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IMMUNOAGING – THE EFFECT OF AGE ON SERUM LEVELS OF NET BIOMARKERS IN MEN: A PILOT STUDY

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Abstract

Objectives: The study aimed to evaluate the impact of aging on the formation of neutrophil extracellular traps (NETs). The impaired formation of NETs is the cause of an abnormal innate immune response. **Material and Methods:** The study included a total of 45 healthy male subjects of different age groups. Whole blood was collected from the subjects, and the concentration of myeloperoxidase (MPO), the main biocidal protein in NETs, was determined in serum using ELISA. The serum levels of circulating free DNA (cfDNA), which are the structural basis of NETs, were also measured by fluorescence. In addition, the white blood cell count was determined, whole blood smear was evaluated, and the neutrophil-lymphocyte ratio was calculated. The variations in the levels of NET biomarkers were analyzed in different age groups. **Results:** The low levels of MPO (243.70 ng/ml) and cfDNA (6.24 ng/100 µl) in boys indicated neutrophil insufficiency for NETosis in children. A progressive increase in the levels of MPO and cfDNA with age was observed among adolescents (420.91, $p = 0.04$; 13.55, $p = 0.03$, respectively), with the highest level noted in the healthy adult group (466.58, $p = 0.01$; 14.07, $p = 0.01$, respectively). The levels of the studied parameters were comparable in adolescents and young adults, which proved that the NETosis process was appropriate and suggested the attainment of neutrophil maturity for the release of NETs in adolescence. The levels of MPO and cfDNA were low in older men (225.46, $p < 0.01$; 5.19, $p < 0.01$, respectively) indicating impaired NET formation. **Conclusions:** Data on the generation of NETs in different age groups obtained in this study can allow a better understanding of the ontogenesis of the immune system in terms of the course of NETosis, and also indicate the need to support nonspecific responses in children and adults. Further research should be performed to determine the possibility of regulating the NETosis process. Int J Occup Med Environ Health. 2023;36(3):333–48

Key words:
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INTRODUCTION

The aging of the body is closely related to the aging of the immune system components [1,2]. Several theories of aging have been proposed so far [3]. A large amount of scientific evidence supports the theory of accumulation of multiple damages and irreversible changes at the molecular level within the cell [4–6]. In contrast, the immunological theory of aging is centered on impaired lymphocyte immune memory. Impaired lymphocyte function results in, on the one hand, ineffective killing of pathogens and, on the other hand, recognition of own cells as foreign, which initiates self-destruction processes. Numerous authors have indicated that aging is associated with significant changes not only in the function but also in the number of lymphocytes [7–10].

The immune response, both cellular and humoral, deteriorates over time [11]. Abnormal antigen presentation, which is caused by abnormal functioning of T and B lymphocytes due to frequent gene mutations in lymphocytes, results in inappropriate response to tumor cells [12,13]. Prolonged infections, metabolic comorbidities, limited physical activity, and aging lead to chronic inflammation [14]. Constant immune stimulation exacerbates ongoing inflammation and increases the autoimmunization reactions [15–18]. On the other hand, increased immune-competent cells exhaustion leads to their failure, which worsens with age. Moreover, immune system depletion may increase sensitivity to infections [19,20].

Hematopoietic stem and progenitor cells, leukocytes, and their microenvironment also take part in aging-related changes [21]. Aging causes significant changes in the proportions of stem cell subpopulations, including a decrease in their number, and inhibits lymphoid differentiation in favor of myeloid cells. The dominance of the myeloid lineage in the bone marrow may be related to a longer survival time or a greater capacity for self-renewal, which seems to be independent of the age of these cells [22,23]. There are no reports in the literature indicating rapid changes in the number of polymorphonuclear neutrophils (PMNs), which suggests the preservation of a normal nonspecific response in elderly patients [24,25]. However, it has been found that neutrophil sensitivity to G-CSF and expression of TLR2 and TLR4 receptors decrease with normal TLR4 receptor signaling [26,27]. Furthermore, the expression of adhesion-related molecules CD16 and CD11b was decreased, and neutrophil chemotaxis was impaired in response to GM-CSF while normal chemotaxis was observed in response to LPS. Many authors have demonstrated a decline in the phagocytic capacity of neutrophils and their aerobic mechanism of pathogen killing [28]. In the elderly, impaired apoptosis is a remarkable functional change of neutrophils, which disrupts the survival time of these cells [29,30].

NETosis is a type of neutrophil death that differs from apoptosis and results in the release of genetic material linked to bactericidal proteins [31]. Neutrophils initiate the NETosis pathway in order to eliminate the source of infection as well as virulence factors. Neutrophil extracellular traps (NETs) were first described as a trap formed by activated neutrophils, but also monocytes/macrophages, eosinophils and even basophils can do this. Neutrophils produce and release NETs in different NETosis pathways, e.g., vital or suicide, depending on the stimulus of the microenvironmental. NETs are made of filaments 15–17 nm in diameter that form thickenings 25 nm in diameter aggregating into structures up to 50 nm in diameter. These compositions are primarily made of neutrophil nuclear DNA. Neutrophil extracellular traps also contain histones and proteins derived from neutrophil granules [32,33]. Proteolytic analysis of spontaneously produced or induced NETs confirmed the presence of 300 different proteins [34]. Subsequent studies on the structure of NETs revealed that DNA filaments of 15–17-nm diameter are composed of smaller parallel filaments 2 nm in diameter which correspond in diameter to the DNA double-helix structure. Neutrophil extracellular traps have slits with an area of $0.03\pm0.04\ \mu m^2$ and fibers with a length of 50–256 nm, and these structures allow them to mechanically trap pathogens [35,36].

Due to the lack of data on changes in the formation of neutrophil extracellular traps with age, the pilot project aimed to evaluate the important biomarkers of released neutrophil traps in whole blood serum obtained from healthy males of different age groups. The study determined the concentrations of circulating free DNA (cfDNA), the structural basis of NETs, and myeloperoxidase (MPO), the main biocidal protein involved in neutrophil traps. In addition, the neutrophil-lymphocytes ratio (NLR) was determined. The neutrophil to lymphocyte ratio is used as a marker of the immune response, especially neutrophils, and recently also in terms of NETs, as it may mirror the extent of degraded neutrophils in favor of NETs.

MATERIAL AND METHODS

The study was approved by the Bioethics Committee of the Medical University of Bialystok (resolution No. APK.O 02.440.2020).

Study participants

The study involved a total of 45 healthy male subjects who were divided based on age into 4 groups. The first group consisted of 5 healthy boys aged 7–11 years. The second group consisted of 10 healthy adolescents aged 12–15 years. Material from these 2 groups was obtained as part of a project conducted in 2014, entitled "Evaluation of the ability of lymphocytes to blastic transformation and expression of PI3K and ERK1 kinases in children with hypercholesterolemia" (No. 143-26590L), for which approval was obtained from the Bioethics Committee of the Medical University of Bialystok (resolution No. R-I-002/388/2014). Written consent was obtained from the parents each time before the material was collected from minors. The collected material was stored in accordance with the principles of Good Laboratory Practice.

The third group of study subjects consisted of 15 healthy adult men aged 23–36 years, while the fourth group consisted of 15 healthy older men aged 57–64 years. Material from these groups was obtained as a part of the project conducted in 2009, entitled "Cardiovascular diseases and cancer in the Podlaskie region – epidemiological situation, prevention and health promotion." The analyses were approved by the Bioethics Committee of the Medical University of Bialystok (resolution No. R-I-002/159/2009). The collected material was stored following the principles of Good Laboratory Practice. Written consent was obtained from all adult subjects before whole blood sampling.

The participants of the study were divided into 4 groups taking into account the age of reaching immune maturity around 12 years of age (for boys and teenage boys) and the variability of the functioning of the immune system related to the productive age (for men and older men). The authors tried to ensure that the groups were similar in age, the correctness of which was confirmed by similar research results in these groups.

The study participants did not take any medications or dietary supplements at least 48 h before the study. The health status of all subjects was assessed by a physician.

Research material

The study material was whole blood collected in 2 tubes: 9 ml with EDTA-K3 and 6 ml with a coagulation activator.

Determination of the total leukocyte count in whole blood samples

The white blood cell (WBC) count in the blood samples obtained from boys and adolescents was determined using a hematology analyzer during routine follow-up.

The WBC count in the blood samples of adults and older adults was determined by the chamber method. Briefly, whole blood was diluted with Türk's reagent (Aqua-Med, Łódź, Poland) and applied to a Bürker chamber, and then WBCs were counted using a light microscope (Olympus, Tokyo, Japan).

Evaluation of leukogram

The leukograms of the blood samples of boys were evaluated using a hematology analyzer during routine follow-up.

The leukograms of the blood samples of adults and elderly men were evaluated by microscopy. For this purpose, whole blood smears were made on basic slides and then stained with May-Grunwald-Giemsa reagent (Aqua-Med). The percentages of leukocytes were determined using a light microscope (Olympus).

Determination of MPO concentration by ELISA

The concentration of MPO in whole blood serum was determined using a Human Myeloperoxidase Quantikine ELISA Kit (R&D Systems, Minneapolis, USA), according to the manufacturer's instructions.

Evaluation of cfDNA levels by fluorescence

The concentration of cfDNA in whole blood serum was determined using a Circulating DNA Quantification Kit (Abcam, Cambridge, UK), according to the manufacturer's instructions.

Statistical analysis

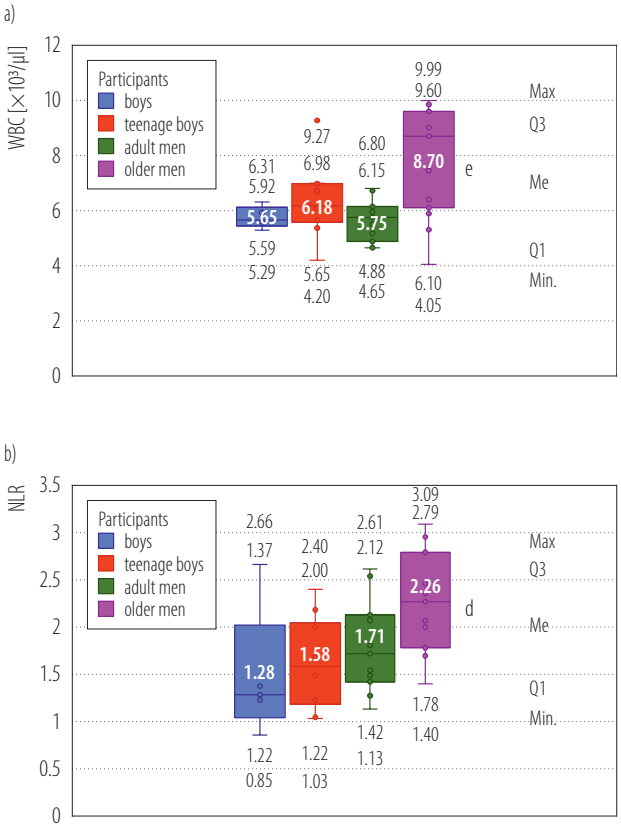
The obtained results were statistically analyzed using Microsoft Excel spreadsheet and Statistica v. 13.3 package. Data were presented as median with minimum and maximum values. The normality of data distribution was verified using the Shapiro-Wilk test. Statistical significance was tested using the Kruskal-Wallis test for comparisons between multiple groups. In cases of statistical association, post-hoc pairwise comparisons of mean ranks were conducted using a method such as the Dunn-Bonferroni test. The minimum level of statistical significance was set at $p = 0.05$. The interdependence of the studied parameters was analyzed using Spearman's method.

RESULTS

Evaluation of WBC and NLR

Since the leukocyte count reflects the state of the immune response, the WBC was tested. In order to verify the changes in the ratio of neutrophils to lymphocytes, the NLR was determined. Both WBC and NLR depend on the number of neutrophils, and their changes may be indirect indicators of NETosis. The aim was to assess the changes in WBC and NLR depending on age.

The total leukocyte count of each participant was found to be within the reference values for the specific age group. A statistically significant difference in the amount of WBC was observed only between adult men and older men (Figure 1, Table 1).



The WBC count in the blood samples obtained from boys (N = 5) and teenage boys (N = 10) were determined using a hematology analyzer or counted using a light microscope in Bürker chamber in the blood samples of adults (N = 15) and elderly men (N = 15). In addition, the NLR was determined.
Statistically significant difference between: [e] adult men and older men and [d] teenage boys and older men

Figure 1. The total leukocyte (WBC – white blood cells) counts and neutrophil–lymphocytes ratio (NLR) values in the study on impact of aging on the formation of neutrophil extracellular traps (NETs) (45 healthy male subjects, Medical University of Bialystok, Poland)

Table 1. Summary of the basic result values in the studied 45 healthy male subjects, divided into 4 groups, based on age – material from the boys and healthy adolescents groups was obtained as part of a project conducted in 2014; the adult men and older men groups was obtained as a part of the project conducted in 2009; Medical University of Bialystok, Poland

Variable	Participants (N = 45)				Kruskal-Wallis H test[1]		
	boys (N = 5)	teenage boys (N = 10)	adult men (N = 15)	older men (N = 15)	p	z	R
Age [years]							
Me	11	13	25	61			
range	7–11	12–15	23–36	57–64			
White blood cells [$\times 10^3/\mu l$]							
Me	5.65	6.18	5.75	8.70[e]	<0.01[e]	3.31[e]	16.70
							24.10
							15.73
							31.63
range	5.29–6.31	4.20–9.27	4.65–6.80	4.05–9.99			
Leukogram							
neutrophils							
Me	$2.97\times10^3/\mu l$	$3.45\times10^3/\mu l$	56%	64%			
range	$2.09–3.73\times10^3/\mu l$	$1.90–5.28\times10^3/\mu l$	51–68%	56–69%			
eosinophils							
Me	$0.15\times10^3/\mu l$	$0.09\times10^3/\mu l$	2%	1%			
range	$0.01–0.19\times10^3/\mu l$	$0.03–0.84\times10^3/\mu l$	0–7%	0–4%			
basophils							
Me	$0.07\times10^3/\mu l$	$0.02\times10^3/\mu l$	0%	0%			
range	$0.01–0.18\times10^3/\mu l$	$0.01–0.11\times10^3/\mu l$	0–1%	0–1%			
monocytes							
Me	$0.51\times10^3/\mu l$	$0.60\times10^3/\mu l$	5%	8%			
range	$0.41–0.70\times10^3/\mu l$	$0.29–0.74\times10^3/\mu l$	1–9%	1–11%			
lymphocytes							
Me	$2.16\times10^3/\mu l$	$2.20\times10^3/\mu l$	32%	28%			
range	$1.40–2.44\times10^3/\mu l$	$1.58–2.77\times10^3/\mu l$	26–45%	22–40%			
Neutrophil–lymphocytes ratio							
Me	1.28	1.58	1.71	2.26[d]	0.03[d]	2.79[d]	13.70
							16.55
							21.86
							31.53
range	0.85–2.66	1.03–2.40	1.13–2.61	1.40–3.09			

R – mean rank; z – the Dunn-Bonferroni test statistic for pairwise comparisons between each group's mean ranks.
Statistically significant difference between: [d] teenage boys and older men, [e] adult men and older men.
[1] $p = 0.0000$

The NLR value was the highest in older males, which was statistically significantly higher compared to that determined in adolescents (Figure 1, Table 1).

Analysis of leukograms

The assessment of the leukogram in the study groups was carried out in order to verify the changes in the percentage of individual leukocyte populations depending on age, which may be related to the formation of NETs.

The analysis of leukograms did not show statistically significant differences in the abundance of leukocyte populations – neutrophils, eosinophils, basophils, monocytes, and lymphocytes – in the studied groups. The mean values of the distribution of individual cell groups are shown in Table 1 and Figure 2.

Analysis of MPO concentration

In order to assess the variability of the NET formation phenomenon in different age groups, the concentration of MPO, the main biocidal trap protein, was assessed as an indirect marker of NETosis.

An increase in serum MPO concentrations was observed with age, with the highest concentration found in the adult male group.

The median level of MPO in adult males was statistically significantly higher compared to that in boys or older men. In contrast, the level of MPO in older men was statistically significantly lower compared to that in the adolescent group, and approached the level determined in boys. The serum level of MPO in adolescents was statistically significantly higher compared to that in boys (Figure 3a, Table 2).

Analysis of cfDNA level

In order to assess the age-related variability of NET formation, the concentration of cfDNA, which is the basis of neutrophil traps, was assessed as an indirect marker of NETosis.

The analysis of serum cfDNA concentration showed an increasing trend with age, similar to MPO.

The highest median level of cfDNA was observed in the adult male group, which was statistically significantly higher compared to that in boys and older men. In contrast, the lowest level of cfDNA was observed in older men which was statistically lower compared to that in adolescents. The level of cfDNA in the adolescent group was statistically significantly higher compared to that in boys (Figure 3b, Table 2).

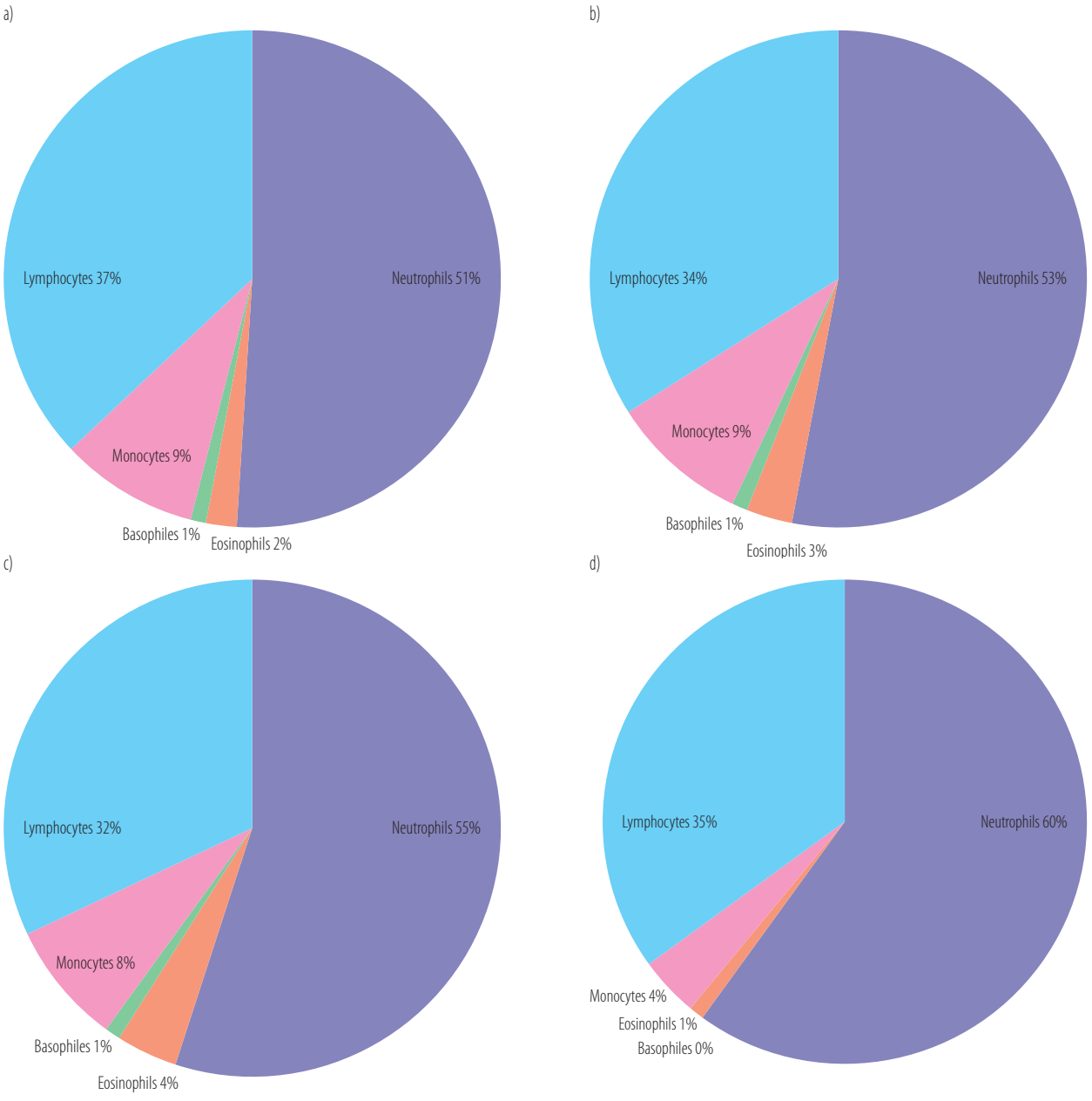
Assessment of relationships between the analyzed parameters

In order to assess the relationship between the parameters related to neutrophils and NETs, statistical analysis was performed taking into account the age of the participants.

A strong positive correlation between MPO and cfDNA concentrations (Figure 4d) and NLR index and neutrophil count (Figure 4a) was found in the group of boys. In addition, the MPO negatively correlated with lymphocyte count (Figure 4e) in this group. A positive correlation was also observed between age and cfDNA (Figure 4g), as well as between age and MPO (Figure 4f) levels, in boys.

The analysis of the values of parameters determined in adolescents showed a positive correlation between the WBC count and the count of neutrophils (Figure 4i) and lymphocytes (Figure 4h). Moreover, the neutrophil count correlated positively with the NLR index (Figure 4a) in this group.

A positive correlation was found between the levels of MPO and cfDNA (Figure 4d) in adult men. In addition, a positive relationship between the NLR index and neutrophil count (Figure 4a) and a negative correlation between the NLR index and lymphocyte count (Figure 4b) were observed in this group. A negative correlation was also observed between the neutrophil count and the lymphocyte count (Figure 4c) in these subjects.

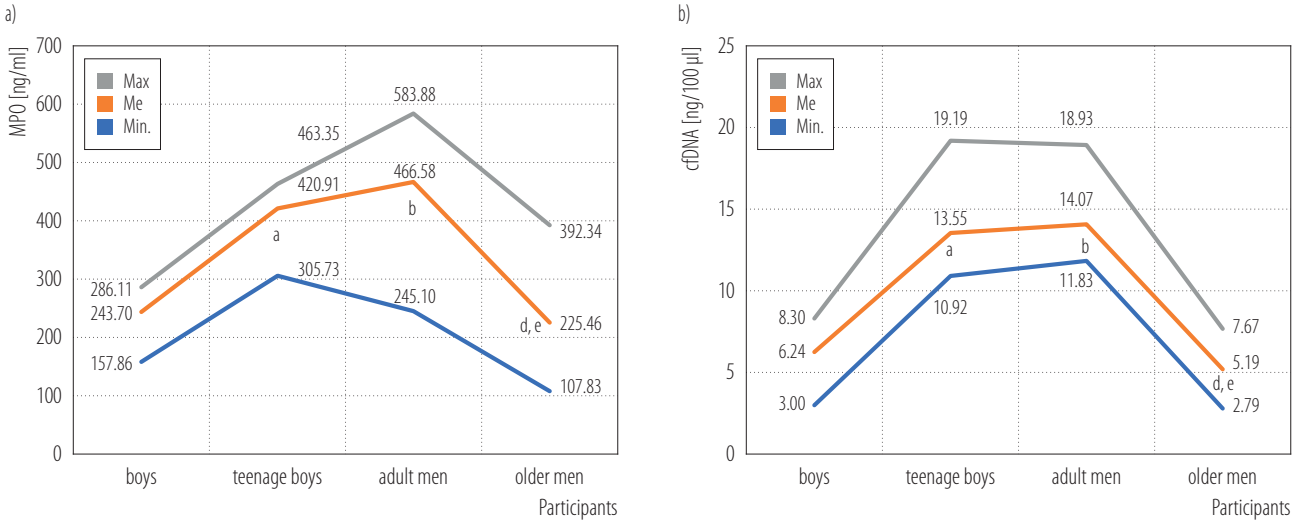


The leukograms of the blood samples of boys (N = 5) and teenage boys (N = 10) were evaluated using a hematology analyzed or determined using a light microscope whole blood smears of adults (N = 15) and elderly men (N = 15).

Figure 2. The leukograms diagrams: a) boys, b) teenage boys, c) adult men, d) older men, in the study on impact of aging on the formation of neutrophil extracellular traps (NETs) (45 healthy male subjects, Medical University of Bialystok, Poland)

Similar to other study groups, a positive correlation between the NLR index and the neutrophil count (Figure 4a) was observed in older men. In addition, a negative correlation between the NLR index and the lymphocyte count (Figure 4b) and between the lymphocyte count and the neutrophil count (Figure 4c) was observed.

The results of the correlation analysis are presented in Figure 4 and Table 3.



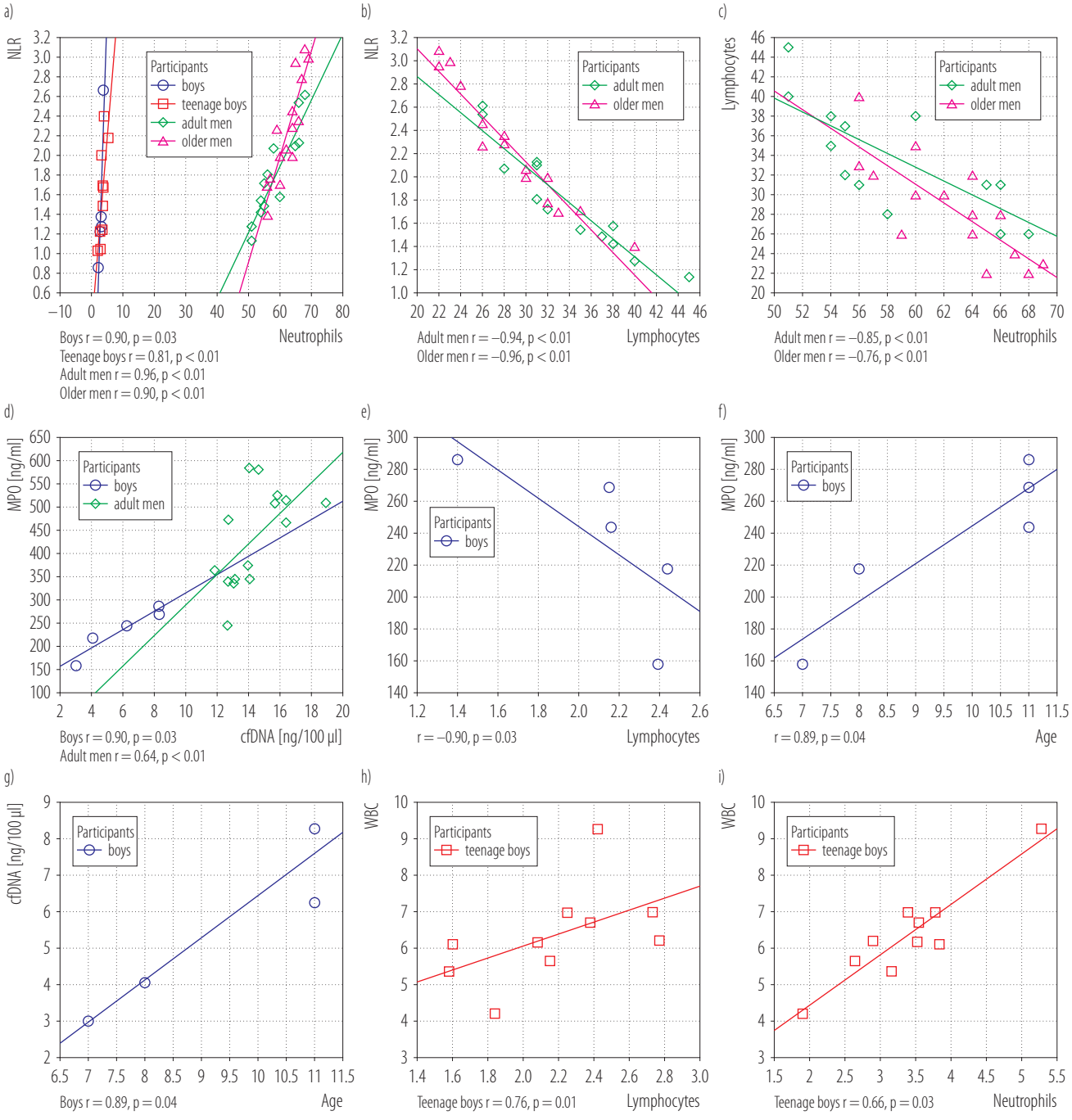
The concentration of MPO was determined using the ELISA kit, and the levels of cfDNA were evaluated by fluorescence method in whole blood serum obtained from boys (N = 5), teenage boys (N = 10), adults (N = 15) and elderly men (N = 15).
Statistically significant difference between: [a] boys and teenage boys; [b] boys and adult men; [d] teenage boys and older men; [e] adult men and older men.

Figure 3. The results of the a) myeloperoxidase (MPO) and b) circulating free DNA (cfDNA) analysis in the studied 45 healthy male subjects, divided into 4 groups (based on age), Medical University of Bialystok, Poland

Table 2. Summary of the result values for neutrophil extracellular traps (NETs) biomarkers in the study on impact of aging on the formation of neutrophil extracellular traps (NETs) (45 healthy male subjects, Medical University of Bialystok, Poland)

NETs biomarker	Participants (N = 45)				Kruskal-Wallis H test[1]		
	boys (N = 5)	teenage boys (N = 10)	adult men (N = 15)	older men (N = 15)	p	z	R
Circulating free DNA (cfDNA) [ng/100 µl]							
M±SD	5.97±2.41	14.59±3.03	14.40±1.91	4.96±1.59			
GM	5.54	14.31	14.29	4.72			
min.–max	3.00–8.30	10.92–19.19	11.83–18.93	2.79–7.67			
Me	6.24	13.55[a]	14.07[b]	5.19[d,e]	0.03[a]	2.72[a]	12.80
					0.01[b]	3.03[b]	32.40
					<0.01[d]	4.22[d]	33.40
					<0.01[e]	4.93[e]	9.73
Myeloperoxidase (MPO) [ng/ml]							
M±SD	234.77±50.17	412.53±44.39	433.73±103.79	224.18±69.58			
GM	229.97	410.13	421.44	214.42			
min.–max	157.86–286.11	305.73–463.35	245.10–583.88	107.83–392.34			
Me	243.70	420.91[a]	466.58[b]	225.46[d,e]	0.04[a]	2.71[a]	12.20
					0.01[b]	3.05[b]	31.70
					<0.01[d]	3.88[d]	32.93
					<0.01[e]	4.60[e]	10.86

GM – geometric mean; R – mean rank; z – the Dunn-Bonferroni test statistic for pairwise comparisons between each group's mean ranks.
Statistically significant difference between: [a] boys and teenage boys, [b] boys and adult men, [d] teenage boys and older men, [e] adult men and older men.
[1] p = 0.0000



The interdependence of the studied parameters was analyzed using the Spearman method for the results obtained in the group of boys (N = 5), teenage boys (N = 10), adults (N = 15) and elderly men (N = 15).

Figure 4. The results of the correlation analysis: a) positive correlation of neutrophil–lymphocytes ratio (NLR) and neutrophils in 4 study groups of men; b) negative correlation of NLR and lymphocytes in adults and elderly men; c) negative correlation of lymphocytes and neutrophils in adults and elderly men; d) positive correlation of myeloperoxidase (MPO) and circulating free DNA analysis (cfDNA) in boys and adults; e) negative correlation of MPO and lymphocytes in boys; f) positive correlation of MPO and age in boys; g) positive correlation of cfDNA and age in boys; h) positive correlation of WBC and lymphocytes in teenage boys; i) positive correlation of total leukocyte counts – white blood cells (WBC) and neutrophils in teenage boys (45 healthy male subjects, Medical University of Bialystok, Poland)

Table 3. Spearman's correlation in the studied 45 healthy male subjects, divided into 4 groups, based on age, Medical University of Bialystok, Poland

Variable	WBC		Lymphocytes		NLR		cfDNA		MPO	
	r	p	r	p	r	p	r	p	r	p
Boys (N = 5)										
age							0.89	0.04	0.89	0.04
neutrophils					0.90	0.03				
lymphocytes									–0.90	0.03
cfDNA									0.90	0.03
Teenage boys (N = 10)										
neutrophils	0.66	0.03			0.81	<0.01				
lymphocytes	0.76	<0.01								
Adult men (N = 15)										
neutrophils			–0.85	<0.01	0.96	<0.01				
NLR			–0.94	<0.01						
cfDNA									0.64	<0.01
Older men (N = 15)										
neutrophils			–0.79	<0.01	0.90	<0.01				
NLR			–0.96	<0.01						

cfDNA – circulating free DNA; MPO – myeloperoxidase; NLR – neutrophils to lymphocytes ratio; WBC – total leukocyte counts (white blood cells).
Summary of statistically significant Spearman's rank correlations observed in analyzed male groups.
The value of the r and p coefficients was presented.

DISCUSSION

Efficient functioning of nonspecific response cells is critical for the immunity of young organisms as the immune system, particularly the mechanisms of specific response, is immature in the early stages of life [37–40]. Neutrophils are the predominant population of nonspecific response cells. They are the first among leukocytes to reach the site of infection, where they recognize and eliminate pathogens. These multinucleated leukocytes employ several combative strategies, involving reactive oxygen species (ROS) or microbe-killing proteins packed into their cytoplasmic granules, and eliminate pathogens via different mechanisms such as phagocytosis, degranulation, or the formation of extracellular traps [41,42].

A key protein in the neutrophil arsenal is MPO, which takes part in almost every type of response of these cells [43]. It has been shown that in patients with complete MPO deficiency neutrophils fail to generate NETs, resulting in severe immune dysfunction [44,45]. Since NETs have a high amount of MPO, this protein is considered as their marker, in addition to cfDNA which acts as a scaffold for proteins and histones [46]. The positive correlation between the serum levels of MPO and cfDNA observed in the study confirms the close relationship between the parameters related to the release of neutrophil extracellular traps into the extracellular space.

Given the immaturity of specific defense mechanisms in neonates, the role of phagocytes is important to ensure the immune homeostasis of the body [47]. However, the low concentrations of MPO and cfDNA observed among young boys in the study indicate the failure of neutrophils to perform NETosis, which is probably related to

the immaturity and/or low number of these cells, as confirmed by the lowest neutrophil percentage determined in this group. In newborns, the absolute neutrophil count in the blood progressively increases within the first 14 h after birth and stabilizes only on the third day. It is also worth noting that immature neutrophil developmental forms can be found in the blood of a child [48].

The functions of neutrophils in newborns are impaired during inflammation. The chemotaxis of granulocytes as well as their ability to polarize is significantly lower in newborns than that observed in adults [49]. The bactericidal properties of neutrophils, including phagocytosis and degranulation, are also impaired at this age [50,51]. Furthermore, neutrophil granules in newborns have a very low content of biocidal protein compared to that in adults [47]. However, the level of ROS production is similar to that observed in adults [50].

The results of this study are consistent with the observations of other authors. Studies on neutrophils from premature neonates have demonstrated the inability of PMNs to perform NETosis. In contrast, neutrophils from full-term neonates released negligible amounts of NETs. Researchers have also examined the level of expression of the TLR4 receptor on neonatal neutrophils to identify the cause of NETosis disorder. Lipopolysaccharide (LPS), which is a ligand of the TLR4 receptor, is also an activator of NETosis. It was found that TLR4 expression in newborns was comparable to that in adults. In addition, neonatal neutrophils failed to form NETs after incubation with *Escherichia coli* or *Staphylococcus aureus* bacteria, and their ability of extracellular elimination of bacteria was impaired [52]. Similar results were reported by Lipp et al. [53] confirming the inability of neutrophils to activate the NETosis pathway. The impaired ability of neutrophils to initiate NETosis may be attributed to the presence of immature neutrophil developmental forms in neonatal blood. Martinelli et al. [54] demonstrated that stimulation of neutrophil precursors by IFN-α or IFN-γ followed by C5a did not result in the release of NETs as done by mature neutrophils. In addition, Yost et al. [55] indicated that a protein derived from cord blood showed the ability to inhibit the NETosis process. Neonatal NET-inhibitory factor inhibits PAD4 activity, histone citrullination, and nuclear chromatin decondensation, all of which are important for NETosis. This factor has not been detected in the blood of adults, which may explain the deficit of NETs in neonates and young children [55,56].

The positive correlation between age and the MPO and cfDNA levels observed in the group of boys in this study suggests that neutrophils acquire the ability to form NETs during the maturation of the immune system. This thesis is supported by the fact that the levels of MPO and cfDNA in the group of adolescents were similar to that in adult men and, at the same time, significantly higher than in boys. The progressive increase in the levels of MPO and cfDNA with age and the comparable levels of NET biomarkers in adolescent boys and adult men confirm appropriate NETosis and suggest that neutrophils reach maturity in terms of NETs release during adolescence.

The low serum levels of MPO and cfDNA observed in older men in this study indicate that the formation of NETs is impaired in this age group. The aging process is associated with chronic low-grade inflammation, which may be related to the increase in total leukocyte count as observed in older men [57]. Moreover, the percentages of particular populations of immune cells change with age [48]. In the study, the tendency of the NLR index to increase with age, with the highest value observed in the group of older men, confirms the changes in the percentage of the leukocyte population toward neutrophils. The negative correlation observed between the count of neutrophils and lymphocytes and between the NLR index and the lymphocyte count justifies the conclusions drawn.

The significantly higher NLR index found in older men compared to boys seems to suggest that nonspecific mechanisms play a greater role in the immune response in the elderly.

However, the functional efficiency of leukocytes has been shown to reduce with age. Immunosenescence is epitomized by the low efficiency of the immune system in combating not only previously encountered pathogens but also new ones [58]. Neutrophils from the elderly have been characterized by a lower capacity of chemotaxis in comparison to neutrophils from younger people [59]. They also continuously stimulate the CXCL8 receptor expressed by the increased activity of PI3K. Neutrophil degranulation has been found to be reduced in the elderly [60]. Moreover, PMNs are characterized by a reduced ability to phagocytose opsonized pathogens in this age group. The reason for this dysfunction is the low expression of CD16 molecules [28]. Furthermore, regression in the neutralization of phagocytosed bacteria was observed in the elderly. The data on the generation of ROS by neutrophils in the elderly are conflicting, suggesting minimally elevated ROS production as well as its regression [28,60].

The low levels of MPO and cfDNA observed in the study in elderly subjects, which were comparable to that in boys, indicate an impaired ability to form NETs, which may be the cause of the impaired antipathogenic response shown by numerous studies. The findings of the study are in line with those of Hazeldine et al. [61] who demonstrated a lower ability of neutrophils from elderly subjects to initiate NETosis. Neutrophils stimulated with IL-8 or LPS were also characterized by a lower ability to produce ROS, in addition to impaired generation of NETs, which the authors linked with the failure of NETosis, with normal expression of receptors for IL-8 and LPS [61]. The reduced ability of neutrophils to release NETs may be related to the lower expression of Atg5 protein, which is involved in autophagocytosis. In a study on an experimental model, stimulation of the TLR2 receptor on the neutrophils of older mice resulted in a lower degree of activation of NETosis compared to that in young mice. Induction of the TLR2 receptor increases the lifespan of neutrophils, but in older mice it results in the induction of the apoptosis pathway [62]. Furthermore, differences between young and old mice in the distribution of lipid rafts after TLR2 stimulation were observed, which may account for the impaired generation of NETs in older individuals [63].

CONCLUSIONS

In both early life and old age, the immune system presents a number of abnormalities. Leukocytes differ in their number and function contributing to increased morbidity and even mortality in children as well as the elderly. However, the comparable levels of MPO and cfDNA in young boys and older men are associated with different mechanisms: immaturity of the immune system in adolescence and impairment in the elderly. The preliminary data on the levels of NET biomarkers in different age groups provide valuable information on the course of NETosis, a pathogen elimination process, and also indicates the necessity to support the nonspecific response in children and adults.

The strongest point of this manuscript are the pioneering results of the research, important from the point of view of the need to support the therapy of children and the elderly due to NET formation disorders. The data obtained provide important information for clinicians about the immune responses of neutrophils in different age groups, which is translatable into medical practice.

The limitations of the study: The cellular origin of the cfDNA and the process by which it was released have not been determined in these studies. Other biomarkers of NETosis assessment are also recommended.

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REFERENCES

1. Miller RA. The aging immune system: primer and prospectus. Science 1996 Jul 5;273(5271):70–4. https://doi.org/10.1126/science.273.5271.70.
2. Fülöp T. Signal transduction changes in granulocytes and lymphocytes with ageing. Immunol Lett 1994 Jun;40(3):259–68. https://doi.org/10.1016/0165-2478(94)00064-6.
3. Weyand CM, Goronzy JJ. Aging of the Immune System. Mechanisms and Therapeutic Targets. Ann Am Thorac Soc 2016 Dec;13 Suppl 5:S422–8. https://doi.org/10.1513/AnnalsATS.201602-095AW.
4. Mailloux RJ. Teaching the fundamentals of electron transfer reactions in mitochondria and the production and detection of reactive oxygen species. Redox Biol 2015;4:381–98. https://doi.org/10.1016/j.redox.2015.02.001.
5. Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol 1956 Jul;11(3):298–300. https://doi.org/10.1093/geronj/11.3.298.
6. Santos AL, Sinha S, Lindner AB. The Good, the Bad, and the Ugly of ROS: New Insights on Aging and Aging-Related Diseases from Eukaryotic and Prokaryotic Model Organisms. Oxid Med Cell Longev 2018;2018:1941285. https://doi.org/10.1155/2018/1941285.
7. Goronzy JJ, Fang F, Cavanagh MM, Qi Q, Weyand CM. Naive T cell maintenance and function in human aging. J Immunol 2015 May 1;194(9):4073–80. https://doi.org/10.4049/jimmunol.1500046.
8. Rink L, Cakman I, Kirchner H. Altered cytokine production in the elderly. Mech Ageing Dev 1998 May 15;102(2–3):199–209. https://doi.org/10.1016/s0047-6374(97)00153-x.
9. Hodes RJ. Aging and the immune system. Immunol Rev 1997 Dec;160:5–8. https://doi.org/10.1111/j.1600-065x.1997.tb01022.x.
10. Xu X, Beckman I, Ahern M, Bradley J. A comprehensive analysis of peripheral blood lymphocytes in healthy aged humans by flow cytometry. Immunol Cell Biol 1993 Dec;71 (Pt 6):549–57. https://doi.org/10.1038/icb.1993.61.
11. Whiting CC, Siebert J, Newman AM, Du H-W, Alizadeh AA, Goronzy J, et al. Large-Scale and Comprehensive Immune Profiling and Functional Analysis of Normal Human Aging. PLoS One 2015;10(7):e0133627. https://doi.org/10.1371/journal.pone.0133627.
12. Yang X, Wang X, Lei L, Sun L, Jiao A, Zhu K, et al. Age-Related Gene Alteration in Naïve and Memory T cells Using Precise Age-Tracking Model. Front Cell Dev Biol 2020;8: 624380. https://doi.org/10.3389/fcell.2020.624380.
13. Foster AD, Sivarapatna A, Gress RE. The aging immune system and its relationship with cancer. Aging health 2011 Oct 1; 7(5):707–18. https://doi.org/10.2217/ahe.11.56.
14. Cevenini E, Monti D, Franceschi C. Inflamm-ageing. Curr Opin Clin Nutr Metab Care 2013 Jan;16(1):14–20. https://doi.org/10.1097/MCO.0b013e32835ada13.
15. Goronzy JJ, Weyand CM. Immune aging and autoimmunity. Cell Mol Life Sci 2012 May;69(10):1615–23. https://doi.org/10.1007/s00018-012-0970-0.
16. Weyand CM, Yang Z, Goronzy JJ. T-cell aging in rheumatoid arthritis. Curr Opin Rheumatol 2014 Jan;26(1):93–100. https://doi.org/10.1097/BOR.0000000000000011.
17. Minciullo PL, Catalano A, Mandraffino G, Casciaro M, Crucitti A, Maltese G, et al. Inflammaging and Anti-Inflammaging: The Role of Cytokines in Extreme Longevity. Arch Immunol Ther Exp (Warsz) 2016 Apr;64(2):111–26. https://doi.org/10.1007/s00005-015-0377-3.
18. Pinti M, Appay V, Campisi J, Frasca D, Fülöp T, Sauce D, et al. Aging of the immune system: Focus on inflammation and vaccination. Eur J Immunol 2016 Oct;46(10):2286–301. https://doi.org/10.1002/eji.201546178.
19. Ventura MT, Casciaro M, Gangemi S, Buquicchio R. Immunosenescence in aging: between immune cells depletion and cytokines up-regulation. Clin Mol Allergy 2017;15:21. https://doi.org/10.1186/s12948-017-0077-0.

20. Wherry EJ, Kurachi M. Molecular and cellular insights into T cell exhaustion. Nat Rev Immunol 2015 Aug;15(8):486–99. https://doi.org/10.1038/nri3862.
21. Pritz T, Weinberger B, Grubeck-Loebenstein B. The aging bone marrow and its impact on immune responses in old age. Immunol Lett 2014 Nov;162(1 Pt B):310–5. https://doi.org/10.1016/j.imlet.2014.06.016.
22. Chung SS, Park CY. Aging, hematopoiesis, and the myelodysplastic syndromes. Blood Adv 2017 Dec 12;1(26):2572–8. https://doi.org/10.1182/bloodadvances.2017009852.
23. Beerman I, Maloney WJ, Weissmann IL, Rossi DJ. Stem cells and the aging hematopoietic system. Curr Opin Immunol 2010 Aug;22(4):500–6. https://doi.org/10.1016/j.coi.2010.06.007.
24. Schneider EL. Infectious diseases in the elderly. Ann Intern Med 1983 Mar;98(3):395–400. https://doi.org/10.7326/0003-4819-98-3-395.
25. Butcher S, Chahel H, Lord JM. Review article: ageing and the neutrophil: no appetite for killing? Immunology 2000 Aug;100(4):411–6. https://doi.org/10.1046/j.1365-2567.2000.00079.x.
26. Chatta GS, Andrews RG, Rodger E, Schrag M, Hammond WP, Dale DC. Hematopoietic progenitors and aging: alterations in granulocytic precursors and responsiveness to recombinant human G-CSF, GM-CSF, and IL-3. J Gerontol 1993 Sep;48(5):M207-212. https://doi.org/10.1093/geronj/48.5.m207.
27. Bailey KL, Smith LM, Heires AJ, Katafiasz DM, Romberger DJ, LeVan TD. Aging leads to dysfunctional innate immune responses to TLR2 and TLR4 agonists. Aging Clin Exp Res 2019 Sep;31(9):1185–93. https://doi.org/10.1007/s40520-018-1064-0.
28. Butcher SK, Chahal H, Nayak L, Sinclair A, Henriquez NV, Sapey E, et al. Senescence in innate immune responses: reduced neutrophil phagocytic capacity and CD16 expression in elderly humans. J Leukoc Biol 2001 Dec;70(6):881–6.
29. Tortorella C, Piazzolla G, Spaccavento F, Pece S, Jirillo E, Antonaci S. Spontaneous and Fas-induced apoptotic cell death in aged neutrophils. J Clin Immunol 1998 Sep;18(5):321–9. https://doi.org/10.1023/a:1023286831246.
30. Fülöp T, Fouquet C, Allaire P, Perrin N, Lacombe G, Stankova J, et al. Changes in apoptosis of human polymorphonuclear granulocytes with aging. Mech Ageing Dev 1997 Jun;96(1–3):15–34. https://doi.org/10.1016/s0047-6374(96)01881-7.
31. Mesa MA, Vasquez G. NETosis. Autoimmune Dis 2013;2013:651497. https://doi.org/10.1155/2013/651497.
32. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science 2004 Mar 5;303(5663):1532–5. https://doi.org/10.1126/science.1092385.
33. Azzouz D, Palaniyar N. ApoNETosis: discovery of a novel form of neutrophil death with concomitant apoptosis and NETosis. Cell Death Dis 2018 Aug 6;9(8):839. https://doi.org/10.1038/s41419-018-0846-9.
34. Petretto A, Bruschi M, Pratesi F, Croia C, Candiano G, Ghiggeri G, et al. Neutrophil extracellular traps (NET) induced by different stimuli: A comparative proteomic analysis. PLoS One 2019;14(7):e0218946. https://doi.org/10.1371/journal.pone.0218946.
35. Obermayer A, Stoiber W, Krautgartner W-D, Klappacher M, Kofler B, Steinbacher P, et al. New aspects on the structure of neutrophil extracellular traps from chronic obstructive pulmonary disease and in vitro generation. PLoS One 2014;9(5):e97784. https://doi.org/10.1371/journal.pone.0097784.
36. Pires RH, Felix SB, Delcea M. The architecture of neutrophil extracellular traps investigated by atomic force microscopy. Nanoscale 2016 Aug 7;8(29):14193–202. https://doi.org/10.1039/c6nr03416k.
37. Ozyürek E, Cetintaş S, Ceylan T, Oğüş E, Haberal A, Gürakan B, et al. Complete blood count parameters for healthy, small-for-gestational-age, full-term newborns. Clin Lab Haematol 2006 Apr;28(2):97–104. https://doi.org/10.1111/j.1365-2257.2006.00767.x.
38. Cheng CK-W, Chan J, Cembrowski GS, van Assendelft OW. Complete blood count reference interval diagrams derived

from NHANES III: stratification by age, sex, and race. Lab Hematol 2004;10(1):42–53. https://doi.org/10.1532/lh96.04010.

39. Kelly D, Coutts AG. Early nutrition and the development of immune function in the neonate. Proc Nutr Soc 2000 May; 59(2):177–85. https://doi.org/10.1017/s0029665100000197.
40. Basha S, Surendran N, Pichichero M. Immune responses in neonates. Expert Rev Clin Immunol 2014 Sep;10(9): 1171–84. https://doi.org/10.1586/1744666X.2014.942288.
41. Lehman HK, Segal BH. The role of neutrophils in host defense and disease. J Allergy Clin Immunol 2020 Jun;145(6):1535–44. https://doi.org/10.1016/j.jaci.2020.02.038.
42. Rosales C, Demaurex N, Lowell CA, Uribe-Querol E. Neutrophils: Their Role in Innate and Adaptive Immunity. J Immunol Res 2016;2016:1469780. https://doi.org/10.1155/2016/1469780.
43. Aratani Y. Myeloperoxidase: Its role for host defense, inflammation, and neutrophil function. Arch Biochem Biophys 2018 Feb 15;640:47–52. https://doi.org/10.1016/j.abb.2018.01.004.
44. Papayannopoulos V, Metzler KD, Hakkim A, Zychlinsky A. Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol 2010 Nov 1; 191(3):677–91. https://doi.org/10.1083/jcb.201006052.
45. Metzler KD, Fuchs TA, Nauseef WM, Reumaux D, Roesler J, Schulze I, et al. Myeloperoxidase is required for neutrophil extracellular trap formation: implications for innate immunity. Blood 2011 Jan 20;117(3):953–9. https://doi.org/10.1182/blood-2010-06-290171.
46. Masuda S, Nakazawa D, Shida H, Miyoshi A, Kusunoki Y, Tomaru U, et al. NETosis markers: Quest for specific, objective, and quantitative markers. Clin Chim Acta 2016 Aug 1; 459:89–93. https://doi.org/10.1016/j.cca.2016.05.029.
47. Petrova A, Mehta R. Dysfunction of innate immunity and associated pathology in neonates. Indian J Pediatr 2007 Feb; 74(2):185–91. https://doi.org/10.1007/s12098-007-0013-2.
48. Manroe BL, Weinberg AG, Rosenfeld CR, Browne R. The neonatal blood count in health and disease. I. Reference values for neutrophilic cells. J Pediatr 1979 Jul;95(1):89–98. https://doi.org/10.1016/s0022-3476(79)80096-7.
49. Yegin O. Chemotaxis in childhood. Pediatr Res 1983 Mar; 17(3):183–7. https://doi.org/10.1203/00006450-198303000-00002.
50. Muniz-Junqueira MI, Peçanha LMF, da Silva-Filho VL, de Almeida Cardoso MC, Tosta CE. Novel microtechnique for assessment of postnatal maturation of the phagocytic function of neutrophils and monocytes. Clin Diagn Lab Immunol 2003 Nov;10(6):1096–102. https://doi.org/10.1128/cdli.10.6.1096-1102.2003.
51. Wolach B, Sonnenschein D, Gavrieli R, Chomsky O, Pomeranz A, Yuli I. Neonatal neutrophil inflammatory responses: parallel studies of light scattering, cell polarization, chemotaxis, superoxide release, and bactericidal activity. Am J Hematol 1998 May;58(1):8–15. https://doi.org/10.1002/(sici)1096-8652(199805)58:1%3C8::aid-ajh2%3E3.0.co;2-x.
52. Yost CC, Cody MJ, Harris ES, Thornton NL, McInturff AM, Martinez ML, et al. Impaired neutrophil extracellular trap (NET) formation: a novel innate immune deficiency of human neonates. Blood 2009 Jun 18;113(25):6419–27. https://doi.org/10.1182/blood-2008-07-171629.
53. Lipp P, Ruhnau J, Lange A, Vogelgesang A, Dressel A, Heckmann M. Less Neutrophil Extracellular Trap Formation in Term Newborns than in Adults. Neonatology 2017;111(2):182–8. https://doi.org/10.1159/000452615.
54. Martinelli S, Urosevic M, Daryadel A, Oberholzer PA, Baumann C, Fey MF, et al. Induction of genes mediating interferon-dependent extracellular trap formation during neutrophil differentiation. J Biol Chem 2004 Oct 15;279(42):44123–32. https://doi.org/10.1074/jbc.M405883200.
55. Yost CC, Schwertz H, Cody MJ, Wallace JA, Campbell RA, Vieira-de-Abreu A, et al. Neonatal NET-inhibitory factor and related peptides inhibit neutrophil extracellular trap formation. J Clin Invest 2016 Oct 3;126(10):3783–98. https://doi.org/10.1172/JCI83873.
56. Wadehn H, Raluy LP, Kolman J, Duecker C, Trochimiuk M, Appl B, et al. Time- and dose-dependent inhibition of

neutrophil extracellular trap formation by blocking of the interleukin-1 receptor. Cent Eur J Immunol 2021;46(4):419–26. https://doi.org/10.5114/ceji.2021.111493.

57. Fülöp T, Dupuis G, Witkowski JM, Larbi A. The Role of Immunosenescence in the Development of Age-Related Diseases. Rev Invest Clin 2016 Apr;68(2):84–91.
58. Fulop T, Larbi A, Dupuis G, Le Page A, Frost EH, Cohen AA, et al. Immunosenescence and Inflamm-Aging As Two Sides of the Same Coin: Friends or Foes? Front Immunol 2017;8: 1960. https://doi.org/10.3389/fimmu.2017.01960.
59. Wenisch C, Patruta S, Daxböck F, Krause R, Hörl W. Effect of age on human neutrophil function. J Leukoc Biol 2000 Jan;67(1):40–5. https://doi.org/10.1002/jlb.67.1.40.
60. Sapey E, Greenwood H, Walton G, Mann E, Love A, Aaronson N, et al. Phosphoinositide 3-kinase inhibition restores neutrophil accuracy in the elderly: toward targeted treatments for immunosenescence. Blood 2014 Jan 9;123(2):239–48. https://doi.org/10.1182/blood-2013-08-519520.
61. Hazeldine J, Harris P, Chapple IL, Grant M, Greenwood H, Livesey A, et al. Impaired neutrophil extracellular trap formation: a novel defect in the innate immune system of aged individuals. Aging Cell 2014 Aug;13(4):690–8. https://doi.org/10.1111/acel.12222.
62. Xu F, Zhang C, Zou Z, Fan EKY, Chen L, Li Y, et al. Aging-related Atg5 defect impairs neutrophil extracellular traps formation. Immunology 2017 Aug;151(4):417–32. https://doi.org/10.1111/imm.12740.
63. Fulop T, Larbi A, Douziech N, Fortin C, Guérard K-P, Lesur O, et al. Signal transduction and functional changes in neutrophils with aging. Aging Cell 2004 Aug;3(4):217–26. https://doi.org/10.1111/j.1474-9728.2004.00110.x.