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ORIGINAL ARTICLE

Establishing biological variation for plasma D-dimer from 25 healthy individuals

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ABSTRACT

D-dimer is considered to be a reliable marker of both coagulation activation and fibrinolysis. However, data on biological variation (BV) of D-dimer is still limited, causing the use of empiric analytical performance specifications and lack of other implications related to BV. This study aimed to estimate the BV of plasma D-dimer employing a study design compliant with The Biological Variation Data Critical Appraisal Checklist. Blood samples were collected from a cohort of 25 healthy subjects (16 females, 9 males; age range, 19–61 years) from Turkey once weekly for 3 consecutive weeks. All plasma samples were analyzed in duplicate within a single run on Roche Cobas c501. The results were assessed for outliers, variance homogeneity, normal distribution, and trend, followed by nested ANOVA to determine BV and analytical variation estimates with confidence intervals (CIs). Gender stratified BV estimates were also calculated. Within-subject (CV_I) and between-subject (CV_G) BV estimates with 95% CIs were for D-dimer 21.2% (17.8-25.9) and 30.9% (21.3-46.2), respectively. No significant BV differences were observed between females and males. The index of individuality (II) and the reference change value (RCV) were calculated as 0.71 and 60.4%, respectively. Analytical performance specifications for desirable imprecision, bias, and total error were 10.6, 9.4, and 26.8%, respectively. This study provides well-characterized BV estimates for D-dimer, which may be helpful for setting objectively analytical performance specifications. Moreover, RCV should be preferred to decide whether a significant difference is present between serial D-dimer measurements from an individual.

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Analytical performance specification; biological variation; D-dimer; index of individuality; reference change values

Introduction

There are mainly three sources that contribute to variation in a test result, namely, analytical variation, pre-analytical variation, and biological variation [1]. Biological variation (BV) is often considered to be the most important source of variation in results over time [1]. Many measurands of clinical interest have random fluctuations around homeostatic set points, which is termed within-subject biological variation (CV_I) [1,2]. Different individuals may have different homeostatic set points for the same measurand, which is termed between-subject biological variation (CV_G) [1,2].

Biological variation data has received increasing attention due to its potential applications in the diagnosis and monitoring of disease and for quality assessment in clinical laboratory medicine [2]. Attempts to compile existing publications related to BV have resulted in the generation of the BV database by the Analytical Quality Commission of the Spanish Society of Laboratory Medicine [3]. This database is still available online but was last updated in 2014 [4].

Currently, a new online database has been established by the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) group on biological variation [5]. The EFLM Biological Variation Database includes BV estimates resulting from a meta-analysis of the studies taking into account the criteria presented in the Biological Variation Data Critical Appraisal Checklist (BIVAC) [6,7]. However, there is currently no data in the EFLM database for some measurands of interest, e.g. D-dimer.

D-dimer, a specific degradation product of cross-linked fibrin, is considered a biomarker of coagulation activation and fibrinolysis [8,9]. In normal physiological conditions, a small amount of fibrinogen is converted into fibrin [8,9]. D-dimer is therefore measurable in low levels in healthy subjects [8,9].

D-dimer is commonly used for exclusion of venous thromboembolism (VTE) as well as for prediction of VTE recurrence, for prediction of thrombosis in medically hospitalized patients, and in diagnosis and monitoring of disseminated intravascular coagulation [8,9]. D-dimer testing has now become an integral part of the validated algorithm for the diagnosis of deep vein thrombosis and pulmonary embolism [9]. Hence, to ensure patient safety, it is important to determine the factors in total testing processes which may impair the clinical efficiency of D-dimer testing. For this purpose, at first, a clinical criterion is required to assess the impact of the variables on D-dimer measurements, especially in pre-analytical and analytical phases [8]. However, there is no official recommendation or an evidence-based specification for D-dimer, and an arbitrary threshold is generally used [8,10].

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The present study was aimed to estimate the components of BV and the aspects related to BV data including analytical performance specifications (APS), the index of individuality (II), and reference change value (RCV) for D-dimer in a group of healthy subjects by applying a study protocol compliant with the BIVAC.

Materials and methods

Participants

Twenty-five healthy adult volunteers from Turkey were enrolled in the study. Exclusion criteria were history of VTE, diagnosed diabetes or fasting serum glucose >7.0 mmol/L, infection [C-reactive protein(CRP) >5 mg/L], liver disease [alanine aminotransferase (ALT) >40 U/L or aspartate aminotransferase (AST) >40 U/L or gamma-glutamyltransferase (GGT) >60 U/L], renal disease (estimated glomerular filtration rate <60 mL/min/1.73 m^2), prolonged prothrombin time (PT) (international normalized ratio >1.20) or activated partial thromboplastin time (APTT) (>40 sec), history of being a hospital inpatient within the last year, female subjects who were pregnant, surgery within the last year, history of recent medication, or history of tobacco or alcohol abuse (cigarette consumption ≥20/day or >60 gr ethanol/day).

Information on current health status and drug use was obtained from participants at each blood sampling visit. The subjects were requested not to alter their lifestyle during the study period.

The research related to human use has been complied with all the relevant national regulations, institutional policies, and the tenets of the Helsinki Declaration, and has been approved by the Local Ethics Committee of Harran University School of Medicine (Approval Number: 31.08.2020/HRU/20.15.21).

Blood collection

Blood collection was carried out three times for each participant using an evacuated blood collection system following EFLM recommendation for venous blood sampling [11], with a 1-week interval between sampling and at the same time of day (between 8:30 and 10:30 in the morning) (September-October 2020).

Fasting blood samples were drawn into a 2 mL tube containing 3.2% sodium citrate (VACUETTE®, Greiner Bio-One, Austria) for D-dimer, PT, and APTT measurements, and an 8 mL serum tube with gel separator (VACUETTE® Z Serum Sep Clot Activator, Greiner Bio-One, Austria) for biochemical measurements including glucose, creatinine, ALT, AST, GGT, and CRP.

Plasma and serum were obtained by centrifugation at 2000 *g* for 15 min and 1500 *g* for 10 min, respectively, within 1 h of collection. D-dimer and CRP levels were determined in each sample, while other analytes were only measured in the samples obtained at the first visit. For D-dimer measurements, plasma samples were transferred to microtubes and stored at −20 °C for 6 weeks from the collection of the first sample to the analysis of the samples. D-dimer is stable for up to 24 months if frozen [12]. Other measurements were carried out in fresh samples. The visual examination was performed for lipemia, icterus, or hemolysis in all samples.

Analytical method

For D-dimer, all samples for all enrolled subjects were tested in the same analytical run, in duplicate. D-dimer was analyzed on Cobas 6000 c501 analyzer (Roche Diagnostics, Germany) by particle-enhanced immunoturbidimetric assay using Tina-quant D-dimer Gen.2 reagents. Two levels of quality control material (D-Dimer Gen.2 Control I/II, Roche Diagnostics) were analyzed before running the samples. In addition to this, based on the last 20 control results, between-day imprecision for D-dimer was determined to be 4.8% and 2.3% at concentrations 850 µg/L and 3750 µg/L, respectively.

Plasma samples were also tested for PT (MTI-1101 PT Thromboplast reagent) and APTT (MTI-1120 APTT S reagent) on MTI Novae coagulation analyzer (Tokra Medikal, Turkey) by the optical method. Serum glucose (GLUC3 reagent with the hexokinase method), creatinine (CREJ2 reagent with the Jaffe method), ALT (ALTL reagent with the enzymatic method according to IFCC without pyridoxal phosphate activation), AST (ASTL reagent with the enzymatic method according to IFCC), GGT (GGT-2 reagent with the enzymatic method standardized against IFCC) and CRP (CRP4 reagent with the particle-enhanced immunoturbidimetry method) values were determined on Cobas 6000 c501 analyzer (Roche Diagnostics, Germany) according to manufacturer's instructions.

Statistical analysis

The data set was examined for outliers at three levels. The Cochrane C test [13] was performed for outlier identification among duplicate measurements from subjects as well as for searching the outliers in the variance of the means of duplicates among the subjects. Outliers among the mean values (the mean of the mean of duplicates) of subjects were examined by the Dixon-Reed criterion [14].

After exclusion of the outliers, the normal distribution assumption was checked by the D'Agostino Pearson test [15] for within-subject data and by the Shapiro-Wilk test [16] for between-subject data. To assess the homogeneity of within-subject and analytical variances, the Cochrane C test was performed. Moreover, the steady-state of the subjects was determined by linear regression analysis of the mean value of the measurements obtained from all subjects in each blood collection week against the number of corresponding points (1-3) [17]. If the 95% confidence interval (CI) of the slope of the regression line included 0, the subjects were considered to be at the steady-state [17].

CV_I, CV_G, and CV_A were dissected by using analysis of variance (ANOVA) with a nested random design in two levels. To present variances as the coefficient of variance, the square root of the respective variance was divided by the

overall mean. The 95% CIs of BV estimates were calculated using the formulas proposed by Burdick and Graybill [18].

BV estimates were determined for the whole group as well as separately for female and male subgroups. When the 95% CI for BV estimates of females and males overlapped, it was considered that there was no significant difference between gender subgroups. Besides, the Student t-test was used for comparison between two means. $p < 0.05$ was considered to be statistically significant.

APS for imprecision, bias, and total error was calculated from BV data using the formula proposed by Fraser et al. at three quality levels, namely, minimum, desirable, and optimal [19].

From the BV components, the II was derived using the formula defined by Harris [20] as $(CV_A^2 + CV_I^2)^{1/2}/CV_G$. RCV for D-dimer was calculated as $RCV = 2^{1/2} \times Z \times (CV_A^2 + CV_I^2)^{1/2}$, where the bidirectional Z-scores of 1.96 for a 95% probability was used. By considering the number of participants, samples, and replicates as well as the ratio between analytical standard deviation (SD_A) and within-subject standard deviation (SD_I), the 95% CIs of RCV were calculated using the tables shown by Røraas et al. [21].

The power of the study design for estimation of CV_I was determined based on the number of replicates, samples, and individuals as well as the ratio of SD_A to SD_I [21].

Data analyses were performed using Minitab (Minitab Inc, version 17.1.0, PA, USA), XLSTAT (Addinsoft Inc, version 2014.5.03, New York, USA), Graphpad Prism (GraphPad Software, version 7.04, CA, USA).

Table 1. General characteristic of the study population.

Characteristic	Value (Median with Minimum to Maximum)
Sex (Female/Male) (*n*)	16/9
Age (Years)	35 (19 to 61)
Body mass index (kg/m^2)	25 (18 to 33)
Prothrombin time (INR)	1.03 (0.9 to 1.15)
Activated partial thromboplastin time (s)	29.7 (25.0 to 36.6)
Glucose (mmol/L)	5.0 (4.4 to 6.2)
eGFR (mL/min/1.73 m^2)	107 (84 to 133)
Aspartate aminotransferase (U/L)	15 (11 to 25)
Alanine aminotransferase (U/L)	8 (6 to 30)
Gamma-glutamyltransferase (U/L)	10 (6 to 43)
C-reactive protein (mg/L)	0.9 (0.3 to 3.9)

eGFR: Estimated glomerular filtration rate calculated using the equation of Chronic Kidney Disease Epidemiology Collaboration based on creatinine; INR: International normalized ratio; s:seconds.

Results

Participants

All of the 25 enrolled subjects adhered to scheduled blood collections at weekly intervals for three weeks. The study population had a median age of 35 years and 64% were female. Alcohol intake was relatively low, with 76% reporting no alcohol intake and no subjects reporting heavy consumption. 20% of individuals were moderate smokers (<10 cigarettes/day). There was no history of any drug use during the blood collection phase.

Based on the results of biochemical and coagulation measurands, which are presented in Table 1, no subject was excluded.

Outliers, normality, and steady-state

When the homogeneity of the 75 duplicate variance was examined by the Cochrane C test [13], no outlier was identified. However, 1 individual was excluded according to the Cochrane C within-subject outliers test, and in addition to this, the identification of outliers among mean values of subjects by Reed-Dixon criteria [14] led to exclusion of one subject (Figure 1). Therefore, a total of 138 D-dimer results from 23 subjects were used in the final data analysis.

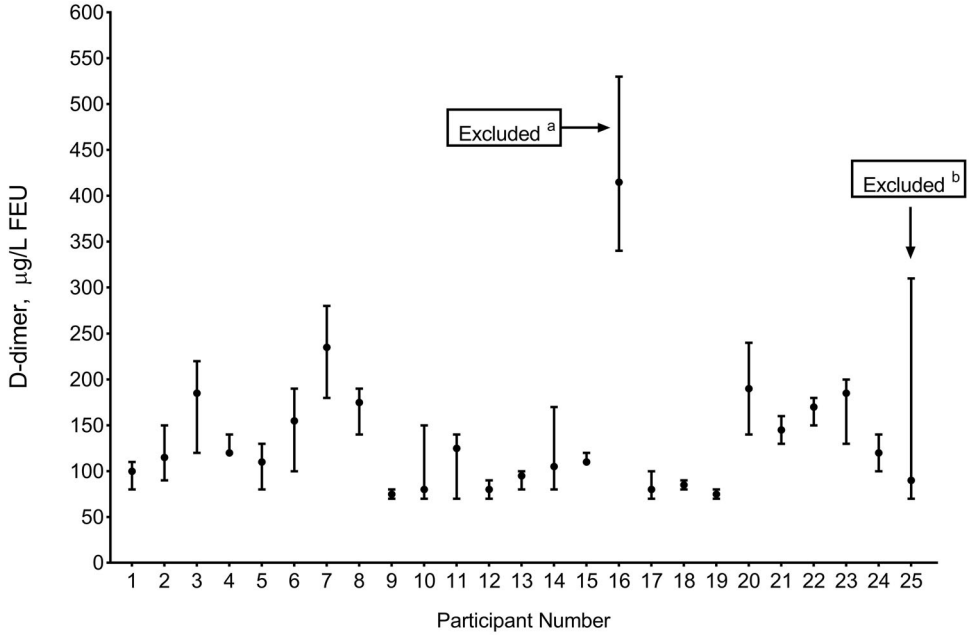

Figure 1. Median values with range (minimum to maximum) for D-dimer for each participant ordered by gender. Individuals 1 to 16 were female and individuals 17 to 25 were male. The arrows indicate excluded subjects because of Dixon-Reed criterion ([a]) or Cochrane C test ([b]).

Table 2. Biological variation estimates for D-dimer with 95% CI, accompanied by the II and RCV.

Parameters	All subjects ($n = 23$)	Female ($n = 15$)	Male ($n = 8$)
Mean (95% CI), (µg/L FEU)*	125.9 ± 46.9	123.8 ± 47.1	129.8 ± 46.7
CV_I (95% CI), %	21.2 (17.8 to 25.9)	23.5 (18.6 to 31.6)	16.4 (11.8 to 25.7)
CV_G (95% CI), %	30.9 (21.3 to 46.2)	30.5 (20.0 to 49.9)	33.6 (21.2 to 70.5)
CV_A (95% CI), %	5.20 (4.4 to 6.3)	5.24 (4.4 to 6.4)	5.09 (4.3 to 6.2)
II	0.71		
RCV (95% CI), %	60.4 (51.4 to 67.6)		

CI: Confidence interval, *: Mean value (95% CI) determined after elimination of outliers, CV_I: Within-subject biological variation, CV_G: Between-subject biological variation, CV_A: Analytical coefficient of variation, II: Index of individuality, RCV: Reference change value.

Table 3. Analytical performance specifications for imprecision, bias, and total error based on the biological variation estimates as reported in Table 2.

Quality Level	APS for imprecision, %	APS for bias, %	APS for total error, %
Optimal Performance	5.3	4.7	13.4
Desirable Performance	10.6	9.4	26.8
Minimal Performance	15.9	14.0	40.22

APS: Analytical performance specification.
Optimal performance: Imprecision= $0.25 \times CV_I$, Bias= $0.125\ (CV_A^2 + CV_G^2)^{1/2}$, Total Error= $0.125\ (CV_A^2 + CV_G^2)^{1/2} + 1.65\ (0.25 \times CV_I)$.
Desirable performance: Imprecision= $0.50 \times CV_I$, Bias =$0.250\ (CV_A^2 + CV_G^2)^{1/2}$, Total Error= $0.250\ (CV_A^2 + CV_G^2)^{1/2} + 1.65(0.5 \times CV_I)$.
Minimal performance: Imprecision = $0.75 \times CV_I$, Bias = $0.375\ (CV_A^2 + CV_G^2)^{1/2}$, Total Error= $0.375\ (CV_A^2 + CV_G^2)^{1/2} + 1.65\ (0.75 \times CV_I)$.

After removal of outliers, the normal distribution assumption for D-dimer was confirmed for the whole population as well as for the female and male subgroups by D'Agostino Pearson [15] and Shapiro-Wilk test [16].

Regression analysis revealed that no significant trend was detected for D-dimer among the participants during the 3 weeks of blood collection, confirming that the variation reported in the current study is physiological.

The BV data and applications

The mean, CV_I, CV_G, and CV_A estimates of D-dimer for all subjects as well as separately female and male subgroups are shown in Table 2. For CV_I and CV_G estimates, the 95% CI of females and males was found to be overlapping. Also, there was no significant difference in mean values of D-dimer between the two subgroups ($p = 0.47$).

Hence, overall CV_I and CV_G estimates were applied in the calculation of II, RCV, and APSs. The II value of D-dimer was found to be 0.71, and RCV was 60.4% with 95% CI 51.4 to 67.6.

APS including minimum, desirable and optimal targets for precision, bias, and total error for D-dimer derived BV data were given in Table 3.

For our study design with 23 individuals, 3 samples, 2 replicates and the ratio of 0.25 between SD_A and SD_I, the power of our study was 1.0.

Discussion

To our knowledge, this study is the first to estimate the BV for D-dimer largely in compliance with BIVAC. In the current study, we observed the BV of D-dimer as relatively high, with CV_I and CV_G estimates of 21.2% and 30.9%, respectively. In the online 2014 BV database [4], only 1 study delivering BV estimates for D-dimer in a healthy population is included. On the other hand, up to the present, there is no data on BV estimates of D-dimer in the EFLM BV database [5].

In one study fulfilling inclusion criteria for the online 2014 BV database, blood samples had been collected from a cohort of 18 healthy women once monthly for 10 consecutive months [22]. The authors transformed D-dimer concentrations to multiples of the median and then calculated the components of BV using these. Despite the modified data and differences in sampling interval, authors reported CV_I (23.8, 95%CI: 18.2 to 29.6) and CV_G (26.5%, 95%CI: 18.2 to 42.8) estimates of a similar magnitude to that observed in the present study.

Apart from the study cited in the 2014 database [4], only two studies have estimated the BV of D-dimer in healthy or diseased populations. One of these studies was carried out in 1995 by Atherosclerosis Risk in Communities study group in adults from U.S. communities [23]. They collected blood samples three times from 39 healthy volunteers, with a 1or 2-week interval between each sampling. For D-dimer, CV_I was reported to be 17.4%, which is lower than that estimated in our study. However, BV data are not given with CI, making direct comparison difficult. The other study by Engelberger et al. [24] assessed BV of biomarkers relevant to atherosclerosis, including D-dimer, by comparing the results obtained from blood samples drawn at baseline visit and three follow-up visits at 3, 6, and 12 months. When comparing the CV_I (25.2%, 95%CI: 20.2 to 30.5) and CV_G (30.6%, 95%CI: 19.2 to 42.9) estimates from the study by Engelberger et al. [24] with those in our study, CIs was observed to be overlapping. However, the authors have not

taken into account CV_A for estimation of CV_I estimate, and in addition to this, the sampling interval is very different between the two studies. Hence, the comparison of BV estimates from the two studies is not reasonable.

The Stockholm conference held in 1999 proposed a hierarchy model for setting performance specifications [25]. In 2014, a follow-up conference organized in Milan, this approach was simplified and described by three models, namely, clinical outcome model, BV based model, and a state of art based model [26]. APS derived from the clinical outcome is considered to be ideal, but the application of this model is possible for just a few measurands. For D-dimer, data for the clinical outcome model is not available, therefore, the BV model may be the best approach at present. The CV_A obtained from duplicate measurements in our study met the APS for imprecision, defined as half the CV_I [1]. Hence, the APSs provided in the present study for D-dimer may safely serve many purposes during laboratory quality management.

In the current study, marked individuality was observed for D-dimer, with the II of 0.71. Harris stated that the use of conventional reference intervals for monitoring patients is limited when II <0.6 and acceptable when II >1.4 [20]. In this situation, if available, comparing the current result with the previous result is considered to be more beneficial than population-based reference intervals. The most appropriate tool to determine whether a difference between consecutive measurements is significant is by use of RCV [27]. The II for D-dimer was calculated to be approximately 0.6 in our study. For this reason, the use of RCV is more helpful in making clinical decisions during patient monitoring by measuring D-dimer, such as determining the optimal duration of anticoagulation therapy in VTE. The RCV can also be used to determine both delta check limits and autoverification rules [27]. Additionally, it should be kept in mind that the RCV of D-dimer obtained using CV_A based on duplicate analysis of samples as in the present study is not universal. Hence, each laboratory must calculate the local RCV of D-dimer using own CV_A estimates.

In a recent review [8], authors reported that a clinically acceptable cut-off of 10% has frequently been used to assess a significant preanalytical change in interference and stability studies regarding D-dimer. The authors emphasized that this empirical threshold should be validated by performing BV studies. The data from this study may contribute to fill this requirement. In this context, APS for desirable bias from our BV data was observed to be close to the cut-off of 10%.

Similarly, in another recent paper by Lippi et al. [10], a threshold of 10% has been suggested for the inter-assay CV of D-dimer immunoassays. This is in concordance with the APS for desirable imprecision derived from BV data provided in the present study.

Ricós et al. have stated that the number of subjects studied and samples collected is not of great importance for BV studies, but even so, they have recommended at least 10 subjects and 5 samples [28]. Besides, Røraas et al. demonstrated that the width of the CI of estimated CV_I, which is a good indicator of the reliability of the estimate, is influenced by the number of individuals, the number of samples, the number of replicates as well as the ratio of CV_A and CV_I [21]. In the current study, 23 subjects were included to estimate BV data, but only 3 samples could be collected from each individual. However, for our design with 23 individuals, 3 samples, and a ratio of 0.25 between CV_A and CV_I, the width of CI for CV_I was 8.1%. When increasing the number of samples from 3 to 4, using the table created by Røraas et al. [21], the expected width of CI for CV_I (8.04%) was observed to be unchanged. Also, there was no great reduction (from 8.1% to 6.15%) in the expected width of CI by increasing the number of samples from 3 to 6. In addition to the width of CI for CV_I, Røraas et al. [21] have stated that the power of the study, which is the probability to detect a CV_I different from 0, should be estimated to evaluate the reliability of estimates of CV_I. They have shown that the power will be 1.0 for most designs if the ratio between SD_A and SD_I is below 1.0. Accordingly, for our study design, the power was determined to be 1.0.

Our study has some limitations. Firstly, D-dimer analysis was performed using a single instrument and reagents from only one manufacturer. The reagent and instruments from different manufacturers may represent different performances, but BV estimates are probably not affected by this [29,30]. Secondly, the number of subjects in gender subgroups was not equal, with predominantly female in the study. However, there were no evident differences in the BV estimates between females and males. Moreover, even if taking into account only the male subgroup, the power of the study was calculated to be 1.0. Third, the participants were mainly young individuals, thus it is uncertain whether the findings of our study can be generalized to older individuals. Finally, our D-dimer data was based on the latex-enhanced immunoturbidimetric assay. Although microplate ELISA has long been considered the reference method, immunoturbidimetric assay shows good agreement with this technique and is widely used in the diagnostic laboratory [8,31].

In conclusion, the present study has provided the BV estimates of D-dimer following a highly powered experimental design and a rigorous statistical approach complying with the BIVAC [6,7]. The APS derived from this BV data may therefore allow clinical laboratories to define objectively quality requirements for D-dimer. Moreover, based on the BV data, clinicians should prefer the RCV to judge whether any change observed in D-dimer results over time is significant.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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