Influence of preanalytical and analytical factors on the quantification of six regulatory serum proteins

Manuscript Number:

Article Type: Research Article

Full Title: Influence of preanalytical and analytical factors on the quantification of six regulatory serum proteins

Short Title: Factors influencing the quantification of proteins in serum

Corresponding Author: Felix Menne, PhD
Predemtec AG
Berlin, GERMANY

Keywords: blood serum; BDNF; IGF-1; VEGF-A; TGF-β1; MCP1; IL-18; blood tube; ELISA; diagnostics; Alzheimer's biomarkers; method comparison; immunoassay

Abstract: Objectives Differing preanalytical and analytical procedures regarding liquid biopsy samples may result in varying test results. This study analyzes differences in six protein concentrations quantified from human blood serum depending on the tube material, and on the immunoassay platform used. Methods First, blood samples of 315 individuals were collected in glass and polypropylene tubes. Second, serum concentrations of six proteins (i.e., BDNF, IGF-1, VEGF-A, TGF-β1, MCP-1, and IL-18) were assessed by using classical enzyme-linked immunosorbent assays (ELISAs) and a novel fully automated immunoassay platform (Ella), respectively. Bland-Altman analyses were conducted to investigate intra-sample variability of protein concentrations depending on sampling tube and immunoassay platform. Results Regarding the between-tube comparison, there was little influence of the tube type on concentrations for two proteins, i.e., mean biases -0.45% (IGF-1), -0.69% (IL-18). The other four proteins (VEGF-A, MCP-1, TGF-β1, and BDNF) exhibited higher mean biases, ranging between -32.17% and -70.64%. Regarding the between-platform comparison, for two of the proteins we found less pronounced influences of the assay type on concentrations, i.e., mean biases of -7.68% for VEGF-A and 11.74% for TGF-β1. Values for the other proteins deviated more strongly with biases between 21.04% and -128.10%. Conclusion Protein concentrations can vary significantly depending on the types of tube and immunoassay used with protein-specific differences. Our results support the need for standardization and harmonization of preanalytical and analytical laboratory measuring conditions. Method-specific reference intervals and clinical decision points should be considered.

Order of Authors:

Felix Menne, PhD
Nicolas A Henzen, MSc
Anja Weber, BSc
Marc Sollberger, MD
Andreas U Monsch, PhD
Carola G Schipke, PhD

Opposed Reviewers: Michael Heneka
Deutsches Zentrum für Neurodegenerative Erkrankungen eV: Deutsches Zentrum für Neurodegenerative Erkrankungen eV
michael.heneka@dzne.de
**Potential bias due to prior collaboration**

### Additional Information:

<table>
<thead>
<tr>
<th>Question</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Financial Disclosure</strong></td>
<td>Enter a financial disclosure statement that describes the sources of funding for the work included in this submission. Review the submission guidelines for detailed requirements. View published research articles from <em>PLOS ONE</em> for specific examples. This statement is required for submission and will appear in the published article if the submission is accepted. Please make sure it is accurate.</td>
</tr>
</tbody>
</table>

#### Unfunded studies

Enter: *The author(s) received no specific funding for this work.*

#### Funded studies

Enter a statement with the following details:

- Initials of the authors who received each award
- Grant numbers awarded to each author
- The full name of each funder
- URL of each funder website
- Did the sponsors or funders play any role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript?

- **NO** - Include this sentence at the end of your statement: The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
- **YES** - Specify the role(s) played.

*I typeset*

### Competing Interests

Use the instructions below to enter a competing interest statement for this submission. On behalf of all authors, disclose any competing interests that could be perceived to bias this work—acknowledging all financial support and any other relevant financial or non-financial competing interests.

I have read the journal's policy and the authors of this manuscript have the following competing interests: FM and CGS are employees of Predemtec AG.
This statement is required for submission and will appear in the published article if the submission is accepted. Please make sure it is accurate and that any funding sources listed in your Funding Information later in the submission form are also declared in your Financial Disclosure statement.

View published research articles from PLOS ONE for specific examples.

**NO authors have competing interests**

Enter: The authors have declared that no competing interests exist.

**Authors with competing interests**

Enter competing interest details beginning with this statement:

I have read the journal's policy and the authors of this manuscript have the following competing interests: [insert competing interests here]

* typeset

**Ethics Statement**

Enter an ethics statement for this submission. This statement is required if the study involved:

- Human participants
- Human specimens or tissue
- Vertebrate animals or cephalopods
- Vertebrate embryos or tissues
- Field research

Write "N/A" if the submission does not require an ethics statement.

General guidance is provided below. Consult the submission guidelines for detailed instructions. Make sure that all 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 in writing by the authors' Institutional Review Board (Ethikkommission Nordwest- und Zentralschweiz, EKNZ; No. 2019-00386).
information entered here is included in the Methods section of the manuscript.

Format for specific study types

Human Subject Research (involving human participants and/or tissue)
• Give the name of the institutional review board or ethics committee that approved the study
• Include the approval number and/or a statement indicating approval of this research
• Indicate the form of consent obtained (written/oral) or the reason that consent was not obtained (e.g. the data were analyzed anonymously)

Animal Research (involving vertebrate animals, embryos or tissues)
• Provide the name of the Institutional Animal Care and Use Committee (IACUC) or other relevant ethics board that reviewed the study protocol, and indicate whether they approved this research or granted a formal waiver of ethical approval
• Include an approval number if one was obtained
• If the study involved non-human primates, add additional details about animal welfare and steps taken to ameliorate suffering
• If anesthesia, euthanasia, or any kind of animal sacrifice is part of the study, include briefly which substances and/or methods were applied

Field Research

Include the following details if this study involves the collection of plant, animal, or other materials from a natural setting:
• Field permit number
• Name of the institution or relevant body that granted permission

Data Availability

Authors are required to make all data underlying the findings described fully available, without restriction, and from the time of publication. PLOS allows rare exceptions to address legal and ethical

Yes - all data are fully available without restriction
concerns. See the [PLOS Data Policy](https://plos.org/practices/data-policy) and [FAQ](https://plos.org/practices/data-policy/faq) for detailed information.

A Data Availability Statement describing where the data can be found is required at submission. Your answers to this question constitute the Data Availability Statement and **will be published in the article**, if accepted.

**Important:** Stating ‘data available on request from the author’ is not sufficient. If your data are only available upon request, select ‘No’ for the first question and explain your exceptional situation in the text box.

Do the authors confirm that all data underlying the findings described in their manuscript are fully available without restriction?

Describe where the data may be found in full sentences. If you are copying our sample text, replace any instances of XXX with the appropriate details.

- If the data are **held or will be held in a public repository**, include URLs, accession numbers or DOIs. If this information will only be available after acceptance, indicate this by ticking the box below. For example: *All XXX files are available from the XXX database (accession number(s) XXX, XXX).*
- If the data are all contained **within the manuscript and/or Supporting Information files**, enter the following: *All relevant data are within the manuscript and its Supporting Information files.*
- If neither of these applies but you are able to provide **details of access elsewhere**, with or without limitations, please do so. For example:

  Data cannot be shared publicly because of [XXX]. Data are available from the XXX Institutional Data Access / Ethics Committee (contact via XXX) for researchers who meet the criteria for access to confidential data.

| Data are fully accessible under [https://doi.org/10.7910/DVN/9RZWAG](https://doi.org/10.7910/DVN/9RZWAG) |
The data underlying the results presented in the study are available from (include the name of the third party and contact information or URL).

- This text is appropriate if the data are owned by a third party and authors do not have permission to share the data.

* typeset

Additional data availability information:
Dear Dr. Chenette,

please find enclosed our original research article titled “Influence of preanalytical and analytical factors on the quantification of six regulatory serum proteins”, which we would like to submit for publication to PLOS ONE.

A physician’s diagnosis often relies on biomarkers analyzed from specimens such as blood or cerebrospinal fluid. However, there is evidence how differing preanalytical and analytical procedures regarding liquid biopsy samples may result in varying test results. In this study, we investigated the influence of different blood tube materials and measuring platforms on concentrations of brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor A (VEGF-A), transforming growth factor-beta type 1 (TGF-β1), monocyte chemoattractant protein 1 (MCP-1), and interleukin-18 (IL-18) concentrations in human blood serum. We found that the aforementioned factors can influence the quantitative results of different regulatory proteins in human serum samples, which might be used as biomarkers.

With this work, we strengthen the need to consider preanalytical and analytical factors when comparing and interpreting absolute biomarker values. We feel that our findings contribute significant findings and support the fact that there is an overall need for standardization and harmonization of laboratory measuring conditions.

This manuscript has not been submitted to PLOS ONE before. As potential academic editors, we suggest Pal Bela Szecsi or Jialin Charles Zheng. We would like to exclude Prof. M. Heneka as potential reviewer.

Thank you very much in advance for your consideration, we are looking forward to receiving your comments.

Sincerely yours,

Felix Menne
Influence of preanalytical and analytical factors on the quantification of six regulatory serum proteins

Short title: Factors influencing the quantification of proteins in serum

Felix Menne1*, Nicolas A. Henzen2, Anja Weber2, Marc Sollberger2, Andreas U. Monsch2, and Carola G. Schipke1

1Predemtec AG, Berlin, Germany
2Memory Clinic, University Department of Geriatric Medicine FELIX PLATTER, Basel, Switzerland

*Corresponding author:
E-Mail: menne@predemtecdx.com (FM)
Abstract

Objectives

Differing preanalytical and analytical procedures regarding liquid biopsy samples may result in varying test results. This study analyzes differences in six protein concentrations quantified from human blood serum depending on the tube material, and on the immunoassay platform used.

Methods

First, blood samples of 315 individuals were collected in glass and polypropylene tubes. Second, serum concentrations of six proteins (i.e., BDNF, IGF-1, VEGF-A, TGF-β1, MCP-1, and IL-18) were assessed by using classical enzyme-linked immunosorbent assays (ELISAs) and a novel fully automated immunoassay platform (Ella), respectively. Bland-Altman analyses were conducted to investigate intra-sample variability of protein concentrations depending on sampling tube and immunoassay platform.

Results

Regarding the between-tube comparison, there was little influence of the tube type on concentrations for two proteins, i.e., mean biases -0.45% (IGF-1), -0.69% (IL-18). The other four proteins (VEGF-A, MCP-1, TGF-β1, and BDNF) exhibited higher mean biases, ranging between -32.17% and -70.64%.

Regarding the between-platform comparison, for two of the proteins we found less pronounced influences of the assay type on concentrations, i.e., mean biases of -7.68% for VEGF-A and 11.74% for TGF-β1. Values for the other proteins deviated more strongly with biases between 21.04% and -128.10%.
Conclusion

Protein concentrations can vary significantly depending on the types of tube and immunoassay used with protein-specific differences. Our results support the need for standardization and harmonization of preanalytical and analytical laboratory measuring conditions. Method-specific reference intervals and clinical decision points should be considered.
Introduction

Sixty to seventy percent of physicians’ medical decisions are based on laboratory test results, while the clinical usefulness and need of such biomarkers vary among the different medical fields [1]. As a measurable indicator of a biological state or condition, biomarker values analyzed in blood or blood fractions are the most common and most convenient types of biomarkers. However, there are several technical issues in blood analyses. Different preanalytical and analytical procedures including clotting (or non-clotting when desired) of samples, storage period and conditions, biomarker assays and platforms, and types of tubes used can result in different test results (for a review see Revuelta-López et al., 2021 [2]). For instance, in the diagnostic process of Alzheimer’s disease (AD) where the use of biomarkers is becoming increasingly important [3,4], it was shown that a lack of standardization in the sampling and processing of cerebrospinal fluid (CSF) [5] and serum [6] biomarkers can lead to varying results. An especially pronounced preanalytical effect is known concerning the tube type used for the collection of cerebrospinal fluid to quantify amyloid-beta peptides [7]. Overall, it is estimated, that up to 75% of total laboratory deviations originate in the preanalytical phase [8–10], of which up to 26% may have a negative impact on appropriate patient care [11].

There is evidence that plain tubes from the same material, but from different manufacturers, result in different concentrations of the same biomarker [12]. The same is true for tubes with and without additives, with variation in biomarker values between tubes of up to 215% [13]. Studies comparing plain tubes with tubes containing clotting activators showed differences in serum protein compositions with up to 2.66-fold difference [14]. These and further findings [15,16] indicate that preanalytical factors and their influence on biomarker values need to be assessed to know whether these values are reliable.
Yet another factor potentially yielding varying absolute results is the use of different biomarker assays and analytic platforms. Especially when implementing immunoassays for the quantification of biological molecules, the use of different antibodies and technical details may lead to varying absolute results for a given marker [17]. This may not hinder a reliable utilization of a given biomarker, but a comparison of values between different assays needs to be assessed consistently to compare results concerning their diagnostic meaningfulness.

The aims of the current study were, (1) to analyze potential intra-individual differences of six biomarker values quantified from blood serum samples taken in blood tubes made from different materials (polypropylene and glass) without additives, and (2), to examine differences in quantitative results from commonly used enzyme-linked immunosorbent assays (ELISAs) and from a novel fully automated immunoassay measuring platform. The six considered biomarkers brain-derived neurotrophic factor (BDNF), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor A (VEGF-A), transforming growth factor-beta type 1 (TGF-ß1), monocyte chemoattractant protein 1 (MCP-1), and interleukin-18 (IL-18) may be instrumental in the biomarker-aided diagnosis of AD [13,18]. Thus, to be beneficial in the diagnostic regimen, the susceptibility of absolute values for these markers depending on varying preanalytical and analytical factors needs to be assessed.

We hypothesize that (1) when analyzing the biomarkers in human blood serum, there will be significant differences in laboratory values according to different blood tubes the sample was filled into, and (2) when analyzing one respective sample on two different immunoassay measuring platforms, laboratory values will be significantly different.
Materials, subjects, and methods

Participants

The sample (N=315) consisted of participants of the study “Earlier and Differential diagnosis of Alzheimer’s Disease” (EDAD). Cognitively impaired patients (n=214) were consecutive referrals to the Memory Clinic of the University Department of Geriatric Medicine FELIX PLATTER, Basel, Switzerland. They were required to have obtained at least 20/30 points on the Mini-Mental Status Examination [19]. Patients were diagnosed according to the fifth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5 [20]). Cognitively healthy subjects (n=101) were participants of the Memory Clinic's "Registry of Cognitively Healthy Individuals Interested to Participate in Research". All patients and healthy controls were of German/Swiss-German native language and were required to be 50 years or older to participate in the trial.

We did not include participants with severe or unstable medical conditions that might interfere with the clinical or neuropsychological study procedures, such as infectious diseases (e.g., HIV, COVID-19), hepatic dysfunction or renal insufficiency, psychotic disorder, bipolar disorder, substance addiction at present or in the past, history of stroke with residual symptoms, current or past unstable malignant disease within the last two years or chronic use of psychoactive drugs with sedative or anticholinergic effects.

Written informed consent was obtained from all participants. The local ethics committee (Ethikkommission Nordwest- und Zentralschweiz, EKNZ; No. 2019-00386) approved the study.
**Blood sampling**

Blood samples were taken by trained nurses and collected in neutral polypropylene 7.5 ml S-Monovette® tubes without additives (Sarstedt, Nümbrecht, Germany) as well as in glass 5.0 ml BD Vacutainer® tubes without additives (BD, Franklin Lakes, United States) in random order. The samples were given time to clot for 60 min and protected from heat before being centrifuged at room temperature for 10 min at 2000×g to segregate the serum. The maximal time from taking the blood sample until freezing of the serum was 120 minutes. The obtained serum was frozen and stored in 500 µl aliquots in 1.5 ml polypropylene tubes at -80°C until analysis. Hemolytic, lipemic or icteric samples were excluded, as such quality deficits can potentially influence assay results. Samples were shipped on dry ice. Storage time between sample taking and analyses ranged between several days and a maximum of 3.5 months for samples analysed with ELISA and up to a maximum of 9 months for samples measured on Ella.

**Measurement of BDNF, IGF-1, VEGF-A, TGF-β1, MCP-1 & IL-18 on ELISA platform**

The protein levels of the six biomarkers BDNF, IGF-1, VEGF-A, TGF-β1, MCP-1, and IL-18 were assessed using ELISAs, described in detail elsewhere [13]. Briefly, the quantifications were performed according to the manufacturer's instructions with the application of the specific sample dilutions. These sample dilutions were optimized and established for each biomarker by our workgroup. In addition to the assay-specific protein standards, an internal control was measured within each assay run. Samples from both blood tube types were assayed within the same runs. Assay results were quantified using a microplate reader (Versamax, Molecular Devices, San Jose, CA, USA). The protein concentrations for BDNF, IGF-1, and TGF-β1 are expressed in ng/ml and pg/ml for VEGF, MCP-1, and IL-18.
Measurement of BDNF, VEGF-A, TGF-β1, MCP-1 & IL-18 on Ella platform

The concentrations of the five biomarkers BDNF, VEGF-A, TGF-β1, MCP-1, and IL-18 were assessed using the platform Ella (ProteinSimple, San Jose, CA, USA), a fully automated immunoassay workbench system working with a closed cartridge system. Since there is no commercially available assay for the quantification of IGF-1 on this platform, no data is available for this marker. Samples for the measurement on Ella platform were solely taken from Sarstedt tubes. The protein concentrations are expressed in pg/ml for all biomarkers.

Statistical Analyses

All statistical analyses were conducted with GraphPad Prism 9.1.0 (GraphPad Software, San Diego, USA).

To assess differences between the six serum biomarker values quantified in serum collected in Sarstedt and BD tubes and the five biomarkers quantified applying classical ELISAs or the Ella platform, respectively, we calculated median, interquartile ranges, and minimum/maximum values, as well as mean and standard deviation.

Furthermore, for the intra-individual differences between tube values and between assay values, respectively, we conducted Bland-Altman analyses and plotted the respective values with the average of two values depicted on the x-axis and the percentual difference (100*(Sarstedt value-BD value)/average) or (100*(ELISA value-Ella value)/average) respectively, on the y-axis. For Bland-Altman analyses, mean bias values close to zero with narrow 95% limits of agreement are considered favorable. However, results need to be interpreted from a clinical point of view for every protein, as there are no valid standard ranges.
Results

Sample sizes & demographic characteristics

Data from 214 patients and 101 healthy controls were merged, equal to a total N of 315. With serum analyses from two different blood tubes, this would amount to a total of 315 pairs of data for each of the 6 biomarkers. For some biomarkers, no data were available due to the respective biomarker value being below the quantification threshold or gaining too little blood from some participants. Overall, 292 participants had data available for all six biomarkers from the two respective tube types. These participants had a mean age of 72.0±8.5 years, 45.5% were female.

For the comparison of different measuring platforms, a sample of n=211 had data available for all 5 biomarkers measured on both platforms.

Descriptive statistics and Bland-Altman analysis of serum biomarkers – blood tube comparison

A full description of the values for the respective biomarkers measured in serum from Sarstedt and BD tubes is depicted in table 1. For biomarkers BDNF, VEGF-A, TGF-β1 and MCP-1 medians are lower in the Sarstedt group than in the BD group. In contrast, medians are similar between both groups for IGF-1 and IL-18, respectively.
### Table 1 - Concentrations of biomarkers quantified from serum collected in different tube types (Sarstedt tubes and BD tubes)

<table>
<thead>
<tr>
<th></th>
<th>BDNF (ng/ml)</th>
<th>IGF-1 (ng/ml)</th>
<th>VEGF-A (pg/ml)</th>
<th>TGFβ-1 (ng/ml)</th>
<th>MCP-1 (pg/ml)</th>
<th>IL-18 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sarstedt (n=297)</td>
<td>BD (n=297)</td>
<td>Sarstedt (n=297)</td>
<td>BD (n=297)</td>
<td>Sarstedt (n=297)</td>
<td>BD (n=297)</td>
</tr>
<tr>
<td>Minimum</td>
<td>1.348</td>
<td>2.33</td>
<td>47.25</td>
<td>24.76</td>
<td>8.536</td>
<td>18.49</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>7.816</td>
<td>19.26</td>
<td>308</td>
<td>108.7</td>
<td>113.4</td>
<td>197</td>
</tr>
<tr>
<td>Median</td>
<td>10.95</td>
<td>24.1</td>
<td>137</td>
<td>138.2</td>
<td>199.8</td>
<td>298.6</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>16.5</td>
<td>29.53</td>
<td>169</td>
<td>168.3</td>
<td>307.1</td>
<td>479.7</td>
</tr>
<tr>
<td>Maximum</td>
<td>34.49</td>
<td>62.96</td>
<td>279.9</td>
<td>276</td>
<td>1320</td>
<td>1688</td>
</tr>
<tr>
<td>Range</td>
<td>33.14</td>
<td>60.63</td>
<td>232.6</td>
<td>251.2</td>
<td>1311</td>
<td>1670</td>
</tr>
<tr>
<td>Mean</td>
<td>12.41</td>
<td>24.92</td>
<td>138.6</td>
<td>139.3</td>
<td>242.8</td>
<td>364.7</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>6.749</td>
<td>8.481</td>
<td>42.25</td>
<td>42.25</td>
<td>189.4</td>
<td>246.4</td>
</tr>
</tbody>
</table>

BDNF=brain-derived neurotrophic factor; IGF-1=insulin-like growth factor 1; VEGF-A=vascular endothelial growth factor A; TGF-β1=transforming growth factor-beta type 1; MCP-1=monocyte chemoattractant protein 1; IL-18=interleukin-18.
According to Bland-Altman analyses, the mean bias was low for the biomarkers IGF-1 (-0.45% with 95% limits of agreement between -20.86% and 19.96%) and IL-18 (-0.69% with 95% limits between -18.04% and 16.66%). Values from the Bland-Altman analyses indicate markedly differing values depending on the type of blood tube for the remaining biomarkers (Fig 1).

Fig 1. Bland-Altman plots of percentage difference (Sarstedt value-BD value, y-axis) against mean (x-axis) of biomarker concentrations quantified from blood serum collected in different tube types. Mean bias and 95% limits of agreement (±1.96 standard deviations) are depicted with dotted lines. BDNF=brain-derived neurotrophic factor; IGF-1=insulin-like growth factor 1; VEGF-A=vascular endothelial growth factor A; TGF-beta1=transforming growth factor-beta type 1; MCP-1=monocyte chemoattractant protein 1; IL-18=interleukin-18.

Normality tests, mean differences and correlations – measuring platform comparison

A full description of the absolute values for the respective biomarkers measured in serum using ELISAs and the Ella platform is shown in table 2. Medians were similar between the two groups for biomarkers BDNF and TGF-β1, respectively. For VEGF-A, the median was higher in the ELISA group, whereas for MCP-1 and IL-18, median biomarker values in the ELISA group were lower, respectively.
Table 2 – Concentrations of biomarkers quantified using different immunoassay platforms (ELISAs and Ella platform)

<table>
<thead>
<tr>
<th></th>
<th>BDNF (pg/ml) ELISA (n=213)</th>
<th>VEGF-A (pg/ml) ELISA (n=213)</th>
<th>VEGF-A (pg/ml) Ella (n=213)</th>
<th>TGFβ-1 (pg/ml) ELISA (n=213)</th>
<th>TGFβ-1 (pg/ml) Ella (n=213)</th>
<th>MCP-1 (pg/ml) ELISA (n=212)</th>
<th>MCP-1 (pg/ml) Ella (n=213)</th>
<th>IL-18 (pg/ml) ELISA (n=211)</th>
<th>IL-18 (pg/ml) Ella (n=213)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minimum</td>
<td>1348</td>
<td>1163</td>
<td>8.54</td>
<td>8.26</td>
<td>4119</td>
<td>4381</td>
<td>12.53</td>
<td>28.1</td>
<td>27.4</td>
</tr>
<tr>
<td>25% Percentile</td>
<td>7710</td>
<td>9208</td>
<td>120.1</td>
<td>109</td>
<td>19475</td>
<td>15531</td>
<td>47.67</td>
<td>254</td>
<td>173</td>
</tr>
<tr>
<td>Median</td>
<td>11262</td>
<td>12932</td>
<td>214</td>
<td>188</td>
<td>25800</td>
<td>22510</td>
<td>67.98</td>
<td>332</td>
<td>224.2</td>
</tr>
<tr>
<td>75% Percentile</td>
<td>16949</td>
<td>20921</td>
<td>305.1</td>
<td>290.5</td>
<td>33323</td>
<td>30204</td>
<td>93.56</td>
<td>405</td>
<td>307</td>
</tr>
<tr>
<td>Maximum</td>
<td>34492</td>
<td>45108</td>
<td>1320</td>
<td>1180</td>
<td>55079</td>
<td>64552</td>
<td>396.8</td>
<td>2207</td>
<td>3269</td>
</tr>
<tr>
<td>Range</td>
<td>33144</td>
<td>43945</td>
<td>1311</td>
<td>1172</td>
<td>50960</td>
<td>60171</td>
<td>384.2</td>
<td>2179</td>
<td>3242</td>
</tr>
<tr>
<td>Mean</td>
<td>12583</td>
<td>15441</td>
<td>246.6</td>
<td>226.9</td>
<td>26589</td>
<td>24325</td>
<td>79.21</td>
<td>352.6</td>
<td>318.5</td>
</tr>
<tr>
<td>Std. Deviation</td>
<td>6958</td>
<td>9078</td>
<td>193.7</td>
<td>172.8</td>
<td>10330</td>
<td>11855</td>
<td>47.96</td>
<td>173.1</td>
<td>386.6</td>
</tr>
</tbody>
</table>

BDNF=brain-derived neurotrophic factor; VEGF-A=vascular endothelial growth factor A; TGF-β1=transforming growth factor-beta type 1; MCP-1=monocyte chemoattractant protein 1; IL-18=interleukin-18; ELISA= enzyme-linked immunosorbent assay.
After conducting Bland-Altman analyses for each biomarker, we found a mean bias of -7.68% with 95% limits of agreement between -29.84 and 45.2% for VEGF-A. For TGF-β1 the overall mean bias was comparable in magnitude with 11.74%, however with wider 95% limits (-60.21% to 83.7%). For all other biomarkers, the biases were larger, the most extreme mean bias was found for MCP-1 with -128.1% and 95% limits of agreement between -175.9% and -80.32% (Fig 2), indicating that values obtained for the same marker on the two different platforms largely differ.

**Fig 2. Bland-Altman plots of percentage difference (ELISA value-Ella value, y-axis) against mean (x-axis) of biomarkers from different platforms Ella and ELISA.** Mean bias and 95% limits of agreement (±1.96 standard deviations) are depicted with dotted lines. BDNF=brain-derived neurotrophic factor; VEGF-A=vascular endothelial growth factor A; TGF-beta1=transforming growth factor-beta type 1; MCP-1=monocyte chemoattractant protein 1; IL-18=interleukin-18; ELISA= enzyme-linked immunosorbent assay.
**Discussion**

Our data show, that depending on (1) blood tubes a specimen is taken into and (2) depending on the type of immunoassay used, obtained concentrations of the six serum protein investigated here can markedly differ. Importantly, results varied depending on the type of biomarker. Given the fact that laboratories in everyday use may implement different tubes and assays, our findings substantiate that absolute biomarker values within and between laboratories and clinical institutions need to be compared with great caution. For these reasons, it is of utmost importance to rule out as many unknown variables as possible to evaluate the quantitative result for a given biomarker.

Our results underline that biomarker values are not directly displaying actual concentrations in the human body but can be confounded by preanalytical handling, whereby the confounding effect of the preanalytical handling varies between biomarkers. We found that usage of tubes of two different manufacturers influenced the obtained serum concentrations of TGF-β1 and BDNF in a non-linear manner as indicated by the most extreme Bland-Altman mean biases and wide 95% limits of agreement (i.e., mean bias of 70.64% for BDNF and -49.5% for TGF-β1). In contrast, biases were lower, and 95% limits narrow for the quantifications of IL-18 and IGF-1. We assume that depending on hydrophobicity as well as the surface charges of molecules of these biomarkers and the surfaces themselves, some molecules are more prone to stick to the surface of certain materials (polypropylene vs. glass). Indeed, findings for the quantification of CSF amyloid-beta42 peptides which adhere differently to plastic surfaces dependent on the composition of the material also showed differing quantitative results dependent on different tube types [7,12,21].

Furthermore, we found varying absolute values for the same markers dependent on the measuring immunoassay platforms, in this case, a commonly used ELISA and a highly automated immunoassay platform (Ella). Whereas for the serum biomarkers BDNF and VEGF-
A mean biases were low and 95% limits rather narrow (mean biases of -18.62% and -7.68%, respectively), results for the remaining biomarkers showed either a comparatively low mean bias paired with wide 95% limits of agreement (TGF-β1, IL-18) or an extermer mean bias with more narrow limits of agreement (MCP-1). From a laboratory point of view, the numbers found for BDNF and VEGF-A compare very well, and the high correlation of the values might most probably be explained by the fact that the highly automated immunoassay platform (Ella) assays for these biomarkers have been developed based on the exact two ELISAs used for the quantification of these analytes (ProteinSimple, San Jose, CA, USA, personal communication). Thus, the antibody pairs used in these assays are identical, and our results show that when using the same antibody pairs in different immunoassay systems, results can be highly comparable. Within the Ella assays for TGF-β1, MCP-1, and IL-18 different antibody pairs than within the ELISAs are used, thus yielding different absolute values and varying non-linear effects within the assays.

To focus on the concentration differences caused by the use of varying tube and assay types, we aimed at ruling out as many other confounding factors as possible in the preanalytical and analytical handling of the specimen by strictly sticking to standard operating procedures (SOP) and technical instructions provided by the assay manufacturers. However, minor deviations cannot be ruled out completely. To exclude effects of length of storage before analysis, we independently monitored the stability of the biomarkers over the time of up to 15 months and did not find and influence of storage time [13]. Additionally, we examined if there are batch effects, i.e., if mean values differ significantly between batches of measured samples and found no significant influence (data not shown).

Our results support the need for standardization and harmonization of preanalytical and analytical laboratory measuring conditions. While from our data we cannot deduct that one type of blood tube or measuring platform is less suited than the other, we here quantify the impact
of two possible influencing factors. However, it cannot be excluded that there are more confounding factors not analyzed in this study.

Consequently, every diagnostic lab may need to assess limiting values, like cut-off thresholds to be regarded as indicative for the presence of a certain condition, separately. There is a need for trials with large cohorts to compare and standardize test results. These trials would need to determine values for different technical systems categorically. Additionally, standard substances need to be made available internationally.
Acknowledgments

We want to thank all study participants and their caregivers.
Bibliography


**BDNF**

- Mean: -70.64%
- +1.96 SD: 20.09%
- -1.96 SD: -161.4%

**IGF-1**

- Mean: -0.45%
- +1.96 SD: 19.96%
- -1.96 SD: -20.86%

**VEGF-A**

- Mean: -44.31%
- +1.96 SD: 19.47%
- -1.96 SD: -108.1%

**TGF-beta1**

- Mean: -49.5%
- +1.96 SD: 21.54%
- -1.96 SD: -120.5%

**MCP-1**

- Mean: -32.17%
- +1.96 SD: 25.27%
- -1.96 SD: -89.61%

**IL-18**

- Mean: -0.69%
- +1.96 SD: 16.66%
- -1.96 SD: -18.04%
**BDNF**

Mean: \(-18.62\%\)

\(\pm 1.96\, SD\): 
- \(-58.05\%\)
- \(+20.81\%\)

**VEGF-A**

Mean: \(-7.68\%\)

\(\pm 1.96\, SD\): 
- \(-29.84\%\)
- \(+45.2\%\)

**TGF-beta1**

Mean: \(+11.74\%\)

\(\pm 1.96\, SD\): 
- \(-60.21\%\)
- \(+83.7\%\)

**MCP-1**

Mean: \(-128.1\%\)

\(\pm 1.96\, SD\): 
- \(-175.9\%\)
- \(-80.32\%\)

**IL-18**

Mean: \(+21.04\%\)

\(\pm 1.96\, SD\): 
- \(-76.23\%\)
- \(+118.3\%\)
Click here to download Data Review URL
https://doi.org/10.7910/DVN/9RZWAG