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Biological variation of proprotein convertase subtilisin/kexin type 9 (PCSK9) in human serum

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ABSTRACT

Background: Proprotein convertase subtilisin/kexin type 9 (PCSK9) is involved in the regulation of LDL receptors. Inhibition of PCSK9 increase uptake of LDL-particles and pathogen-associated molecular patterns (PAMPs). The aim of our study was to evaluate biological variation of serum PCSK9.

Methods: Within-subject (CV_I) and between-subject (CV_G) biological variations were assessed in 14 healthy volunteers in a 6-week protocol (7 samples, equidistant time intervals). Serum concentration of PCSK9 was measured by a Quantikine ELISA assay (R&D systems, Bio-Techne Ltd., UK) on a DS2 ELISA reader (Dynex Technologies GmbH, Germany). Precision (CV_A) was assessed by duplicate measurements. Two methods with different levels of robustness were used for the estimation of CV_I, SD-ANOVA and CV-ANOVA method. We calculated the index of individuality and reference change values. The experiment was fully compliant with EFLM database checklist.

Results: The within-subject values of PCSK9 in healthy persons, as calculated by two statistical methods, were 23.2% (SD-ANOVA with CV_A of 5.6%) and 26.6% (CV-ANOVA with CV_A of 4.8%). The CV_G was 10.9% (SD-ANOVA), index of individuality and RCV were 2.13 and 66.3%, respectively.

Conclusions: The high index of individuality indicates that common reference intervals can be used to interpret serum PSCK9 values.

1. Introduction

Proprotein convertase subtilisin/kexin type 9 (PCSK9) plays an important role in the lipid metabolism, namely regulation of LDL receptors [1]. Inhibition of enzyme activity of PCSK9 (e.g., by means of evolocumab or alirocumab, potent PCSK9 inhibitors, PSCK9-i) represents a powerful tool in the treatment of hypercholesterolemia [2]. Lower plasma PCSK9 levels are probably responsible not only for an increased surface density of LDL receptors on hepatocytes and similar receptors on adipocytes, but also for increased lipoprotein and bacterial lipopolysaccharide clearance and thereby decreased probability of sepsis development [3]. Concentrations of PCSK9 in plasma increase after administration of statins, fenofibrate, and supervised physical activity [4,5], whereas fasting reduces plasma concentrations of PCSK9 [4]. In spite of the necessity to incorporate biological variation into preanalytical (number of samples), analytical (analytical quality specifications), and postanalytical phase (interpretation), data on biological variation of PCSK9 in plasma are rather scarce. Direct methods of biological variation estimation (within-subject, CV_I, and between-subject, CV_G, biological variation) are based on recommendations and check list described by European Federation of Clinical Chemistry and Laboratory Medicine (EFLM), namely EFLM Working Group on Biological

Abbreviations: CV, coefficient of variation; CV_A, analytical variation (imprecision); CV_G, between-subject variation; CV_I, within-subject variation; II, index of individuality; PCSK9, proprotein convertase subtilisin/kexin type 9; PCSK9-i, proprotein convertase subtilisin/kexin type 9 inhibitors; RCV, reference change value; RI, reference interval.

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Variation (WG-BV) and the Task Group for the Biological Variation Database (TG-BVD) [6]. An indirect elegant method for CV_I estimation, as described by Jones [7], requires large data in the laboratory information systems and cannot be used for, "new" or less frequently requested analytes. The aim of our study was to evaluate the biological variation (CV_I and CV_G) of PCSK9 together with the assessment of CV_A and calculation of reference change value (RCV) and index of individuality (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 life style, without any clinical and laboratory signs of acute or chronic disease, without statin or any other hypolipidemic treatment) were invited to participate in a study. There were aged 20–50 years (median age: 32 years; range: 28–40). Detailed description of the study subjects including liver function tests, renal function, nutritional and metabolic status and humoral immunity was given elsewhere [8]. 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 [9]. 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 (8 and Supplementary material - Methods).

2.2. Analytical methods

We used a Human Proprotein Convertase 9/PCSK9 Quantikine ELISA diagnostic kit (R&D systems, Bio-Techne Ltd., Abingdon Science Park, Abingdon, UK) and DS2 ELISA reader (Dynex Technologies GmbH, Denkendorf, Germany). The assay uses pre-coated microplates with immobilized monoclonal antibody specific for human PCSK9. Standards or samples (20-fold dilution) are pipetted into the wells and incubated (for 2 h at room temperature). The unbound substances are washed out thereafter. An enzyme-linked polyclonal antibody is applied and incubated again. The unbound antibody-enzyme complexes are washed out, the substrate with chromogen (tetramethylbenzidine) is added and optical density of developed color is measured at 450 nm. The assay recognizes free and LDL receptor-bound PCSK9, and recombinant human PCSK9. We used lot of R&D diagnostic kit No. P259882 (reagents and calibrators), and R&D controls No. P195791 (Low: 3.07–5.01 μg/L; mid: 8.91–14.5 μg/L; high: 17.2–28.1 μg/L). According to the manufacturer, patient serum samples underwent a 20-fold dilution.

Repeatability (within-run CV), as declared by the manufacturer, was between 4.1% (at the concentration of 4.82 μg/L) and 6.5% (27.7 μg/L). These concentrations represent 96 μg/L and 554 μg/L in real samples. Intermediate imprecision was between 4.1% (27.9 μg/L) and 5.9% (4.64 μg/L). These concentrations represent 558 μg/L and 93 μg/L in real samples.The reference range declared by the manufacturer was 313 ± 71.5 μg/L (mean ± SD) or 177–460 μg/L (range, data for serum), based on 37 apparently healthy volunteers (donors without medical histories). Traceability was not provided by the manufacturer.

All measurements were performed in duplicate in three runs within three consecutive working days by the same person (author TV). 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 [9] was used, details were described elsewhere [8]. Briefly, we tested homogeneity of individual variance (Fligner-Killeen test as recommended by Røraas et al.) [10,11,12], then we detected outliers among the mean values of subjects (Reed test). Normality of individual data sets and normality of mean values of subjects were assessed by the Shapiro-Wilk test. Due to the sufficient normality of both within subjects and between subjects 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 [10,11,12]. The components of biological variation (CV_I and CV_G) were calculated using a mixed linear regression model with R software version 3.4.0 [13]. RCV and II (based on the CV_I and CV_G, without considering the CV_A) were calculated. RCV was calculated by the 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. [14].

Summarized description of the experimental design, as recommended by EFLM database team, is given in Table 1.

3. Results

Table 2 describes data as requested by EFLM database team. Mean and median of all 98 values of serum PCSK9 were 322.9 and 311.4 μg/L, respectively, with interquartile range (IQR) of 269.2–374.5 μg/L. There was a normal distribution of the PCSK9 data (Shapiro-Wilk test, D'Agostino-Pearson test). We also measured serum cholesterol in study subjects from the first sample of the experiment: median was 4.90 mmol/L, IQR 4.45–5.48 mmol/L.

Fig. 1 describes data in 14 study subjects during 6-week experiment (median, minimum–maximum). Minimum and maximum values were selected according to Carobene [15]. Similarly Supplementary Fig. 2 displays CV-transformed data in 14 study subjects.

There were no outliers as assessed from the raw and CV transformed data and data were normally distributed (Fligner-Killeen test, Reed test, Shapiro-Wilk test). Also duplicates of the measurements were acceptable (Supplementary Fig. 1). Therefore all 98 values were involved in the calculation of biological variation components. PCSK9 was similar in men (subjects 1–7) and women (subjects 7–14) and all individual medians were within reference range as recommended by the manufacturer

Table 1
Description of the experimental design (items as requested by EFLM database team).

Methodology	Serum PCSK9 (μg/L), measured by ELISA kit (Quantikine ELISA Human Proprotein Convertase 9/PCSK9, R&D systems, Bio-Techne Ltd., Abingdon Science Park, Abingdon, UK) on the DS2 ELISA reader (Dynex Technologies GmbH, Denkendorf, Germany), all measurement were performed in duplicates by one of the authors (TV) in three runs within three consecutive working days in random order generated by MS Excel function
Study subjects	14 healthy subjects (7 men, 7 nonpregnant women), 14 of them were included in the BV estimation, Caucasian race, median age 32 years (minimum 28, maximum 40 years)
Study protocol	Duration 6 weeks, equidistant sampling, one sample per week (total of 7 samples per subject, all 7 samples were used for BV estimation), sampling time between 08:00 to 10:00 a.m., fasting state
Statistical methods	Fligner-Killeen and Cochran tests (assessment of homogeneity of variances), Reed test (detection of outliers among the mean values of subjects), Shapiro-Wilk test (assessment of normality of individual data-sets and of mean values of subjects), SD-ANOVA on raw data, CV-ANOVA on CV-transformed data), estimation of CV_A (including confidence interval) from duplicate measurements, calculation of confidence intervals both for CV_I and CV_G, RCV calculated by means of standard formula and according to Fokkema

Table 2
Descriptive statistics of serum PCSK9 in study subjects. All values (N = 98) in μg/L. Table lists items requested by EFLM database team.

Measurand mean / median (μg/L)	322.9 / 311.4	Calculated from all means of duplicate measurement
Measurand minimum / maximum (μg/L)	143.9 / 554.1	Values of means of duplicate masurement
Measurand SD (μg/L)	83.4	Based on all means of duplicate masurement
Measurand IQR (μg/L)	269.4–372.2	Calculated from all means of duplicate measurement

(177–460 μg/L). Three values out of 98 were bellow the lower reference limit and 6 values (in 5 persons) were above the upper reference limit (see also Supplementary Fig. 1). This data supports the reference interval as supplied by the manufacturer.

The repeated measurement (duplicates) enabled both the calculation of the CV_A and estimation of a power of the study. The results of the biological variation are given in Table 3 both for SD-ANOVA and CV-ANOVA method. The CV_G was calculated only using the SD-ANOVA method, and the resulting value of 10.9% produced an II of 2.13. Similarly, RCV was calculated only for SD-ANOVA method, as recommended by Røraas [11]. RCV- and RCV+, calculated according to Fokkema [14], were similar.

4. Discussion

Our analysis revealed that the CV_A for the ELISA method (below 6%) met the optimal standard relative to the CV_I of a group of healthy volunteers. We found CV_I of 23.2% (95% CI 20.0–27.4), CV_G of 10.9% (3.1–18.7), and RCV of 66.3%. Higher value of CV_I than CV_G produced II of 2.13, which is above the limit 1.4 enabling reliable use of reference intervals. We used two statistical methods to estimate the CV_I 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) method [11]. However, values of CV_I estimated by the two respective methods were similar (23.2% and 26.6%, Table 3)

Reference change value (RCV), based on analytical variation (CV_A)

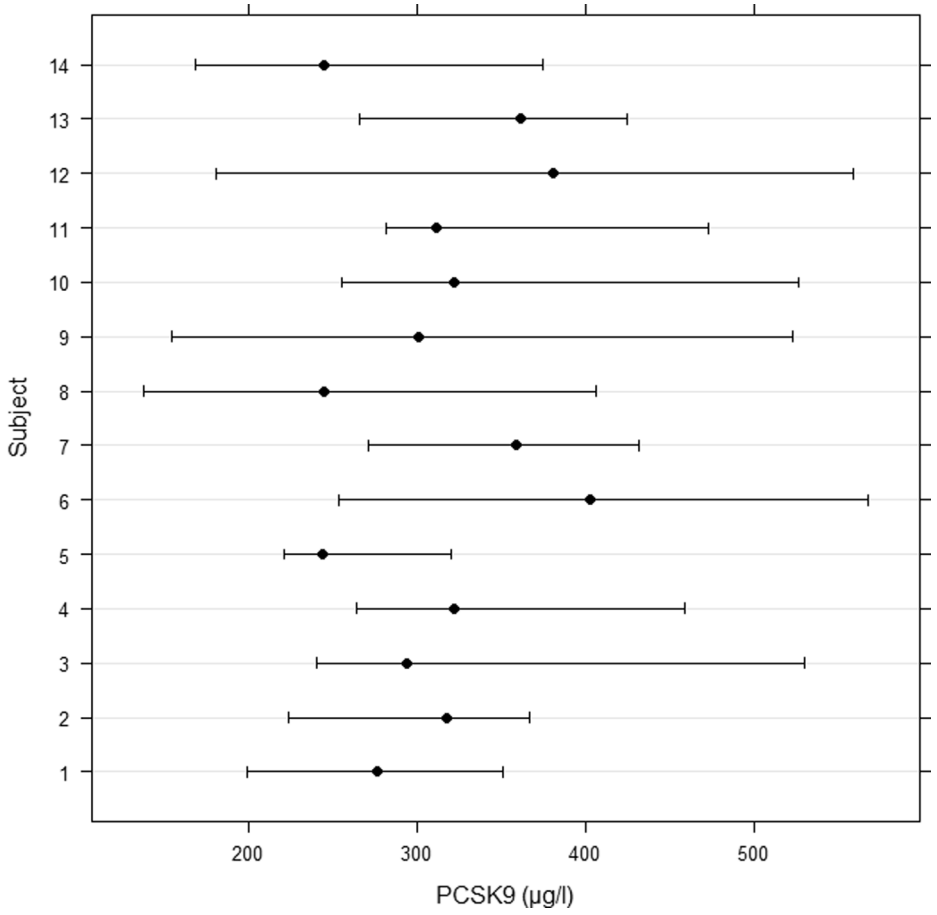

Fig. 1. Biological variation of serum PCSK9 in 14 healthy volunteers: six-week experiment, 7 samples per person (1–7 men, 8–14 women); medians (minimum-maximum) are given for all subjects.

Table 3
Comparison of the two methods to estimate the biological variation of serum PCSK9: CV-ANOVA and SD-ANOVA. The resulting CV_A, CV_I, CV_G, II, and RCVs are given. CV_A was estimated from duplicate measurements in each subject. N.R. - not recommended for used method of calculation.

Statistical method	Mean (95% CI)	CV_A% (95% CI)	CV_I% (95% CI)	CV_G% (95% CI)	II	RCV (%)	RCV- (%)	RCV+ (%)
SD-ANOVA	323.0 μg/L (298.4–347.5)	5.6 (4.9–6.5)	23.2 (20.0–27.4)	10.9 (3.1–18.7)	2.13	66.3	−48.0	92.2
CV-ANOVA	1.00 (0.96–1.04)	4.8 (4.0–5.3)	26.6 (24.1–31.4)	N.R.	–	N.R.	−52.1	109.0

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 [16]. 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 (RI) can be used for similarly tested population as was used for the derivation of RI. Vice versa, with II bellow 0.6, RI are of limited value and monitoring is essential. Value of II 2.13 as estimated in our study is not usual, but can be found for some biomarkers. Table 4 lists examples of biomarkers with II above 1.0. Some of analytes listed in Table 4 are based on EFLM database [17] with proven CV_I and CV_G and their confidence intervals. However, there are also data with conflicting results. In case of serum lipase, lipoprotein (a), osmolality and parathyroid hormone (PTH) there are different ($CV_I > CV_G$) data in Westgard biobase [18] and EFLM database ($CV_I < CV_G$). Further, there are other data for serum analytes in Westgard biobase with higher CV_I than CV_G (e.g., 5′ nucleotidase, iron, lactate dehydrogenase isoforms 2 and 4, LDL receptor mRNA, myeloperoxidase, SCC antigen, superoxide dismutase and tumor necrosis factor alpha). However, these data were not validated sufficiently yet.

Higher values of CV_I require repeated sampling for the estimation of homeostatic point of biomarker. Number of samples to estimate the homeostatic point with selected uncertainty can be calculated according to the Fraser formula

$$n = \left[Z.\left(CV_A^2 + CV_I^2\right)^{0,5}/D\right]^2$$

where n is number of samples, Z is 1.96 (probability of 95%) and D (%) represents interval for the assessment of homeostatic point. Fig. 2 displays the relationship between D and n for CV_A of 5.6% and CV_I of 23.2% from our experiment (Table 3).

Table 4
Some examples of analytes with higher CV_I than CV_G. Sorted according to the II = index of individuality (CVI/CVG).

Matrix	Analyte	CV_I% (95% CI)	CV_G% (95% CI)	II	Source
U	Adrenaline (ratio to creatinine)	135.0 (129.2–141.3)	54.0 (36.9–93.8)	2.50	17
B	Hydrogen ion (H +) (*)	3.5 (3.0–4.1)	2.0 (1.2–3.5)	1.75	17
B / P	Lactate	27.2 / 31.0	16.7 / 29.0	1.63	17 (two sources in EFLM database)
S	Tumor Necrosis Factor-alpha	43.0	29.0	1.48	18 (no data in EFLM database)
S	Calcium - ionised	2.18 (1.95–2.38)	1.58 (1.26–2.03)	1.38	17 (no metaanalysis)
S	Estradiol	15.0 (13.3–16.5)	13.0 (9.9–18.1)	1.15	17
S	Iron	26.5	23.2	1.14	18 (no data in EFLM database)
S	SCC antigen	39.4	35.7	1.10	18 (no data in EFLM database)
P	Fibroblast growth factor-23 (FGF23)	14.2 (12.3–16.8)	13.4 (9.0–22.1)	1.06	8,17
P	Aldosteron	36.6 (32.2–42.3)	34.7 (25.5–49.3)	1.05	17
S	Superoxide dismutase	17.1	16.7	1.02	18 (no data in EFLM database)

(*)Remark: unit for hydrogen ion is nmol/L.

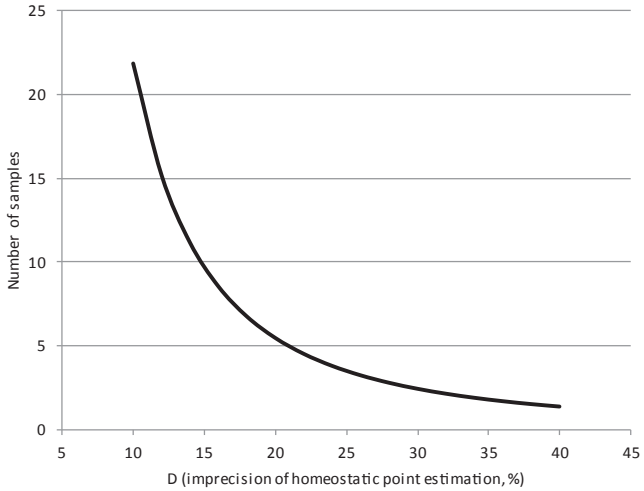

Fig. 2. The relationship between D (imprecision of homeostatic point estimation) and number of samples needed to estimate homeostatic point of PCSK6. CV_A = 5.6% and CV_I = 23.2% were taken from Table 3.

An imprecision of homeostatic point assessment of about 25–30% gives a realistic number of 2–3 samples for the measurement of PCSK9 in serum. Such level of homeostatic point imprecision corresponds well with the number of different factors influencing plasma concentration of PCSK9.

Except for widely used statins and fenofibrate there are other factors connected with upregulation of PCSK9 [4,19]. Atorvastatin (80 mg for 4 weeks) increased plasma PCSK9, decreased total cholesterol and LDL cholesterol [19]. Circulating levels of PCSK9 are reduced by fasting [4,19]. This metabolic effect is bound to the reduced cholesterol synthesis, the common pathway regulated by steroid regulatory binding protein (SREBP-2) is subject to diurnal rhytm of PCSK9 [19]. Moreover, treatment with growth hormone significantly decreased PCSK9 in plasma [19].

In septic patients higher concentrations of PCSK9 were found, so PCSK9 inhibition leading to increased clearance of bacterial lipopolysaccharide was suggested to be a promising way of treatment to decrease their mortality [3]. The measurement of PCSK9 concentration in serum could possibly help in risk stratification of these patients in terms of prediction of complications [5]. However, concentrations of PCSK9 changed in septic patients with higher mortality in the first quartile of PCSK9 concentrations during ICU stay [20]. Patients with septic shock and lower plasma PCSK9 had poor outcome with increased 28-day and 90-day mortality [21]. Therefore, immune response, lipid metabolism, and PCSK9 should be studied in more details in these patients [22].

Due to many abovementioned factors influencing PCSK9 concentration in plasma our results present valuable data on biological variation under physiological conditions. However, more data are needed before routine measurement of PCSK9 in real clinical circumstances is introduced.

5. Conclusions

We revealed the imprecision (CV_A) bellow 6% during our experimental estimation of biological variation of serum PCSK9. The CV_I values of PCSK9 in healthy persons, as calculated by two statistical methods, were 23.2% (SD-ANOVA) and 26.6% (CV-ANOVA). The CV_G was 10.9%, RCV was 66.3%. Values of CV_I and CV_G gave high index of individuality of 2.13, thus the reference intervals derived from general population by means of specific analytical procedure can be used for PCSK9 in plasma. The estimation of homeostatic point can be based on at least 3 blood samples to assess its value within ± 25%. More data are needed to verify the transferability of these results into clinical practice.

CRediT authorship contribution statement

An tonín Jabor: Conceptualization, Methodology, Software, Supervision, Writing - original draft, Writing - review & editing. **Tereza Vacková:** Data curation, Formal analysis, Writing - review & editing. **Zdenek Kubíček:** Conceptualization, Data curation, Formal analysis, Methodology, Software, Writing - review & editing. **Jitka Komrsková:** Data curation, Formal analysis, Writing - review & editing. **Marek Protuš:** Writing - review & editing. **Janka Franeková:** Methodology, Writing - review & editing.

Ethical approval

The study protocol corresponds to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the IKEM Ethics Committee. All subjects signed an informed consent form.

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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.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2021.06.023.

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