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An Italian national multicenter study for the definition of a reference ranges for normal values of peripheral blood lymphocyte subsets in healthy adults

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ABSTRACT

Background and Objective. **Reference ranges are necessary in clinical chemistry and hematology to compare an observed value and to provide meaningful information. The aim of this multicenter study was the definition of reference ranges of the relative and absolute numbers of lymphocyte subsets by evaluating a large cohort of healthy adults and by using a standard protocol to reduce the variability in both sample preparation methodology and flow cytometer operation. Other aims of this study were the evaluation of the influence of sex, age, obesity, smoking, sport and some methodological variables on lymphocyte subsets and the comparison of differential white blood cell values obtained by flow cytometry and those obtained by hematology counters.**

Design and Methods. **Blood samples from 1311 healthy adults (blood donors and volunteers chosen according to the Italian law for donor selection) were analyzed to study, by flow cytometry, the immunophenotype of lymphocyte subsets and their distribution in terms of percentages and absolute values. Pre-analytical and analytical phases were performed according to the guidelines of the *International Federation of Clinical Chemistry* (IFCC) and the *Italian Group of Cytometry* (GIC). T cells were defined by the expression of CD3; T subpopulations by the coexpression of CD4 or CD8 or HLA-DR; B-lymphocytes were identified by the expression of CD19 while natural killer lymphocytes were identified by positivity of CD16 and/or CD56 without CD3. We calculated for each laboratory and for all data collected the frequency distribution percent values and absolute values of each lymphocyte subset. The influence of age, sex, smoking, obesity and sport was calculated by the t-test. The influence of some methodological variables was calculated by the t-test and multiple regression test.**

Results. **Fifty-three flow cytometry laboratories at different institutions in Italy participated in this study. Data was obtained from 1311 healthy adults aged from 18 to 70; 968 phenotype analyses (74%) were considered eligible for statistical analysis. Significant results were found as regards sex, smoking and some methodological variables (quantity of sample, washing procedures, brand of monoclonal antibodies and kind of instruments used). The comparison between hematology counters and cytometers showed no difference for any of the parameters considered.**

Interpretation and Conclusions. **The large number of cases, the different kind of laboratories and their distribution throughout the country make our sample representative of the Italian adult population. The standardization criteria of pre-analytical and analytical phases (the most important issues in evaluating reference values for an indicator) have assured good reproducibility among laboratories so that the obtained reference ranges may be useful for inter-laboratory comparison of results; furthermore, the instruments and the brand of monoclonal antibodies may represent an inevitable cause of variability.**

Key words: flow cytometry, immunophenotype, standardization of analytical process, lymphocyte subpopulations, reference ranges

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The more extensive use of flow cytometry to detect peripheral blood lymphocyte subpopulations needs a careful definition of reference ranges in terms of subset percentage and absolute numbers in order to permit inter-institutional comparison of results. Therefore methodological criteria must be more accurately defined in order to reduce pre-analytical and analytical causes of variability.[1-3] The aim of this multicenter study was the definition of reference ranges of relative and absolute numbers of lymphocyte subsets by evaluating a large cohort of healthy adults and by using a standard protocol to reduce the variability in both sample preparation methodology and flow cytometer operations, so that the data might reflect inherent biological variability in the population and not simply variation in methods. The effort of standardization in order to reach an inter-laboratory reproducibility was extended to every step of the method: sample collection, conser-

vation and preparation; staining technique; panel of monoclonal antibodies; ways of analysis with particular attention to the purity of the lymphocyte gate, to the lymphocyte recovery and to the consistency of some check sums (lymphosum, correspondence of the sum of $CD3^+/CD4^+$ and $CD3^+/CD8^+$ with CD3) as internal quality control. These efforts were in accordance with guidelines from the *International Federation of Clinical Chemistry* (IFCC)[4] and the *Italian Group of Cytometry* (GIC).[5]

This kind of approach, together with the analysis of absolute values of single cell subpopulations, may be considered the contribution of this study to the better definition of reference ranges of lymphocyte subsets just published in other studies.[6-9]

Another aim of this study was the evaluation of the influence of sex, age, obesity, smoking and sport on lymphocyte subsets and the comparison of leukocyte formulas obtained by hematology counters with those calculated by cytometers.

To conclude, we wanted to evaluate the influence of some methodological variables which were not subject to any restriction criteria due to a lack of a wide consensus such as time and temperature of sample incubation, fixative employment and use of washing procedures; furthermore we analyzed the importance of the brand of monoclonal antibodies and of the kind of cytometer utilized.

Design and Methods

Fifty-three flow cytometry laboratories at different institutions in Italy participated in this study. Data was obtained from 1311 healthy adults; most of them were blood donors, the other volunteers were selected according to Italian law which specifies criteria of donor selection including case history, and physical and laboratory examinations. The criteria for standardization of pre-analytical and analytical phases, fixed to ensure reproducible data, are summarized in Table 1.

Each laboratory had to specify some aspects of the technique utilized: quantity of sample employed; time and temperature of incubation; which kind of lysant was employed; use of washing procedures after lysis; use of fixative; brand of monoclonal antibodies and kind of cytometer utilized.

The panel of monoclonal antibodies was defined to evaluated B-lymphocytes ($CD19^+$), T-lymphocytes ($CD3^+$), T subpopulations ($CD3^+/CD4^+$, $CD3^+/CD8^+$, $CD3^+/HLA\text{-}DR^+$), and natural killer (NK) lymphocytes ($CD16^+$ and/or $CD56^+$, CD3 negative).

Statistical analysis was performed with Windows CSS; for each laboratory we calculated mean, standard deviation (SD), median and range of lymphocyte subset percent values and of their absolute count. Subsequently we pooled the data and analyzed the frequency distribution of each variable; data with a Gaussian distribution were described by mean and standard deviation and the reference range was defined by the mean±2 SD, while, if the result of the Kolmogorov-Smirnov test was significant, the data was considered to have a non-Gaussian distribution and the reference range was defined as the central 95% of the area under the distribution curve of values (from 2.5 to 97.5%). The influences of age, sex, smoking, obesity and sport were evaluated by the t-test; the comparison between leukocyte differential counts obtained with hematolgy counters and cytometers was made by a paired T-test. Furthermore to display the results of this comparison, we chose the Altman method,[10] so we have plotted the difference between the results of the two instruments against the average. From this type of plot it is much easier to assess the magnitude of disagreement and to see the spot outliers and whether there is any trend.

The differences in the results due to the methodological variables, to the brand of monoclonal antibodies or to the kind of cytometer were evaluate by the t-test and multiple regression test.

Table 1. Criteria for standardization of pre-analytic and analytic phases.

1. Tests must be performed on whole blood samples collected in EDTA no more than 6 hours before.
2. Cell staining: each sample must be stained with the following panel of monoclonal antibodies (MoAb): a) CD45/CD14; b)Isotype negative control; c) HLA-DR/CD3; d) CD3/CD4; e) CD3/CD8; f) CD3/CD16+ CD56; g) CD3/CD19. MoAb may be conjugated with fluoroscein isothiocyanate (FITC) or phycoerythrin (PE). The NK subset may be identified with CD16 or with a blend of CD16+56. Absolute counts: hematology counters must be employed for WBC absolute and differential counts. Lymphocyte subset counts (number of cells/μL) are calculated by the formula: WBC x lymphocyte percentage x subset percentage. The cytometric formula must be calculated excluding debris on the dot-plot CD45 vs CD14 with a gate on positive events and detecting lymphocytes, monocytes and granulocytes on the dot-plot CD14 vs SSC.
3. Results: for each tube 10,000 events must be acquired; a lymphocyte gate must be drawn on physical parameters; lymphocyte percentage in the gate must be over 95% (gate purity); the gate must contain at least 90% of all lymphocytes (recovery). The percentage of CD3+ must be the same in each tube (±3%); The sum of CD3+CD4+% and CD3+ CD8+% should be close to the total amount of CD3+ T lymphocyte (±10%). The sum of CD3+%, CD19+%, CD16/CD56+% should be over 95%. If the CD4 absolute count is lower than 400 cells/μL it is necessary to repeat the analysis immediately and after one month.

Results

Fifty-three flow-cytometry laboratories at different institutions in Italy participated in this study; they comprised 27 transfusional services, 9 analysis laboratories, 7 microbiological laboratories, 3 anatomopathological services and 7 institutions of hematology. Thirty-eight were situated in northern Italy, 5 in central regions and 10 in southern Italy.

We collected 1311 samples for phenotype analysis of which only 968 (74%) fulfilled the requirements and were considered eligible. Data from 343 individuals was excluded from statistical analysis due to: a) lym-

Table 2. Lymphocyte subset percent and absolute number reference ranges.

	No.	*Mean*	*SD*	*Median*	*Reference ranges*
CD3	965	73.7	6.72	75	60-87
CD3 c/µL*	965	1532.91	463.49	1455	605-2460
CD4	946	45.09		45	32-61
CD4 c/µL	965	940.49		885	493-1666
CD8	965	26.44		26	14-43
CD8 c/µL	965	551.37		511	224-1112
NK	965	13.15		12	4-28
NK c/µL	965	278		240	73-654
CD19	916	11.07		11	5-20
CD19 c/µL	916	230.6		211	72-520
DR	711	16.77		16	6-31
DR c/µL	760	341.29		308	86-799
CD3/DR	769	5.79		5	1-18
CD3/DR c/µL	770	122.19		87.5	14-411

**c/µL=cells/µL.*

Table 3. Variability of results according to some biological parameters.

	Male (532)	*Female (436)*	*Overweight (140)*	*Normal weight (728)*
DR (%)	16.5	15.7	14.7*	16.0*
DR (c./µL)	344	337	338	355
CD 3 (%)	72.8*	74.7*	73.7	73.7
CD 3 (c./µL)	1490*	1590*	1652*	1517*
CD 4 (%)	44.2*	46.5*	45.1	45.3
CD 4 (c./µL)	902*	989*	1046*	926*
CD 8 (%)	26.7	26.0	25.7	26.6
CD 8 (c./µL)	548	557	573	549
NK (%)	13.9*	12.1*	12.9	13.2
NK (c./µL)	293*	259*	288	276
CD 19 (%)	10.7	10.7	10.9	10.6
CD 19 (c./µL)	225	226	266*	218*

**Significant differences.*

Table 4. Variability of results according to some life-style parameters.

	Smokers (224)	*Non smokers (650)*	*Sports (282)*	*No sports (590)*
DR (%)	17.3*	15.7*	16.0	15.9
DR (c./µL)	388*	320*	326	337
CD 3 (%)	73.8	73.6	73.5	73.8
CD 3 (c./µL)	1656*	1482*	1498	1537
CD 4 (%)	46.8*	44.8*	45.0	45.6
CD 4 (c./µL)	1044*	904*	916	950
CD 8 (%)	25.0*	26.9*	26.4	26.3
CD 8 (c./µL)	563	542	534	551
NK (%)	11.7*	13.7*	13.3	13.1
NK (c./µL)	260	285	278	277
CD 19 (%)	11.9*	10.4*	10.8	10.6
CD 19 (c./µL)	266*	213*	217	229

**Significant differences.*

phosum ($CD3^+CD19^+$ NK) <95 and/or purity of lymphocyte gate <95% (141 cases); b) lymphocyte collection <90% of total lymphocytes (70 cases); c) difference of ±10% between CD3 and $CD4^+CD8$ (45 cases); d) missed data (purity,CD19, NK) (70 cases); e) recent infections (4 cases); f) vaccination (2 cases); g) WBC over 15×10^9/L (1 case); h) WBC=1.5×10^9/L (1 case); i) eosinophil count over 20% (2 cases); l) monocyte count over 25%, (1 case); m) NK>40% (1 case); n) CD4 value < 400 cells/mL without a second determination (5 cases). The 968 eligible subjects comprised 532 males and 436 females aged from 18 to 70 years (mean±SD: 37±10.8; median: 36 years).

All laboratories sent data on lymphocyte subpopulation $CD3^+$, $CD3^+/CD4^+$, $CD3^+/CD8^+$. Data on $CD19^+$ subset and NK were missing in the data base of two laboratories; only 42 sites analyzed lymphocyte DR^+ and $CD3^+/DR^+$. The reference ranges obtained are shown in Table 2; only in the case of CD3 percent value the ranges were defined by mean±SD (Gaussian distribution).

The results of the analysis of the influence of sex, age, obesity, smoking and sport are shown in Tables 3 and 4. We found significant differences in results divided according to sex: as reported in previous studies,[8,9] we confirmed the presence of higher values of CD3 and CD4 lymphocytes and a lower value of NK cells in the female sex. Furthermore, we noted higher values of CD3, CD4 and CD19 subsets in chronic smokers, while their percentage of NK was lower.

Age, within this adult population, did not influence the results; in fact we saw only a small decrement in the CD4 percent values with increasing age.

The results of the comparison of the leukocyte differential counts obtained with hematology counters and with cytometers showed no differences for any of the considered parameters (neutrophils, lymphocytes and monocytes). The results of the comparison of lymphocyte and neutrophil counts, plotted according to the Altman method, are shown in Figures 1 and 2. In both figures it is possible to see that there are very few outliers and a good correlation between the results obtained by the two instruments (the paired t-test was not significant).

Further analysis of the data was performed in order to evaluate the importance of the methodological variables not submitted to standardization criteria. Table 5 shows the distribution of the cases analyzed according to the differences in the techniques employed; most of the laboratories used a sample volume of 100 µL, incubation at 4°C for more than 20 minutes, no washing method and no fixative. The amount of sample used influenced the percent and the absolute value of lymphocyte subsets DR^+ and $CD3^+/DR^+$; the laboratories that utlilized 100 µL obtained lower values of HLA-DR (15% and 334 c/µL vs 20% and 432 c/µL; p= 0.000 and p=0.003) and of $CD3^+/DR^+$ (5% and 107 c/µL vs 8.6% and 187 c/µL; p=0.000). The duration and the temperature of the

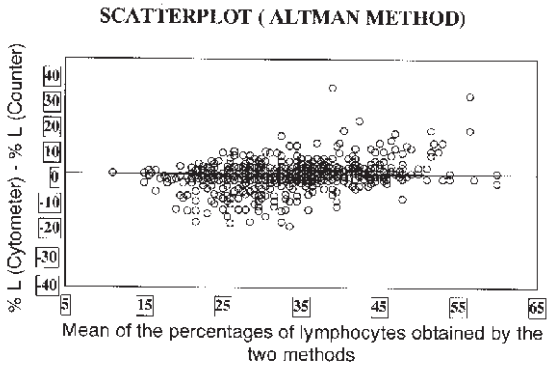

Figure 1. Comparison of the lymphocyte counts obtained by the two instruments plotted according to the Altman method.

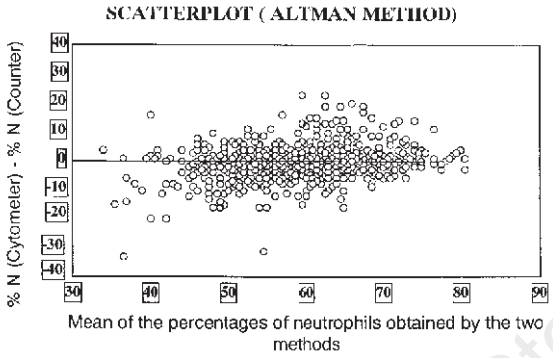

Figure 2. Comparison of the neutrophil counts obtained by the two instruments plotted according to the Altman method.

incubation did not influence the results, nor did the use of fixatives, while washing techniques led to a selective loss of lymphocyte subsets; infact after washing procedures a lower percent and absolute value of HLA-DR$^+$, CD19$^+$ and CD3$^+$/DR$^+$ lymphocytes and a lower percentage of NK cells was noted (HLA-DR: 16.1% and 332 c/µL vs 18.5% and 419 c/µL; CD19: 10.3% and 211 c/µL vs 11.1% and 289 c/µL; CD3/DR: 5.5% and 114 c/µL vs 7.5% and 168 c/µL; NK: 12.7% vs 13.8%).

The results of the comparison between the percent and absolute value obtained with different brand of monoclonal antibodies or with different kinds of instruments are summarized in Tables 6 and 7. Cytometers were grouped only on the basis of the manufacturing company, the instrument model was not considered because it was not always specified and also because we did not want to make the single groups too small to allow meaningful statistical comparison.

We obtained significant results in both comparisons; in particular, the kind of instrument appears

Table 5. Differences in techniques employed.

Quantity of sample	*Time of incubations*
100 µL: 1081 cases (82% 50 µL: 151 cases (12%) ND: 79 cases (6%)	30 m: 787 cases (60%) 20 m: 233 cases (18%) 15 m: 147 cases (12%) ND: 102 cases (9%)

Temperature of incubation	*Washing*	*Fixation*
4°C: 788 cases (60%)	Yes: 761 (58%)	Yes: 357 (27%)
20°C: 421 cases (32%)	No: 448 (34%)	No: 792 (60%)
ND: 102 cases (8%)	ND: 102 (8%)	ND: 162: (13%)

Table 6. Lymphocyte subsets: variations according to the brand of monoclonal antibodies.

		Becton Dickinson (668)	*Coulter (126)*	*Ortho (155)*	*Dako (19)*	*p*
DR	(%)	17.3	13.3	13.5	12.7	0.0000
	c./µL	363	297	283	293	0.0000
CD3	(%)	73.3	74.9	73.9	76.3	0.02
	c./ µL	1475	1504	1388	1400	N.S.
CD3/DR	(%)	6.9	2.1	3.7	2.3	0.02
	c./ µL	146	43	80	54	0.0000
CD4	(%)	44.5	47.7	46.4	46.5	0.001
	c./ µL	924	993	958	1056	0.03
CD8	(%)	26.8	24.8	26.0	28.5	0.01
	c./ µL	560	516	535	660	0.03
NK	(%)	13.4	10.8	13.8	11.1	0.0000
	c./ µL	285	225	289	247	0.006
CD19	(%)	10.7	12.1	9.5	10.4	0.0000
	c./ µL	227	249	201	237	0.05

**c/µL=cells/µL.*

Table 7. Lymphocyte subsets: variations according to the cytometer.

		Becton Dickinson (617)	*Coulter (133)*	*Ortho (168)*	*p*
DR	(%)	18.5	13.3	10.3	0.0000
	c./µL	386	294	231	0.0000
CD3	(%)	72.6	75.0	76.3	0.0000
	c./µL	1497	1561	1673	0.0006
CD3/DR	(%)	7.1	2.1	2.9	0.0000
	c./µL	152	43	64	0.0000
CD4	(%)	44.2	47.8	47.3	0.0000
	c./µL	909	993	1037	0.0003
CD8	(%)	26.5	25.0	26.9	0.03
	c./µL	550	520	586	0.04
NK	(%)	13.6	10.7	13.1	0.0008
	c./µL	286	221	298	0.006
CD19	(%)	11.2	12.1	7.9	0.0000
	c./µL	236	248	181	0.0002

Table 8. Differences in techniques employed, analysis performed by multiple regression.

Response variables	*Significative variables*
DR	
(%)	cytometer and microliters of sample
(absolute count)	cytometer, microliters of sample and washing
CD3	
(%)	cytometer, brand of monoclonal antibodies
(absolute count)	cytometer, microliters of sample and washing
CD3/DR	
(%)	cytometer, microliters of sample and brand of monoclonal antibodies
(absolute count)	cytometer, microliters of sample, brand of monoclonal antibodies and washing
CD4	
(%)	cytometer and washing
(absolute count)	cytometer
CD8	
(%)	washing
(absolute count)	cytometer, microliters of sample and washing
NK	
(%)	no significative variable
(absolute count)	cytometer and washing
CD19	
(%)	cytometer and brand of monoclonal antibodies
(absolute count)	cytometer, brand of monoclonal antibodies and washing

Table 9. Differences in techniques employed, analysis performed by multiple regression.

	Dependent variables
Cytometer	DR, DR (c./µL), CD3, CD3 (c./µL), CD3DR, CD3DR (c./µL), CD4, CD4 (c./µL), CD8 (c./µL), NK (c./µL), CD19, CD19 (c./µL)
Brand of antibodies	CD3, CD3DR, CD3DR (c./µL), CD19, CD19 (c./µL)
µL of samples	DR, DR (c./µL), CD3 (c./µL), CD3DR, CD3DR (c./µL), CD8 (c./µL)
Washing	DR (c./µL), CD3 (c./µL), CD3DR (c./µL), CD4, CD8, CD8 (c./µL) CD3DR (c./µL)

the most important cause of variability. There was a good correspondence between the kind of instrument utilized and the brand of monoclonal antibodies employed, although we did not see the same correspondence in the results of the comparison of the single lymphocyte subsets performed in multivariate analisys, which indicates that the two variables may be independent of each other. The results of multiple regression analysis are summarized in Tables 8 and 9; it is clear that the kind of cytometer is an important cause of variability for all considered values, while the brand of monoclonal antibodies influences only some of the variables. Multivariate analysis did not show any evidence of results being influenced by time, temperature of incubation or use of fixative, while it confirmed the importance of no-washing techniques and of the amount of sample required.

Discussion

Reference values for lymphocyte subpopulations, the main end-point of this study, were obtained gathering data collected by 53 laboratories. Particular attention was paid to the standardization of pre-analytical and analytical phases in order to reduce the variability of results and to ensure inter-laboratory reproducibility. We collected 968 samples that were eligible for analysis (74% of all samples), but it is important to note that the main cause of ineligibility, 95% of eliminated analyses, was the lack of fulfilment of requirements for internal quality control (lymphosum, purity of lymphocyte gate, lymphocyte recovery, difference between CD3 and the sum of CD4 and CD8). This result underlines that routine internal quality control is not common.

The variability of the results of some subpopulations was also influenced by the biological parameters analyzed and by some of the methodological variables considered (quantity of sample, washing procedures and above all brand of monoclonal antibody and kind of instrument).[11,12] This data confirms the importance of multicenter studies in order to define reference ranges for a common interpretation of results as it is not possible to standardize the kind of instruments and the brand of monoclonal antibodies used.

Concerning the determination of the leukocyte differential count obtained by cytometers, this study has demonstrated the uselfulness of this instrument in calculating the absolute count with the double platform method.

In conclusion the large number of cases, the different kind of laboratory and their distribution throughout the country make our sample representative of the Italian adult population; moreover the standardization criteria of pre-analytical and analytical phases assured good reproducibility among laboratories, even though some differences in the brands of monoclonal antibodies employed and in the cytometers utilized may be inevitable causes of some variability.

Appendix

This work was made with the collaboration by:
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AS, GG, VB and MG were responsible for the design of the study. AS, GG, GC and PP contributed to the realization of the study and data collection. AP was responsible for the statistical analysis. AS contributed to the analysis and writing of the paper. The order in which the names appear is based on the conceptual contribution given to the paper and the time spent for data collection and analysis.The names which appear in the list of collaborating centers are those reponsible for the flow cytometry laboratories which participated in this study.

Disclosures

Conflict of interest: none.

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