Reference values of lymphocyte subsets from healthy Polish adults

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Abstract

The flow cytometry method could support physicians' decisions in the diagnosis and treatment monitoring of immunodeficient patients. Most clinical recommendations are focused on the search for alterations in T- and B-lymphocyte subsets, less commonly natural killer (NK) cells and granulocytes. While reference values for clinically meaningful lymphocyte subsets have been published ubiquitously among numerous countries, we have not found significant data for a population of adult Polish habitats; thus we determined reference values for T, B, and NK subsets according to sex and age. The female group showed a higher percentage of lymphocytes (CD45^{++}), T helper lymphocytes with a higher absolute count, as well as CD4/CD8 ratio, marginal zone-like B cells, class-switched B cells, and CD21^{low} B cells than the male group. The male group was found to have elevated percentages of naïve B lymphocytes, transitional B cells, and plasmablasts. A weak positive correlation with age was found among double positive T lymphocytes, natural killer T cells (NKT) lymphocytes, and CD21^{low} B cells. A negative correlation with age for double negative T lymphocytes, marginal zone-like B cells, and plasmablasts was noted. The results indicated the importance of creating distinct reference ranges regarding sex and age concerning immunophenotype.

Key words: *B lymphocytes subsets, class-switching B lymphocytes, CVID – common variable immunodeficiency, flow cytometry, healthy adults, immunophenotype, lymphocyte subsets, peripheral blood, NK – natural killer, NKT – natural killer T cells, primary immunodeficiency – PID, reference values.*

Introduction

Flow cytometry (FC) is a single-cell analysis technique routinely used in diagnostic procedures of hematological and immunological disorders. The clinical use began in the 1980s, from diagnosis of patients with acquired immunodeficiency syndrome (AIDS) [1]. The technique is still an inseparable tool for monitoring CD4+ T cell count in patients with AIDS, as a predictor of disease progression [2]. Currently, the applications of FC are significant, and it constitutes a fundamental tool assisting the diagnosis and monitoring of secondary (SID) and primary immunodeficiencies (PID) [3, 4] as well as in hematological disorders, e.g. leukemias [5, 6]. Less commonly, FC is a helpful tool in the diagnosis of infectious diseases [7], such as Lyme disease [8], allergology (e.g. basophil activation test) [9, 10], and reproductive health.

Primary immunodeficiencies are a heterogenous group of genetically determined disorders which cause an impaired, improper, or absent immune response from one or more leucocyte compartments. Primary immunodeficien-

cies commonly manifest recurrent infections [16]. Over 400 gene mutations have been associated with the occurrence of PID [17]. Mostly, PIDs are associated with diagnosis in infancy or childhood. Due to a better understanding of these diseases and a wider range of tests available for diagnosis, the number of inborn immune disorders is rising every year [18]. However, in the last decades more attention has been given to cases of PID in adults [19]. Data indicate that 40-60% of patients with PID are diagnosed as an adult [20, 21], with 8% of the patients being older than 65 years [21]. PIDs that have an onset in adulthood are different from those diagnosed in childhood, e.g. thymoma with immunodeficiency (infancy Good's syndrome). Most widespread are immunoglobulin (Ig) A deficiency, common variable immunodeficiency (CVID), late onset combined immunodeficiency (LOCID), idiopathic CD4+ lymphocytopenia, and GATA2 deficiency [21].

Common variable immunodeficiency, alongside selective IgA deficiency, is the most common immune deficiency, with heterogenous etiology, respecting adults. Recent

retrospective studies have shown that the median age in adult male patients is around 30 years, 33.5 years for females, and the average age of diagnosis CVID is around 35 years [20-22]. The onset of symptoms preceded diagnosis of CVID by 6-10 years [22-25]. In addition to clinical symptoms of CVID, such as recurrent infections, enlarged lymph nodes, persistent inflammation, patients have a deficiency or an absence of serum IgG (below 2 standard deviations of the mean according to age) and IgA or, in minority, IgM levels, as well as a poor response to vaccines [26-28]. Importantly, in the diagnosis of CVID, in addition to clinical manifestations and determination of antibody levels, flow cytometric phenotyping of lymphocyte subsets and evaluation of peripheral maturation of B lymphocytes are recommended [22]. Reduced CD4$^+$/CD8$^+$ ratio, lower numbers of CD45RA$^+$ T helper lymphocytes, lack or low numbers of B cells, elevated CD19$^+$CD21^{low} B lymphocyte subset, lack or low numbers of class-switched memory B lymphocytes (CD19$^+$CD27$^+$IgM$^-$IgD$^-$), and elevated transitional B cells (CD19$^+$CD38^{++}IgM$^-$) were observed in CVID patients [23, 27-32].

Evaluation of lymphocyte subsets is one of the most used laboratory techniques in the diagnosis of immunodeficiencies, and helps to distinguish between primary and secondary immunodeficiency [30, 33]. The method is rapid and helps to choose further steps in the diagnostic process, e.g., targeted genetic testing; thus in our research we used flow cytometry to establish reference values according to age for men and women. Primarily, we focused on establishing reference values of relative frequencies and absolute counts of T lymphocytes (including CD8 and CD4 subsets), B lymphocytes (including transitional, naïve, marginal zone-like, class-switched memory, CD21^{low} B lymphocytes, and plasma blasts), NK cells, and NKT lymphocytes, in healthy adults. Considering the wide onset age range, and evidence that women are diagnosed later than men [36], we aimed to assess differences in lymphocyte populations regarding sex and age.

Material and methods

Ethics approval

The Bioethics Committee of the Medical University of Warsaw approved the study. All measurements, interventions, and blood collections were performed after obtaining informed consent. The number of the Bioethics Committee statement is AKBE/83/2023.

Subjects and sample

Peripheral blood samples stored in 1.3 ml EDTA anticoagulant tubes were commercially obtained from the Regional Blood Centre in Warsaw. All participants had to meet the health criteria for healthy blood donors and were more than 18 years old at the time of sample collection. Finally, a group of 61 volunteers (31 women, 30 men) was included in the study. Information about age and gender was collected. All tests were performed within 4 hours of specimen collection.

Immunophenotyping

Multitest

Peripheral EDTA blood samples were mixed gently. 50 µl aliquots were transferred into Trucount tubes (BD Biosciences, USA) via reverse pipetting and 20 µl of antibody cocktail – BD Multitest 6-color TBNK (BD Biosciences, USA), containing CD3$^-$FITC/CD16$^+$CD56$^-$PE/CD45$^-$PerCP-Cy5.5/CD4$^-$APC/CD19$^-$PE-Cy7/CD8$^-$APC-Cy7, was added to each tube, then vortexed gently for 3-5 seconds and incubated at room temperature in absence of light for 15 minutes. After incubation, 450 µl of BD FACS Lysing Solution (BD Biosciences, USA) was added and left for further incubation (15 min, no light, room temperature) to lyse erythrocytes. A Becton Dickinson FACS-Canto II cytometer (BD FACS Canto II, Becton Dickinson, Franklin Lakes, NJ, USA) was used to collect data, and then analysis was performed with BD FACS Diva 6.1.3 software. For an example of analysis of the results, see Supplementary Materials Figure S1.

Staining for B lymphocyte subsets

Prior to the experiment, each antibody was titrated to determine the concentration with the highest signal-to-noise ratio (Table 1). Compensation was calculated using single antibody-stained live peripheral blood cells in previously determined concentrations. Additionally, fluorescence-minus-one (FMO) tests were performed to determine cut-off values of antigen-positive cell populations.

Table 1. Volumes of antibodies that were added to each tube, established by previous titration experiments; BD – Becton Dickinson – manufacturer

	FITC		PE		PerCP		APC	
	Antibody (clone)	Volume	Antibody (clone)	Volume	Antibody (clone)	Volume	Antibody (clone)	Volume
Tube 1	anti-IgM (G20-127) BD	5 µl	anti-IgD (IA6-2/δ-IA6-2) BD	2 µl	anti-CD19 (4G7) BD	6 µl	anti-CD27 (L128) BD	3 µl
Tube 2	anti-IgM (G20-127) BD	5 µl	anti-CD21 (B-ly4) BD	2 µl	anti-CD19 (4G7)	6 µl	anti-CD38 (HIT2) BD	5 µl

Sample preparation and staining were performed according to the procedure described by Piątosa *et al.* [34]. In order to minimize the staining interference of cell-bound antibodies, 300 µl aliquots of blood were washed in PBS containing 2% bovine serum and 0.1% sodium azide, and after centrifugation the supernatant was discarded. This step was repeated twice. Erythrocyte and leukocyte precipitate was resuspended in 200 µl of PBS containing 0.1% sodium azide. Aliquots of 100 µl were transferred to tube 1 and tube 2 and monoclonal antibodies were added as described in Table 1. Both tubes were incubated for 30 minutes at 4 degrees Celsius in the dark, then 1 ml of BD FACS Lysing Solution was added to each tube and incubated for 15 minutes, at room temperature, in the dark. Then, cells were washed twice and resuspended in 180 µl of PBS supplemented with 0.1% sodium azide.

Data acquisition

Cell readouts were acquired using a Becton Dickinson FACS Canto II cytometer (BD FACS Canto II, Becton Dickinson, USA). For automatic calculation of lymphocyte absolute count in a Multitest Trucount tube, FACS Canto Software (Becton Dickinson, USA) was used. Further analysis was performed with BD FACS Diva 6.1.3. software (Becton Dickinson, USA). Examples of data analyses are shown in supplementary data, Figure S1 for Multitest and Figure S2 for B cell subclasses, respectively.

Absolute counts of lymphocyte subpopulations were calculated as:

lymphocyte absolute count $\left(\frac{\text{cell}}{\mu l}\right) \times$ percentage of lymphocytes of lymphocyte subpopulation (%),

and absolute counts of B lymphocyte subpopulations were calculated as:

B lymphocyte absolute count $\left(\frac{\text{cell}}{\mu l}\right) \times$ percentage of lymphocytes of B lymphocyte subpopulation (%).

Statistical analysis

For each data set of lymphocyte subpopulation, the mean, standard deviation, standard error, median, and 5-95 percentile range were calculated for the 1) whole studied population and 2) with distinction for males and females. Data are shown in the tables. To analyze the differences between the male and female groups, an unpaired Student's *t*-test was used since all data sets were bigger than or equal to 30. To assess the correlation of each studied subpopulation with age, the Spearman correlation test was performed. The α level was set at 0.05; *p*-values below 0.05 were considered statistically significant. All statistical analyses were conducted with GraphPad Prism 8.3.4 and the power of each Student's *t*-test was determined with PQStat 1.8.0.

Results

A summary of our results is presented in Table 2 and Table 3 as mean, standard deviation, standard error, median, and reference range presented as 5 to 95 percentiles.

In the studied population, we found a higher frequency of lymphocytes in the female volunteers' group than in the male one (test power = 0.5082), higher frequency (test power = 0.7501) with higher absolute count (test power = 0.5488) of T helper lymphocytes, and higher CD4/CD8 ratio (test power = 0.5319) in the female group. In the male population, we found a higher frequency of CD4⁻CD8⁻ T cells in comparison to the female group (test power = 0.4473).

Considering B lymphocyte subpopulations, we found the following differences between male and female populations: lower frequency of naïve B lymphocytes (test power = 0.9656) and transitional B lymphocytes (test power = 0.8091) among the female group in comparison to men; higher frequency of marginal zone-like B lymphocytes (test power = 0.7221), class-switched B lymphocytes (test power = 0.8672), CD21^{low} B lymphocytes (test power = 0.6122) in the female in comparison to the male group. Interestingly, no difference was found in the absolute counts of the mentioned subpopulations between these two groups, except for transitional B cells, which were higher in the male group (power = 0.6132).

We found that the populations of CD4⁺CD8⁺ T and NKT lymphocytes had a weak positive correlation with age, and the population of CD4⁻CD8⁻ T cells had a weak negative correlation with age for both frequency and absolute count (Fig. 1A, C). A CD4⁻CD8⁻ T lymphocyte correlation with age was observed in the female population, whereas the same analysis showed no significance in the male group ($p = 0.14$, $R = -0.2719$). Additionally, among the studied B lymphocyte subsets, we found a negative correlation of absolute counts of marginal zone-like B cells (CD19⁺CD27⁺IgD⁺) and plasmablasts (CD19⁺CD38⁺⁺IgM⁻) with age (Fig. 2A, B) and a positive correlation of CD21^{low}CD38⁻ B cell frequency with age (Fig. 2C). Surprisingly, in the male group we found no correlation of any studied B lymphocyte subset with age and shifts in the subsets were observed in the female group only.

Discussion

Over the last thirty years a few similar studies have been performed in various parts of the globe, differing in the equipment and repertoire of antibodies used. One of the recent congruous studies was performed in Germany on healthy hospital staff volunteers, where a wide range of lymphocyte counts and frequencies were studied regarding immunosenescence, and differences in reference range according to gender [35]. The reference ranges of T lymphocytes in the mentioned study were similar to our results, excluding the CD8⁺ T lymphocyte percentage, which in our

Table 2. Summary of distinct leukocyte population frequencies and absolute counts from Multitest

Parameter	Mean	Std. dev.	Std. error	Median	Reference range (5-95 percentile)		FvsMSD
Age (years)							
Female	36.35	11.77	2.11	35.00	19.80	– 60.60	Not diff.
Male	35.77	10.54	1.92	35.50	20.20	– 52.90	
Male and female	36.07	11.09	1.42	35.00	21.10	– 54.90	
Granulocytes (% of WBC)							
Female	50.59	8.88	1.59	52.40	36.16	– 65.48	Not diff.
Male	53.89	7.26	1.33	53.40	39.96	– 67.71	
Male and female	52.21	8.23	1.05	53.00	36.74	– 67.09	
Monocytes (% of WBC)							
Female	9.52	2.14	0.38	9.30	6.26	– 13.62	Not diff.
Male	10.18	2.30	0.42	9.80	6.69	– 14.82	
Male and female	9.85	2.23	0.28	9.50	6.36	– 13.89	
Lymphocytes CD45^{++} (% of WBC)							
Female	39.83	8.27	1.49	38.20	25.10	– 54.16	*
Male	35.71	7.69	1.40	35.35	20.56	– 50.26	$p = 0.0486$
Male and female	37.80	8.19	1.05	37.00	20.86	– 53.35	
Lymphocytes abs. count							
Female	2092.67	438.68	78.79	2062.00	1241.60	– 2987.80	Not diff.
Male	1922.77	645.16	117.79	1839.00	1209.50	– 3860.95	
Male and female	2009.11	552.03	70.68	1958.00	1227.20	– 3183.80	
T lymphocytes/CD3$^+$ (% of lymph.)							
Female	73.51	6.37	1.14	72.10	61.88	– 82.38	Not diff.
Male	71.43	7.79	1.42	73.50	56.22	– 80.80	
Male and female	72.49	7.12	0.91	73.00	59.60	– 82.18	
T lymphocytes/CD3$^+$ abs. count							
Female	1538.79	361.80	64.98	1501.58	926.33	– 2308.71	Not diff.
Male	1377.32	486.37	88.80	1322.37	742.18	– 2603.01	
Male and female	1459.38	431.75	55.28	1408.50	771.29	– 2184.92	
Th lymphocytes/CD3$^+$CD4$^+$ (% of lymph.)							
Female	44.91	5.97	1.07	44.20	35.34	– 59.42	**
Male	40.38	7.20	1.31	39.35	29.35	– 55.11	$p = 0.0097$
Male and female	42.68	6.93	0.89	42.40	32.31	– 56.46	
Th lymphocytes/CD3$^+$CD4$^+$ abs. count							
Female	935.24	286.56	51.47	899.03	364.38	– 1449.97	*
Male	779.22	289.02	52.77	746.50	383.52	– 1418.34	$p = 0.0385$
Male and female	858.51	296.00	37.90	842.31	394.05	– 1360.57	
Tc lymphocytes/CD3$^+$CD8$^+$ (% of lymph.)							
Female	24.39	6.17	1.11	24.60	14.72	– 36.32	Not diff.
Male	25.69	6.09	1.11	24.95	16.33	– 38.44	
Male and female	25.03	6.11	0.78	24.80	15.27	– 35.92	
Tc lymphocytes/CD3$^+$CD8$^+$ abs. count							
Female	504.50	155.55	27.94	476.06	294.43	– 869.37	Not diff.
Male	495.28	189.45	34.59	492.40	212.03	– 949.74	
Male and female	499.97	171.66	21.98	485.58	230.73	– 888.87	

Table 2. Cont.

Parameter	Mean	Std. dev.	Std. error	Median	Reference range (5-95 percentile)			FvsMSD
Double positive lymphocytes CD3$^+$CD4$^+$CD8$^+$ (% of lymph.)								
Female	0.79	0.43	0.08	0.60	0.30	–	1.84	Not diff.
Male	0.66	0.50	0.09	0.45	0.10	–	1.70	
Male and female	0.73	0.46	0.06	0.60	0.20	–	1.70	
Double positive lymphocytes CD3$^+$CD4$^+$CD8$^+$ abs. count								
Female	16.29	9.38	1.68	13.18	7.12	–	39.22	Not diff.
Male	12.42	10.13	1.85	9.51	1.84	–	38.77	
Male and female	14.39	9.87	1.26	11.75	2.48	–	38.54	
Double negative lymphocytes CD3$^+$CD4$^-$CD8$^-$ (% of lymph.)								
Female	3.41	2.57	0.46	2.70	1.26	–	9.98	Not diff.
Male	4.69	2.81	0.51	4.25	1.28	–	11.24	
Male and female	4.04	2.74	0.35	3.20	1.32	–	11.03	
Double negative lymphocytes CD3$^+$CD4$^-$CD8$^-$ abs. count								
Female	70.87	55.99	10.06	51.41	25.12	–	222.44	Not diff.
Male	90.27	79.99	14.60	70.47	30.11	–	305.31	
Male and female	80.41	68.96	8.83	61.24	27.74	–	179.86	
CD4/CD8 T lymphocyte ratio								
Female	1.99	0.68	0.12	1.80	1.11	–	3.28	*
Male	1.67	0.51	0.09	1.69	0.82	–	2.70	*p* = 0.0411
Male and female	1.83	0.62	0.08	1.75	1.04	–	3.12	
NK cells (% of lymph.)								
Female	14.01	5.56	1.00	13.60	6.40	–	26.30	Not diff.
Male	14.15	5.36	0.98	13.55	6.25	–	25.43	
Male and female	14.08	5.42	0.69	13.60	6.43	–	24.72	
NK abs. count								
Female	288.84	119.61	21.48	270.25	115.07	–	554.41	Not diff.
Male	267.04	119.04	21.73	257.57	109.36	–	525.26	
Male and female	278.12	118.84	15.22	266.22	119.55	–	510.82	
NKT/CD3$^+$CD56$^+$ (% of lymph.)								
Female	5.22	3.44	0.62	3.90	1.56	–	13.00	Not diff.
Male	4.57	2.49	0.45	3.55	1.62	–	9.34	
Male and female	4.90	3.00	0.38	3.70	1.80	–	12.39	
NKT/CD3$^+$CD56$^+$ abs. count								
Female	108.94	75.57	13.57	75.01	28.66	–	283.06	Not diff.
Male	86.04	50.12	9.15	65.47	26.61	–	188.25	
Male and female	97.68	64.83	8.30	65.84	33.86	–	263.71	
B lymphocytes/CD19$^+$ (% of lymph.)								
Female	11.16	3.48	0.62	10.40	5.80	–	18.10	Not diff.
Male	12.93	4.99	0.91	11.50	7.03	–	25.29	
Male and female	12.03	4.35	0.56	10.90	6.24	–	22.29	
B lymphocytes/CD19$^+$ abs. count								
Female	236.98	99.44	17.86	205.30	94.14	–	461.44	Not diff.
Male	249.56	160.68	29,34	221.50	132.03	–	680.32	
Male and female	243.16	132.15	16.92	217.17	109.59	–	424.70	

Female n = 31, male n = 30, N = 61. NK – natural killer, NKT – natural killer T lymphocytes, lymph. – lymphocytes, abs. – absolute, Std. dev. – standard deviation, Std. error – standard error, FvsMSD – female vs. male statistical difference, Student's t-test.

Table 3. Summary of distinct B lymphocyte population frequencies and absolute counts

Parameter	Mean	Std. dev.	Std. error	Median	Reference range (5-95 percentile)			FvsMSD
Age								
Female	36.35	11.77	2.11	35.00	19.80	–	60.60	Not diff.
Male	35.77	10.54	1.92	35.50	20.20	–	52.90	
Male and female	36.07	11.09	1.42	35.00	21.10	–	54.90	
Naïve B cells IgD$^+$CD27$^-$ (% CD19$^+$)								
Female	57.93	12.49	2.24	56.50	31.90	–	78.04	***
Male	69.29	10.47	1.91	68.95	46.13	–	85.07	$p = 0.0003$
Male and female	63.52	12.80	1.64	67.10	38.36	–	82.79	
Naïve B cells IgD$^+$CD27 abs. count								
Female	142.66	80.93	14.54	128.30	36.64	–	324.02	Not diff.
Male	173.60	115.20	21.03	145.29	74.02	–	475.37	
Male and female	157.87	99.66	12.76	135.86	47.21	–	297.51	
Marginal zone-like B cells IgD$^+$CD27$^+$ (% CD19$^+$)								
Female	19.37	8.61	1.55	18.30	7.56	–	40.92	*
Male	14.60	5.33	0.97	13.90	4.74	–	24.69	$p = 0.0121$
Male and female	17.02	7.52	0.96	15.80	6.92	–	30.67	
Marginal zone-like B cells IgD$^+$CD27$^+$ abs. count								
Female	49.74	63.93	11.48	34.16	16.70	–	213.12	Not diff.
Male	36.93	31.41	5.73	29.01	11.99	–	132.61	
Male and female	43.44	50.62	6.48	31.58	16.05	–	101.84	
Class switched memory B cells IgM$^-$IgD$^-$CD27$^+$ (% CD19$^+$)								
Female	18.22	6.69	1.20	16.60	5.86	–	32.32	**
Male	13.29	5.56	1.01	11.35	5.56	–	25.34	$p = 0.0027$
Male and female	15.79	6.59	0.84	15.00	5.73	–	28.26	
Class switched memory B cells IgM$^-$IgD$^-$CD27$^+$ abs. count								
Female	42.53	29.34	5.27	36.13	10.09	–	113.66	Not diff.
Male	32.55	22.20	4.05	26.78	10.18	–	97.34	
Male and female	37.62	26.34	3.37	32.31	10.46	–	81.64	
Transitional B cells CD38^{++}IgM^{++} (% CD19$^+$)								
Female	1.83	1.54	0.28	1.40	0.56	–	5.78	**
Male	2.88	1.29	0.24	2.75	0.91	–	5.19	$p = 0.0058$
Male and female	2.35	1.51	0.19	1.80	0.61	–	5.10	
Transitional B cells CD38^{++}IgM^{++} abs. count								
Female	4.52	3.80	0.68	3.12	0.64	–	13.07	*
Male	7.09	4.93	0.90	6.22	1.25	–	20.15	$p = 0.0258$
Male and female	5.79	4.54	0.58	4.52	0.83	–	14.81	
Plasmablasts IgM$^-$CD38^{++} (% CD19$^+$)								
Female	1.28	1.23	0.22	0.90	0.20	–	4.50	Not diff.
Male	1.50	1.23	0.22	1.20	0.60	–	4.88	
Male and female	1.39	1.22	0.16	1.10	0.50	–	2.90	

Table 3. Cont.

Parameter	Mean	Std. dev.	Std. error	Median	Reference range (5-95 percentile)			FvsMSD
Plasmablasts IgM⁻CD38⁺⁺ abs. count								
Female	2.96	2.61	0.47	1.98	0.28	–	9.41	Not diff.
Male	3.49	2.71	0.49	2.66	1.07	–	10.73	
Male and female	3.22	2.65	0.34	2.56	0.62	–	8.32	
CD21^{low}CD38⁻ (% CD19⁺)								
Female	5.88	2.74	0.50	5.50	2.62	–	11.13	*
Male	4.11	3.30	0.60	3.05	1.57	–	13.02	$p = 0.0273$
Male and female	4.99	3.14	0.41	3.90	1.70	–	10.88	
CD21^{low}CD38⁻ abs. count								
Female	12.62	8.71	1.59	10.06	6.42	–	35.51	Not diff.
Male	9.55	7.40	1.35	7.10	3.07	–	30.14	
Male and female	11.09	8.16	1.05	8.95	3.21	–	29.80	

Female n = 31, male = 30, N = 61; abs. count – absolute count, Std. dev. – standard deviation, Std. error – standard error, FvsMSD – female vs. male statistical difference, Student's t-test.

study was lower, probably because we distinguished and measured the population of CD4⁺CD8⁺ cells, which was not included in the study of Kverneland *et al.* [35]. Additionally, we achieved narrower reference ranges, while the cohort size was comparable, which suggests that our studied population was more homogeneous. Kverneland *et al.* did not find any of the gender-related differences in the proportions of B lymphocyte subsets that we found, with the exception of the percentage of transitional B lymphocytes, which we found to be higher in males, whereas Kverneland *et al.* found the reverse dependency [35].

Similar studies performed in France showed that women had significantly lower levels of CD4⁻CD8⁻ and CD8⁺CD4⁺ T lymphocytes, naïve and transitional B lymphocytes, and higher levels of T lymphocytes, B lymphocytes, including class-switched B lymphocytes, and NK cells in comparison to the male group [36]. Some of the findings confirm our observations, e.g. elevated lymphocyte levels, class-switched B cells, and diminished transitional and naïve B cell levels. We did not confirm some of the differences. The results we obtained seem to be coherent with other published papers regarding means and reference ranges of percentages and absolute counts of studied immune cells [35, 37-40]. Concerning B lymphocyte subsets, our results were consistent with the Piątosa *et al.* study, performed on a group of young Polish adults and adolescents (more than 16 years old), although our reference ranges were wider and the age of participants was higher [34]. Shahal-Zimra *et al.* observed a lower average proportion of lymphocytes (28.43 ±6.04% vs. 37.80 ±8.19% of WBC) and a lower number (193.20 ±102.00 cells/µl vs. 278.12 ±118.84 cells/µl) as well as proportion (9.82 ±5.05% vs. 14.08 ±5.42%) of NK cells in comparison to our observations [37]. Likewise, Valiathian *et al.*

reported lower levels of NK cells [39]. In the research performed by Al-Thani *et al.*, the absolute count of CD8⁺ T lymphocytes was higher (967 ±364 cells/µl vs. 499.97 ±171.66 cells/µl) than in our research [38].

Our study confirmed previous observations, where females tended to have higher proportions of CD4⁺ T lymphocytes in comparison to males [35-38, 40-43]. Nonetheless, the observation is not reproduced in each study [39]. Consequently, a replicated elevated CD4/CD8 ratio in the female population was also found [35, 36, 39, 41, 43]. However, we failed to verify the finding that CD8⁺ T lymphocyte frequency is higher in the male than in the female population, which was reported in several research papers [35, 37, 41]. Nevertheless, a few other studies did not find that dependency [42-44]. The discrepancies in these results might depend on the origin of the studied population.

Our results showed age-dependency of NKT-like cells, CD4⁻CD8⁻ and CD4⁺CD8⁺ T lymphocytes and IgD⁺-CD27⁺CD21^{low} B lymphocytes as well as plasmablast frequency and absolute count. In the work of Apoil *et al.*, negative correlations of CD4⁻CD8⁻ and plasmablasts with age were observed, similarly to our work [36]. Double-negative T lymphocytes are often overlooked in publications establishing reference ranges. In a recent study by Villegas-Valverde *et al.*, it was noted that the population might have a negative correlation with age, significantly deteriorating after 50 years old, which supports our findings [45]. However, in our research, it concerns only the female population, while Villegas-Valverde *et al.* suggested that the correlation occurs in both groups, males and females [45]. It was reported that plasma and memory B cells decrease with age [46]. We partially confirmed that phenomenon in our study, since we found a negative correlation between age and memory CD27⁺IgD⁺ B
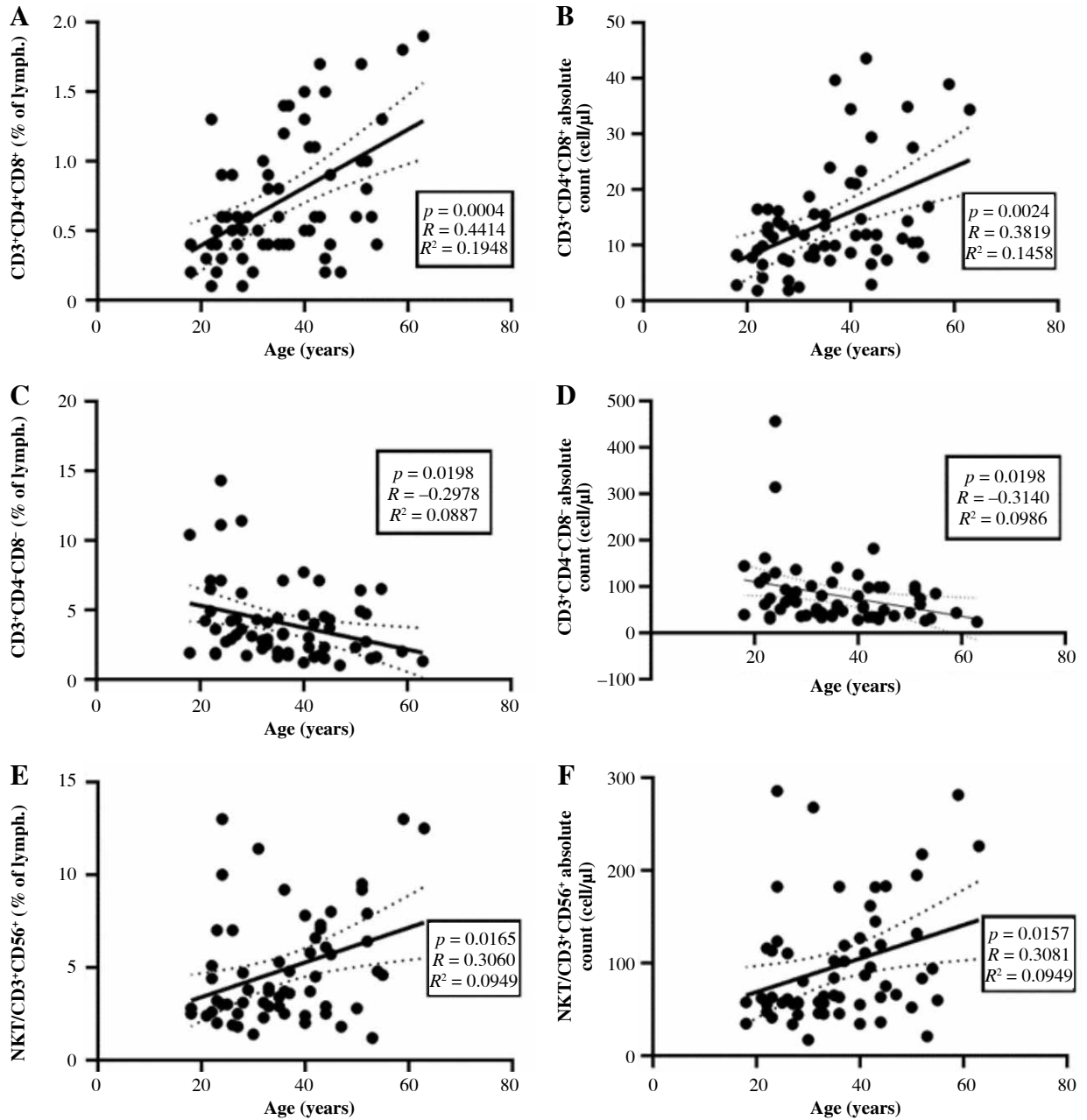
Fig. 1. Correlation of distinct lymphocyte subpopulations. **A**) Correlation of CD3$^+$CD4$^+$CD8$^+$ T lymphocyte frequency; **B**) Correlation of CD3$^+$CD4$^+$CD8$^+$ T absolute count with age; **C**) Correlation of CD4$^-$CD8$^-$ T lymphocyte frequency with age; **D**) Correlation of CD4$^-$CD8$^-$ absolute count with age; **E**) Correlation of CD3$^+$CD56$^+$ cell frequency with age; **F**) Correlation of CD3$^+$CD56$^+$ cell absolute count with age. Spearman correlation tests were performed due to the ordinal character of the age. The studied population consists of both men and women, $N = 61$
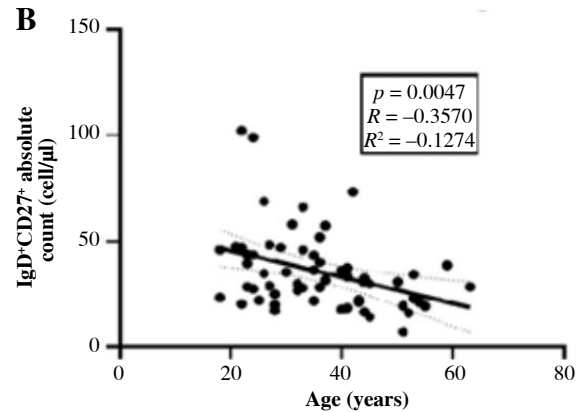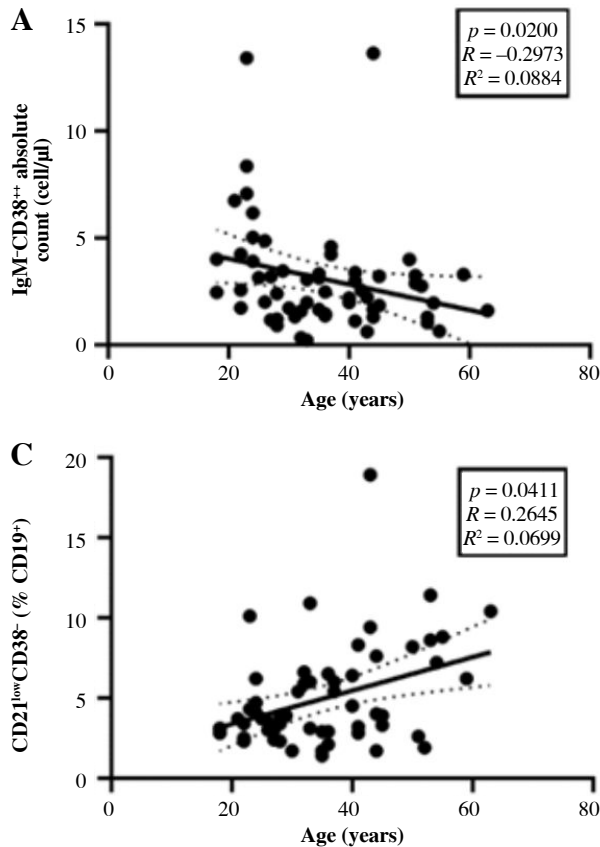
NK-T – natural killer T lymphocytes

lymphocytes. We noted the expansion of double-positive CD4$^+$CD8$^+$ T lymphocytes with age, as was reported by Laux *et al.* [47]. However, the double positive CD4$^+$CD8$^+$ T lymphocyte population is still poorly studied. The data of the role of CD4$^+$CD8$^+$ are controversial; cytotoxic and suppressive activity was reported [48]. Nevertheless,

the population has been associated with numerous types of cancer and infectious diseases [48].

In previous studies concerning immunosenescence it was found that CD27$^+$ B cells and exhausted memory B cells decline with age, which was reflected in our study, and CD21^{low}CD38$^-$ B lymphocyte count is higher with age [49].

A

p = 0.0200
R = –0.2973
R^2 = 0.0884

B

p = 0.0047
R = –0.3570
R^2 = –0.1274

C

p = 0.0411
R = 0.2645
R^2 = 0.0699

Fig. 2. Charts represent the correlation of distinct B lymphocyte subpopulations with age. **A**) Correlation of plasmablasts absolute count with age; **B**) Marginal zone-like B cell absolute count with age; **C**) CD21^{low}CD38$^-$ B lymphocyte frequency with age. Spearman correlation tests were performed due to the ordinal character of the age in this experiment. The population showed on the graphs consists of both men and women, $N = 61$

The data we gathered indicating a different composition of B lymphocyte subsets regarding sex seem to support the general observation that women tend to have a stronger immune response to infection and a better response to vaccination than men [50, 51]. The observation is usually explained by the immunosuppressive influence of testosterone, while inter alia estradiol enhances the survival of naïve B cells and contributes to the production of high-affinity immunoglobulins, which might be supported by our data showing higher proportions of class-switching B cells in the female group [51, 52]. Moreover, the age-related changes in the composition of B lymphocytes that we observed in females, but not in the male population, might be explained by hormonal changes in the post-menopausal phase in females [52]. Additionally, elevated proportions of naïve and transitional B lymphocytes have been associated with a higher survival rate of allogenic transplant, due to elevated immunoregulatory subsets [53]. This might also reflect poorer humoral immune responses in men.

We failed to find other studies that would reflect our findings in this matter, since only a small number of researchers have carried out such analyses.

Conclusions

There is a great benefit of better reference adaptation of reference ranges regarding age and sex, since peripheral blood lymphocyte populations are not distributed equally among men and women, which should be taken into consideration during diagnosis of PID or SID. Our results may contribute to a better understanding of immunosenescence and sex-related susceptibility to various diseases as well as secondary immunodeficiencies.

Declarations

Informed consent of participants was obtained. This study was performed in accordance with the principles of the Declaration of Helsinki. Ethical review and approval were not required to publish the case details in accordance with the local legislation and institutional requirements.

Supplementary material is available on the journal website.

The authors declare no conflict of interest.

References

1. Mandy FF (2004): Twenty-five years of clinical flow cytometry: AIDS accelerated global instrument distribution. Cytometry 58: 55-56.
2. Giorgi JV, Lyles RH, Matud JL, et al. (2002): Predictive value of immunologic and virologic markers after long or short du-

ration of HIV-1 infection. J Acquir Immune Defic Syndr 29: 346-355.

3. van Dongen JJM, van der Burg M, Kalina T, et al. (2019): EuroFlow-based flowcytometric diagnostic screening and classification of primary immunodeficiencies of the lymphoid system. Front Immunol 10: 1271.

4. Boldt A, Borte S, Fricke S, et al. (2014): Eight-color immunophenotyping of T-, B-, and NK-cell subpopulations for characterization of chronic immunodeficiencies. Cytometry B Clin Cytom 86: 191-206.

5. Matarraz S, Almeida J, Flores-Montero J, et al. (2017): Introduction to the diagnosis and classification of monocytic-lineage leukemias by flow cytometry. Cytometry B Clin Cytom 92: 218-227.

6. Theunissen P, Mejstrikova E, Sedek L, et al. (2017): Standardized flow cytometry for highly sensitive MRD measurements in B-cell acute lymphoblastic leukemia. Blood 129: 347-357.

7. van Eeden SF, Klut ME, Walker BA, et al. (1999): The use of flow cytometry to measure neutrophil function. J Immunol Methods 232: 23-43.

8. Nielsen CM, White MJ, Goodier MR, et al. (2013): Functional significance of CD57 expression on human NK cells and relevance to disease. Front Immunol 4: 422.

9. Bridts CH, et al. (2014): Flow cytometric allergy diagnosis: basophil activation techniques. In: Basophils and mast cells: Methods and protocols. Gibbs BF, Falcone FH (Eds.). Springer New York, New York, NY; 147-159.

10. Torres MJ, Padial A, Mayorga C, et al. (2004): The diagnostic interpretation of basophil activation test in immediate allergic reactions to betalactams. Clin Exp Allergy 34: 1768-1775.

11. Azargoon A, Mirrasouli Y, Barough MS, et al. (2019): The state of peripheral blood natural killer cells and cytotoxicity in women with recurrent pregnancy loss and unexplained infertility. Int J Fertil Steril 13: 12-17.

12. Vaquero E, Lazzarin N, Caserta D, et al. (2006): Diagnostic evaluation of women experiencing repeated in vitro fertilization failure. Eur J Obstet Gynecol Reprod Biol 125: 79-84.

13. Jerzak M, Kniotek M, Mrozek J, et al. (2008): Sildenafil citrate decreased natural killer cell activity and enhanced chance of successful pregnancy in women with a history of recurrent miscarriage. Fertil Steril 90: 1848-1853.

14. Kwak JY, Kwak FM, Ainbinder SW, et al. (1996): Elevated peripheral blood natural killer cells are effectively downregulated by immunoglobulin G infusion in women with recurrent spontaneous abortions. Am J Reprod Immunol 35: 363-369.

15. Ahmadi M, Abdolmohammadi-Vahid S, Ghaebi M, et al. (2017): Effect of Intravenous immunoglobulin on Th1 and Th2 lymphocytes and improvement of pregnancy outcome in recurrent pregnancy loss (RPL). Biomed Pharmacother 92: 1095-1102.

16. McCusker C, Warrington R (2011): Primary immunodeficiency. Allergy Asthma Clin Immunol 7 Suppl 1 (Suppl 1): S11.

17. Tangye SG, Al-Herz W, Bousfiha A, et al. (2020): Human inborn errors of immunity: 2019 update on the classification from the International Union of Immunological Societies Expert Committee. J Clin Immunol 40: 24-64.

18. Picard C, Gaspar HB, Al-Herz W, et al. (2018): International Union of Immunological Societies: 2017 Primary Immunodeficiency Diseases Committee Report on Inborn Errors of Immunity. J Clin Immunol 38: 96-128.

19. Bousfiha AA, Jeddane L, Ailal F, et al. (2013): Primary immunodeficiency diseases worldwide: more common than generally thought. J Clin Immunol 33: 1-7.

20. Schulman R (1995): Primary immune deficiency diseases in America: The first national survey of patients and specialists. Available from: https://primaryimmune.org/publication/surveys/primary-immune-deficiency-diseases-america-first-national-survey-patients-and.

21. Rosenberg E, Dent PB, Denburg JA (2016): Primary immune deficiencies in the adult: a previously underrecognized common condition. J Allergy Clin Immunol Pract 4: 1101-1107.

22. Chapel H, Lucas M, Lee M, et al. (2008): Common variable immunodeficiency disorders: division into distinct clinical phenotypes. Blood 112: 277-286.

23. Resnick ES, Moshier EL, Godbold JH, et al. (2012): Morbidity and mortality in common variable immune deficiency over 4 decades. Blood 119: 1650-1657.

24. Cunningham-Rundles C, Bodian C (1999): Common variable immunodeficiency: Clinical and immunological features of 248 patients. Clin Immunol 92: 34-48.

25. Ziętkiewicz M, Więsik-Szewczyk E, Matyja-Bednarczyk A, et al. (2020): Shorter diagnostic delay in polish adult patients with common variable immunodeficiency and symptom onset after 1999. Front Immunol 11: 982.

26. Ghafoor A, Joseph SM (2020): Making a diagnosis of common variable immunodeficiency: A review. Cureus 12: e6711.

27. Locke BA, Dasu T, Verbsky JW (2014): Laboratory diagnosis of primary immunodeficiencies. Clin Rev Allergy Immunol 46: 154-168.

28. Ameratunga R, Woon ST, Gillis D, et al. (2013): New diagnostic criteria for common variable immune deficiency (CVID), which may assist with decisions to treat with intravenous or subcutaneous immunoglobulin. Clin Exp Immunol 174: 203-211.

29. Patuzzo G, Barbieri A, Tinazzi E, et al. (2016): Autoimmunity and infection in common variable immunodeficiency (CVID). Autoimmun Rev 15: 877-882.

30. Abinun M, Albert M, Beaussant S, et al. (2011): ESID Registry – Working Definitions for Clinical Diagnosis of PID. 2020 November 2019 (cited 2020 Jan 11). Available from: https://esid.org/Working-Parties/Registry-Working-Party/Diagnosis-criteria.

31. Warnatz K, Denz A, Dräger R, et al. (2002): Severe deficiency of switched memory B cells (CD27(+)IgM(–)IgD(–)) in subgroups of patients with common variable immunodeficiency: a new approach to classify a heterogeneous disease. Blood 99: 1544-1551.

32. Wehr C, Kivioja T, Schmitt C, et al. (2008): The EUROclass trial: defining subgroups in common variable immunodeficiency. Blood 111: 77-85.

33. de Vries E; European Society for Immunodeficiencies (2012): Patient-centred screening for primary immunodeficiency, a multi-stage diagnostic protocol designed for non-immunologists: 2011 update. Clin Exp Immunol 167: 108-119.

34. Piątosa B, Wolska-Kuśnierz B, Pac M, et al. (2010): B cell subsets in healthy children: Reference values for evaluation of B cell maturation process in peripheral blood. Cytometry B Clin Cytom 78B: 372-381.

35. Kverneland AH, Streitz M, Geissler E, et al. (2016): Age and gender leucocytes variances and references values generated using the standardized ONE-Study protocol. Cytometry A 89: 543-564.

36. Apoil PA, Puissant-Lubrano B, Congy-Jolivet N, et al. (2017): Influence of age, sex and HCMV-serostatus on blood lymphocyte subpopulations in healthy adults. Cell Immunol 314: 42-53.

37. Shahal-Zimra Y, Rotem Z, Chezar J, et al. (2016): Lymphocyte subset reference ranges in healthy Israeli adults. Isr Med Assoc J 18: 739-743.

38. Al-Thani A, Hamdi WS, Al-Marwani A, et al. (2015): Reference ranges of lymphocyte subsets in healthy Qatari adults. Biomark Med 9: 443-452.

39. Valiathan R, Deeb K, Diamante M, et al. (2014): Reference ranges of lymphocyte subsets in healthy adults and adolescents with special mention of T cell maturation subsets in adults of South Florida. Immunobiology 219: 487-496.

40. Kokuina E, Breff-Fonseca MC, Villegas-Valverde CA, et al. (2019): Normal values of T, B and NK lymphocyte subpopulations in peripheral blood of healthy Cuban adults. MEDICC Rev 21: 16-21.

41. Uppal SS, Verma S, Dhot PS (2003): Normal values of CD4 and CD8 lymphocyte subsets in healthy Indian adults and the effects of sex, age, ethnicity, and smoking. Cytometry B Clin Cytom 52: 32-36.

42. Gize A, Mathewos B, Moges B, et al. (2014): Establishment of normal reference intervals for CD3(+), CD4(+), CD8(+), and CD4(+) to CD8(+) ratio of T lymphocytes in HIV negative adults from University of Gondar Hospital, North West Ethiopia. AIDS Res Treat 2014: 267450.

43. Kamallou A, Haji Abdolbaghi M, Mohraz M, et al. (2014): Reference values of lymphocyte sub-populations in healthy human immunodeficiency virus-negative Iranian adults. Iran J Immunol 11: 221-232.

44. Al-Mawali A, Pinto AD, Busaidi RA, et al. (2013): Lymphocyte subsets: Reference ranges in an age- and gender-balanced population of Omani healthy adults. Cytometry A 83: 739-744.

45. Villegas-Valverde CA, Kokuina E, Breff-Fonseca MC (2020): Determination of reference values for double-negative T lymphocytes in Cuban adults. MEDICC Rev 22: 48-50.

46. Caraux A, Klein B, Paiva B, et al. (2010): Circulating human B and plasma cells. Age-associated changes in counts and detailed characterization of circulating normal CD138- and CD138+ plasma cells. Haematologica 95: 1016-1020.

47. Laux I, Khoshnan A, Tindell C, et al. (2000): Response differences between human CD4(+) and CD8(+) T-cells during CD28 costimulation: implications for immune cell-based therapies and studies related to the expansion of double-positive T-cells during aging. Clin Immunol 96: 187-197.

48. Overgaard NH, Jung JW, Steptoe RJ, et al. (2015): CD4+/ CD8+ double-positive T cells: more than just a developmental stage? J Leukoc Biol 97: 31-38.

49. Frasca D, Diaz A, Romero M, et al. (2011): Age effects on B cells and humoral immunity in humans. Ageing Res Rev 10: 330-335.

50. Fish EN (2008): The X-files in immunity: sex-based differences predispose immune responses. Nat Rev Immunol 8: 737-744.

51. Giefing-Kröll C, Berger P, Lepperdinger G, et al. (2015): How sex and age affect immune responses, susceptibility to infections, and response to vaccination. Ageing Cell 14: 309-321.

52. Gubbels Bupp MR, Potluri T, Fink AL, et al. (2018): The confluence of sex hormones and aging on immunity. Front Immunol 9: 1269.

53. Newell KA, Asare A, Kirk AD, et al. (2010): Identification of a B cell signature associated with renal transplant tolerance in humans. J Clin Invest 120: 1836-1847.