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Significant age- and gender-related variability of main lymphocyte subsets in paediatric patients: Latvian data

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Abstract

Age- and gender-related variability of main lymphocyte subsets (T, B and NK cell absolute counts and percentages from Ly; T4, T8 and DN cell absolute counts and percentages from lymphocytes and from T cells; T4:T8 and T:B ratios) was studied in a large cohort of paediatric patients (2 days-17 years) at yearly intervals. A total of 4128 six-color TBNK tests performed on BD FACSCanto II flow cytometer were assessed; patients with immune deficiencies and tumours were not included. The study revealed significant age- and gender-related changes in all subsets. Absolute counts of T, B, T4 cells dropped from neonates to adolescents, decrease in T8 and NK cells was milder; relative count of T cells increased with age and that of B cells decreased; T4:T8 ratio went down and T:B ratio grew. Total T, T4 cells and T4:T8 ratio were significantly higher in girls, while T8, NK and DN cells were significantly higher in boys; significantly higher relative and absolute B cell counts in boys appeared in adolescence. We compared our results with reference values for healthy children (Tosato et al, *Cytometry A*. 2015;87:81); there was a good concordance, except for DN cells. Advantages of using patient cohort instead of healthy children as reference, possibilities for adjusting age- and gender-specific reference ranges and potential international data pooling are discussed.

1 | INTRODUCTION

Main subpopulations of lymphocytes (Ly) that are routinely detected in clinical setting include $CD3^+$ T cells that are further subdivided into $CD3^+CD4^+$ T Ly (T4 cells), $CD3^+CD8^+$ T Ly (T8 cells) and $CD3^+CD4^-CD8^-$ double-negative T Ly (DN cells); $CD19^+$ B Ly and $CD3^-CD16/CD56^+$ natural killer cells (NK cells). Variations of relative and absolute counts of the main subsets are used as a marker of abnormal/damaged cellular immunity in patients with both primary and secondary immune deficiencies. Multicolor flow cytometry (FC) is the routine method for enumerating the subsets; it provides stable reproducible results with good discrimination of populations and has long been standardized.[1,2]

Reference ranges for the subsets have been established by studies performed on normal donors.[3-5] There are few published cohort-based studies of cytometrically defined normal Ly subsets in children,[6-8] paediatric cohorts being small due to ethical and technical issues. Normal cohorts demonstrated major age-related changes in all Ly subsets, indicating dynamic maturation of cellular immunity during childhood[3,5-8] and ageing effect in adults[9]; ethnic variability has been reported.[3,4,10]

Significant gender differences, mostly within T cell compartment, have been found in adults,[5,11] including HIV-positive adult patients.[12] Surprisingly, although different relative and absolute total Ly count in boys and girls is a well-established phenomenon, only fragmentary and sometimes contradictory data on gender-related variations in children have been published. Higher relative and absolute T and T4 cell counts in females of all ages were found in Brazilian population,[8] higher T4 cell count was reported in African American and Hispanic adolescent girls in the United States[13]; on the other hand, no statistical differences were found in an adolescent cohort from South Florida[5] or in children born from HIV-infected mothers.[14]

Screening for cellular primary immune deficiency (PID) and clinically manifested secondary immune deficiencies is the main purpose for the assessment of Ly subsets by FC in paediatric practice.[15] Of note, the majority of patients with clinical suspicion of PID are severely ill; thus, "normal" controls may be not truly applicable for the cohort with cellular immunity compromised by infections, toxic and inflammatory factors and autoimmune conditions. Paediatric patients without rough immunity defects rather than normal donors seem to be a more reasonable reference population in this setting.

The aim of the study was to characterize the main Ly subsets in a representative cohort of paediatric patients at yearly time points from neonates until adulthood for both genders, using a single standardized multicolor FC platform.

2 | MATERIALS AND METHODS

A total of 4128 tests performed in Children's Clinical University Hospital Laboratory (Riga, Latvia) for paediatric patients in 2012-2016 were retrospectively analysed. Patients with proven PIDs, HIV infection and haematological tumours had been excluded.

The patients' age was from 2 days to 18 years. 2210 samples were obtained from boys, 1918 from girls.

EDTA anticoagulated peripheral blood samples were tested by a lyse-no-wash method on routinely calibrated FACSCanto II flow cytometer (Becton-Dickinson, Franklin Lakes, New Jersey, USA), using IVD certified BD Multitest 6-color TBNK kit and BD FACSCanto clinical software. The reports contained T, T4, T8, B and NK cell percentages from Ly and absolute counts, DN percentage from T Ly ("% T-sum") and T4:T8 ratio. A small population of $CD4^+CD8^+$ double-positive T Ly was below the method's sensitivity and poorly discriminated, it was not included in the study. Absolute leucocyte count and Ly relative count were obtained from the same tube by Sysmex XN-1000 (Sysmex Co, Kobe, Japan) haematology analyser.

For further analysis, the worklists were exported into MS Excel database. Additional parameters (T4 and T8 percentage from T Ly, DN cell percentage from Ly and absolute count and T:B ratio) were calculated. The data were further subdivided by gender and by age (1-year groups, infants younger than 1 year further split into groups of 0-5 months and 6-11 months old).

Statistics were performed by IBM SPSS v.23, Armonk, New York, USA. Kolmogorov-Smirnov Z test was used for differences, with P values in age groups adjusted according to the Benjamini-Hochberg procedure at false discovery rate 0.25. Age-corrected influence of gender on the studied parameters was calculated by Univariate Analysis of Variance (ANCOVA).

3 | RESULTS

The study revealed major age-related changes in all studied subsets (Tables 1 and 2, Figures S1-S14). Absolute counts of T, B, T4 cells and T4:T8 ratio dropped steeply from neonates to adolescents, T8 and NK cells' decrease was milder. Relative count of T cells increased with age, while percentage of B Ly decreased; T:B ratio went up.

There were more boys (53.5%) in the cohort and they were significantly younger (median 4 vs 5 years, $P = 0.001$). Analysis of the whole cohort after age adjustment revealed significant gender-related differences in all subpopulations: total T and T4 cells as well as T4:T8 ratio were higher in girls, while T8, NK and DN cells were higher in boys (Table 3). Although B cell did not differ statistically in the total cohort, significant gender differences in both relative and absolute B cell counts appeared in adolescence.

We compared our results with reference values for healthy children recently published by Tosato et al[7] Overall, there was a good concordance (Table 4), except for DN cells that needed special consideration. DN excluded, difference between medians above 20% (22%-32%) was found in only a minority of parameters (eight of 72; 12%), mostly in age groups with smaller number of normal children studied; in 28 of 66 parameters (42%) the difference between medians was below 5%. Medians of relative T cell count and absolute NK and B cell counts were consistently higher in all age groups in our study, differences in medians of other parameters varied randomly. Our study returned broader 10%-90% ranges for majority of parameters (54 of 66; 82%); of note, seven of 12 parameters with broader ranges for normal children were in the adolescent age group.

TABLE 1 Percentages: medians and 10-90 percentile ranges

Age	Gender	T % Ly	T4% Ly	T4% T Ly	T8% Ly	T8% T Ly	DN % Ly	DN % T Ly	NK % Ly	B % Ly
0-5 mo	Boys (n = 166)	62.8 (49.9-74.7)	44.8 (33.0-55.0)	72.7 (58.0-79.9)	15.9 (11.0-24.3)	26.0 (18.5-38.8)	0.8 (0.2-1.8)	1.3 (0.4-2.9)	9.3 (4.6-19.1)	25.1 (14.9-38.2)
	Girls (n = 110)	64.2 (55.6-75.3)	45.8 (35.1-57.3)	73.1 (59.2-80.6)	16.4 (10.5-24.4)	24.8 (18.0-38.7)	0.7 (0.1-1.6)	1.2 (0.2-2.7)	10.0 (4.6-18.4)	22.9 (13.1-34.0)
	P	0.144	0.540	0.677	0.965	0.528	0.674	0.335	0.461	0.150
6-11 mo	Boys (n = 141)	66.6 (57.3-74.5)	45.0 (34.0-54.4)	68.5 (55.0-76.8)	18.6 (12.2-26.6)	28.0 (19.8-41.7)	1.4 (0.5-2.5)	2.0 (0.7-3.6)	7.9 (4.5-15.5)	23.1 (16.6-31.9)
	Girls (n = 118)	64.7 (53.6-75.0)	44.7 (35.6-54.6)	70.6 (60.4-78.7)	16.2 (11.3-25.2)	25.4 (18.4-36.7)	1.2 (0.4-2.3)	1.8 (0.8-3.5)	7.9 (4.3-14.2)	25.2 (15.0-35.7)
	P	0.193	0.998	0.082*	3.7E-04*	0.041*	0.466	0.505	0.954	0.135
1 y	Boys (n = 238)	64.6 (54.1-73.0)	41.2 (30.9-49.1)	63.7 (51.8-73.4)	19.4 (13.8-27.9)	31.1 (22.4-44.2)	1.7 (0.9-3.9)	2.8 (1.4-6.1)	7.9 (4.7-16.0)	25.3 (15.5-35.3)
	Girls (n = 169)	65.3 (54.7-74.3)	41.2 (29.6-52.9)	65.1 (52.7-75.4)	18.7 (13.1-28.5)	29.4 (20.3-42.4)	1.7 (0.8-3.6)	2.7 (1.3-5.2)	7.8 (4.0-15.4)	25.2 (16.4-35.4)
	P	0.398	0.232	0.149*	0.362	0.384	0.877	0.899	0.351	0.913
2 y	Boys (n = 234)	65.6 (56.5-73.9)	39.0 (29.8-47.5)	59.5 (49.0-69.4)	21.3 (16.0-29.3)	33.0 (25.4-44.0)	2.5 (1.4-4.9)	3.9 (2.2-7.1)	9.0 (5.0-16.9)	23.6 (16.2-30.4)
	Girls (n = 167)	66.1 (55.8-74.1)	40.1 (30.9-48.6)	61.2 (49.0-71.5)	19.8 (14.8-27.9)	31.2 (23.7-43.2)	2.5 (1.2-5.1)	4.0 (1.9-7.8)	9.1 (4.6-17.0)	23.1 (16.5-32.0)
	P	0.671	0.361	0.116*	0.023*	0.067*	0.592	0.282	0.967	0.939
3 y	Boys (n = 197)	65.8 (57.2-74.0)	37.9 (28.6-46.2)	58.5 (46.2-66.8)	22.3 (17.1-29.5)	33.6 (26.6-44.8)	3.0 (1.5-5.9)	4.6 (2.3-8.5)	9.9 (5.2-19.2)	21.7 (15.6-30.4)
	Girls (n = 172)	66.1 (56.0-75.2)	39.2 (30.8-48.2)	60.3 (51.0-68.0)	21.2 (15.8-29.2)	33.0 (24.9-42.2)	2.7 (1.4-4.9)	4.1 (2.1-7.2)	9.2 (4.9-18.6)	21.5 (13.9-30.8)
	P	0.917	0.456	0.014*	0.17443	0.352	0.417	0.144	0.463	0.846
4 y	Boys (n = 167)	67.2 (58.9-74.7)	37.3 (30.2-46.9)	56.1 (46.3-67.3)	23.3 (17.0-29.8)	35.5 (27.0-44.4)	3.4 (1.6-6.4)	5.2 (2.5-8.9)	11.1 (5.6-19.0)	20.0 (14.5-26.1)
	Girls (n = 150)	67.6 (59.0-75.7)	39.8 (31.1-49.6)	59.7 (50.0-68.5)	21.4 (16.6-28.5)	31.7 (25.2-41.5)	3.1 (1.5-5.6)	4.7 (2.3-8.2)	9.6 (5.4-18.0)	20.4 (14.9-26.9)
	P	0.595	0.005*	1.5E-04*	0.002*	2.4E-04*	0.220	0.128	0.083	0.841
5 y	Boys (n = 148)	68.9 (60.5-76.6)	38.1 (29.7-46.0)	56.2 (44.3-64.8)	23.9 (18.7-31.3)	36.0 (28.4-44.2)	3.4 (1.9-6.7)	5.1 (2.8-9.5)	11.2 (5.9-22.3)	17.8 (11.5-25.3)
	Girls (n = 139)	67.7 (59.2-75.7)	39.9 (30.1-46.7)	57.6 (48.0-66.8)	22.5 (16.6-29.8)	33.4 (26.5-42.5)	3.3 (1.6-5.7)	5.1 (2.5-8.7)	11.5 (5.1-19.9)	19.0 (13.4-26.8)
	P	0.842	0.139	0.114*	0.052*	0.043*	0.737	0.643	0.609	0.093
6 y	Boys (n = 144)	69.3 (60.8-76.3)	36.4 (29.9-44.6)	53.1 (44.3-64.0)	25.6 (18.1-33.2)	37.5 (27.7-46.5)	3.8 (1.7-7.7)	5.7 (2.4-10.6)	12.2 (6.7-18.6)	17.0 (12.4-22.8)
	Girls (n = 105)	69.9 (61.9-77.2)	40.0 (30.3-49.2)	57.2 (47.5-66.6)	23.9 (17.0-30.2)	33.6 (25.2-44.0)	3.8 (1.6-7.4)	5.6 (2.4-10.2)	9.9 (5.0-16.8)	18.3 (12.9-24.5)
	P	0.685	0.007*	0.001*	0.010*	6.9E-04*	0.791	0.984	0.008*	0.104
7 y	Boys (n = 107)	66.9 (58.0-76.7)	36.1 (28.4-44.4)	54.7 (43.0-62.9)	23.9 (18.3-31.2)	36.4 (28.0-46.5)	4.0 (1.8-6.6)	6.1 (2.6-9.6)	13.0 (7.4-21.8)	16.8 (11.3-26.6)
	Girls (n = 99)	70.6 (62.5-76.6)	38.6 (30.9-47.0)	56.0 (44.8-65.3)	25.2 (17.6-32.4)	35.7 (26.1-46.8)	3.5 (2.2-6.9)	5.2 (3.2-9.5)	11.0 (6.2-16.9)	17.2 (11.8-23.9)
	P	3.9E-04*	0.019*	0.320	0.367	0.232	0.266	0.043	0.019*	0.774
8 y	Boys (n = 90)	70.6 (61.3-77.5)	37.6 (29.0-44.4)	54.8 (42.7-64.0)	26.1 (18.9-35.2)	37.9 (29.9-46.2)	3.4 (1.7-7.8)	5.2 (2.4-10.0)	10.7 (6.2-20.1)	16.8 (10.8-25.0)
	Girls (n = 88)	69.3 (60.8-77.3)	38.4 (28.8-47.2)	54.1 (45.2-64.8)	25.1 (19.6-32.8)	37.3 (28.8-47.0)	3.2 (1.6-6.1)	4.7 (2.5-9.6)	11.8 (6.7-22.2)	16.7 (10.8-23.0)
	P	0.858	0.525	0.342	0.628	0.953	0.2658	0.120	0.179	0.472

(Continues)

TABLE 1 (Continued)

Age	Gender	T % Ly	T4% Ly	T4% T Ly	T8% Ly	T8% T Ly	DN % Ly	DN % T Ly	NK % Ly	B % Ly
9 y	Boys (n = 79)	69.8 (57.7-76.8)	37.7 (29.1-46.7)	55.2 (44.7-64.3)	25.2 (18.7-32.5)	36.8 (28.3-46.3)	3.5 (1.9-6.4)	5.3 (2.7-8.8)	12.3 (5.6-19.5)	17.8 (10.4-25.1)
	Girls (n = 79)	70.9 (57.8-78.9)	40.6 (31.5-49.9)	58.1 (46.4-68.5)	23.0 (16.8-31.4)	33.7 (24.9-46.1)	3.3 (1.8-6.3)	4.8 (2.7-8.7)	11.9 (6.2-21.3)	15.9 (10.9-22.7)
	P	0.428	0.052*	0.052*	0.322	0.167	0.322	0.235	0.685	0.021*
10 y	Boys (n = 98)	70.5 (63.0-79.0)	38.7 (29.6-45.3)	55.9 (42.8-62.8)	25.4 (19.3-34.5)	34.9 (28.8-49.3)	3.7 (1.6-8.6)	5.1 (2.5-11.9)	10.4 (6.1-19.5)	16.7 (10.9-22.8)
	Girls (n = 61)	70.6 (58.6-77.4)	40.5 (30.5-47.4)	57.9 (47.2-64.3)	24.4 (18.3-29.9)	35.4 (27.7-45.3)	2.8 (1.3-5.7)	4.1 (1.9-8.1)	11.7 (6.8-18.9)	16.2 (11.4-27.7)
	P	0.315	0.268	0.054*	0.372	0.511	0.080	0.136	0.406	0.743
11 y	Boys (n = 76)	70.7 (60.9-77.3)	36.4 (29.6-45.6)	54.0 (44.9-63.1)	26.0 (19.9-35.4)	37.1 (30.0-47.1)	3.6 (1.2-6.6)	5.4 (2.0-9.2)	11.7 (6.0-20.6)	16.1 (11.2-23.3)
	Girls (n = 70)	71.1 (57.5-78.6)	40.8 (30.4-50.3)	58.0 (48.6-67.7)	24.4 (17.8-31.6)	34.9 (26.4-45.2)	2.8 (1.0-5.3)	4.2 (1.4-7.1)	11.2 (6.3-20.1)	17.0 (11.3-24.6)
	P	0.859	0.018*	0.003*	0.427	0.132	0.023	0.053	0.414	0.675
12 y	Boys (n = 59)	68.6 (61.4-75.1)	37.9 (31.5-44.6)	55.3 (47.2-66.2)	24.0 (17.7-31.4)	36.6 (27.9-43.6)	3.6 (1.4-6.7)	5.0 (2.1-9.1)	11.9 (6.9-20.8)	17.0 (12.1-23.6)
	Girls (n = 69)	71.9 (62.1-76.9)	40.9 (32.4-48.9)	57.8 (49.7-65.8)	23.8 (18.9-30.9)	34.3 (27.6-41.4)	3.2 (1.4-5.4)	4.4 (2.2-7.4)	11.6 (6.4-18.8)	16.4 (11.5-22.7)
	P	0.027*	0.023*	0.059*	0.818	0.332	0.548	0.61481	0.717	0.368
13 y	Boys (n = 59)	66.7 (58.5-77.6)	38.1 (28.6-43.1)	56.0 (40.2-65.9)	24.9 (18.8-34.6)	38.1 (28.3-47.6)	2.5 (1.5-6.1)	3.6 (2.4-8.7)	10.2 (5.5-26.1)	18.7 (10.9-26.3)
	Girls (n = 61)	71.7 (61.6-77.7)	40.2 (32.9-48.9)	59.2 (50.0-66.8)	24.6 (18.4-31.6)	35.3 (27.1-44.9)	2.4 (1.4-6.0)	3.3 (2.1-8.1)	11.5 (6.5-19.0)	15.2 (10.1-21.3)
	P	0.006*	0.012*	0.044*	0.778	0.049*	0.943	0.490	0.402	0.005*
14 y	Boys (n = 71)	69.5 (61.5-77.0)	37.4 (30.4-47.0)	57.4 (42.2-65.5)	23.8 (18.8-33.9)	36.0 (28.7-48.4)	2.9 (1.6-6.6)	4.4 (2.4-9.7)	11.5 (6.3-20.2)	16.4 (10.6-24.6)
	Girls (n = 76)	73.1 (64.8-79.9)	43.2 (34.4-49.4)	59.3 (49.0-68.5)	25.1 (19.4-33.6)	34.4 (27.3-44.5)	2.7 (1.2-5.2)	3.8 (1.8-7.5)	9.7 (5.7-17.2)	15.6 (10.0-22.0)
	P	0.011*	8.8E-05*	0.116*	0.856	0.582	0.343	0.225	0.523	0.072
15 y	Boys (n = 52)	71.8 (58.2-80.3)	37.8 (28.5-45.4)	55.2 (40.7-65.1)	27.0 (20.3-39.1)	38.1 (30.9-52.8)	2.9 (1.1-4.8)	4.3 (1.7-6.9)	11.4 (6.8-19.6)	16.8 (10.6-24.7)
	Girls (n = 59)	73.6 (63.6-80.6)	41.9 (35.5-51.8)	57.8 (49.0-67.1)	24.7 (19.8-32.3)	34.3 (28.0-42.7)	3.1 (1.3-6.2)	4.2 (1.7-8.3)	11.0 (6.4-19.6)	13.7 (8.5-19.9)
	P	0.050	0.007*	0.016*	0.045*	0.007*	0.923	0.965	0.940	0.004*
16 y	Boys (n = 37)	70.6 (63.8-77.6)	40.3 (29.7-50.9)	55.8 (46.0-67.5)	24.7 (19.0-32.4)	34.4 (25.6-44.0)	3.2 (1.3-6.9)	4.3 (2.0-9.7)	11.3 (6.3-18.1)	16.3 (10.4-24.3)
	Girls (n = 68)	73.7 (62.1-80.4)	42.0 (34.9-50.7)	58.1 (50.6-67.7)	25.5 (20.2-33.7)	36.0 (28.8-44.8)	2.2 (0.8-4.2)	3.1 (1.2-6.1)	11.8 (6.1-19.8)	13.3 (8.3-21.2)
	P	0.180	0.256	0.474	0.402	0.648	0.014	0.017	0.974	0.026*
17 y	Boys (n = 45)	72.4 (63.1-79.2)	37.7 (31.0-45.3)	55.7 (42.3-63.2)	26.4 (20.1-39.5)	37.1 (30.7-50.2)	2.7 (1.3-6.4)	3.9 (1.8-9.0)	12.8 (7.1-22.4)	14.0 (9.2-18.7)
	Girls (n = 60)	75.1 (64.6-82.2)	43.2 (33.8-52.0)	59.3 (49.1-66.2)	26.2 (21.5-33.1)	35.2 (29.1-44.6)	2.4 (1.2-5.1)	3.3 (1.6-7.0)	12.1 (5.7-19.7)	11.9 (8.8-16.2)
	P	0.202	0.004*	0.080*	0.875	0.285	0.704	0.473	0.937	0.032*

*Difference significant after Benjamini-Hochberg adjustment.

TABLE 2 Absolute counts × 10E6/L and T4:T8 and T:B ratio: medians and 10-90 percentile range

Age	Gender	T cells	T4 cells	T8 cells	DN cells	NK cells	B cells	T4:T8 ratio	T:B ratio
0-5 mo	Boys (n = 166)	3990 (2256-5775)	2784 (1528-4044)	999 (495-1740)	49 (10-128)	574 (259-1326)	1599 (751-2614)	2.8 (1.5-4.3)	2.5 (1.4-4.9)
	Girls (n = 110)	4112 (1990-5778)	2740 (1409-4198)	952 (459-1735)	41 (5-116)	529 (243-1206)	1437 (641-2591)	3.0 (1.5-4.4)	2.8 (1.7-5.7)
	P	0.740	0.894	0.782	0.418	0.246	0.038	0.551	0.098*
6-11 mo	Boys (n = 141)	3860 (2580-5736)	2559 (1629-3988)	1030 (566-1818)	78 (25-163)	448 (222-1031)	1329 (733-2467)	2.4 (1.3-3.9)	2.8 (1.9-4.8)
	Girls (n = 118)	4194 (2233-6260)	2860 (1558-4375)	1113 (490-1927)	65 (25-173)	508 (206-1060)	1442 (679-2797)	2.7 (1.7-4.2)	2.6 (1.5-5.0)
	P	0.198	0.041*	0.477	0.199	0.458	0.2140	0.060*	0.035*
1 y	Boys (n = 238)	3176 (1963-5219)	2017 (1179-3136)	988 (505-1908)	89 (35-209)	383 (204-933)	1272 (652-2104)	2.1 (1.2-3.3)	2.5 (1.6-4.6)
	Girls (n = 169)	3343 (2245-5115)	2155 (1350-3373)	999 (554-1754)	89 (40-200)	378 (197-1061)	1254 (699-2074)	2.2 (1.3-3.7)	2.6 (1.5-4.4)
	P	0.106	0.166	0.970	0.630	0.897	0.454	0.364	0.764
2 y	Boys (n = 234)	2765 (1758-4199)	1613 (1002-2562)	911 (517-1587)	109 (53-211)	351 (175-826)	940 (584-1637)	1.8 (1.1-2.7)	2.8 (1.9-4.5)
	Girls (n = 167)	2838 (1842-4309)	1731 (1053-2793)	915 (516-1430)	107 (47-226)	396 (185-787)	1013 (606-1597)	1.9 (1.2-3.0)	2.9 (1.8-4.3)
	P	0.386	0.310	0.984	0.263	0.338	0.069	0.073*	0.951
3 y	Boys (n = 197)	2570 (1590-4073)	1460 (881-2407)	910 (471-1434)	112 (55-227)	363 (180-850)	864 (529-1456)	1.7 (1.1-2.5)	3.0 (1.9-4.6)
	Girls (n = 172)	2490 (1416-3801)	1465 (806-2274)	812 (421-1327)	105 (50-189)	337 (151-889)	787 (423-1441)	1.8 (1.2-2.8)	3.0 (1.9-5.0)
	P	0.384	0.852	0.033	0.150	0.633	0.080	0.143	0.700
4 y	Boys (n = 167)	2126 (1467-3415)	1226 (778-2023)	758 (488-1264)	116 (51-202)	342 (155-835)	650 (374-1170)	1.6 (1.0-2.5)	3.3 (2.4-5.1)
	Girls (n = 150)	2371 (1439-3511)	1431 (807-2257)	775 (468-1154)	109 (48-200)	341 (165-787)	723 (454-1102)	1.9 (1.2-2.7)	3.4 (2.3-4.9)
	P	0.015*	5.4E-04*	0.673	0.809	0.586	0.208	5.8E-05*	2.6E-04*
5 y	Boys (n = 148)	2198 (1333-3063)	1199 (696-1820)	758 (448-1256)	108 (49-238)	371 (142-678)	571 (306-898)	1.5 (1.0-2.2)	3.8 (2.5-6.2)
	Girls (n = 139)	2198 (1217-3220)	1299 (692-1924)	739 (392-1193)	106 (48-190)	325 (155-696)	587 (325-973)	1.7 (1.1-2.5)	3.6 (2.3-5.5)
	P	0.885	0.683	0.123	0.776	0.216	0.354	0.022*	1.6E-05*
6 y	Boys (n = 144)	2029 (1250-3111)	1071 (710-1656)	743 (403-1166)	115 (46-267)	346 (157-712)	477 (322-793)	1.4 (1.0-2.2)	4.1 (2.8-6.0)
	Girls (n = 105)	2096 (1374-3287)	1166 (753-2017)	696 (450-1128)	111 (59-223)	283 (142-623)	561 (314-886)	1.7 (1.1-2.6)	3.8 (2.5-5.9)
	P	0.899	0.155	0.378	0.680	0.015*	0.020	2.1E-04*	0.002*
7 y	Boys (n = 107)	1865 (1216-2743)	1001 (653-1425)	674 (431-1066)	119 (43-200)	380 (172-681)	480 (236-802)	1.5 (0.9-2.1)	4.0 (2.3-6.7)
	Girls (n = 99)	1988 (1284-2724)	1061 (654-1532)	673 (412-1181)	98 (54-206)	288 (140-570)	462 (269-808)	1.6 (1.0-2.4)	4.1 (2.6-6.3)
	P	0.708	0.662	0.940	0.234	0.003*	0.641	0.206	0.821
8 y	Boys (n = 90)	1892 (1166-2710)	954 (581-1444)	683 (442-1127)	102 (34-221)	291 (123-522)	452 (237-777)	1.4 (0.9-2.1)	4.2 (2.4-6.8)
	Girls (n = 88)	1856 (1135-2970)	1013 (680-1581)	687 (365-1116)	88 (34-188)	297 (147-648)	455 (229-720)	1.4 (1.0-2.2)	4.2 (2.8-7.1)
	P	0.536	0.757	0.930	0.196	0.349	0.676	0.558	0.470

(Continues)

TABLE 2 (Continued)

Age	Gender	T cells	T4 cells	T8 cells	DN cells	NK cells	B cells	T4:T8 ratio	T:B ratio
9 y	Boys (n = 79)	1622 (824-2334)	891 (442-1316)	591 (335-869)	86 (29-176)	289 (123-489)	412 (195-668)	1.5 (1.0-2.2)	4.1 (2.3-7.4)
	Girls (n = 79)	1865 (1270-2907)	1058 (698-1685)	675 (337-1057)	91 (41-180)	313 (165-456)	408 (280-694)	1.7 (1.0-2.8)	4.4 (2.6-7.4)
	P	0.116	0.013*	0.167	0.916	0.428	0.235	0.052*	0.078*
10 y	Boys (n = 98)	1738 (1203-2483)	942 (618-1359)	635 (359-1120)	87 (39-213)	273 (136-540)	413 (219-675)	1.5 (0.9-2.1)	4.3 (2.8-6.8)
	Girls (n = 61)	1781 (969-2643)	1009 (553-1525)	611 (301-1000)	71 (39-148)	293 (158-492)	408 (255-653)	1.6 (1.1-2.3)	4.1 (2.0-6.9)
	P	0.743	0.232	0.401	0.150	0.234	0.966	0.181	0.578
11 y	Boys (n = 76)	1752 (1062-2522)	914 (529-1361)	630 (363-1024)	88 (28-193)	319 (137-563)	394 (199-677)	1.5 (0.9-2.1)	4.5 (2.8-6.6)
	Girls (n = 70)	1662 (935-2266)	984 (509-1369)	551 (311-864)	66 (24-118)	240 (118-512)	419 (218-592)	1.7 (1.1-2.6)	4.2 (2.6-8.0)
	P	0.937	0.2260	0.388	0.007	0.070	0.732	0.028*	0.717
12 y	Boys (n = 59)	1618 (1027-2097)	876 (622-1174)	548 (344-836)	73 (30-155)	265 (126-611)	404 (223-590)	1.5 (1.1-2.4)	3.9 (2.5-5.7)
	Girls (n = 69)	1634 (1175-2299)	935 (646-1396)	548 (386-806)	78 (34-136)	260 (139-442)	365 (221-596)	1.6 (1.2-2.4)	4.5 (2.6-6.4)
	P	0.623	0.344	0.388	0.824	0.760	0.368	0.132	0.436
13 y	Boys (n = 59)	1596 (900-2184)	809 (485-1205)	599 (329-912)	64 (26-176)	242 (122-721)	415 (223-772)	1.5 (0.9-2.3)	3.7 (2.4-6.8)
	Girls (n = 61)	1688 (1244-2341)	965 (738-1335)	573 (387-941)	60 (33-153)	295 (138-500)	362 (226-602)	1.7 (1.1-2.4)	4.5 (3.1-7.3)
	P	0.464	0.003*	0.836	0.935	0.296	0.178	0.079*	0.010*
14 y	Boys (n = 71)	1464 (923-2255)	819 (496-1227)	506 (302-917)	63 (30-143)	234 (122-492)	325 (179-608)	1.6 (0.9-2.2)	4.2 (2.5-7.5)
	Girls (n = 76)	1594 (1130-2211)	960 (627-1266)	516 (348-881)	58 (25-109)	209 (113-453)	328 (201-565)	1.8 (1.1-2.5)	4.8 (2.9-7.5)
	P	0.113	0.004*	0.571	0.241	0.612	0.454	0.329	0.074*
15 y	Boys (n = 52)	1343 (973-2107)	743 (458-1142)	534 (304-852)	55 (21-116)	244 (124-415)	331 (165-538)	1.4 (0.8-2.1)	4.4 (2.4-7.9)
	Girls (n = 59)	1583 (1004-2182)	923 (577-1339)	564 (348-811)	66 (24-142)	237 (134-452)	296 (160-427)	1.7 (1.1-2.4)	5.3 (3.2-9.5)
	P	0.010*	0.017*	0.793	0.330	0.948	0.202	0.02221*	0.010*
16 y	Boys (n = 37)	1495 (991-1964)	839 (604-1316)	475 (357-728)	62 (26-168)	239 (104-375)	323 (174-625)	1.7 (1.1-2.7)	4.3 (2.5-7.8)
	Girls (n = 68)	1517 (928-2302)	862 (565-1344)	523 (341-989)	42 (15-100)	235 (117-405)	290 (140-501)	1.6 (1.1-2.4)	5.7 (3.2-9.8)
	P	0.150	0.319	0.122	0.114	0.910	0.807	0.774	0.017*
17 y	Boys (n = 45)	1469 (752-2172)	792 (432-1171)	553 (323-838)	47 (22-131)	237 (134-475)	285 (133-441)	1.5 (0.9-2.0)	5.4 (3.2-8.8)
	Girls (n = 60)	1526 (1103-2092)	902 (615-1275)	529 (375-855)	47 (24-116)	265 (116-466)	243 (164-490)	1.7 (1.2-2.3)	6.2 (4.3-9.9)
	P	0.517	0.139	0.909	0.959	0.909	0.122	0.202	0.044*

*Difference significant after Benjamini-Hochberg adjustment.

TABLE 3 Gender-related differences after age adjustment (ANCOVA)

	F	*P*
T % Ly	8.3	**0.004**
age 13-17	21.0	**<1.0E-10**
T × 10E6/L	3.5	0.061
T4% Ly	82.2	**<1.0E-10**
T4% T Ly	76.8	**<1.0E-10**
T4 × 10E6/L	21.9	**<1.0E-10**
T8% Ly	26.6	**<1.0E-10**
T8% T Ly	50.2	**<1.0E-10**
T8 × 10E6/L	4.5	**0.034**
DN % Ly	22.6	**<1.0E-10**
DN % T Ly	27.3	**<1.0E-10**
DN × 10E6/L	11.4	**0.001**
NK % Ly	6.6	**0.010**
NK × 10E6/L	1.5	0.216
B % Ly	0.9	0.349
age 13-17	24.9	**<1.0E-10**
B × 10E6/L	1.0	0.319
age 13-17	7.2	**0.007**
T4:T8 ratio	53.9	**<1.0E-10**
T:B ratio	2.7	0.100

Significant differences are in black.

4 | DISCUSSION

4.1 | The cohort selection

Overall, there was an excellent match between the study data and results obtained from normal donors. The majority of variations (Table 4) were not critical and are probably due to combination of underlying diseases in our study, relatively small numbers of normal children reported and cross-platform variations (BD FACSCanto II vs Beckman-Coulter Navios); ethnic differences could not be completely excluded.

The only significant differences were found in DN counts. The problem does not seem to be technical: we have previously demonstrated that automatic 6-TBNK platform provides very accurate assessment of DN cells,[16] the counts being negligibly lower than obtained by manual analysis of FC files on a leukaemia-oriented software.

Aside from the DN cell counts discrepancy, there are evident advantages in using patients instead of normal donors for defining reference ranges. The cohort of children with health problems actually may be a more suitable reference group for PID and other clinically relevant immunity defects than normal donors. First, the former are as a rule clinically symptomatic patients, usually with severe infections and/or autoimmune phenomena, so screening for PID is usually performed in ill children. Furthermore, the use of routine clinical tests provides bulk data that are impossible to acquire from healthy children for ethical and technical reasons. Exclusion of rough pathology that could directly influence immune status (like occasional cases of leukaemias, chemotherapy, PIDs, mononucleosis, etc.) would still be necessary for reliable results.

Finally, definition of "normality" could be tricky in paediatric population. For example, the 10%-90% range reported by Tosato et al in healthy adolescents was actually broader than in our study that could point to unreported health problems, resulting in the cohort heterogeneity.

Thus, the study demonstrated that for Ly subsets the results of routine testing of a relatively large cohort of paediatric patients could be a good substitution for the ethically and technically complicated procedure of enrolling a reference population of healthy children.

4.2 | Age-related dynamics

The study confirmed the existence and general pathways of age-related dynamic changes of main Ly subsets, earlier described for normal cohorts.[3,5-8] Like in normal children, absolute counts of all subsets in paediatric patients gradually decreased with age. The grade of this decrease differed between subsets, resulting in change of proportions: T:B ratio rose with age, T4:T8 ratio decreased, while percentage of NK cells remained nearly stable.

The curves were followed at yearly intervals; this detailed view demonstrated two patterns. One group of parameters (relative and absolute counts of T cells and percentage of T8 and NK cells) followed the general linear drop of Ly from infancy to adulthood without evident extremums. Other subsets had a critical point near the age of 6-7 years: relative counts of T8 and NK cells and T4:T8 ratio formed plateau afterwards; absolute T4 as well as relative and absolute B cell counts decrease turned more gradual. The change was particularly manifest for relative T4 cell counts that changed previous descent into mild increase, and for absolute counts and percentage of DN cells that turned from upward to downward trend at this point.

4.3 | Gender differences

The study demonstrated significant gender-related differences in all subsets, particularly in relative counts; such consistency must have a solid biological background.

TABLE 4 Comparison with a normal cohort (1—our data, 2—Tosato et al[7])

		0-2 mo (n1 = 129, n2 = 16)				3-11 mo (n1 = 349, n2 = 24)				1 y (n1 = 407, n2 = 22)			
		Median	10%	90%	Range	Median	10%	90%	Range	Median	10%	90%	Range
Ly × 10E9/L	1	6277	3457	9051	5594	6169	3736	9380	5644	5057	3195	7833	4638
	2	5740	4054	7048	2994	5690	3320	7006	3686	4685	3873	6141	2268
d (1-2)%		9	−17	22	46	8	11	25	35	7	−21	22	51
T % Ly	1	64.8	52.9	75.4	23	64.9	53.7	74.6	21	65.0	54.1	73.9	20
	2	72.0	62.7	81.6	19	66.8	51.8	74.2	22	67.5	60.7	75.8	15
d (1-2)%		−11	−19	−8	16	−3	4	1	−7	−4	−12	−3	24
T × 10E6/L	1	4153	1993	5848	3855	4005	2317	5974	3657	3227	1976	5197	3221
	2	4040	3180	5401	2221	3833	2284	4776	2492	3133	2642	4933	2291
d (1-2)%		3	−60	8	42	4	1	20	32	3	−34	5	29
T4% Ly	1	45.9	34.1	56.9	23	44.2	33.9	54.3	20	41.2	30.6	50.2	20
	2	53.2	42.8	65.7	23	43.6	34.9	53.1	18	41.2	35.0	51.9	17
d (1-2)%		−16	−26	−16	0	1	−3	2	11	0	−14	−3	14
T4 × 10E6/L	1	2792	1201	4171	2970	2662	1559	4117	2558	2051	1266	3269	2003
	2	3079	2330	3617	1287	2492	1523	3472	1949	1866	1573	2949	1376
d (1-2)%		−10	−94	13	57	6	2	16	24	9	−24	10	31
T8% Ly	1	16.1	11.2	27.2	16	17.0	11.3	25.8	15	18.9	13.4	28.3	15
	2	18.4	15.0	23.0	8	16.2	12.8	27.1	14	19.3	16.1	29.4	13
d (1-2)%		−15	−33	15	50	5	−13	−5	1	−2	−20	−4	11
T8 × 10E6/L	1	1031	465	1738	1272	1028	513	1891	1378	998	523	1820	1297
	2	1048	712	1361	649	976	524	1583	1059	884	656	1432	776
d (1-2)%		−2	−53	22	49	5	−2	16	23	11	−25	21	40
NK % Ly	1	10.9	4.7	19.0	14	8.1	4.4	15.5	11	7.8	4.3	15.7	11
	2	8.2	4.2	14.8	11	7.9	4.0	15.1	11	6.8	4.0	13.8	10
d (1-2)%		25	10	22	26	2	9	3	0	13	7	12	14
NK × 10E6/L	1	598	254	1241	987	498	217	1070	853	381	200	998	798
	2	408	201	870	669	381	230	801	571	296	186	724	538
d (1-2)%		32	21	30	32	23	−6	25	33	22	7	27	33
B % Ly	1	22.1	11.4	34.5	23	24.9	15.8	35.7	20	25.3	15.9	35.4	20
	2	17.0	7.4	21.3	14	23.4	17.0	37.2	20	24.0	14.3	28.2	14
d (1-2)%		23	35	38	40	6	−8	−4	−2	5	10	20	29
B × 10E6/L	1	1449	506	2463	1957	1481	734	2752	2018	1265	666	2109	1443
	2	1032	315	1383	1068	1123	776	2238	1462	1152	733	1338	605
d (1-2)%		29	38	44	45	24	−6	19	28	9	−10	37	58
T4/T8 ratio	1	2.84	1.55	4.40	3	2.61	1.45	4.13	3	2.13	1.21	3.45	2
	2	2.90	1.93	4.19	2	2.64	1.48	3.77	2	2.00	1.34	3.04	2
d (1-2)%		−2	−24	5	21	−1	−2	9	15	6	−11	12	24
DN % T	1	2.8	1.6	4.4	3	1.8	0.6	3.5	3	2.7	1.4	5.7	4
	2	2.4	1.1	4.0	3	4.7	3.1	6.7	4	5.9	4.0	10.2	6
d (1-2)%		15	29	9	−2	−161	−417	−91	−24	−119	−186	−79	−44

Divergence of most gender-related subset curves was clearly seen in adolescence (difference in percentage of total T, T4 and B cells and T:B ratio being particularly striking) and thus will probably persist into adulthood; the results support previously published observations.[8,13] In some instances (like T and B cell percentage in

2-5 y (n1 = 1374, n2 = 57)				6-11 y (n1 = 1096, n2 = 56)				12-17 y (n1 = 716, n2 = 20)			
Median	10%	90%	Range	Median	10%	90%	Range	Median	10%	90%	Range
3716	2301	5721	3420	2681	1698	4004	2306	2203	1461	3124	1663
3800	2340	5028	2688	2500	1662	3448	1786	2285	1340	3173	1833
−2	−2	12	21	7	2	14	23	−4	8	−2	−10
66.8	57.6	74.9	17	69.9	60.4	77.4	17	71.5	61.9	79.2	17
68.6	59.7	77.6	18	71.7	63.2	77.8	15	73.0	62.6	80.4	18
−3	−4	−4	−3	−3	−5	−1	14	−2	−1	−2	−3
2480	1485	3866	2381	1865	1137	2768	1631	1558	1021	2245	1224
2580	1578	3707	2129	1793	1239	2611	1372	1629	954	2332	1378
−4	−6	4	11	4	−9	6	16	−5	7	−4	−13
38.7	30.1	47.8	18	38.3	29.3	47.0	18	40.1	31.6	49.0	17
38.0	31.1	47.4	16	39.9	31.7	47.0	15	44.0	32.6	51.5	19
2	−3	1	8	−4	−8	0	14	−10	−3	−5	−9
1410	815	2320	1505	1008	603	1538	935	872	550	1284	734
1448	870	2144	1274	1030	646	1515	869	887	610	1446	836
−3	−7	8	15	−2	−7	1	7	−2	−11	−13	−14
22.1	16.3	29.7	13	24.9	18.2	32.8	15	25.0	19.3	33.7	14
21.0	16.0	26.9	11	24.0	17.1	30.0	13	23.0	19.0	29.0	10
5	2	9	19	4	6	9	12	8	2	14	31
827	455	1365	910	661	374	1098	724	540	343	879	536
804	472	1107	635	595	365	945	580	518	282	749	467
3	−4	19	30	10	2	14	20	4	18	15	13
9.8	5.2	18.8	14	11.5	6.2	20.0	14	11.4	6.2	19.8	14
8.0	4.7	16.2	12	9.8	5.4	18.6	13	11.7	4.3	16.2	12
18	10	14	15	15	13	7	4	−3	31	18	13
352	163	811	648	299	142	598	456	247	121	460	339
299	155	565	410	262	120	483	363	230	87	504	417
15	5	30	37	12	15	19	20	7	28	−10	−23
21.1	14.5	29.6	15	16.9	11.3	24.2	13	15.1	9.9	22.9	13
22.0	12.9	29.2	16	15.6	12.0	24.0	12	14.0	11.9	21.0	9
−4	11	1	−8	8	−6	1	7	7	−20	8	30
772	409	1397	988	453	246	766	520	330	178	561	383
730	434	1274	840	403	276	640	364	321	173	685	512
5	−6	9	15	11	−12	16	30	3	3	−22	−34
1.76	1.10	2.66	2	1.53	0.97	2.31	1	1.60	1.04	2.33	1
1.77	1.26	2.90	2	1.67	1.18	2.65	1	2.05	1.21	2.64	1
−1	−15	−9	−5	−9	−22	−15	−10	−28	−16	−13	−11
4.4	2.3	8.3	6	5.2	2.4	9.7	7	4.0	1.8	8.1	6
9.3	6.7	14.0	7	8.0	6.0	14.6	9	7.4	4.3	10.7	6
−111	−191	−69	−22	−54	−150	−51	−18	−85	−139	−32	−2

adolescents, relative T4 counts after age 2-3 and T4:T8 ratio in the whole cohort) the differences may be sufficient to require separate reference ranges.

Another age group of potential interest are infants younger than 1 year when most curves in our study crossed. Evidently, partitioning of the interval into only two subgroups (0-5 months and 6-11 months) is not sufficient for detailed categorization of the curves, but further subdivision was limited by the size of the patients' cohort.

4.4 | General considerations

6-TBNK is a standardized and widely used platform, so patients' data may be internationally pooled, further refining the curves, particularly their behaviour near critical points and in cohorts less frequently tested for Ly subsets (adolescents and especially small infants). Multicentre database of routine tests could be an excellent reference tool and an instrument to evaluate the scope of ethnic-related variability.

About 10%-90% reference ranges obtained both in healthy children and in our study are rather crude instrument that is usable for a rough screening only; for example, 10% margin for absolute T4 cell count is far above the clinically relevant values for the acquired immune deficiency. Bulk data may help to adapt the ranges to clinical needs.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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