



## Research paper

## Biological variation of peripheral blood T-lymphocytes

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## ABSTRACT

**Background:** Flow cytometric analysis of the lymphocyte subsets has become one of the most commonly used techniques in the routine clinical laboratory. It is frequently used in monitoring lymphocyte recovery after hematopoietic stem cell transplantation (HSCT), as well as diagnosis and treatment of acquired immunodeficiency syndrome (AIDS). Reliable biological variation (BV) data is needed for safe clinical application of these tests. In this study, similar preanalytical and analytical protocols to the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) checklist were followed and a stringent statistical approach was applied to define BV of T-lymphocytes.

**Methods:** During the 10 weeks study period, weekly blood samples were obtained from 30 healthy individuals (20 females, 10 males) and analyzed with Facs Canto (BD Biosciences, San Jose, CA, USA) analyzer using 4-colour BD Multitest CD3/CD8/CD45/CD4 reagents. Data were assessed in terms of normality, tendencies, outliers and variance homogeneity prior to applying coefficient of variance (CV)- analysis of variance (ANOVA) test. Sex-stratified within-individual (CV<sub>i</sub>) and between-individual (CV<sub>G</sub>) BV estimates of CD3+, CD3 + CD4+, CD3 + CD8+, and CD3 + CD4 + CD8+ T lymphocytes were calculated.

**Results:** No difference was found between males and females. Except for the CD3 + CD4 + CD8+ subset, stable BV was found for CD3+, CD3 + CD4+, and CD3 + CD8+ subsets.

**Conclusion:** Instead of using the conventional reference ranges of CD3+, CD3 + CD4+ and CD3 + CD8+ counts for monitoring HIV positive or post-HSCT patients, RCV should be used. Because individuality characteristic of lymphocytes subsets RCVs should be used instead of RIs for patient monitoring.

## 1. Introduction

Flow cytometric analysis of the lymphocyte subsets has become one of the most commonly used techniques in the routine clinical laboratory. Lymphocyte subsets analysis is used in evaluating immunological state, diagnosing immune diseases such as primary immunodeficiencies, autoimmune diseases and malignant tumors, and evaluating the efficacy of medical treatment or monitoring the course of the disease. Additionally, it is frequently used in monitoring lymphocyte recovery following hematopoietic stem cell transplantation (HSCT), and diagnosis and treatment of acquired immunodeficiency syndrome (AIDS) (Miyawaki et al., 1984; Abo et al., 1985; Termorshuizen et al., 2002).

Previous studies have documented that lymphocyte subsets can be influenced by race, age, medications, gender, stress, physical activity, and lifestyle. It follows a circadian rhythm, and has been shown to vary between different seasons and over years (Afoke et al., 1993; Paglieroni and Holland, 1994; Mazzoccoli et al., 2010). Establishing reliable criteria for accurate interpretation of these tests is a major task for clinical laboratories. In order to assure safe and valid clinical interpretation of these tests, objective analytical performance specifications (APS) are necessary together with robust knowledge of their biological variations (BV). Two components of BV are the between-individual variation, which occurs due to heterogeneity of the physiological effects between individuals, and the within-individual variation, which is the result of the biological variability in the same individual over time (Ceriotti

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et al., 2017; Sandberg et al., 2015).

There have been concerns regarding the quality of the earlier studies on BV (Afoke et al., 1993; Paglieroni and Holland, 1994) and consequently, the reliability of the BV estimates that were reported in these studies as well as the estimates published in the online 2014 BV database (Carobene, 2015). Moreover, the online database only provides estimates for the percent value of CD4 subset and no other data regarding other lymphocyte subsets (Minchinella et al., 2019).

The present study aims to determine BV estimates for absolute values of CD45 + CD3+ that are commonly used for monitoring HIV(+) and post-HSCT patients, T helper cells (CD45 + CD3 + CD4+), cytotoxic T cells (CD45 + CD3 + CD8+) and double positive T (CD45 + CD3 + CD4 + CD8+) cells, using 4 colored flow cytometry in a single platform (BD TruCount tube Biosciences, San Jose, CA, USA). In order to assure derivation of highest quality BV estimates for calculation of APSs and other BV-related applications, similar preanalytical and analytical protocols with the European Federation of Clinical Chemistry and Laboratory Medicine (EFLM) checklist were followed and a rigorous statistical approach was applied.

## 2. Material and methods

This study was conducted at the Hematology Laboratories of Ankara Numune Training and Research Hospital, Ankara, Turkey. A total of 30 subjects (10 male, 20 female) with a mean age of 35 years (range 22–54) were recruited to estimate the biological variations of T cells (CD3+), helper T cells (CD3 + CD4+), suppressor T cells (CD3 + CD8+), double positive (DP) T cells (DT) CD3 + CD4 + CD8+ cells. The participants were checked to be in healthy state, and did not take any medications or herbal supplements or smoke. Individuals having one of the following combined diseases/situations were not included in the study: 1) concomitant autoimmune or autoinflammatory disease; 2) acute or chronic infection; 3) malignancy; 4) systemic diseases such as diabetes mellitus or heart failure; 5) pregnancy or postpartum 6 months. During the study period, the participants maintained their normal life styles. They did not eat a specific diet, and avoided exhaustive physical exercise. Serum alanine aminotransferase (ALT), creatine kinase (CK), triglyceride (TG) and C-reactive protein (CRP) levels of all participants were monitored weekly during the 10 weeks period. Whenever a participant did not feel good on the day of sampling, blood sampling was postponed, and all blood samples were drawn when the participants felt they were completely in a healthy state. Blood collection was performed under standardized conditions to minimize pre-analytical variation. The specimens were collected after 10 min of rest in a seated position. Venous blood samples were collected by the same phlebotomist between 8 and 10 am on the same day for 10 consecutive weeks between April 2017 and June 2017. 2 mL of blood was drawn after 12 h of fasting, into K<sub>3</sub>EDTA containing tubes (Becton Dickinson BD, Germany) of the same lot number. All blood samples were transported to the laboratory under identical conditions in terms of temperature and elapsed time. The study protocol was approved by the Ethical Committee of Ankara Numune Training and Research Hospital. All participants signed informed consent.

## 3. Methods of flow cytometric determination

Absolute count of peripheral blood lymphocyte subsets was measured using a 4-colour BD Multitest CD3/CD8/CD45/CD4 reagent (BD Biosciences, San Jose, CA, USA) (containing anti-CD3- FITC, anti-CD8-PE, anti-CD45-PerCP, and anti-CD4-APC monoclonal antibodies). Each sample was transferred into a BD TruCount tube (BD Biosciences, San Jose, CA, USA), and was processed with a “lyse no wash” protocol. Briefly, 50 µL of freshly drawn peripheral EDTA blood were added into TruCount tube using reverse pipetting and stained with 20 µL CD3/CD8/CD45/CD4 BD Multitest reagents and incubated at room temperature in the dark for 15 min. Then, erythrocytes were lysed with

450 µL BD FACS lysing buffer (BD Biosciences, San Jose, CA, USA) incubated at room temperature in the dark for 15 min. The mixture was analyzed using a FACS Canto analyzer (BD Biosciences, San Jose, CA, USA). Prior to sample analysis, a series of quality control microspheres and reagents in a flow cytometry system were used to complete the instrument calibration and compensation settings. After loading the samples, the instrument automatically collected at least 20,000 cells; the data were analyzed with BD FACSDiva software. The proportion and absolute count of lymphocyte subsets including T cells (CD3+), helper T cells (CD3 + CD4+), suppressor T cells (CD3 + CD8+), DP T cells CD3 + CD4 + CD8+ populations were determined. Each sample was measured in at least duplicates. Internal quality control procedures were used to assess instrument parameters and ensure accurate results. Before each run, Cytometer Setup and Tracking beads of the same lot were used for optimizing the system. The beads allow the software to automatically characterize, track, and report measurements of supported BD digital flow cytometers. The laboratory participated in external quality programs for leukocyte immunophenotyping (UK-Neqas) to ensure accurate CD3, CD4, CD8 results during the study period.

### 3.1. Statistical analysis

#### 3.1.1. Identification of the outliers

Prior to analysis of data, outlier values were checked in three steps: (i) presence of outliers between the replicate measurements was checked with Cochran test; (ii) in order to determine whether an individual's distribution was greater or smaller than those of the group as a whole, variance distributions of each individual were examined, and the outlying individuals were identified using Cochran test (Cochran, 1941; Snedecor and Cochran, 1989); (iii) whether a mean value of any individual was significantly different compared to other individuals was investigated using Reed criteria (Dixon, 1953). In each outlier identification step, those individuals that are observed to have outlier value were excluded from the analysis.

#### 3.1.2. Normality assessment

For each analyte, normality assessment was performed separately for each individual using Shapiro-Wilk test (Shapiro and Wilk, 1965). In case > 50% of the individuals did not fit in normal distribution, logarithmic transformation was applied to all data. Normality was checked once again with Shapiro-Wilk test after logarithmic transformation. Additionally, whether the arithmetic means of the individuals were distributed normally was checked with Shapiro-Wilk test. After checking for assumption of normal distribution, it was found that > 50% of the individuals showed normal distribution for every analyte. Thus, as recommended by Braga and Panteghini (Braga and Panteghini, 2016), it was decided to continue the analyses with ANOVA.

#### 3.1.3. Comparison for gender

In this step, Student *t*-test was used to examine whether there was a significant difference between the two sexes regarding each analyte. In addition, homogeneity of variances was examined with Bartlett test.

#### 3.1.4. Analysis of variance (two-fold nested ANOVA)

Data were analyzed using analysis of variation (ANOVA)-coefficient of variation (CV), a type of ANOVA in which data is first subjected to CV-transformation (Røraas et al., 2016). Data from males and females were analyzed separately. The difference between male and female CV<sub>1</sub>s, and between individual BV (CV<sub>G</sub>) estimates were calculated as described by Burdick and Graybill, in consideration of the overlap between 95% confidence intervals (CI) (Burdick and Graybill, 1992). When there was no overlap between the 95% CIs of male and female mean values, the lower of the two CV<sub>G</sub> estimates was used for calculating APS. In case there was no significant difference between males and females, CV<sub>1</sub> and CV<sub>G</sub> values were reported for all individuals, and these estimates were used in application of BV data.

### 3.1.5. Analytical performance specifications (APS) and other applications

$CV_I$  and  $CV_G$  data were used for calculating the number of samples required to estimate performance specifications desired for imprecision (CVAPS) and bias (BiasAPS), individuality index (II) and RCV. The following equations were used for this purpose: CVA refers to the analytical variation, (Fraser, 2001a; Aarsand et al., 2015)

$$\begin{aligned} CV_{APS} &< 0.5 CV_I \\ Bias_{APS} &< 0.25 (CV_I^2 + CV_G^2)^{1/2} \\ II &= CV_I / CV_G \\ RCV &= 2^{1/2} * Z * (CV_A^2 + CV_I^2)^{1/2} \end{aligned}$$

and D refers to the allowable percent deviation from the true homeostatic set point; Z is 1.96 (for  $p$  value < .05). We calculated with 5%, 10%, and 20% deviations from the true homeostatic set points.

## 4. Results

The mean ages of the females and males were 32 (range, 22–53) and 40 (range 25–54) years, respectively. During the course of the study, 1 case was excluded due to elevation in CRP level, and 1 case was excluded due to initiation of antibiotic treatment for dental abscess. The study was completed with the remaining 28 participants (20 female, 8 male). According to Shapiro-Wilk test, > 50% of the samples did not meet normal distribution. Therefore, logarithmic transition was applied, and data were evaluated once again for normality with Shapiro-Wilk test. The data showed normal distribution after logarithmic transformation (normality percents were 51%, 50%, 50%, 52%, and 52% for CD3+, CD3 + CD4+, CD3 + CD8+, CD3 + CD4 + CD8 respectively). All individuals were presumed to be in a steady state throughout the study period.

Student  $t$ -test results showed no significant differences between the two sexes regarding CD3+, CD3 + CD4+, CD3 + CD8+, and CD3 + CD4 + CD8+ ( $p = .89$ ,  $p = .10$ ,  $p = .78$  and  $p = .29$ , respectively). Therefore, the results didn't give separately for men and women. Variance homogeneity was analyzed with Bartlett-test, and no difference was found between the sexes. Therefore, male and female subjects were evaluated as a whole. In order to determine whether an individual's distribution was greater or smaller than those of the group as a whole, variance distributions of each individual were examined, and the outlying individuals were identified using Cochran test.

### 4.1. Within-individual and between-individual variations

$CV_I$ ,  $CV_G$ ,  $CV_A$  and II are presented in Table 1. For all parameters,  $CV_A$  was < 0.5%. The highest  $CV_I$  and  $CV_G$  values were found for CD3 + CD4 + CD8+ cells/ $\mu$ L (18.15% and 57.93%, respectively), whereas the lowest  $CV_I$  and  $CV_G$  values were found for CD3+ and CD3 + CD4+ (10.78–26.18% and 11.54–20.69%, respectively) (Table 1).

### 4.2. Determination of reference change value or critical difference

CIs and RCVs of all parameters were calculated (Table 1). RCV was used to determine whether there was a significant difference (increase or decrease) between two consecutive test results of an individual. While 95% probability ( $p < .05$ ) indicates presence of significant difference (probability of randomness is 5%), 99% ( $p < .01$ ) probability represents highly significant difference (probability of randomness 1%). According to the results, RCV of CD3 + CD4 + CD8+ cells/ $\mu$ L was higher than RCVs of other parameters. We could not compare our BV estimates since there is no data in online 2014 BV database. All parameters that were examined in this study were found to have II values lower than 0.60 (Table 1). Our quality specifications related to BV,  $CV_{APS}$ , is shown in Table 2. While CD3 + CD4 + CD8+ showed the highest  $CV_{APS}$  (10.4%), the  $CV_{APS}$  of CD3+, CD3 + CD4+ and

CD3 + CD8+ were within a narrower range (5.6%, 5.8%, and 7.1%, respectively).

## 5. Discussion

Flow cytometric analysis of absolute counts of lymphocyte subsets is a non-invasive and rapid method frequently used in the clinical practice especially for monitoring HIV and immune recovery after HSCT. Clinical decisions are based on the results of these analyses. Total variation in the laboratory results consists of preanalytical, analytical and the inherently present biological variations. Although there is a great deal of data related to preanalytical and analytical variations of lymphocyte count, there is no clear data on the biological variation of lymphocytes. In this study, we documented biological variation of T lymphocyte subsets in healthy individuals. We believe our findings will contribute greatly to the existing database, as well as help physicians particularly when monitoring patients with HIV or post-HSCT. Our study becomes more important when we consider the fact that majority of previous studies on biological variation of lymphocyte subsets are outdated (Backteman and Ernerudh, 2007). Therefore, their outdated data has lost its significance considering the major technical advances in analytical methods for counting lymphocyte subsets and in production of monoclonal antibodies as well. Moreover, those previous studies did not evaluate the differences between the sexes (Backteman and Ernerudh, 2007; Tosato et al., 2013; Huang et al., 2015; Sekiguchi et al., 2011). We performed a sex-stratified analysis of BV estimates, but could not find any difference in this study.

Very recently, Tosato et al. (Tosato et al., 2013) and Huang et al. (2015) investigated the BV of lymphocyte subsets using flow cytometric analysis, and demonstrated low within- and between- individual BVs in lymphocyte subsets. Whereas Huang et al. investigated BV by taking three samples a day for a three day period and performing measurements flow cytometrically with dual platforms, Tosato et al. calculated BV with a single platform at first week, first month and third month by taking four samples a day. Our study is different from these two studies in terms of timing of sampling, analytical method, and also the statistical approach used. Namely; (i) the measurements were performed on a single platform, with 'no lyse no wash' method; (ii) we collected samples during 10 weeks (iii)  $CV_A$  estimates were calculated by measuring all samples at least in duplicates and (iv) a very stringent statistical approach was applied. Due to all these reasons, comparison of our findings with those two studies mentioned above becomes difficult. Our study is the first study on T lymphocyte subsets that is conducted in accordance with the recommendations of EFLM BV working group.

As shown in Table 1, the calculated  $CV_I$  and  $CV_G$  values and the individuality index (II) that is calculated as the  $CV_I/CV_G$  ratio provided information regarding the individuality of a certain laboratory result, and primarily the utility of reference ranges calculated for a population made of apparently healthy individuals (Sandberg et al., 2015). In our study, all calculated II values were < 0.6. Population-based reference ranges are not useful for interpreting the results when II is lower than 0.6. In such a case, RCV should be used to evaluate the changes in T lymphocyte subset counts.

In our study, we observed the highest RCV values for CD3 + CD4 + CD8+ cells, whereas other parameters had lower RCV values. A possible cause may be that CD3 + CD4 + CD8+ cells constitute a very small portion (0.5%) of lymphocytes in the peripheral blood. RCV varies according to the changes in the analytical precision. RCV can be used to determine whether there is a significant difference (increase or decrease) between the two consecutive test results of an individual if variations between two consecutive measurements occur in the analytical stage. This greatly aids especially while assessing clinical prognosis and treatment efficacy in HIV cases. In other words, RCV can detect whether an external factor (acute disease, therapy, physical exercise, diet, etc.) can affect the results of a certain parameter, and whether the observed variation is independent of the instrumental

**Table 1**

Within-subject (CVI) and between (CVG) biological variation estimates  $\pm$  95% CIs, mean concentrations, the associated estimates of analytical variation (CVA), the total number of subject and the average number of samples of Lymphocyte, CD3+, CD3 + CD4+, CD3 + CD8+ and CD3 + CD4 + CD8 tests.

Parameters	Number of subjects	Total number of results	Mean (95% CI)	CV <sub>A</sub> , % (95% CI)	CV <sub>I</sub> , % (95% CI)	CV <sub>G</sub> , % (95% CI)	II	RCV%	p value*	p value <sup>#</sup>
CD3+ (cells/ $\mu$ L)	All	380	1644.49	0.00	10.78	26.18	0.41	29.88		
	19		(1598.70–1690.28)	(0.0001–0.0003)	(9.75–12.06)	(19.65–38.89)				
	Females	260	1657.52	0.00	10.88	17.45	0.60	30.17	0.89	0.24
	13		(1617.22–1697.83)	(0.0001–0.0003)	(9.65–12.48)	(12.27–29.16)				
Males	120	1616.26	0.0002	10.54	42.22	0.25	29.21			
6		(1500.29–1732.23)	(0.0001–0.0003)	(9.65–12.48)	(26.22–1.00)					
CD3 + CD8+ (cells/ $\mu$ L)	All	480	541.13	0.0009	13.81	25.29	0.55	38.28		
	24		(527.40–554.86)	(0.0008–0.0010)	(12.62–15.25)	(19.46–35.73)				
	Females	340	571.44	0.0008	13.54	22.40	0.60	37.52	0.10	0.98
	17		(555.88–587.00)	(0.0007–0.0009)	(12.17–15.24)	(16.43–34.45)				
Males	140	467.52	0.0009	14.54	29.68	0.49	40.29			
7		(443.39–491.64)	(0.0008–0.0010)	(12.38–17.61)	(18.79–65.97)					
CD3 + CD4 + CD8+ (cells/ $\mu$ L)	All	320	16.51	0.0000	18.15	57.93	0.31	50.31		
	15		(15.44–17.58)	(0–0)	(16.28–20.52)	(42.61–89.91)				
	Females	200	17.05	0.0000	18.27	53.72	0.34	50.65	0.78	0.64
	10		(15.77–18.34)	(0–0)	(15.95–21.40)	(36.71–98.47)				
Males	120	15.60	0.0000	17.86	70.76	0.25	49.49			
5		(13.72–17.48)	(0–0)	(15.03–22.00)	(43.94–1.00)					
CD3 + CD4+ (cells/ $\mu$ L)	All	460	941.70	0.0004	11.54	20.69	0.56	31.99		
	23		(921.64–961.76)	(0.0003–0.0005)	(10.53–12.77)	(15.83–29.51)				
	Females	300	974.23	0.0004	11.41	19.71	0.58	31.62	0.29	0.93
	15		(949.73–998.72)	(0.0003–0.0005)	(10.19–12.95)	(14.22–31.40)				
Males	160	880.71	0.0004	11.81	22.48	0.53	32.73			
8		(847.74–913.68)	(0.0004–0.0006)	(10.15–14.11)	(14.59–46.24)					

Common estimate for both sexes. F, females; M, males; NA, Not Assessment.

\* p value for Student's *t* test for mean differences of gender.

# p value for F test for average within-subject total variance.

**Table 2**

Optimal, desirable, and minimum analytical goals for imprecision, bias, and total error calculated in relation to biological variability.

Analyte	Quality specifications								
	Imprecision %			Bias %			Total error %		
	Optimal	Desirable	Minimum	Optimal	Desirable	Minimum	Optimal	Desirable	Minimum
CD3+ ( <i>n</i> = 380)	< 2.8	< 5.6	< 8.4	< 7.0	< 3.5	< 10.5	< 11.6	< 12.7	< 24.4
CD3 + CD8+ ( <i>n</i> = 480)	< 3.5	< 7.1	< 10.7	< 7.6	< 3.8	< 11.5	< 25.4	< 15.6	< 17.4
CD3 + CD4 + CD8+ ( <i>n</i> = 320)	< 5.2	< 10.4	< 15.5	< 14.8	< 7.2	< 22.2	< 40.5	< 24.5	< 30.8
CD3 + CD4+ ( <i>n</i> = 460)	< 2.9	< 5.8	< 8.8	< 6.0	< 3.0	< 9.0	< 20.4	< 12.6	< 13.8

or biological variation. This is indeed very important when monitoring post-HSCT or HIV patients. It should be noted here that RCV calculated from biological variation in healthy individuals may not be the same that is observed in the presence of a disease (Fraser, 2001b). RCV should be used with caution in certain situations. RCV is calculated from CV<sub>A</sub> and CV<sub>I</sub> values; using RCV might not be appropriate when APS is not satisfactory for imprecision criteria. In our study, we found that APSs for CD3 + CD4 + CD8+ count was unsatisfactory (CV<sub>APS</sub> = 10.4%). Therefore, it is not appropriate to use RCV for evaluating CD3 + CD4 + CD8+ results. Whereas for CD3 + CD4+ and CD3 + CD8+ cells, which are the principle T cell subsets, BV data should always be considered when monitoring patients.

## 6. Study limitations

One possible limitation of the present study is that all analyses were performed using flow cytometer and monoclonal antibody from a single manufacturer. We did not find difference between BV estimates of the two sexes, although the number of males could be a little higher in our study.

## 7. Conclusion

The BV database does not include any data on T lymphocyte subset counts measure with flow cytometry on a single platform. The present study applied stringent preanalytical protocols and statistical methods and no difference was found between male and female subjects. Except for CD3 + CD4 + CD8+ subset, stable BV was found for CD3+, CD3 + CD4+ and CD3 + CD8+ T lymphocytes. Instead of using the conventional reference ranges of CD3+, CD3 + CD4+ and CD3 + CD8+ counts for monitoring HIV positive or post-HSCT patients, RCV should be used. Because individuality is characteristic of lymphocytes subsets RCVs should be used instead of RIs for patient monitoring. We believe our findings will provide reference and aid for both the database and future studies on this subject.

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