IMMUNE SYSTEM PARAMETERS IN CHILDREN OF CENTRAL AND EASTERN EUROPE: THE CESAR STUDY

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SUMMARY

Objective of this paper is to compare observed values of immune parameters obtained in the CESAR study (The Central Europe Study of Air Pollution and Respiratory Health, funded by EC PHARE program) with ranges derived from other large population-based studies.

Study design: Data were collected in healthy school children aged 9–11 years, in 6 countries: Bulgaria, the Czech Republic, Hungary, Poland, Romania and the Slovak Republic with the same standard approach in 1996. Random samples of 85 children per country, from 19 communities were selected from children having completed the health questionnaire, in total 495 children were analyzed. Lymphocyte subsets were determined by two-colour flow cytometric immunophenotyping using the lysed whole blood method (Becton-Dickinson). For determination of immunoglobulin concentration in sera nephelometric method (Behring Nephelometer system) was used.

Results: Medians, (5^{th}–95^{th} percentiles) of the lymphocyte subsets absolute count (x 10^9/l) were as follows: $CD19^+$ B cells 0.36 (0.13–0.66), total $CD3^+$ T cells 1.74 (0.98–2.90), $CD3^+CD4^+$ helper-inducer T cells 0.95 (0.47–1.78), $CD3^+CD8^+$ suppressor/cytotoxic T cells 0.71 (0.38–1.22), $CD3^-CD16^+56^+$ NK cells 0.36 (0.14–0.78), and for $CD3^+CD4^+$/ $CD3^+CD8^+$ ratio 1.4 (0.8–2.4). Medians, (5^{th}–95^{th} percentiles) of percentages of lymphocyte subpopulations (%) were as follows: $CD19^+$ B 13 (7–22), $CD3^+$ T 70 (59–80), $CD3^+CD4^+$ T 38 (27–48), $CD3^+CD8^+$ T 28 (20–39), $CD3^-CD16^+56^+$ NK cells 14 (6–27). Medians, (2.5^{th}–97.5^{th} percentiles) of the total immunoglobulin [g/l] were 11.7 (7.4–18.2) for IgG, 1.2 (0.5–2.5) for IgM, and 1.5 (0.5–3.4) for IgA.

Based on the aspects of the size of the CESAR immune biomarker study and on the use of the standardized protocols we recommend to use the reference ranges on lymphocyte subsets and immunoglobulin in Europe as provided by this study.

Key words: lymphocyte subsets, flow cytometry, immunoglobulin, reference intervals

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INTRODUCTION

Measurements of lymphocyte subsets and total immunoglobulin are generally considered of great value to the evaluation of the immune status. The quantity of immune biomarkers together with their functional capacity and their reactivity indicate the quality of the immune response (normal, suppressed, stimulated).

The accurate interpretation of lymphocyte subsets as well as immunoglobulin requires reliable reference ranges. Many reports of reference ranges of lymphocyte subsets and immunoglobulin were published, but few met criteria of sufficient sample size, description of health status, the use of standard analytical procedure, reference materials and statistical methods of assessment (1, 2, 3, 4). In addition, there is a lack of comparable data collected in different countries with the same standard approach.

From this point of view, data on the distribution of immune biomarkers (IBM) were collected within framework of the CESAR study (The Central European Study on Air Pollution and Respiratory Health) using the same approach in 6 Central and Eastern European countries, which resulted in one of the largest data sets.

METHODS

Study population. The CESAR study was conducted in Bulgaria, the Czech Republic, Hungary, Poland, Romania, and the Slovak Republic (5, 6). A total of 25 study urban areas were selected on the basis of differences in ambient air pollution level. For logistic reasons, information on immune biomarkers was collected in 19 of

the 25 study areas. In the areas, children aged 7 to 11 were invited to participate in the CESAR study. After obtaining the parental consent to participate (71.8% of invited), the parents of children filled out in a questionnaire on respiratory health, family history of respiratory health, passive smoking, and socio-economic status. Random samples of 85 children per country, 523 children in total aged 9–11 year with a completed health questionnaire were selected for the immune biomarker study. Samples were collected using the same protocol in all 6 countries between 11 April and 10 May 1996.

A venous blood sample was taken from each child in the morning and transported to the laboratory at a temperature of 4 ºC. Blood for immunophenotyping was drawn into Vacutainers containing Li-heparin as anticoagulant and analyses started within 10 hours after collection. The 495 children for whom blood samples were analysed for lymphocyte subsets or total immunoglobulin are included in the analyses.

Information about the presence of a common cold and medication use within last 24 hours and respiratory illnesses and medication use within 2 weeks prior to blood test were collected with a questionnaire among the children just before the blood sampling.

Laboratory parameters and methods. Laboratory assays have been chosen on the ground of WHO recommendation of assays panel for assessment of human immune status and immunotoxicity (7, 8).

Immune biomarkers were determined by standard operation procedure in selected laboratories. Since this report concerns investigation and results of lymphocyte subsets and serum immunoglobulin A, G, M, the used laboratory methods of immunophenotyping and nephelometry are described only.

Leukocyte subsets. Two-color flow cytometric immunophenotyping using the lysed whole blood method (Becton-Dickinson) was performed to determine lymphocytes subsets. Cells were analysed on Becton-Dickinson (FACScan, FACSCalibur) or Coulter flow cytometry system (EPICS XL Coulter) with automated or manual gating procedure.

Lymphocyte subsets were analysed after binding of specific monoclonal antibodies to cells surface receptors. For this purpose 100 µl aliquots whole blood were incubated at room temperature with 20 µl fluorescein isothiocyanate and phycoerythrin conjugated monoclonal antibodies (Becton-Dickinson). After incubation, the erytrocytes were lysed by Optilyse C and cells subsequently analysed by flow cytometer.

The lymphocytes gate was checked for its purity by use of CD 14^+/$CD45^+$ double staining and was regarded to be correct, if the gate included at least 95% of all lymphocytes and contained less than 5% contamination with monocytes, granulocytes, or cell debris. The following lymphocyte subsets were determined: $CD19^+$ B cells, total $CD3^+$ T cells, $CD3^+CD4^+$ helper-inducer T cells, $CD3^+CD8^+$ supressor/cytotoxic T cells, $CD3^-CD16^+56^+$ NK cells. The data were calculated both for the ratio of a particular subset, as a percentage of total lymphocytes and also as the absolute number of cells per microliter of blood.

The total leukocyte count was determined by hematocytometer and cell differential was performed manually. The total lymphocyte count was calculated from the lymphocyte percentage and the total leukocyte count.

Immunoglobulin levels. Due to standardisation, the nephelometric method using Behring Nephelometer System was chosen for determination of immunoglobulin concentration in sera. The equipment is fully automated with built-in software that performs automatically appropriate dilutions and mixtures of samples, antisera and reaction buffers. It has also built-in basic control system. All reagents, buffers, standards and controls used were of Behring origin. Calibration curves were constructed for all proteins using predefined standards.

Statistical methods. One of the aims was to compare the observed ranges of relative and absolute size of lymphocyte subsets and immunoglobulin with ranges derived from other large population-based studies. For this reason we have chosen the descriptive statistics that were used also in largest datasets published so far (2, 9).

Median and percentiles with their 95% confidence intervals without assumption about the distribution were determined for the absolute and relative size of blood cell counts, lymphocyte subsets and the concentrations of immunoglobulin. The 95% range for absolute size of lymphocyte subsets, as well as for immunoglobulin concentration was calculated by determining the antilog of the outcomes of the logarithmically transformed data, according to the mean ± 1.96 SD, assuming lognormal distribution.

A normal immune biomarker concentration is defined as a value within the 95 range, a low immune biomarker concentration as a value below the lower limit of the 95% range and a high immune biomarker concentration as value above the upper limit of the 95% range.

RESULTS

A total of 495 children aged 9–11 years had samples analysed, their mean age was 9.8 years. The distribution of the participants by gender and country is shown in Table 1. The overall percentage of girls was 52.5%.

Summary statistics for absolute counts and percentages are shown in Tables 2–3: white blood cells, neutrophils and lymphocytes in Table 2 (analysed for 471 children), lymphocyte subsets $CD19^+$, $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$, $CD3^-CD16^+56^+$, and $CD3^+CD4^+$/ $CD3^+CD8^+$ ratio in Table 3 (analysed for 457 children). Values of total immunoglobulin IgG, IgM, and IgA (analysed for 478 children) are summarized in Table 4.

When using the CESAR 95% range as the reference range for the leukocyte subpopulations, we compared the percentages of children with low or high immune biomarkers between countries. The highest percentage of children with low absolute number of leukocytes were observed similarly in both the Czech Republic (6.3%), and in Slovakia (5.9%). The most children with low absolute number of neutrophils were found in the Slovakian sample (15.3%), and with low absolute number of lymphocytes in the Czech sample.

The sample of children in Hungary has the highest percentage children with low $CD19^+$ B (21.5%), $CD3^+$ T (7.7%), $CD3^+CD4^+$ T lymphocytes (15.4%), and also $CD3^-CD16^+56^+$ NK cells (6.2%). For $CD3^+CD8^+$ T lymphocytes, the highest percentage of children with low values was observed in the sample from Romania (7.7%). As for total immunoglobulin, the highest percentages of children with low IgG (8.9%), IgA (11.4%), and also IgM (10.1%) were found in Romania. Similarly also in Romania we observed the

Table 1. *Description of the study population, CESAR immune biomarker survey, 1996*

	Bul	Cze	Hun	Pol	Rom	Slo	Total
Age (mean)	9.9	9.7	10.7	9.3	9.3	9.5	9.8
Boys (%)	48.6	53.2	43.3	46.3	47.3	47.1	47.5
No of participants	72	79	93	80	84	87	495

Table 2. *Summary statistics of selected blood cells (N=471)*

	Percentiles (P)						Median	95% range
	2.5%P	5%P	25%P	75%P	95%P	97.5%P		
Leukocytes								
[x 10^9/l]	3.8	4.0	5.0	7.2	9.0	10.0	6.1	3.6–10.0
Neutrophils								
[x 10^9/l]	1.2	1.4	2.3	4.0	5.5	6.2	3.1	1.3–6.9
% Leuk	26	31	45	59	68	71	52	
Lymphocytes								
[x 10^9/l]	1.4	1.5	2.1	3.1	4.2	4.3	2.5	1.4–4.5
% Leuk	23	31	45	59	68	69	42	

Table 3. *Summary statistics of the lymphocyte subsets, absolute sizes and relative frequencies within the lymphocyte population (N=457)*

	Percentiles (P)						Median	95% range
	2.5%P	5%P	25%P	75%P	95%P	97.5%P		
$CD19^+$ B								
[x 10^9/l]	0.10	0.13	0.24	0.47	0.66	0.75	0.34	0.12–0.91
% Lymph	5	7	11	16	22	25	13	
$CD3^+$ T								
[x 10^9/l]	0.90	0.98	1.45	2.16	2.90	3.2	1.74	0.93–3.30
% Lymph	55	59	66	74	80	82	70	
$CD3^+CD4^+$ T								
[x 10^9/l]	0.43	0.47	0.76	1.21	1.78	1.89	0.95	0.45–1.98
% Lymph	24	27	33	42	48	50	38	
$CD3^+CD8^+$ T								
[x 10^9/l]	0.32	0.38	0.54	0.91	1.22	1.42	0.71	0.34–1.44
% Lymph	18	20	24	32	39	41	28	
CD4/CD8 ratio	0.7	0.8	1.1	1.7	2.2	2.4	1.4	
$CD3^-CD16^+56^+$ NK								
[x 10^9/l]	0.13	0.14	0.25	0.49	0.78	0.93	0.36	0.13–0.95
% Lymph	5	6	11	18	27	32	14	

Table 4. *Summary statistics of the total immunoglobulin A, G, and M (N=478)*

	Percentiles (P)						Median	95% range
	2.5%P	5%P	25%P	75%P	95%P	97.5%P		
IgA [g/l]	0.5	0.8	1.2	1.9	2.9	3.4	1.5	0.7–3.4
IgG [g/l]	7.4	8.2	10.2	13.8	17.2	18.2	11.7	7.7–18.3
IgM [g/l]	0.5	0.6	0.9	1.6	2.4	2.5	1.2	0.5–2.8

highest percentages of children with high IgG (12.7%), and IgA (5.1 %).

Reference values of our study were compared with values of similar studies performed elsewhere. The results of the comparison are found in Tables 6–8.

White Blood Count (WBC): The statistical analysis of the 95% range of total WBC (3.6–10.0) and the absolute number of neutrophils (1.3–6.9) showed modestly lower limits of the 95% range compared with other published reference values. As for the absolute number of lymphocytes the 95% range (1.4–4.5) is comparable with other reference values (Table 6).

Lymphocyte subsets (Table 3): CESAR immunophenotyping data showed a lower percentage (median 13, 5Percentile 7, 95Percentile 22) and an absolute number (median 0.34, 5Perc 0.13, 95Perc 0.66) of $CD19^+$ B cells. The percentage and absolute number of $CD3^+$ (percentage: median 70, 5Perc 59, 95Perc 80; absolute number: median 1.74, 5Perc 0.98, 95Perc 2.9) and $CD3^+CD8^+$ (percentage: median 28, 5Perc 20, 95Perc 39; absolute number: median 0.71, 5Perc 0.38, 95Perc 1.22) were similar, the percentage of $CD3^+CD4^+$ (percentage: median 38, 5Perc 27, 95Perc 48; absolute number: median 0.95, 5Perc 0.47, 95Perc 1.78) slightly higher, and the percentage and absolute number of $CD3^-CD16^+56$ NK cells (percentage: median 14, 5Perc 6, 95Perc 27; absolute number: median 0.36, 5Perc 0.14, 95Perc 0.78) were also higher in comparison with other published reference values (2, 9 and Table 7).

Table 5. *Percentages of children with low/high immune biomarkers by country*

		Bul	Cze	Hun	Pol	Rom	Slo	Total
Leukocytes	*Low*	0.0	6.3	0.0	1.3	0.0	5.9	2.3
	High	4.3	2.5	1.3	3.8	2.5	0.0	2.3
Neutrophils	*Low*	0.0	3.8	0.0	5.0	0.0	15.3	4.3
	High	1.4	0.0	1.3	1.3	4.9	0.0	1.5
Lymphocytes	*Low*	2.9	6.3	5.3	1.3	4.9	0.0	3.4
	High	1.4	1.3	1.3	2.5	0.0	2.3	1.5
CD 19^+ B Lymphocytes	*Low*	1.4	1.3	21.5	0.0	2.6	0.0	3.9
	High	0.0	0.0	0.0	2.5	0.0	3.5	1.1
$CD3^+$ T Lymphocytes	*Low*	0.0	3.8	7.7	1.3	6.4	0.0	3.1
	High	2.9	0.0	1.5	3.8	1.3	2.4	2.0
$CD3^+CD4^+$ T Lymphocytes	*Low*	0.0	2.5	15.4	1.3	6.4	0.0	3.9
	High	1.4	2.5	1.5	2.5	0.0	1.2	1.5
$CD3^+CD8^+$ T Lymphocytes	*Low*	2.9	6.3	3.1	1.3	7.7	0.0	3.5
	High	0.0	0.0	1.5	3.8	1.3	3.5	1.8
$CD3^-CD16^+56^+$NK cells	*Low*	2.9	0.0	6.2	0.0	2.6	4.7	2.6
	High	0.0	3.8	3.8	5.0	0.0	1.2	2.2
IgA	*Low*	2.9	7.6	3.3	3.8	11.4	2.5	5.2
	High	2.9	0.0	2.2	1.3	5.1	1.2	2.1
IgG	*Low*	4.4	1.3	1.1	0.0	8.9	2.5	2.9
	High	0.0	0.0	0.0	1.3	12.7	0.0	2.3
IgM	*Low*	1.5	6.3	4.4	1.3	10.1	1.2	4.2
	High	2.9	0.0	0.0	3.8	2.5	0.0	1.5

Table 6. *Comparison of the CESAR observed ranges for selected blood cells with published data*

	CESAR	Bain, 1995 (18)		Dallman,1977 (19)
	95% range	95% range		95% range
	Age 9–11 years N=471	Age 9–10 years	Age 11 years	Age 10 years
Leukocytes				
[x 10^9/l]	3.6–10.0	3.9–9.9	4.5–13.5	4.5–13.5
Neutrophils				
[x 10^9/l]	1.3–6.9	1.5–5.9	1.5–5.9	1.8–8.0
Lymphocytes				
[x 10^9/l]	1.4–4.5	1.4–3.8	1.4–3.8	1.5–3.5

Table 7. *Comparison of the CESAR observed ranges for lymphocyte subpopulations with published data*

		CESAR	Hannet, 1992(9)		CESAR	Comans-Bitter, 1997 (2)	
		Age 9–11 years	Age 7–17 years		Age 9–11 years	Age 5–10 years	Age 10–16 years
		N=457	N=22		N=457	N=35	N=23
		Centile (95%CI)	Centile		Centile (95%CI)	Centile	Centile
Lymphocytes	*Median*	2.5 (2.42–2.58)	2.4	*Median*	2.5 (2.42–2.58)	2.8	2.2
[x 10^9/l]	*25Perc*	2.1 (2.0–2.15)	2.0	*5Perc*	1.5 (1.39–1.6)	1.1	1.0
	75Perc	3.1 (2.99–3.2)	2.7	*95Perc*	4.2 (3.93–4.29)	5.9	5.3
$CD19^+$ B	*Median*	0.34 (0.33–0.36)	0.4	*Median*	0.34 (0.33–0.36)	0.5	0.3
[x 10^9/l]	*25Perc*	0.24 (0.23–0.26)	0.3	*5Perc*	0.13 (0.10–0.15)	0.2	0.2
	75Perc	0.47 (0.45–0.49)	0.5	*95Perc*	0.66 (0.61–0.72)	1.6	0.6
% Lymph	*Median*	13 (13–14)	16	*Median*	13 (13–14)	18	16
	25Perc	11 (10–11)	12	*5Perc*	7 (5–7)	10	8
	75Perc	16 (16–17)	22	*95Perc*	22 (20–24)	31	24
$CD3^+$ T	*Median*	1.74 (1.69–1.80)	1.8	*Median*	1.74 (1.69–1.80)	1.9	1.5
[x 10^9/l]	*25Perc*	1.45 (1.39–1.50)	1.4	*5Perc*	0.98 (0.93–1.08)	0.7	0.8
	75Perc	2.16 (2.09–2.27)	2.0	*95Perc*	2.90 (2.83–3.06)	4.2	3.5
% Lymph	*Median*	70 (69–71)	70	*Median*	70 (69–71)	69	67
	25Perc	66 (65–67)	66	*5Perc*	59 (57–61)	55	52
	75Perc	74 (73–75)	76	*95Perc*	80 (79–81)	78	78
$CD3^+CD4^+$ T	*Median*	0.95 (0.93–0.99)	0.8	*Median*	0.95 (0.93–0.99)	1.0	0.8
[x 10^9/l]	*25Perc*	0.76 (0.71–0.80)	0.7	*5Perc*	0.47 (0.44–0.51)	0.3	0.4
	75Perc	1.21 (1.14–1.30)	1.1	*95Perc*	1.78 (1.63–1.87)	2.0	2.1
% Lymph	*Median*	38 (37.6–39)	37	*Median*	38 (37.6–39)	35	39
	25Perc	33 (32.6–34)	33	*5Perc*	27 (26–29)	27	25
	75Perc	42 (42–44)	41	*95Perc*	48 (47.8–50)	53	48
$CD3^+CD8^+$ T	*Median*	0.71 (0.68–0.74)	0.8	*Median*	0.71 (0.68–0.74)	0.8	0.4
[x 10^9/l]	*25Perc*	0.54 (0.51–0.58)	0.6	*5Perc*	0.38 (0.33–0.41)	0.3	0.2
	75Perc	0.91 (0.87–0.96)	0.9	*95Perc*	1.22 (1.17–1.31)	1.8	1.2
% Lymph	*Median*	28 (27–29)	30	*Median*	28 (27–29)	28	23
	25Perc	24 (24–25)	27	*5Perc*	20 (18–21)	19	9
	75Perc	32 (31–32)	35	*95Perc*	39 (37–41)	34	35
CD4/CD8	*Median*	1.4 (1.3–1.4)	1.3	*Median*	1.4 (1.3–1.4)	1.2	1.7
ratio	*25Perc*	1.1 (1.05–1.13)	1.1	*5Perc*	0.8 (0.7–0.82)	0.9	0.9
	75Perc	1.7 (1.6–1.75)	1.4	*95Perc*	2.2 (2.1–2.4)	2.6	3.4
$CD3^-CD16^+56^{+-}$NK	*Median*	0.36 (0.33–0.38)	0.3	*Median*	0.36 (0.33–0.38)	0.3	0.3
[x 10^9/l]	*25Perc*	0.25 (0.23–0.27)	0.2	*5Perc*	0.14 (0.13–0.16)	0.09	0.07
	75Perc	0.49 (0.46–0.51)	0.3	*95Perc*	0.78 (0.72–0.87)	0.9	1.2
% Lymph	*Median*	14 (14–15)	12	*Median*	14 (14–15)	12	15
	25Perc	11 (10–11)	9	*5Perc*	6 (5–7)	4	6
	75Perc	18 (17–19)	16	*95Perc*	27 (25–29)	26	27

Total immunoglobulin: CESAR values of total immunoglobulin are higher in IgG (2.5Perc 7.4, 97.5Perc 18.2) in comparison to published ranges, namely the 97.5th percentile [13 and 14 in (19, 20), 15.8 in (21) 15.3 in (22)] . The IgA (2.5Perc 0.5, 97.5Perc 3.4) and IgM (2.5Perc 0.5, 97.5Perc 2.5) values were modestly higher.

Reference values stratified by age and gender for immunoglobulin were compared with published data by Ritchie, 1998. Results of the comparison are shown in Table 9. Predicted medians, 2.5th and 97.5th percentiles for age 10 and both genders are compared with the calculated characteristics from the CESAR data using the same age group. Medians for all immunoglobulin are higher for the CESAR data for both genders in comparison to predicted values in (10, 11).

DISCUSSION

We have established range values in lymphocyte subpopulations and immunoglobulin levels in blood for children aged 9–11 years from Central and Eastern European countries.

In establishing normal ranges for lymphocyte subsets it is desirable that as large group of healthy children as possible should be studied to minimize the contribution of the various confounding factors even when sampling and analysis procedure are performed according to standard protocols, which takes into consideration health status, physical or emotional stress, use and abuse drug, circadian and seasonal variation in lymphocyte population, possible ethnic and gender differences, nutritional status, etc. (1, 12).

To achieve a standard laboratory protocol, a pilot study was conducted in participating laboratories from each country for laboratory selection. CD-Chex (immunology control for Cluster Designation) which could be analysed on Becton-Dickinson or Coulter flow cytometry system using manual or automated gating procedure was sent for analysis to specialised laboratories in four of the six countries: Bulgaria, the Czech Republic, Hungary and the Slovak Republic. The results showed that all laboratories met the criteria for the FACS procedure. WBC and differential count was performed in one laboratory in each country.

Immunoglobulin levels are also influenced by above mentioned factors. In addition, levels of IgA, IgG and IgM varies with age and IgM varies also with gender (i.e. levels are higher in females). For this reason age and gender specific reference values are necessary for correct result assessment (10, 11).

Table 8. *Comparison of the CESAR observed ranges for immunoglobulin with published data*

		CESAR	Thomas, 1995 (20,21) (Behring)		Frank, 1995 (22)	Bienveu, 1996 (23)
		Age 9–11 years N=478	Age 6–9 years	Age 10–13 years	Age 9–10 years	Age 10 years
		Centile (95%CI)	Centile	Centile	Centile	Centile
IgA [g/l]	2.5Perc	0.5 (0–0.61)	0.6	0.7	0.4	0.52
	97.5Perc	3.4 (3.0–3.6)	2.2	2.3	2.4	2.74
IgG [g/l]	2.5Perc	7.4 (0–7.9)	6.0	7.0	6.1	6.7
	97.5Perc	18.2 (17.5–19.5)	13	14	15.8	15.3
IgM [g/l]	2.5Perc	0.5 (0–0.53)	0.4	0.4	0.5	0.48
	97.5Perc	2.5 (2.4–2.9)	1.6	1.5	2.5	1.79

Table 9. *Comparison of predicted IgA, IgG, and IgM medians and selected centiles stratified by age and gender with the CESAR observed data*

		CESAR	Ritchie, 1998 (10)	CESAR	Ritchie, 1998 (10)
		Age 10 years Males N=95	Age 10 years Males	Age 10 years Females N=106	Age 10 years Females
IgG [g/l]	Median	11.2 (10.4–11.6)	10.3	11.4 (11.1–12.2)	10.1
	2.5Perc	7.5 (0–8.1)	6.6	6.8 (0–7.9)	6.4
	97.5Perc	17.3 (15.6–19.7)	16.2	16.9 (15.9–17.9)	15.9
IgM [g/l]	Median	1.09 (1.02–1.19)	0.95	1.38 (1.26–1.52)	1.19
	2.5Perc	0.40 (0–0.58)	0.38	0.60 (0–0.74)	0.48
	97.5Perc	2.43 (2.02–2.91)	2.35	2.60 (2.26–3.12)	2.94
IgA [g/l]	Median	1.65 (1.49–1.75)	1.35	1.43 (1.30–1.58)	1.23
	2.5Perc	0.40 (0–0.95)	0.57	0.65 (0–0.81)	0.52
	97.5Perc	3.55 (3.04–4.23)	3.18	2.81 (2.63–3.5)	2.90

The pilot study was conducted also for laboratory analyses of immunoglobulin levels. Samples of low, medium, and high concentration of immunoglobulin A, G, M were sent for analysis by nephelometry. Interlaboratory comparison showed a deviation of less than 5% for four laboratories: in the Czech Republic, Hungary, Poland, and Slovakia. These laboratories were selected for the immunoglobulin analysis in the main study.

The CESAR immune biomarkers study fulfills some of these criteria, i.e. the size of the study group, selection of healthy children provided by child's presence at school as well as by the questionnaire, and use of standard protocols for blood collection, data management and statistical analysis.

Enumeration of lymphocyte subsets. So far the largest published group is the European Collaborative Study (13) comprising 459 participating children of 3 years of age. Other published reports of immunophenotyping in healthy children of an age interval which can be compared with the CESAR immune biomarkers study are analysed in (9): 22 participants of age group 7–17; in (2): 35 of age group 5–10, 23 of age group 10–16; in (14): 10 children of age group 8–11; and in (15): 25 children of age group 9–10.

There are some differences in calculated values of lymphocytes subpopulations when comparing reference ranges: the reference intervals (5^{th} percentile –95^{th} percentile) of all lymphocyte subsets in our study are narrower than the reference intervals of the Comans-Bitter study (which is the most similar study regarding age grouping), with exception of $CD19^{+}$. It is expected, that 95% confidence intervals for the compared medians would be within each other sample variability.

Total immunoglobulin: The analyses of the CESAR data show higher total immunoglobulin reference values in comparison to predicted reference values by Ritchie, 1998. It is expected, that if interval ranges for the published data were provided, the ranges for the reference values would be within each other sampling variability.

Differences between countries found in results might be explained by factors influencing immunity status of children, such as environment, air pollution, socio-economic status, environmental tobacco smoking (16). These influences are analyzed in other publications based on the CESAR data (17).

CONCLUSION

Based on the aspects of the size of the CESAR immune biomarker study and on the use of the standardized protocols we recommend to use in Europe the reference ranges on lymphocyte subsets and immunoglobulin as provided by this study.

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REFERENCES

1. Bonilla FA, et al.: Normal ranges for lymphocyte subsets in children. J Pediatr 1997; 130 (3): 347–349.
2. Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WC, Groeneveld K, et al.: Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. J Pediatr 1997; 130 (3): 388–393.
3. Erkeller-Yuksel F, Deneys V, Yuksel B, et al.: Age-related changes in human blood lymphocyte subpopulations. J Pediatr 1992; 120:216–222.
4. Robinson M, et al.: An analysis of the normal ranges of lymphocyte subpopulations in children aged 5–13 years. Eur J Pediatr 1996; 155 (7): 535–539.
5. CESAR: Central European Study on Air Pollution and Respiratory Health. PHARE report; 1998: I. Evaluation. II. Questionnaires. III. Manuals and Protocols. IV. Study Results.
6. Fletcher T, Brunekreef B, Fabianova E, Gurzau, ES, Houthuijs D, Lebret E, et al.: Central European Study on Air pollution and Respiratory health (CESAR): an introduction. Epidemiology 1999; 10: S29.
7. Environmental Health Criteria 180. Principles and methods for assessing direct immune toxicity associated with exposure to chemicals. WHO, Geneva; 1996.
8. van Loveren H, Germolec D, Lowery HS, Luster MI, Nolan C, Repetto R, et al.: Report on the Bilthoven Symposium: Advance of epidemiological studies in assessing human health aspect of immunotoxic agents in the environment and workplace. Biomarkers 1999; 4:135–157.
9. Hannet I, Erkeller-Yuksel F, Deneys V, Lydyard P, De Bruyere M.: Lymphocyte populations as a function of age. Immunol Today 1992.
10. Ritchie RF, Palomaki GE, Neveux LM, Navolotskaia O, Ledue TB, Craig WY.: Reference distributions for immunoglobulin A, G, and M: a practical, simple, and clinically relevant approach in a large cohort. J Clinic Lab Anal 1998; 12 (6):363–370.
11. Ritchie RF, Palomaki GE, Neveux LM, Navolotskaia O.: Reference Distribution for Immunoglobulin A, G, and M: a comparison of a large cohort to the world's literature. J Clin Lab Anal 1998; 12 (6):371–377.
12. McNerlan SE, Alexander HD, Rea IM.: Age-related reference intervals for lymphocyte subsets in whole blood of healthy individuals. Scand J Clin Lab Invest 1999; 59:89–92.
13. The European Collaborative study. Age-related standards for T lymphocyte subsets based on uninfected children born to human immunodeficiency virus 1-infected women. Pediatr Infect Dis J 1992; 11:1018–1026.
14. Heldrup J, Kalm O, Prellner K.: Blood T and B lymphocyte subpopulations in healthy infants and children. Acta Paediatr 1992; 81:125–132.
15. Bofill M, Janossy G, Lee CA, Macdonald-Bums D, Phillips AN, Sabin C, Timms A, et al.: Laboratory control values for CD4 and CD8 T lymphocytes. Implications for HIV–1 diagnosis. Clin Exp Immunol 1992; 88:243–252.
16. Wagner V, Wagnerová M.: Ecoimunology. Avicenum; 1988. (In Czech.)
17. Leonardi GS, Houthuijs D, Steerenberg PA, Fletcher T, Armstrong B, Antova T.: Immune biomarkers in relation to exposure to particulate matter: a cross-sectional survey in 17 cities of Central Europe. Inhalation Toxicol 2000; 12(Supplement 4):1–14.
18. Bain BJ.: Blood cells. A practical guide. Blackwell Science; 1995.
19. Dallman, PR.: Blood cell normal ranges. In: Rudolph AM, editor. Pediatrics, 16^{th} ed. New York: Appleton-Century-Crofts; 1977. p.1178.
20. Thomas L.: Proteindiagnostik, Bestimmung von Serumproteinen, Anderung der Referenzbereiche bei Umstelung der Kalibration ab 12.9.95 CRP470. Views + Reviews, Aktuelle Labor-Information 1995; 1:1–4.
21. Thomas L.: Referenzbereiche für Plasmaproteine. Thomas Views + Reviews, Aktuelle Labor-Information 1998; 5:1–4.
22. Frank MM, Austen KF, Claman HN, Unanue, ER, editors.: Samter's immunology disease. 5^{th} ed. Little Brown and Company; 1995.
23. Bienvenu J, Whicher J, Aguzzi F.: Immunoglobulin *In:* Ritchie RF, Navolotskaia O. Serum proteins in clinical medicine. Scarborouhg Foundation of Blood Research 1996; 11:1–16.

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