRN ELISA kits, from Adipogen (Inc., Seoul, Korea) and R&D Systems (Inc., Minneapolis, MN, USA), were used according to manufacturers’ instructions. Samples were run in duplicate and only samples with a coefficient of variation (CV) below 15% were used in the analyses, except for the assay validation.

**Analytical performance PGRN ELISAs**

**Method comparison PGRN concentrations**

To compare absolute concentrations of PGRN, we measured 17 FTD patient serum samples in both the Adipogen and R&D assays. For CSF, we used the aliquots of pooled CSF that were exposed to pre-analytical conditions during the stability tests explained below.
Stability of progranulin in serum and CSF

**Intra-assay variation**
The intra-assay CVs for CSF and serum were calculated as mean % CV of 4–6 replicates of 1-2 different samples within 1 plate. Acceptance criteria were <15%.

**Inter-assay and inter-lot variation**
For inter-assay and inter-lot variability, 2-3 samples were analyzed in 3–6 plates over 1–3 different lots per manufacturer. Acceptance criteria were <15%.

**Linearity**
The linearity was calculated as the recovery of 2 serially diluted serum or CSF samples, up to 8 times dilution. Acceptance criteria were 85–115%.

**Lower limit of detection (LLOD)**
The lower limit of detection (LLOD) was calculated using the mean of 12 blanks plus 10 times the mean SD of the blanks and calculated according to the calibration curve. The LLOD is indicated in all graphs.

**Stability tests**

**Delayed processing**
To test the effect of delayed processing on PGRN concentrations, 3 serum samples were aliquoted in 2.0 mL PP tubes after collection and before centrifugation, and kept at either RT or at 4°C for 0.5 (reference), 1, 2, 4, or 24 h before centrifugation and storage of the supernatant at −80°C.

For delayed processing in CSF, we were informed directly after CSF collection in our memory clinic (VU Medical Center, Amsterdam) and aliquoted the CSF from the collection tubes (10 mL, Sarstedt, cat. nr. 62.610.018) in fractions of 600 µl within 30 min after lumbar puncture. These aliquots were left for 0 (reference), 1, 2, or 24 h at 4°C or RT, then centrifuged, aliquoted and stored as described above.

**Temperature and time stability**
To test the stability of PGRN concentrations in serum and CSF at different temperatures over different time spans, after processing, aliquots were kept at either −20°C, 4°C, RT, 37°C for 0 (reference), 7, 14, or 21 days before final storage at −80°C.
Freeze/thaw stability PGRN
For testing effects of repeated freezing/thawing, aliquots underwent 1, 3, 5, or 9 freeze/thaw (f/t) cycles. One f/t cycle comprises thawing for 2 h at RT followed by freezing it again at –80°C at least for one night.

Effect of fasting on serum PGRN levels
Blood was collected from 5 healthy volunteers just before lunch (fasted) and 2 h after lunch (non-fasted) and processed as described in the general procedures. Serum PGRN concentrations were measured in the Adipogen kit. To test the effect of fasting on serum PGRN levels, a paired-samples t-test was performed. Data are presented as mean with standard deviation.

Statistics
Statistical analyses were performed using IBM SPSS Statistics 20 (Chicago, IL, USA). When assumptions for normal distribution were not met, non-parametric statistics were applied. Data of pooled serum and CSF used in the stability experiments were presented as medians with ranges, minimum to maximum values. Assay characteristics and fasted and non-fasted serum PGRN levels were normally distributed and expressed as means with standard deviations.

For comparing PGRN levels between the Adipogen and R&D kits in CSF and serum, Passing-Bablok regression analyses were performed in Excel’s Analyse-it. In addition, related-samples Wilcoxon signed rank tests and Spearman correlations were performed.

For the parameters delayed processing and storage stability, linear regression analyses were performed with PGRN concentration as dependent variable and time and temperature as independent variables.

Freeze/thaw stability was statistically tested with repeated measures ANOVA for absolute as well as normalized values.

A p-value below 0.05 was considered significant.

Results
Analytical performance
Method comparison Adipogen and R&D assays
Absolute levels of serum PGRN were higher in the Adipogen kit compared to the R&D kit (related samples Wilcoxon signed rank test: T=–3.6, p<0.001) with a fold-change of 1.9 ± 0.2 (Figure 1).
Passing-Bablok’s regression line was fit at y=5.423 + 0.4749x, with no systematic differences between the methods (95% CI intercept: –21.0; 13.4) but a proportional difference between the methods was found (95% CI slope: 0.38; 0.72). Spearman’s correlation coefficient was 0.86 (95% CI: 0.63–0.95), p<0.0001 (N=17). Remarkably, in parallel CSF samples, we saw no significant

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Stability of progranulin in serum and CSF

change in absolute R&D and Adipogen PGRN levels, the correlation between both levels was moderate with $\rho = 0.51$ (Spearman’s rho, 95% CI: 0.21 – 0.72, $p<0.01$). Passing-Bablok’s regression line was fit at $y = 1.347 + 0.6222x$, with no systematic differences between the methods (95% CI intercept: –0.43; 2.63), neither a proportional difference between the methods was found (95% CI slope: 0.28; 1.10).

**Intra-assay variation**
Both assays show a good repeatability, the R&D assay performing slightly better (CVs: 6.7% for serum and 3.6% ± 0.3 for CSF) than the Adipogen assay (CVs: 8.3% for serum and 12.0% ± 13.6 for CSF) (Table 1).

**Table 1. Characteristics of the Adipogen and R&D Systems ELISA kits for CSF and serum PGRN analysis.**

<table>
<thead>
<tr>
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<th>Intra-assay variation (%)</th>
<th>Inter-assay variation (%)</th>
<th>Inter-lot variation (%)</th>
<th>Linearity (%)</th>
<th>LLLOD (ng/ml)</th>
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<td><strong>CSF</strong></td>
<td></td>
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<td>Adipogen</td>
<td>12.0 ± 13.6</td>
<td>44.5 ± 3.8</td>
<td>n.d.</td>
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<td>16.0 ± 7.0</td>
<td>31.8*</td>
<td>n.d.</td>
<td>3.1</td>
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<td>Adipogen</td>
<td>8.3*</td>
<td>21.0 ± 11.3</td>
<td>26.2*</td>
<td>97.1 ± 11.0</td>
<td>89.8</td>
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<tr>
<td>R&amp;D Systems</td>
<td>6.7*</td>
<td>9.2 ± 3.6</td>
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Figure 1. Correlation of serum (left) and CSF (right) samples measured with the Adipogen and R&D kit. For serum, 17 samples were compared, Spearman’s $r$ was 0.86 (95% CI: 0.63–0.95), $p<0.0001$ and the slope of the Passing-Bablok regression line was 0.47 (95% CI: 0.38; 0.72) indicating proportional differences. For CSF, 36 samples were compared, Spearman’s rho was 0.51 (95% CI: 0.21–0.72), $p<0.01$ and the slope of the Passing-Bablok regression line was 0.62 (95% CI: 0.28; 1.1) indicating no proportional differences.
Linearity

Linearity in serum fell within the acceptance criteria for both assays: 92.0% ± 4.0 in R&D and 97.1% ± 11.0 in Adipogen. We were unable to detect PGRN in diluted CSF samples and thus could not assess linearity in CSF (table 1).

Lower limit of detection (LLOD)

For the R&D assay the LLOD was 12.4 ng/ml for serum and 3.1 ng/ml for CSF, for the Adipogen assay LLOD values were 89.8 ng/ml for serum and 1.8 ng/ml for CSF (table 1).

Figure 2. Stability of PGRN concentrations in serum (A+B) and CSF (C+D) during delayed processing at different temperatures up to 24 hours measured with Adipogen (A+C) and R&D (B+D). Box plots indicate medians with range (n = 3).
Stability of progranulin in serum and CSF

Stability tests

Delayed processing

PGRN concentrations remained stable if either blood or CSF samples were retained for up to 24 h did however surpass the LLOD indicated by the firm itself (LLOD range: 0.05 – 0.54 ng/mL, mean LLOD: 0.17 ng/mL).

Temperature and time stability

Despite some variation, PGRN levels in serum remained stable after 3 weeks of storage at −20°C, 4°C, RT, and 37°C (Figure 3A, B) measured with Adipogen (Figure 3A) or R&D (Figure 3B). The CSF PGRN levels remained stable at all temperatures up to 3 weeks (Figure 3C, D), except for the sample kept at 37°C after 2 weeks, however, this decrease was detected only using the Adipogen assay (Figure 3C). Linear regression analysis with CSF PGRN (ng/ml) as dependent variable in Adipogen indicated temperature as a significant predictor (unstandardized beta = −0.191, p = 0.003). Serum PGRN levels in R&D were also significantly influenced by temperature (unstandardized beta = 1.664, p = 0.01), but not by time, in linear regression analysis.

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Freeze/thaw stability PGRN

In serum as well as CSF, PGRN levels were stable upon multiple freeze/thaw cycles, measured with both Adipogen en R&D assays (Figure 4).

Effect of fasting on serum PGRN levels

As presented in Figure 5, we found no effect of fasting on the serum PGRN levels in a small group of 5 healthy volunteers (150.2 ± 49.4 ng/mL in fasted state and 140.8 ± 43.0 ng/mL in non-fasted state, n.s.) measured with the Adipogen ELISA.
Discussion
In this paper, we show that PGRN is a relatively stable protein in serum and CSF under different pre-analytical conditions. Both Adipogen and R&D kits met most assay validation criteria and correlated well. However, R&D performed slightly more accurate on intra- and inter-assay variation. There was a strong and significant concentration difference between both assays in serum, as demonstrated by 1.9-fold higher serum PGRN levels in Adipogen measures. PGRN measurements in CSF showed higher variation in the majority of parameters tested in this study than in serum. We found higher variation coefficients for serum compared to both Adipogen and R&D performance reports (R&D intra-assay CV: 4.9%, R&D inter-assay CV: 7.9%, Adipogen
intra-assay CV: 5.1%; Adipogen inter-assay CV: 6.4%) and especially for Adipogen we considered these less accurate than promised. Validation parameters for CSF have not been published before; both kits were able to detect CSF PGRN but at the lower detection range of the kit. This issue impeded the assessment of the linearity of PGRN in CSF in both kits. Since we were not satisfied with the LODs for CSF in both kits, we tried a third manufacturer, Cayman Chemical (Ann Arbor, MI, USA), but the LOD was similar to the current kits (LOD = 2.12 ng/mL, intra-assay CV % in CSF = 7.7%) so we did not proceed with this kit. Since CSF levels were detected on the borderline of detection in both kits, the CSF results should be interpreted with caution.

Next, we addressed pre-analytical factors that could occur during laboratory processing. We observed stability of PGRN concentrations exposed to delayed processing, delayed storage up to three weeks at different temperatures, and multiple freeze/thaw cycles, in serum as well as in CSF. This does not exclude other pre-analytical factors from influencing the differences in PGRN concentrations reported in previous studies. These pre-analytical factors could relate to patient factors, such as diet or diurnal rhythm, or assay performance, such as differences in buffer compositions or machine settings. Illustratively, the selection of patient groups could account for the discrepancies between studies, as several patient-related factors, such as hypertension, were recently found to influence blood PGRN levels. Food intake is another patient-related pre-analytical factor possibly influencing PGRN concentrations, since PGRN has a role in the insulin mechanism and in metabolic diseases. In contrast with this idea, a recent study proved that serum PGRN levels remained constant over fasting, as well as during the oral glucose tolerance test and oral lipid tolerance test. In a small subset of healthy volunteers we confirmed that there is no effect of fasting on serum PGRN levels (Figure 5). The effects of age and gender on blood and CSF PGRN levels have been recently studied, results are slightly discrepant showing no or minor effects. Blood and CSF PGRN levels were correlating with each other in these studies to a minor extent. Inter-individual differences in serum PGRN concentrations are strongly determined by the rs5848 polymorphism related to FTD risk, which could have influenced group estimates of PGRN concentrations in studies that did not check for this genetic predisposition. Since we used pools for our stability experiments, our results should not be influenced by these differences, but the effects of age, gender, or genetics might explain some of the variation found between other cohorts measured with the same commercial assay.
Stability of progranulin in serum and CSF

Figure 5. No effect of fasting on serum PGRN concentrations measured with the Adipogen kit. Data are presented as means with standard deviations (N = 5).

Although no differences in stability of PGRN between the kits appeared, the absolute levels of PGRN were biased by kit, shown in the Passing-Bablok regression analyses. For serum, the PGRN levels yielded 1.9-fold higher levels using the Adipogen compared to R&D kit, in contrast to comparable absolute values for CSF. These data suggest an effect of the matrix, changing the affinity of the PGRN antibody to the protein. This might be caused by conformational differences of the protein caused by the matrix, or the presence of components in either serum or CSF that interact with PGRN, thereby altering its binding capacity. The sensitivity to this matrix effect might differ for the antibodies used in both kits, explaining why we see an absolute difference between kits in serum but not CSF. Although our conversion factor in serum is more modest than the one recently described by Almeida and colleagues, which was 2.9, we confirm higher serum PGRN levels measured in Adipogen compared to R&D. To gain insight into the nature of this difference, we measured the levels of the highest calibrator (12 ng/ml) of both kits in the Cayman Chemical kit. The resulting concentration was 1.14-fold higher in Adipogen compared to R&D, suggesting that this fold-change between Adipogen and R&D is indeed partly due to the composition of the protein used as calibrator, which is described as human-derived in the Adipogen kit and recombinant in the R&D kit. Another cause could be difference in affinity of native human PGRN with the antibodies in the kits.

Remarkably, CSF PGRN levels measured by the Adipogen kit dropped after more than two weeks at 37°C, while this was not seen in the same samples measured by the R&D kit. PGRN in serum measured with R&D showed a marginal, however significant, increase in concentration when kept at 37°C which was not detected in Adipogen. This could similarly be explained by detection of different epitopes of PGRN by the kits. A possible explanation may be the fact that Adipogen uses polyclonal antibodies and R&D systems a monoclonal antibody. Part of
the epitopes recognized by the mixture of polyclonal antibodies could be lost after two weeks at 37°C, resulting in the observed reduced PGRN levels, while the epitope targeted by the monoclonal antibody of R&D systems remained intact. Unfortunately, the exact epitopes of the antibodies are not disclosed.

In summary, we first demonstrated that the R&D and Adipogen PGRN kits both showed acceptable assay validation characteristics (according to Andreasson et al. 25), that the R&D kit was slightly more robust than Adipogen, and that both assays just barely detected PGRN levels in CSF. Importantly, for all PGRN measurements and particularly those in clinical samples, awareness of the intra- and inter-assay variation percentages of these kits is required and using different lots (leading to > 20% variability) should be avoided. Second, we proved that variation in pre-analytical conditions cannot explain the large differences in serum PGRN concentration ranges that were found in earlier studies testing clinically similar cohorts with the same assays 16,23, since PGRN was stable under all pre-analytical circumstances in CSF as well as in serum. Moderate degradation of CSF PGRN after two weeks at 37°C in the Adipogen kit, and marginal increase of serum PGRN at 37°C in R&D, were detected, probably due to modifications of an epitope specific for that kit only. Our paper secures stability of PGRN, and although some variability issues still need to be resolved, this leads towards facilitation of collaborative multicenter studies, including samples from historical biobanks, on PGRN as potential biomarker in neurodegenerative diseases.

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References


