Distribution of leukocyte and lymphocyte subsets in peripheral blood. Age related normal values for preliminary evaluation of the immune status in Polish children

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Abstract

Determination of the relative and absolute numbers of various lymphocyte subsets is currently considered significant for diagnosis and monitoring of various pathological inborn, as well as acquired immune-related conditions. The purpose of this study was to establish reference values of basic leukocyte and lymphocyte subsets for pediatric population in Poland useful for accurate interpretation of results in children with inborn and acquired disorders of immunity.

The study was performed on whole cord and peripheral blood samples from a group of 292 healthy newborns and children from very narrow age groups. Multicolor flow cytometry, lyse-no-wash approach, and single platform technology were applied for the determination of distribution of T lymphocytes, their helper and suppressor subsets, B lymphocytes and NK cells. Additional information on maturation of T lymphocytes was obtained from the distribution of helper and suppressor T lymphocyte subsets expressing RA and RO isoforms of the CD45 molecule.

Tremendous changes in the immune system encountering new antigens and gradually acquiring the ability to respond to that challenge were found during the first year of life. Variation in relative sizes of individual cell populations was found not to be directly consistent with the variation in absolute counts. Results of the study provide a useful tool for the interpretation of clinical and laboratory data obtained from the patients with suspected primary immune deficiency.

Key words: *lymphocyte subsets, flow cytometry.*

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Introduction

Lymphocytes play both regulating and effector functions. Determination of relative and absolute numbers of various lymphocyte subsets is currently a routine method, considered significant for diagnosis and monitoring in various pathological inborn, as well as acquired immune-related conditions. Composition of blood lymphocyte subsets depends on age [1-3] and might be changed through stress [4], physical activity [5], lifestyle-related factors [6, 7] the circadian rhythm [8], etc. Accurate interpretation of results requires reliable normal ranges derived from large studies. The purpose of this study was to establish reference values of basic lymphocyte subsets for pediatric population in Poland useful for diagnostics of inborn and acquired disorders of immunity.

Material and methods

Population

The tested population included 292 children and young adults living in municipal or rural area, aged 0-31 years, without infectious, immunologic, hematological, and other

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Table 1. Demographics of the population under study

Age group	Agemedian 5-95 percentile	Total number of subjects	Sex ratio F/M
Cord blood	–	22	14/8
0-7 days	2 days 0-5 days	24	13/11
8 days – 2 months	1.5 months 0.3-1.9 months	12	8/4
2-5 months	3.6 months 2.4-4.8 months	23	11/12
5-9 months	6.5 months 5.2-8.7 months	21	9/12
9-15 months	11.9 months 9.3-14.3 months	20	8/12
15-24 months	20.2 months 15.7-23.5 months	38	14/24
2-5 years	3.0 years 2.1-4.7 years	32	15/17
5-10 years	6.7 years 5.3-9.4 years	30	14/16
10-16 years	12.0 years 10.3-15.7 years	42	26/16
16 years	18.6 years 16.4-25.9 years	28	16/12

chronic diseases or treatment of any kind that could affect the immune system. Sample collection was preceded by physical examination, evaluation of the past medical history based on detailed questionnaire, and obtaining an informed consent from subjects above 16 and from parents in case of children under 16 years of age. Any acute or chronic infection within four weeks before sample donation resulted in exclusion from the study. Subjects were divided into eleven age groups: i.e. samples were taken from cord blood and patients aged 0 to 7 days, 8 to 60 days, 2 to 5 months, 5 to 9 months, 9 to 15 months, 15 to 24 months, 2 to 5 years, 5 to 10 years, 10 to 16 years, and older than 16 years (detailed demographical data see Table 1). The study was approved by the institutional review board at the Children's Memorial Health Institute, Warsaw, Poland and conducted in accordance with the guidelines of Helsinki Declaration.

Blood samples and immunophenotyping

One milliliter peripheral blood samples were taken by venipuncture during morning hours. Cord blood samples were obtained from umbilical cord vessels within few minutes after delivery. All samples were anticoagulated with EDTA-K2. Relative and absolute numbers of T ($CD3^+$), B ($CD19^+$), NK ($CD16.56^+CD3^-$) cells, as well as T helper ($CD3+CD4^+$) and T suppressor ($CD3^+CD8^+$) cells were determined by flow cytometry using lyse-no-wash technique and four-color cocktails of antibodies. Additionally expression of RA and RO isoforms of CD45 molecule was determined on T helper and T suppressor cells. Commercially available compositions of antibody cocktails (see Table 2 for details) were used for determination of relative numbers of individual cell subsets. Trucount tubes (Becton Dickinson) were used to determine absolute lymphocyte counts. Antibody manufacturer's instructions were followed during the staining procedure. Briefly, 0.05 ml aliquots of blood were incubated with optimally titered antibodies for 15 minutes in room temperature. The incubation was followed by erythrocyte lysis using 0.45 ml of BD FACSLysing Solution (Becton Dickinson) diluted according to manufacturer's instructions. At least 15 000 events were acquired to properly calibrated flow cytometer, with lymphocyte gate defined based on CD45 expression and side scatter characteristics. Absolute numbers of individual cell subsets were calculated based

Table 2. Antibody cocktails used to detect basic lymphocyte subsets

Tube	FITC	PE	PerCP	APC
	Specificity/Clone	Specificity/Clone	Specificity/Clone	Specificity/Clone
1	CD3/SK7	CD8/SK1	CD45/2D1(HLe-1)	CD4/SK3
2	CD3/SK7	CD16/B73.1 CD56/NCAM 16.2	CD45/2D1(HLe-1)	CD19/SJ25C1
3	CD45RA/L48	CD45RO/UCHL-1	CD3/SK7	CD4/SK3
4	CD45RA/L48	CD45RO/UCHL-1	CD3/SK7	CD8/SK1

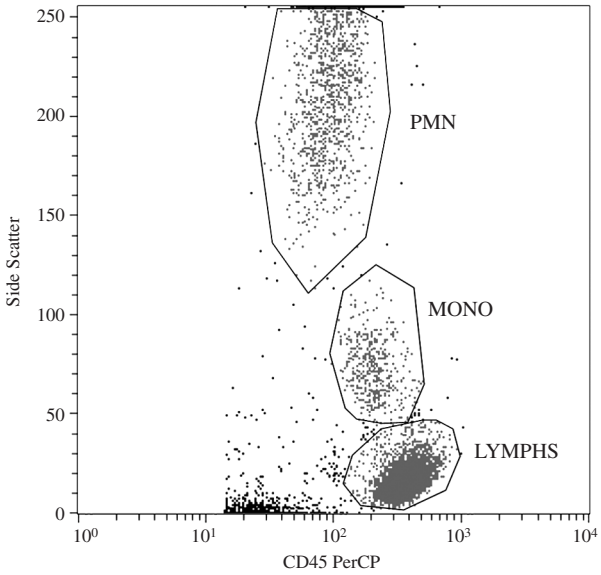

Fig. 1. Distribution of basic leukocyte populations in peripheral blood based on differential expression of CD45 and varying side scatter characteristics. Lymphocytes (Lymphs) demonstrate highest expression of CD45 and lowest side scatter characteristics in contrast to polymorphonuclears (PMN). Monocytes (Mono) demonstrate intermediate CD45 expression and side scatter characteristics

on proportion of the respective cell subpopulation and absolute lymphocyte count.

All analyses were performed on four color FACSCalibur cytometer (Becton Dickinson) equipped with licensed FACSComp, Multiset and Cellquest software, properly calibrated with CaliBRITE and CaliBRITE APC beads (Becton Dickinson). Standard quality control criteria recommended for all immunophenotyping analyses were applied [9]. To ensure data quality immunophenotyping results were reviewed for data consistency within each sample, i.e. the sum of T ($CD3^+$), B ($CD19^+$) lymphocytes and natural killer cells ($CD16.56^+CD3^-$) was required to be 100 ±5 [10].

Calculation of relative and absolute lymphocyte counts

Distribution of three leukocyte populations, i.e. lymphocytes, monocytes, and polymorphonuclears (PMN) was determined based on differential expression of CD45 and side scatter characteristics (see Fig. 1). Determination of absolute lymphocyte count was carried automatically by Multiset software in lymphocyte gate set based on CD45 and side scatter characteristics. Absolute sizes of individual lymphocyte subsets were calculated from the respective relative sizes and absolute lymphocyte counts. All results are presented as median and 5 to 95 percentile values.

Results

Distribution of leukocyte populations

Distribution of lymphocytes and polymorphonuclears, peripheral white blood cell populations essential for the immune response to foreign antigens, significantly varies with age, both in terms of relative and absolute cell counts (see Table 3). Polymorphonuclears (PMN), which compose the major population of peripheral blood leukocytes at birth, rapidly decline during the first year of life, replaced by equally rapidly increasing numbers of lymphocytes. Such variation is not observed for monocytes, which remain almost invariable throughout life, except for the first few days after birth when they rapidly increase from values found in cord blood. Changes in absolute lymphocyte counts significantly affect the absolute counts of individual cell subsets.

T lymphocytes and their subsets

Despite almost invariant relative numbers of T lymphocytes, their absolute count increases rapidly after birth reaching peak numbers in children 5-9 months old. Their number decreases thereafter along with decreasing number of lymphocytes. Although the relative number of $CD4^+$ T lymphocytes demonstrates declining tendency with age, in contrast to the $CD8^+$ subset, the absolute counts of both T cell subset reach peak values before the end of the first year of life and decrease thereafter reaching stable absolute counts in children older than 10 years. Such variation affects also the CD4:CD8 ratio which is highest in newborns and gradually decreases to values observed in adulthood (see Tables 4 and 5).

B lymphocytes

B lymphocytes present limited variability in terms of relative numbers in contrast to significant variation in their absolute counts which increase rapidly during the first 5 months of life and gradually decrease thereafter reaching plateau in children older than 5 years (see Tables 4 and 5).

NK cells

Both the relative and the absolute numbers of NK cells remain almost invariable during early childhood, except for the first days of life when they rapidly decrease from the numbers found in cord blood to those encountered in peripheral blood of newborns. Despite the tendency to increase their proportion in further life their absolute counts remain almost invariable (see Tables 4 and 5)

Expression of CD45 isoforms RA and RO

The relative numbers of both CD4-positive and CD8-positive T cells expressing RA isoform of CD45 molecule decrease gradually from the numbers observed in newborns to those found in adults. However, the absolute counts of

Table 3. Distribution of basic leukocyte populations in peripheral blood in children from various groups determined on cell subsets defined based on differences in CD45 expression and side scatter characteristics. The relative number of polymorphonuclears (PMN), monocytes and lymphocytes are presented as median and 5 to 95 percentile range

		PMN [%]	Monocytes [%]	Lymphocytes [%]	Lymphocytes [× 10^9/l]
Cord blood	median	54.7	10.0	34.3	3.8
	range	42.1-68.7	7.3-13.2	22.1-46.7	2.3-7.6
0-7 days	median	54.0	14.1	31.0	3.1
	range	34.6-70.8	7.6-23.2	18.0-48.6	2.3-5.1
8-60 days	median	29.3	10.8	59.2	5.0
	range	17.5-46.8	6.8-15.7	41.2-75.4	3.8-8.1
2-5 months	median	20.9	7.3	70.5	5.9
	range	11.7-48.3	4.8-14.4	40.0-79.3	3.3-7.6
5-9 months	median	24.2	6.4	68.4	6.2
	range	11.9-40.0	4.6-10.8	57.0-83.6	4.0-8.6
9 - 15 months	median	26.5	6.8	68.4	5.2
	range	12.8-37.4	3.7-11.1	52.0-82.5	2.6-7.8
15-24 months	median	32.5	6.9	58.4	4.6
	range	21.7-50.4	4.4-10.6	43.3-72.3	2.8-7.5
2-5 years	median	41.2	6.8	49.5	3.9
	range	23.5-63.4	4.2-11.7	29.6-69.2	2.3-6.9
5-10 years	median	51.6	8.1	39.7	2.6
	range	41.6-64.1	6.1-12.5	29.6-49.8	1.7-3.6
10-16 years	median	53.9	8.1	38.4	2.3
	range	36.2-64.9	6.1-10.5	24.7-56.0	1.5-3.9
> 16 years	median	53.8	7.9	37.9	2.1
	range	45.7-62.8	5.1-11.7	28.9-46.5	1.3-3.7

cells from both T cell subsets present a different pattern of changes in expression of CD45RA isoform: T $CD8^+$ lymphocytes remain almost invariable for almost 2 years of life and slowly decrease thereafter reaching stable numbers in children older than 5 years. The number absolute of T helper cells expressing CD45RA follows the pattern of changing T helper numbers and increase since the day of birth reaching maximum counts in children 5-9 months old and decrease thereafter, reaching stable counts in children above 10 years of age. Expression of CD45RO molecule on T lymphocytes remains unchanged throughout the first year of life, with the relative numbers of T helper and T suppressor cells expressing CD45RO beginning to increase during the second year of life and reaching plateau around 10 years of life. The absolute counts of T lymphocytes expressing CD45RO molecule remain invariable during the whole childhood (see Table 6).

Discussion

Enumeration of lymphocyte subpopulations in peripheral blood is considered of great significance for the evaluation of the immune status, despite the fact that peripheral blood lymphocytes represent only about 2% of their whole population in the body [11] and several factors affect their recirculation [12]. Interpretation of results requires access to reliable reference ranges, which is difficult in case of pediatric population, where age-related variation must be taken into account. Special effort must be made to obtain samples from healthy children, with age groups no more than few months apart [13]. Based on results of the study performed using a modern approach of multicolor flow cytometry and single platform technology for enumeration of cell counts, we determined normal values for basic lymphocyte subsets in pediatric population of Poland providing a useful tool for interpretation of clinical and laboratory data obtained from patients with suspected primary immune deficiencies.

Over the years composition of lymphocyte subsets has been widely studied in various populations [13-18] with differences that could be explained both by methodological aspects [19-21], life-style related factors [4, 22], as well as environmental conditions significantly affected worldwide due to increasing air pollution [23], race [15], or nutritional status [7]. Until results of this study have been summarized we used widely accepted normal values described by

Table 4. Relative frequencies of basic lymphocyte subsets in children from birth until early adulthood presented as median and 5 to 95 percentile ranges

Lymphocyte subset / Phenotype		T $CD3^+$	T helper $CD3^+4^+$	T suppressor $CD3^+8^+$	CD4 : CD8	NK $CD16.56^+3^-$	B $CD19^+$
Cord blood	median	63.9	45.0	19.2	2.4	16.4	17.2
	range	46.2-76.5	28.4-55.7	11.8-29.6	1.3-3.2	5.8-26.0	13.2-25.4
0-7 days	median	78.5	57.7	22.8	2.5	7.5	10.9
	range	60.1-87.5	41.6-68.2	15.8-30.1	1.8-4.4	2.7-20.3	6.2-24.9
8-60 days	median	69.5	48.4	18.6	2.8	9.9	19.9
	range	54.6 - 80.5	38.1-61.2	10.1-24.7	2.0-4.2	4.0-14.2	10.0-30.7
2-5 months	median	62.4	42.9	19.0	2.3	6.7	27.3
	range	57.1-72.4	37.7-50.3	13.6-24.2	1.8-4.3	4.6-9.6	18.4-37.5
5-9 months	median	65.9	46.2	17.0	2.7	6.2	25.5
	range	58.5-77.1	48.0-57.4	121-21.7	1.9-4.2	3.8-10.5	15.7-34.1
9-15 months	median	71.7	47.8	20.0	2.4	5.1	21.2
	range	54.9-79.2	34.8-59.9	14.6-28.8	1.4-3.6	3.4-14.9	13.9-28.2
15-24 months	median	67.9	44.6	20.4	2.2	6.2	22.7
	range	57.9-75.9	34.5-53.2	13.9-27.7	1.4-3.4	3.5-14.3	16.1-34.4
2-5 years	median	68.9	41.7	22.7	1.8	6.0	21.4
	range	54.9-77.6	32.8-46.9	14.5-30.4	1.1-2.8	2.9-19.8	14.1-28.5
5-10 years	median	70.5	39.4	25.0	1.5	9.8	15.7
	range	52.4-77.9	26.7-46.2	15.0-35.4	0.8-2.5	6.2-29.8	9.7-23.7
10-16 years	median	69.9	40.5	25.1	1.5	13.9	14.2
	range	52.9-79.1	27.4-54.3	18.2-33.2	1.1-2.7	5.2-28.6	9.4-22.8
>16 years	median	72.9	39.2	28.3	1.4	12.3	11.6
	range	59.7-82.0	30.4-51.2	19.0-38.9	0.8-2.5	7.3-24.0	7.2-22.5

Comans-Bitter [3], but due to frequently observed abnormal results in otherwise healthy children we decided to undertake our own study having in mind the differences in type of the population, as well as environmental and living conditions in Poland and western countries. The tested population appeared to be different in comparison to the Dutch one, with generally lower absolute total lymphocyte counts, the phenomenon affecting results for all tested lymphocyte subsets and their interpretation. Explanation of the differences between results of this study and quite similar results obtained in Poland and the Netherlands around 15 years ago analyzed by Zeman [1] and Comans-Bitter [3], respectively is not straightforward and was not the aim of this study.

Results of this study avoid the drawbacks of previous studies carried on Polish population, such as two color technique, lymphocyte gating performed based on side and forward scatter characteristics of cells, double platform approach for enumeration of cells, lack of absolute cell counts, or limited age groups [1, 24]. Patients with suspected severe primary immune deficiencies usually develop clinical symptoms during the first year of age, therefore detailed data regarding distribution of lymphocyte subsets during the first year of life are of greatest significance. Additional information on maturation of the immune system may come from the distribution of T lymphocytes expressing RA and RO isoforms of CD45 molecule considered to represent naive/activated and antigen-experienced/memory cells [25], with abnormalities common in various combined immune deficiencies [26] due to aberrant splicing mechanisms [25]. Children younger than one year, with unusually high proportion of T lymphocytes expressing CD45RO, may suffer from severe combined immune deficiency [27] or abnormal stimulation of lymphocytes [28], while significantly higher proportion of cells expressing CD45RA molecule may indicate e.g. acute EBV infection [29].

This study of normal pediatric lymphocyte population was designed for application in diagnostics of primary immune deficiencies and included samples taken from cord blood, newborns, and children from very narrow age groups to facilitate and advance time to reach the diagnosis, but will also be useful for the assessment of children with suspected secondary depression of the

Table 5. Absolute cell counts (× 10^6/l) for basic lymphocyte subsets in children from birth until early adulthood presented as median and 5 to 95 percentile ranges

Lymphocyte subset / Phenotype		T CD3+	T helper CD3+4+	T suppressor CD3+8+	NK CD16.56+3-	B CD19+
Cord blood	median	2.3	1.7	0.7	0.7	0.9
	range	1.6-4.9	1.1-3.8	0.5-1.4	0.2-1.7	0.4-1.4
0-7 days	median	2.5	1.8	0.7	0.2	0.3
	range	1.6-4.1	1.1-3.2	0.4-1.2	0.1-0.7	0.2-0.8
8-60 days	median	3.5	2.6	1.0	0.5	1.0
	range	2.2-5.5	1.4-4.2	0.4-1.4	0.2-1.0	0.7-1.8
2-5 months	median	3.5	2.3	1.1	0.4	1.4
	range	2.0-4.7	1.5-3.2	0.5-1.4	0.2-0.7	0.7-2.4
5-9 months	median	4.3	3.0	1.0	0.4	1.5
	range	2.8-5.7	1.8-4.4	0.6-1.5	0.2-0.8	0.7-2.8
9-15 months	median	3.6	2.1	1.0	0.3	1.0
	range	1.7-6.0	1.2-4.0	0.3-1.7	0.1-1.1	0.4-2.9
15-24 months	median	3.2	2.0	0.9	0.3	1.1
	range	1.9-5.0	1.3-3.4	0.5-1.7	0.2-0.9	0.6-1.9
2-5 years	median	2.6	1.6	0.9	0.2	0.9
	range	1.4-5.3	0.9-2.8	0.5-1.5	0.1-0.7	0.4-1.7
5-10 years	median	1.8	0.9	0.6	0.2	0.4
	range	1.0-2.6	0.5-1.5	0.3-1.0	0.1-0.7	0.3-0.6
10-16 years	median	1.6	0.9	0.6	0.3	0.3
	range	1.0-2.7	0.5-1.6	0.3-1.1	0.1-0.8	0.2-0.6
> 16 years	median	1.5	0.8	0.6	0.3	0.2
	median	0.9-2.6	0.5-1.6	0.3-1.2	0.1-0.5	0.1-0.6

immune system in course of various diseases and types of treatment. To our knowledge this is the first study carried on such a large and heterogeneous population of Polish children from various age groups, both from municipal as well as rural area, with verified health status, performed on whole blood samples, using multicolor flow cytometry, lyse-no-wash approach, and single platform technology. Our study demonstrates changes in sizes of individual cell populations occurring during childhood, with changes in relative counts that are not directly consistent with variation in absolute numbers. Short intervals between age groups during the first year of life allow demonstration of tremendous changes in the immune system encountering new antigens and gradually acquiring the ability to respond to that challenge. Methodology used in this study, in particular whole blood lysis sample preparation, CD45 vs. side scatter gating strategy and single platform lymphocyte enumeration which allow to use small blood sample without non-specific cell loss due to isolation of lymphocytes on density gradients [20, 30], avoidance of inclusion of nucleated red blood cells in lymphocyte gate that can falsify the ratio by disproportional reduction in lymphocyte subsets is particularly useful for pediatric population. Additional information on the proportion of other than lymphocytes white blood cells may bring faster diagnosis of diseases associated with abnormal monocyte or neutrophil count.

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Table 6. Relative and absolute numbers of T helper and T suppressor cells demonstrating RA and RO isoforms of CD45 molecule. Median relative frequency and 5 to 95 percentile ranges are presented for every cell subset

Age group / T cell subset			CD4+45RO+ (%)	CD4+45RA+ (%)	CD8+45RO+ (%)	CD8+CD45RA+ (%)
Cord blood	(%)	median	13.9	73.8	10.5	81.5
		range	6.1-26.4	43.6-89.3	6.4-21.8	62.2-91.3
	(× 10⁶/l)	median	0.24	1.16	0.10	0.59
		range	0.09-0.52	0.68-3.15	0.03-0.18	0.39-1.12
0-7 days	(%)	median	14.5	78.1	10.3	84.1
		range	6.2-25.1	52.6-90.7	5.4-23.0	67.4-93.6
	(× 10⁶/l)	median	0.26	1.35	0.08	0.59
		range	0.09-0.77	0.83-2.28	0.03-0.19	0.29-1.12
8-60 days	(%)	median	12.4	73.9	9.3	82.8
		range	8.5-19.2	52.9-81.3	4.9-15.1	74.3-91.4
	(× 10⁶/l)	median	0.32	1.59	0.08	0.82
		range	0.18-0.54	1.05-3.23	0.03-0.18	0.32-1.29
2-5 months	(%)	median	12.5	68.4	8.4	77.3
		range	9.8-20.6	57.5-85.2	4.7-30.1	59.4-92.5
	(× 10⁶/l)	median	0.32	1.67	0.08	0.83
		range	0.16-0.39	0.90-2.46	0.03-0.39	0.39-1.14
5-9 months	(%)	median	13.1	74.5	10.6	80.2
		range	8.8-18.0	59.5-85.3	3.8-22.1	67.5-90.1
	(× 10⁶/l)	median	0.35	2.35	0.13	0.79
		range	0.28-0.62	1.14-3.31	0.04-0.30	0.50-1.26
9-15 months	(%)	median	12.9	79.2	9.3	83.0
		range	7.9-21.3	71.0-96.1	4.6-24.3	67.0-98.2
	(× 10⁶/l)	median	0.35	1.74	0.12	0.88
		range	0.13-0.47	0.93-3.18	0.03-0.27	0.27-1.34
15-24 months	(%)	median	18.6	73.5	12.8	79.1
		range	13.4-30.9	60.7-81.9	7.8-22.9	65.7-86.6
	(× 10⁶/l)	median	0.39	1.58	0.14	0.72
		range	0.25-0.62	0.86-2.81	0.05-0.30	0.42-1.23
2-5 years	(%)	median	23.5	69.2	12.9	78.9
		range	14.4-35.1	54.3-78.4	7.5-32.9	61.0-88.5
	(× 10⁶/l)	median	0.38	1.11	0.13	0.70
		range	0.22-0.67	0.54-2.12	0.05-0.33	0.33-1.22
5-10 years	(%)	median	32.3	60.5	25.1	60.7
		range	21.6-47.5	42.9-70.0	14.6-45.3	48.3-77.5
	(× 10⁶/l)	median	0.30	0.53	0.14	0.39
		range	0.16-0.47	0.24-0.99	0.07-0.41	0.18-0.60
10-16 years	(%)	median	42.3	52.7	29.7	64.2
		range	27.2-62.0	31.1-66.3	15.9-46.4	44.1-77.1
	(× 10⁶/l)	median	0.37	0.52	0.16	0.35
		range	0.20-0.57	0.18-0.88	0.08-0.30	0.17-0.73
>16 years	(%)	median	44.7	46.0	29.4	64.5
	(× 10⁶/l)	median	37.9-60.0	26.9-57.2	16.4-49.0	44.9-76.9
	(%)	median	0.18	0.40	0.18	0.37
	(× 10⁶/l)	median	0.20-0.74	0.19-0.74	0.08-0.36	0.19-0.63

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