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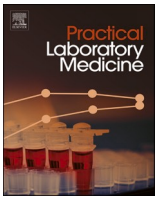

Biological variation of PIVKA-II in blood serum of healthy subjects measured by automated electrochemiluminescent assay

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ABSTRACT

Background: Prothrombin/Protein Induced by Vitamin K Absence-II (PIVKA-II) is a candidate biomarker of hepatocellular cancer, recommended both for diagnostics and monitoring. The aim was to evaluate biological variation (BV) of serum PIVKA-II.
Methods: Within-subject (CV_I) and between-subject (CV_G) BV estimates were assessed in 14 healthy volunteers in a 6-week protocol. Serum concentrations of PIVKA-II were measured by a Roche Elecsys PIVKA-II diagnostic kit (cobas e8000). Precision (CV_A) was assessed from duplicate measurements of all volunteers' samples. Two methods were used for the estimation of CV_I: SD-ANOVA and CV-ANOVA method. We calculated the index of individuality (II) and reference change value. The experiment was fully compliant with EFLM database checklist.
Results: The CV_I of PIVKA-II in healthy persons, as calculated by two statistical methods, were 8.2% (SD-ANOVA with CV_A of 3.2%) and 9.4% (CV-ANOVA) with CV_A of 2.7%). The CV_G was 19.5% (SD-ANOVA), and respective II and RCV were 0.42 and 24.4%.
Conclusions: CV_I and CV_G of PIVKA-II were 8.2% and 19.5%, respectively, with CV_A below 4%. The low II and RCV below 25% enable the use of this biomarker both for diagnostics and monitoring. More data are needed before the introduction of PIVKA-II into clinical practice.

1. Introduction

PIVKA-II (Prothrombin/Protein Induced by Vitamin K Absence-II, also known as des-γ-carboxyprothrombin, DCP) is a candidate biomarker of hepatocellular cancer (HCC). Its production increases during malignant transformation of hepatocytes. Changes in the cytoskeleton of transformed hepatocytes impact uptake of vitamin K with the resulting production of abnormal prothrombin. Similarly, the deficiency of γ-glutamyl carboxylase will also increase levels of abnormal prothrombin. As a result, the overexpression of vascular growth factors leads to malignant transformation of the hepatocyte with microvascular invasion. PIVKA-II is therefore able to reveal malignant transformation of the hepatocyte with the ability to differentiate between HCC and cirrhosis. In addition to being able to identify microvascular invasion, concentrations of PIVKA-II correlate with malignity stage of HCC and higher risk of HCC recurrence after liver transplantation. Thus, PIVKA-II has been used as a diagnostic and prognostic biomarker with a potential to discriminate between HCC and other liver diseases, to differentiate between different stages of HCC, and to indicate high-risk patients with HCC [1].

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List of abbreviations

AFP	alpha-fetoprotein
BV	biological variation
CV	coefficient of variation
CV_A	analytical variation (imprecision)
CV_G	between-subject biological variation
CV_I	within-subject biological variation
D	desired uncertainty
ECLIA	electrochemiluminescent assay
EFLM	European Federation of Clinical Chemistry and Laboratory Medicine
HCC	hepatocellular cancer
IKEM	Institute for Clinical and Experimental Medicine
II	index of individuality
IQR	interquartile range
LoD	limit of detection
LTx	liver transplantation
PIVKA-II	prothrombin induced by vitamin K absence-II (also known as des-γ-carboxy-prothrombine, DCP)
RCV	reference change value
RI	reference interval
TG-BVD	Task Group for the Biological Variation Database
WG-BV	Working Group on Biological Variation

PIVKA-II and alpha-fetoprotein (AFP) do not necessarily correlate in HCC, as the positivity of PIVKA-II was found in more aggressive tumors with AFP negativity. Nevertheless, if AFP is combined with PIVKA-II into GAAD score (Gender, Age, AFP, DCP) [2] (or GALAD score based on PIVKA-II, AFP and its isoenzyme L3 [3], the diagnostic potential of PIVKA-II may further increase.

Primary liver cancer is the 3rd cause of death from malignant diseases in the world with a mortality of 8.3% and the 6th most frequently diagnosed cancer with an incidence of 7% [4]. Liver transplantation (LTx) is one of treatment modalities in patients with hepatocellular cancer. Because of limited access to liver donors and poor prognosis of patients with advanced HCC, the eligibility criteria were introduced to clinical practice. First set of indication criteria for LTx, starting with Milano criteria in 1996 [5], was based on morphology (diameter, volume a number of tumors or cancer lesions). However, expanded criteria incorporating AFP and PIVKA-II were developed to increase probability of better prognosis and survival after LTx [6].

The necessary prerequisites for proper use of every laboratory test involve at least the knowledge of analytical performance and biological characteristics. Biological variation, both within-subject (CV_I) and between-subject (CV_G), has many potential uses in the domain of laboratory medicine. It is possible to calculate the number of necessary samples to estimate the homeostatic point of the biomarker (pre-analytical phase), specify analytical quality performance specification (in terms of optimal, desirable, or minimal performance, analytical phase) or use biological variation for interpretation (post-analytical phase). Reference change value (RCV) defines the minimal difference between two consecutive measurements, which can be clinically important; index of individuality (II) describes the potential of population-based reference intervals for interpretation.

There are direct methods for the estimation of CV_I and CV_G [7]. The Working Group on Biological Variation (WG-BV) and the Task Group for the Biological Variation Database (TG-BVD) of the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) defined detailed protocol for the direct estimation of biological variation [8]. Also, an indirect method of CV_I estimation based on big data analysis is available [9].

The aim of our study was to estimate the biological variation (CV_I and CV_G) of PIVKA-II together with the assessment of CV_A and calculation of RCV and II in healthy subjects by means of direct method.

2. Study subjects and methods

2.1. Study subjects, sampling and sample preparation

Fourteen apparently healthy persons (7 men and 7 women; Caucasian race; healthy nonsmokers with common lifestyle, without any clinical and laboratory signs of acute or chronic disease, without statin or any other hypolipidemic treatment, without treatment with vitamin K or vitamin K antagonists) were invited to participate in a study. They were aged 28–40 years (median age: 32 years). Detailed description of the study subjects including liver function tests, renal function, nutritional and metabolic status and humoral immunity was given elsewhere [10]. Blood samples were taken at equidistant intervals. The study lasted for 6 weeks; thus, 7 venous blood samples were available from every study subject. We followed the algorithm by Braga and Panteghini [11]. The study subjects were instructed to be in a fasting state (no food intake for at least 12 h before sampling); they were in a sitting position for at least 5 min (but not more than 10 min) before and during sampling. Two randomly assigned phlebotomists were responsible for the sampling. Venous blood was taken between 08:00 and 10:00 a.m. on the same day of the week (Tuesday). A detailed description of sampling

system, separation of serum, aliquoting and storing before analysis was given elsewhere [10], see also Supplementary material, Methods. Informed consent was obtained from all individuals included in this study. The study protocol corresponds to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the Institute for Clinical and Experimental Medicine (IKEM) Ethics Committee.

2.2. Analytical methods

We used an Elecsys PIVKA-II diagnostic kit (Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim, Germany) and cobas e8000 analyzer (Roche, Germany). The electrochemiluminescent assay (ECLIA) uses the sandwich principle, calibrated against purified recombinant des-γ-carboxy-prothrombine (DCP). We used lot of Roche diagnostic kit No. 540079 (reagents), Roche calibrators No. 540769, and Roche controls No. 568765 (Low: 16.2–24.8 μg/L; High: 249–381 μg/L).

Repeatability (within-run CV), as declared by the manufacturer, was between 1.0 and 1.8% (at the concentrations 7.42–10563 μg/L); intermediate imprecision was between 4.3 and 6.9% (the same concentration range). Repeatability and total precision in our laboratory were verified by EP15 (CLSI) protocol on two levels of control material. Variation coefficients of repeatability and total precision were 1.5% and 2.8%, respectively, for the low level of control material (20.7 μg/L), and 1.7% and 2.5%, respectively, for the high level of control material (322.2 μg/L).

The medians of reference values declared by the manufacturer in healthy persons were 18.1 μg/L (women, N = 380), 19.0 μg/L (men, N = 431), and 18.7 μg/L (all, N = 811). Corresponding 95th percentiles were 27.8, 28.6, and 28.4 μg/L, respectively. Corresponding ranges of reference values (min-max) were 8.40–54.4, 11.2–131, and 8.40–131 μg/L, respectively.

All measurements in our study were performed in duplicate within one day by the same person (author JČ). All 98 study subjects' samples were measured in random order generated by a Microsoft Excel (2010) function.

2.3. Statistical evaluation

An algorithm recommended by Braga and Panteghini [11] was used, details were described elsewhere [10]. Briefly, we tested homogeneity of individual variance (Fligner-Killeen test with alpha of 0.05, as recommended by Røraas et al. [12,13], then we detected outliers among the mean values of subjects (Reed test). Normality of individual data sets and normality of mean values of the subjects were assessed by the Shapiro-Wilk test. Outliers were detected by the Fligner-Killeen test. Normality of data for both within-subject and between-subject variation calculation was proved after elimination of outliers and the necessary condition to use ANOVA was fulfilled. Two methods were used to estimate CV_I: SD-ANOVA, standard ANOVA performed on raw data, and CV-ANOVA performed on CV transformed data [12,13]. The components of biological variation (CV_I and CV_G) were calculated using a mixed linear regression model with R software version 4.2.2 [14]. RCV and II (based on the CV_I and CV_G, without considering the CV_A) were calculated. RCV was calculated by formula $2^{0.5} \times 1.96 \times (CV_A^2 + CV_I^2)^{0.5}$ with the assumption of two-tailed probability. Additionally, we calculated the lognormal RCV for decreases (RCV-) and lognormal RCV for increases (RCV+) according to Fokkema et al. [15].

Summarized description of the experimental design, as recommended by the EFLM [7,8], is given in the Supplementary Material, Supplementary Table 1.

3. Results

Table 1 describes the data as requested by EFLM [7,8]. The mean and median of all 98 values of serum PIVKA-II were 19.11 and 17.98 μg/L, respectively, with interquartile range (IQR) of 16.11–21.63 μg/L. Similarly, data are given for 77 values after elimination of 3 outliers by the Fligner-Killeen test (subjects S1, S2, and S13). There was a normal distribution of the PIVKA-II individual means (Shapiro-Wilk, D'Agostino-Pearson, and Kolmogorov-Smirnov tests) both before and after elimination of 3 outliers.

Fig. 1 describes data in all 14 study subjects during the 6-week experiment (median, minimum-maximum). Minimum and maximum values were selected according to Carobene [16]. Similarly Supplementary Material, Supplementary Fig. 1 displays CV-transformed data in 14 study subjects.

All measured data were above the limit of detection (LoD), as specified by the manufacturer (3.5 μg/L). Data were normally

Table 1
Descriptive statistics of serum PIVKA-II in study subjects.

Statistical characteristics	All data (14 subjects, 98 results)	Outliers eliminated (11 subjects, 77 results)	Description
Measurand mean/median (μg/L)	19.11/17.98	18.72/17.60	Calculated from all means of duplicate measurements
Measurand minimum/maximum (μg/L)	12.8/30.3	12.8/29.3	Values of means of duplicate masurements
Measurand SD (μg/L)	4.00	3.84	Based on all means of duplicate masurements
Measurand IQR (μg/L)	16.11–21.63	16.0–22.19	Calculated from all means of duplicate measurements

All values (N = 98 results) in μg/L and data after elimination of 3 outliers both in raw or CV-transformed data (N = 77 results). Table lists items as requested by the EFLM.

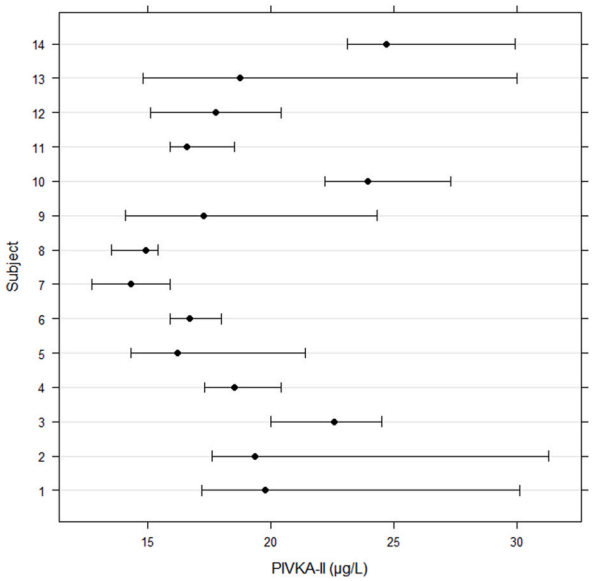

Fig. 1. Biological variation of serum PIVKA-II in 14 healthy volunteers: six-week experiment, 7 samples per person (1–7 men, 8–14 women); medians (minimum-maximum) are given for all subjects. All subjects are displayed; however, subjects S1, S2 and S13 were classified as outliers and were not included in the final statistical evaluation.

distributed after elimination of 3 outliers (Fligner-Killeen test). Therefore, 77 values were involved in the calculation of biological variation components. Also duplicates of the measurements were acceptable (Supplementary Material, Supplementary Fig. 2).

PIVKA II was similar in men (median of 17.8 µg/L, subjects S1–S7) and women (median of 18.0 µg/L, subjects S7–S14) and all individual medians were below the 95th percentile of reference values as recommended by the manufacturer (27.6 and 28.4 µg/L for women and men, respectively). Only 4 values out of 98 were above the 95th percentile of upper reference limit (see also Fig. 1 and Supplementary material, Supplementary Fig. 2).

The repeated measurements **(**duplicates**)** enabled both the calculation of the CV_A and the estimation of a power of the study. According to Røraas [17], the estimated value of a power would be 0.99 for 10 subjects with 6 repeated samples measured in duplicates. Therefore the power of our study is close to 1.0, as the ratio between analytical and within-person biological variation is lower than 1 (Table 2). The results of the biological variation are given in Table 2 both for SD-ANOVA and CV-ANOVA method together with data by Bayart [18] for comparison. The CV_G was calculated only using the SD-ANOVA method, and the resulting value of 19.5% produced an II of 0.42. RCV- and RCV+, calculated according to Fokkema [15], were similar both for SD-ANOVA and CV-ANOVA.

4. Discussion

In a group of healthy volunteers, we estimated components of biological variation of PIVKA-II in serum measured by the Roche Elecsys assay. We revealed satisfactory analytical variation (below 4%) and low within-subject biological variation (CV_I of about 9%) in comparison to between-subject biological variation (CV_G of about 20%).

We used two statistical methods to estimate the components of biological variation with different sensitivities to non-Gaussian distribution: SD-ANOVA (standard nested ANOVA performed on the raw data) and CV-ANOVA (ANOVA after CV transformation, individual data were divided by individual means) methods [12]. Importantly, values of CV_A and CV_I estimated by the two respective methods were similar (Table 2). Specifically, we found CV_A, CV_I, CV_G, and RCV to be 3.2, 8.2%, 19.5%, and 24.4%, respectively (SD-ANOVA). Using CV-ANOVA, CV_A, CV_I, and RCV were 2.7%, 9.4%, and 27.2, respectively. Given that only the SD-ANOVA allows the CV_G to be calculated, the index of individuality was 0.42.

Table 2
Comparison of the two methods to estimate the biological variation of serum PIVKA-II: CV-ANOVA and SD-ANOVA.

Statistical method	Mean (95% CI)	CV_A% (95% CI)	CV_I% (95% CI)	CV_G% (95% CI)	II	RCV (%)	RCV- (%)	RCV+ (%)
SD-ANOVA	18.7 µg/L (16.6–20.9)	3.2 (2.7–3.7)	8.2 (6.6–9.7)	19.5 (10.6–27.8)	0.42	24,4	−21.6	27.5
CV-ANOVA	1.0 (0.98–1.02)	2.7 (2.2–3.0)	9.4 (8.4–11.4)	(*)	–	27.2	−23.7	31.3
Bayart, 2020	32.0 mAU/mL (31.0–33.0)	2.82 (2.46–3.31)	13.4 (11.4–16.0)	16.1 (11.6–25.9)	0.85	37.7	–	–

(*) CV-ANOVA method does not allow the CV_G to be calculated.
The resulting CV_A, CV_I, CV_G, II, and RCVs are given. CV_A was estimated from duplicate measurements on each subject. Calculation after elimination of 3 outliers (77 results). The bootstrap confidence interval was set to 2000 simulations. Data by Bayart, expressed in mAU/mL, are given for comparison.

An important point could be the variation of values in outliers and the values above the upper reference limit. There were 4 values out of 98 above the 95th percentile of upper reference limit (men 27.6 μg/L, women 28.4 μg/L). These 4 values were in 4 patients: patient No. s1 (male, 29.7 μg/L), s2 (male, 30.25 μg/L), s13 (female, 29.25 μg/L) and s14 (female, 29.3 μg/L, not evaluated as outlier); it means, that these values were near the 95th percentile as claimed by the manufacturer, possibly below the 97.5th percentile (not stated by the manufacturer), and deep below the maximal value of the reference population of healthy persons (131 μg/L). These data support the reference interval as supplied by the manufacturer. We assume that the probability of another strong source of variation – except of random fluctuations around homeostatic point – is rather low.

According to our knowledge, this is the first report describing biological variation of PIVKA-II measured by Elecsys Roche electrochemiluminescent (ECLIA) principle. However, Bayart and coworkers in their excellent study assessed biological variation and analytical performance of PIVKA-II measured by the Lumipulse G600II analyzer (Fujirebio). This analytical method (results expressed in mAU/ml) had repeatability lower than 3% and intermediate precision lower than 4% [18]. Also, our study confirmed reliable analytical performance of Elecsys PIVKA-II electrochemiluminescent assay with CV_A below 4%. High level of analytical quality of Elecsys PIVKA-II was also described by Chan and co-workers. It should be stated, however, that Chan and coworkers revealed only moderate agreement between Elecsys and Lumipulse PIVKA-II assays [1].

CV_A in Bayart's study (2.8%, Lumipulse) and CV_A in our study (2.7 and 3.2%, Elecsys) were similar. However, CV_I of PIVKA-II in our experiment (8.2 and 9.4% depending on the statistical model used) was lower than 13.4% as described by Bayart. We suppose, that the difference in CV_I between Bayart's study and our experiment, except for different analytical method, was probably given by strict rules for the selection of study subjects and precise control of preanalytical factors (see Methods and Supplementary material, Supplementary Table 1). For example, alcohol intake up to 10 g/den was allowed, there was a higher number of subjects (19 subjects, 1 outlier), 5 samples were taken during the experimental period, and detailed information on race was missing. One can speculate that a „real life" situation will probably bring CV_I closer to the Bayart's data.

Because of low CV_I in our experiment, precision was not „optimal", but still „desirable", using Fraser's criteria for analytical performance of precision based on CV_I only [19,20]. Total allowable analytical error derived from CV_I and CV_G (according to Westgard) is about 10% in our experiment. Taken together, analytical performance of Elecsys PIVKA-II assay enables both diagnostics and monitoring.

Low values of CV_I enable a minimal number of samples to estimate a homeostatic point of biomarker with desired uncertainty (D, calculation is described in Supplementary material). Taken CV_A of 3%, CV_I of about 9% (Table 2) and D of 20%, only one sample is needed to estimate homeostatic point with that uncertainty, or three samples with an uncertainty of 10%.

Reference change value (RCV), based on analytical variation (CV_A) and within-subject biological variation (CV_I) has been used for years as a tool for the assessment of sequential changes in biomarker serum concentrations [20]. The calculated value of RCV of PIVKA-II (only for SD-ANOVA method, as recommended by Røraas [12]), was about 25%. Such level of RCV is common among the majority of chemistry tests. However, an important feature of PIVKA-II in HCC is an almost exponential increase of concentrations with advanced stage of the cancer [1].

Index of individuality (II) describes the relationship between CV_I and between-subject biological variation (CV_G), with or without CV_A taken into account. With II above 1.4, the reference intervals (RIs) can be used for similarly tested population as was used for the derivation of RI. Vice versa, with II bellow 0.6, RIs are of limited value and monitoring is essential. PIVKA-II had II of 0.42 in our experiment (i.e., the limited value of RI) or 0.85 in Bayart's experiment [18]. Therefore, the monitoring of PIVKA-II concentrations seems to be a reliable tool. Sagar and coworkers [21] advocated the monitoring of PIVKA-II and AFP levels in patients with hepatocellular cancer (HCC) treated with sorafenib. They showed that steady concentrations of PIVKA-II represented stable disease but gradual increase in concentrations was found in patients with the progression of the disease. Patients with microvascular invasion and poorly differentiated cancer had significantly higher concentrations of PIVKA-II, a phenomenon which was not found in AFP concentrations. Similarly, advanced stages of HCC had significantly higher concentrations of PIVKA-II than early stages, and the increase in concentrations with advancing stages was higher than in AFP [21]. To summarize, firstly changes in RCV of only about 25% of previous value are a strong signal of probable harmful disease progression. Secondly, due to the exponential increase of PIVKA-II concentrations in these situations, less time is necessary to receive this signal of the disease progression.

This study has some limitations. There are differences in reference ranges of PIVKA-II e.g., in Japanese and European populations. Similarly, Chinese authors found differences even among ethnicities in China population. Also, data describing CV_I and CV_G in specific cohorts are scarce, and we can only speculate on similar values across different races or ethnicities. We derived CV_I and CV_G for Caucasian population with the use of widely available Roche ECLIA method. Generally, it is advisable to use specific reference limits of the analytical method used and to use the values of CV_G and CV_I on target population similar to that was derived from. We eliminated 3 outliers out of 14 subjects, and final calculations of CV_I and CV_G were done on 5 men and 6 women, without differences in results between sexes. Four values of PIVKA-II in four patients were high in comparison with other individual values. However, these values were close to upper reference limit and therefore random fluctuation is more probable reason for these results. Finally, a general problem of all experiments on biological variation is a transferability of results into clinical settings.

5. Conclusions

We confirmed the satisfactory analytical performance of PIVKA-II measurement by Roche Elecsys assay with precision (CV_A) below 4% during our experiment of biological variation assessment. We used two methods for the calculation of biological variation components and CV_I values of PIVKA-II in healthy persons were 8.2% (SD-ANOVA) and 9.4% (CV-ANOVA), respectively. The CV_G was 19.5%, RCV was 24.4% and index of individuality 0.42. Only one sample is necessary to assess the homeostatic point of PIVKA-II with a

desirable uncertainty of 20%. Values of CV_I and CV_G gave very low index of individuality of 0.42, thus supporting the monitoring of PIVKA-II concentrations. PIVKA-II as a candidate and efficient biomarker requires further attention and more data are needed to verify its potential.

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CRediT authorship contribution statement

Antonín Jabor: Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Zdenek Kubíček:** Conceptualization, Formal analysis, Methodology, Writing – original draft, Writing – review & editing. **Jitka Čásenská:** Investigation, Writing – review & editing. **Tereza Vacková:** Investigation, Writing – review & editing. **Vanda Filová:** Investigation, Writing – original draft, Writing – review & editing. **Janka Franeková:** Conceptualization, Methodology, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2024.e00389.

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