The application of new immune cell activation markers in the diagnosis of various disease states

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Abstract

Introduction: Inflammation is a natural body's response to noxious stimulus present in many diseases with various aetiologies. Our study purpose was to assess quantitative changes in the values of new haematological parameters in patients with diseases coexisting with inflammation and in healthy persons without inflammation. Correlation between Extended Inflammation Parameters (EIP) and classic parameters commonly used in inflammation assessment were also analysed. Particular attention was given to assessment of diagnostic value of using new diagnostic parameters in groups of subjects diagnosed with inflammation in gynaecological, dermatological diseases and in type 2 diabetes.

Methods: Quantitative and qualitative assessment of reactive cells is performed using fluorescence flow cytometry with classic 5-diff differentiation. Thanks to the significant differences in the produced signals it is possible to differentiate between the reactive cells and the resting cells. These signals represent the number of body's inflammatory cells, i.e. the assessment of activated lymphocytes such as RE-LYMP, AS-LYMP and activation state of neutrophils such as NEUT-RI, NEUT-GI.

Results and summary: In patients diagnosed with inflammation higher values of new haematological parameters are reported: NEUT-RI, NEUT-GI, RE-LYMP#, RE- LYMP%. These new analysed haematological parameters may be used to improve and help in inflammation assessment with various aetiology.

Key words:

Reactive lymphocytes, neutrophils, inflammation, flow cytometry

Introduction

Inflammation is a defensive reaction of the body caused by specific and non-specific stimuli (mechanical, chemical or physical injury, foreign body, pathogenic micro-organisms, toxins or radiation). This reaction is based on the stimulation of innate and acquired immunity. Damaging factors may disturb the homeostatic balance, which, in turn, stimulates the immune cells to develop a defence response, i.e. inflammation [1]. The cardinal signs of inflammation include: redness, swelling, pain, increase in body temperature, and loss of function of the damaged tissue. These symptoms derive from the activation of inflammatory mediators comprising cytokines, chemokines, or acute-phase proteins, which are to neutralize the adverse effects of a noxious stimulus on one's body and restore homeostasis. Dependent on the severity of damage, severe and chronic inflammation can be distinguished [2].

For many years, experts have diagnosed inflammation in human body by means of widespread and easily accessible laboratory indicators, such as CRP (C-reactive protein) level, ESR (Erythrocyte Sedimentation Rate) or WBC (white blood cells) count [3-6].

Recently, the laboratory assessment of inflammation has used new haematological parameters informing about the state of activation of immune system cells. These parameters are accessible in SYSMEX XN haematological analysers in the form of "Extended Inflammation Parameters" (EIP) [7]. They indicate the number of inflammatory cells in the body, i.e. activated lymphocytes – RE-LYMP, AS-LYMP and neutrophil activation status – NEUT-RI, NEUT-GI, NEUT-WY. RE-LYMP and AS-LYMP parameters specify the number of all reactive lymphocytes and reactive lymphocytes releasing antibodies in peripheral blood, respectively. Lymphocyte populations are differentiated on the basis of their functionality and differences, which results from their functionality in internal structure, present granularity and size of the analysed cells. The neutrophil parameters, namely NEUT-RI, NEUT-GI, and NEUT-WY, provide the information on the activation stage of neutrophil granulocytes. The measurement considers the metabolic activity of neutrophils, their internal structure and cell size [3].

The aim of our research is to evaluate the changes in the new haematological parameters value, namely, RE-LYMP, AS-LYMP, NEUT-RI, NEUT-GI, and NEUT-WY in patients with psoriasis, type 2 diabetes, endometriosis and uterine myomas, and in healthy subjects. The relationship between Extended Inflammation Parameters (EIP) and classic parameters commonly used in the diagnosis of inflammation, i.e. CRP and WBC, is also meant to be analysed.

Material and methods

Characteristics of the study group

37 patients of the Independent Public Clinical Hospital No. 1 in Lublin were included in the study. The control group consisted of healthy people, represented by 19 volunteers over 18 years old. The absolute inclusion criterion for the study in this group was the absence of inflammation and correct results of routine laboratory tests, which take into account mainly CRP level < 5 mg/l and WBC = 4,000-10,000/µl. The study group consisted of 8 women and 10 men over the age of 18 who met the analysis inclusion criteria. These patients were diagnosed with inflammation, on the basis of classical markers used in the assessment, i.e. CRP level (> 5 mg/l) and WBC level (> 10,000 / µl or < 4,000 /µl). Among the patients of the study group there were 3 subgroups depending on the aetiology of immune cells activation: with inflammatory skin lesions in psoriasis (6 individuals), patients with gynaecological inflammation (3 women diagnosed with endometriosis and 3 patients diagnosed with uterine fibroids), and patients with inflammatory conditions associated diagnosed with type 2 diabetes (6 persons). 10 ml of venous blood samples were taken from all the patients using single-use equipment. The analysis of blood concentration of the selected haematological and biochemical parameters was performed within 2 hours after taking the blood samples. All the subjects participating in the studies had been informed about the course and purpose of the research and had given their written consents to

performance and use of the results. The study was approved by the Bioethics Committee at the Medical University of Lublin, obtaining permission to its implementation by resolution no. KE-0254/232/2019.

Apparatus and methodology

The haematological analysis was conducted with the use of Sysmex XN 1500 apparatus, while biochemical measurements were taken with the use of Roche Cobas Integra 800 apparatus.

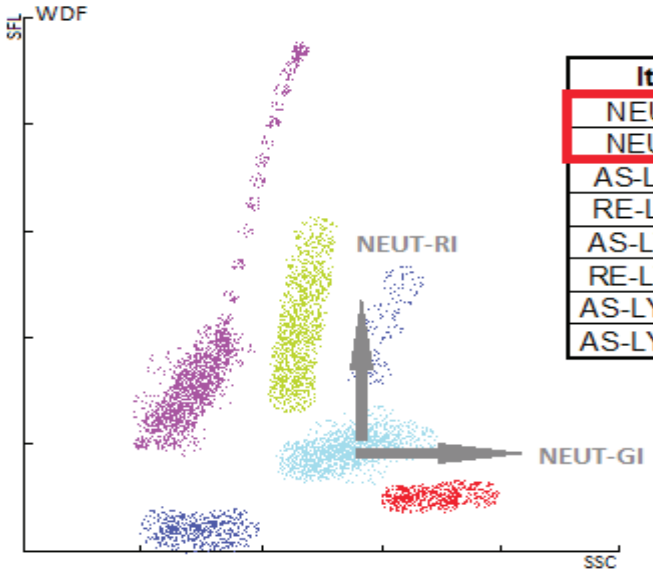
All the blood samples obtained from the ulnar vein was the test material. The blood samples were taken in the morning from patients in their fasting state. First, blood samples (7.6 ml each) were collected to vacutainer serum clot activator tubes, sized 8-16 x 50-100 mm, to determine the concentration of C-reactive protein (CRP). Then, the material was collected to 2.7 ml vacutainer tubes, containing K_3EDTA anticoagulant, to determine the concentrations of selected haematological parameters. The test tubes were left to clot for approximately 20-30 minutes and subsequently centrifuged at 2,500 rpm for 10 minutes. The blood samples for haematological tests, collected to K_3EDTA, were analysed within 2 hours after taking the samples.

The examined haematological parameters included: WBC (White Blood Cells), NEUT (Neutrophils), LYMPH (Lymphocyte), MONO (Monocytes). New laboratory indicators of inflammation were also analysed, viz. RE-LIMPH (Reactive Lymphocytes), AS-LIMPH (Antibody-Secreting Reactive Lymphocytes), NEUT-RI (Neutrophil Reactive Intensity), NEUT-GI (Neutrophil Granularity Intensity). The evaluated biochemical marker was the CRP level (C-reactive Protein).

Quantitative and qualitative assessment of reactive cells was carried out using fluorescent flow cytometry with classical 5-diff differentiation. This technique is applied in Sysmex XN analysers, among others. It allows us to differentiate reactive cells (neutrophils and lymphocytes) from cells at rest, thanks to the differences in the produced signals. The fluorescent flow cytometry makes it possible to measure the functional state of cells during routine blood tests together with CBC [7]. As a result of the laser beam passing through a single cell, the signals of scattered light directly (FSC) and laterally (SSC) are recorded and graphically depicted on a scattergram. Side fluorescence light (SFL) is measured at a 90-degree angle. Signals are recorded and displayed on a scattergram in the form of clouds of individual leukocyte populations. Activated cells differ from the rest, apart from the membrane lipid composition, and the cytoplasm activity through the compounds they produce, e.g. cytokines. The fluorescence signal produced by the cells is therefore more intense than that of the unstimulated cells. The intensity of neutrophil reactivity is illustrated by the NEUT-RI parameter. It is expressed as FI (fluorescence intensity). The SFL detector creates a channel to distinguish white blood cells, providing information on cell density or complexity, which is also reflected in leukocyte granulation and metabolic activity. In the form of changes in the leukocyte cloud position, the NEUT-GI parameter, expressed in the SI unit (scattering intensity), also change. This parameter, recorded by the SSC detector, informs us about the internal structure of the cell. Both the NEUT-RI and NEUT-GI parameters, illustrated in Figure 1, indicate not so much about the exact number of cells as about the fluorescence intensity and lateral dispersion, measured in the central position of NEUT on the scattergram (Fig. 1).

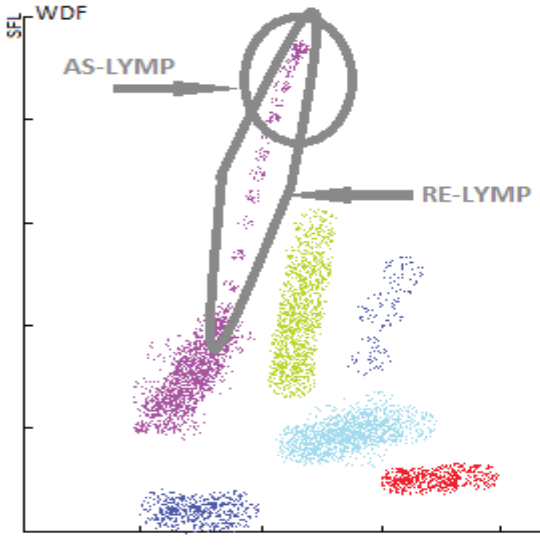
The method, on which the XN-Diff analysis is based, also allows us to isolate the whole population of reactive lymphocytes, as the cells showing the increased cellular activity in cytoplasm. It is expressed quantitatively by the RE-LYMP parameter. Reactive lymphocytes send far more different signals to SSC and FSC detectors than lymphocytes at rest, which results in differences in fluorescence intensity (Fig. 2). In the method using fluorescent flow cytometry we also distinguish AS-LYMP parameter (Fig. 2), which allows us to distinguish B lymphocytes from the remaining population of activated lymphocytes [7].

CRP test was carried out using the immunoturbidimetric method. Latex particles coated with monoclonal antibodies against human C-reactive protein have bonded to human CRP on an agglutination basis. The resulting turbid precipitate was measured turbidimetrically at a wavelength of 552 nm [8].



Item	Data	Unit
NEUT-RI	47.2	FI
NEUT-GI	147.9	SI
AS-LYMP#	0.04	10^3/uL
RE-LYMP#	0.11	10^3/uL
AS-LYMP%	1.2	%
RE-LYMP%	3.3	%
AS-LYMP%L	4.3	%
AS-LYMP%L	11.7	%

Fig. 1.
A sample result of a patient from the test group – NEUT-RI and NEUT-GI parameters on the WDF scattergram (NEUT-RI – Neutrophil Reactive Intensity, NEUT-GI – Neutrophil Granularity Intensity, WDF – White Blood Cell Differential Channel, SFL – Side-Fluorescence Light, SSC – Side-Scattered Light, SI – Scatter Intensity, FI – Fluorescence Intensity)



Item	Data	Unit
NEUT-RI	47.2	FI
NEUT-GI	147.9	SI
AS-LYMP#	0.04	10^3/uL
RE-LYMP#	0.11	10^3/uL
AS-LYMP%	1.2	%
RE-LYMP%	3.3	%
AS-LYMP%L	4.3	%
AS-LYMP%L	11.7	%

Fig. 2.
A Sample result of a patient from the test group – AS-LYMP and RE-LYMP parameters on the WDF scattergram (AS-LYMP – Antibody-Secreting Reactive Lymphocytes, RE-LYMP – Reactive Lymphocytes, WDF – White Blood Cell Differential Channel, SFL – Side-Fluorescence Light, SSC – Side-Scattered Light, SI – Scatter Intensity, FI – Fluorescence Intensity)

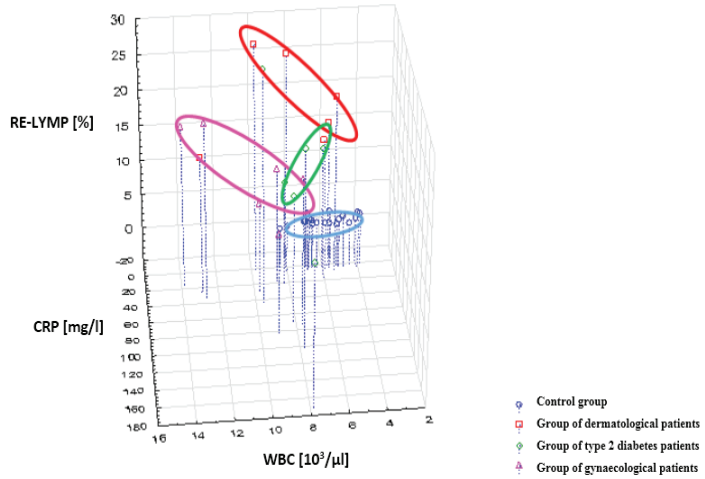


Fig. 3.
3D scattergram showing the relationship between CRP/WBC/RE-LYMPH% with respect to the study groups (RE-LYMP – Reactive Lymphocytes, WBC – White Blood Cells, CRP – C-reactive Protein).

Statistical analysis

The statistical analysis of the test results was carried out using Statistica 13, StatSoft's statistical software. The results were illustrated in the form of arithmetic averages (X-average), standard deviation (SD), and interquartile range (IQR). The Shapiro-Wilk test was used to check the distribution normality of variables. For the groups with normal distribution, the homogeneity of variances was tested using the Brown-Forsyth test. The statistical significance of the differences in the parameters was assessed, by means of: Student's t-test for comparison groups with normal distribution, while maintaining the assumption of the homogeneity of variances, Cochran-Cox's test for comparison groups with normal distribution without maintaining the assumption of the homogeneity of variances, and Mann-Whitney's test for comparison groups with abnormal distribution, respectively. To check the correlation between the selected variables, we used Pearson's linear correlation test for variables with normal distribution or the Kruskal-Wallis rank correlation coefficient test for variables with abnormal distribution. The relationship between the selected parameters was presented in the form of scatterplots. The conclusion error was assumed to be 5%, and the associated statistical significance level $p<0.05$, which indicates the occurrence of statistically significant differences or relationships.

Results

Test results were developed using the basic elements of descriptive statistics. In patients diagnosed with inflammation higher values of new haematological parameters (than in healthy persons) were reported, namely the EIP parameters, which depict the level of activation of lymphocytes and neutrophils and which comprise of NEUT-RI, NEUT-GI, RE-LYMP and AS-LYMP. The highest EIP value of all the study groups was observed in the group of dermatological patients (Table 1, row 10-15). Gynaecological patients had generally a higher EIP value than patients with type 2 diabetes and those of the control group (Table 1, row 10-15). Overall, the lowest EIP value of all the study groups was observed in the control group. The highest scatter plot of the correlation values between quartiles was observed in patients with type 2 diabetes and the lowest one in control group. The largest standard deviation occurred in the group of patients with type 2 diabetes, and the smallest one in the control group. The test results for all the parameters are shown in Table 1. The statistical analysis of the obtained data has revealed that the average EIP values in the control subjects differ significantly from the average EIP values in the subjects with inflammatory conditions occurring in dermatological diseases, type 2 diabetes, and gynaecological diseases. Moreover, it has been shown that NEUT-RI and NEUT-GI

Table 1.
Descriptive characteristics of all parameters in the study groups (WBC – White Blood Cells, RBC – Red Blood Count, PLT – Platelets, NEUT – Neutrophils, LYMPH – Lymphocyte, MONO – Monocytes, RE-LIMPH – Reactive Lymphocytes, AS-LIMPH – Antibody-Secreting Reactive Lymphocytes, NEUT-RI – Neutrophil Reactive Intensity, NEUT-GI – Neutrophil Granularity Intensity, CRP – C-reactive Protein, K – Control group, D – Group of dermatological patients, C – Group of type 2 diabetes patients, G – Group of gynaecological patients).

	Tested parameter	Study group	Number of cases [n]	$X_{average}$	SD	Type of variables distribution
1.	**WBC [10^3/µl]**	K	19	5.91	1.32	normal (p=0.67)
		D	6	7.97	2.94	normal (p=0.28)
		C	6	7.95	1.16	normal (p=0.49)
		G	6	10.26	2.45	normal (p=0.33)
2.	**RBC [10^6/µl]**	K	19	4.66	0.38	normal (p=0.09)
		D	6	4.95	0.53	normal (p=0.57)
		C	6	3.97	0.57	normal (p=0.33)
		G	6	4.29	0.42	normal (p=0.38)
3.	**PLT [10^3/µl]**	K	19	274.68	60.42	normal (p=0.98)
		D	6	301.17	59.61	normal (p=0.54)
		C	6	303.67	71.35	normal (p=0.73)
		G	6	228.50	59.48	normal (p=0.26)
4.	**NEUT [10^3/µl]**	K	19	3.26	1.05	normal (p=0.54)
		D	6	5.27	2.47	normal (p=0.62)
		C	6	5.81	1.39	distribution different from normal (p=0.04)
		G	6	7.56	2.43	normal (p=0.64)
5.	**NEUT [%]**	K	19	54.19	7.52	normal (p=0.90)
		D	6	64.17	11.12	normal (p=0.36)
		C	6	72.48	12.62	normal (p=0.37)
		G	6	72.55	7.46	normal (p=0.37)
6.	**LYMPH [10^3/µl]**	K	19	1.96	0.40	normal (p=0.07)
		D	6	1.96	0.60	normal (p=0.66)
		C	6	1.25	0.75	normal (p=0.15)
		G	6	1.74	0.43	normal (p=0.50)
7.	**LYMPH [%]**	K	19	33.73	5.75	normal (p=0.65)
		D	6	26.23	9.31	normal (p=0.12)
		C	6	16.10	10.47	normal (p=0.39)
		G	6	17.80	6.12	normal (p=0.44)
8.	**MONO [10^3/µl]**	K	19	0.51	0.14	normal (p=0.24)
		D	6	0.54	0.21	distribution different from normal (p=0.02)
		C	6	0.72	0.13	normal (p=0.47)
		G	6	0.78	0.22	normal (p=0.13)

Table 1. Continued

9.	**MONO [%]**	K	19	8.90	2.30	normal (p=0.080)
		D	6	6.95	1.50	normal (p=0.73)
		C	6	9.13	1.37	normal (p=0.97)
		G	6	7.73	1.94	normal (p=0.61)
10.	**NEUT-RI [FI]**	K	19	43.38	1.60	normal (p=0.52)
		D	6	56.50	1.86	normal (p=0.54)
		C	6	49.60	5.72	normal (p=0.05)
		G	6	51.45	4.55	normal (p=0.49)
11.	**NEUT-GI [SI]**	K	19	146.82	4.66	normal (p=0.83)
		D	6	151.90	1.68	normal (p=0.97)
		C	6	149.28	1.76	normal (p=0.51)
		G	6	153.32	4.48	normal (p=0.40)
12.	**RE-LYMP [10^3/µl]**	K	19	0.04	0.02	distribution different from normal (p=0.01)
		D	6	0.43	0.14	normal (p=0.52)
		C	6	0.27	0.17	normal (p=0.74)
		G	6	0.25	0.12	normal (p=0.88)
13.	**RE-LYMP [%]**	K	19	2.09	0.77	normal (p=0.06)
		D	6	20.05	5.74	normal (p=0.12)
		C	6	17.23	7.26	normal (p=0.32)
		G	6	13.83	4.97	normal (p=0.65)
14.	**AS-LYMP [10^3/µl]**	K	19	0.01	0.02	normal (p=0.52)
		D	6	0.02	0.05	normal (p=0.10)
		C	6	0.01	0.01	normal (p=0.33)
		G	6	0.03	0.09	normal (p=0.35)
15.	**AS-LYMP [%]**	K	19	0.07	0.02	normal (p=0.12)
		D	6	0.05	0.01	normal (p=0.22)
		C	6	0.03	0.03	normal (p=0.05)
		G	6	0.01	0.01	normal (p=0.39)
16.	**CRP [mg/l]**	K	19	3.52	3.36	distribution different from normal (p=0.01)
		D	6	9.50	9.95	normal (p=0.25)
		C	6	68.82	61.51	normal (p=0.71)
		G	6	41.57	28.99	normal (p=0.13)

parameters correlate positively with each other and show strong correlation with RE-LYMP and RE-LYMP% values, as well as with NEUT/LYMPH and MONO/LYMPH ratios. The NEUT-RI, NEUT-GI, RE-LYMP# and RE-LYMPH% parameters show a negative correlation with the LYMPH%. It has been evidenced that the average RE-LYMPH% scatter over WBC and CRP was the highest in the group of dermatological patients.

The examination of the scattergrams has revealed that in all the analysed study groups the determination of new haematological parameters NEUT-RI, NEUT-GI, RE-LYMP#, RE-LYMP% may not so much replace, but strongly support the assessment of inflammation. It can be observed that, by applying these parameters additionally, specific groups of patients can be distinguished, which differ from one another by particular parameters (Fig. 3). The scattergrams using the new inflammation parameters have presented that within a given group of subjects the results and the correlations were similarly distributed and differed between the groups, depending on the type and background of the inflammation.

Discussion

The presence of activated lymphocytes and neutrophils with clear cytoplasmic granulation TGN (Toxic Granulation Neutrophils) can be observed in the blood of the patients with an active inflammation after the microscopic examination of manual blood smears, performed for a long time in everyday laboratory practice. Based on the results of their research, Zimmermann et al. [9] suggested replacing the diagnostics using traditional microscopic methods with new methods of measuring the internal structure of inflammatory cells. Currently, in addition to calculating the GI granularity index, the total number of white blood cells, their percentage distribution and platelet and red cell parameters, automatic haematological analysers also enable the determination of the so-called EIP – new parameters of inflammation. They include 4 parameters showing the activation of immune cells: NEUT-GI and NEUT-RI, quantitatively assessing the activation status of neutrophils, and RE-LYMP and AS-LYMP, showing the number of all activated lymphocytes and the number of antibody-secreting activated lymphocytes, respectively.

In the studies on the use of the new parameters of inflammation, carried out in this study, the intensity of neutrophil reactivity (NEUT-RI) was measured both in the study groups and in the control group. In all the patient groups, the values of RE-LYMP and AS-LYMP were statistically insignificant ($p > 0.05$), while the value of NEUT-RI was statistically significantly higher than in the group of healthy subjects ($p = 0.001$, $p = 0.007$, $p = 0.04$).

The highest increase among all the study groups was observed in the dermatological patients, when compared to the control group ($p = 0.001$). Moreover, the value of the NEUT-GI parameter in the dermatological patients group ($p = 0.016$) was also statistically significant. This value may indicate the activation of inflammatory cells, i.e. neutrophils in the peripheral blood [10,11]. Similar changes in the activation of neutrophils were observed as well by Dinsdale et al. [12] in patients with inflammatory skin conditions. Zonneveld et al. [13] also observed that in patients with inflammation, neutrophils differ significantly in morphology – they are larger and characterized by increased granularity. Based on the above studies [12-15], it can be assumed that the NEUT-GI and NEUT-RI indicators will prove helpful as markers of neutrophil activation. Additionally, in our study, significantly higher values of the NEUT-GI parameter were observed in the gynaecological patients ($p = 0.01$) and NEUT-RI ($p = 0.04$). Likewise, in the study of Ottolin et al. [16], a significantly higher number of neutrophils was found in patients with endometriosis compared to the control group. This fact demonstrates a local inflammatory response and the associated activation of immune cells in endometriosis. Significantly higher values of the NEUT-RI parameter ($p = 0.007$) were also observed in the type 2 diabetic patients. This may indicate that the inflammation associated with diabetes may also contribute to the activation of cells of the immune system, including neutrophils [17]. This is confirmed by the study conducted by Petchakup et al. [18], which confirmed the presence of activated neutrophils in the whole blood of patients diagnosed with type 2 diabetes. The

studies led by Dinsdale [12], Ustantseva et al. [14] and Cornet et al. [19], also indicate a high diagnostic value of the NEUT-GI and NEUT-RI parameters in the morphological assessment of neutrophils, e.g. presence of toxic granules, Döhli bodies in the cytoplasm and vacuolization.

In the studies conducted by the authors of this paper, the other parameters from the EIP group, i.e. RE-LYMP and AS-LYMP, did not show any statistically significant differences compared to the control group. However, studies conducted by Bos [20] and Ferenczi [21] revealed that the stimulated CD4 + and CD8 + lymphocytes play an important role in the inflammatory response, due to their increased number in the skin and peripheral blood, e.g. in patients with psoriasis. In addition, histological examinations of the skin in this patient group indicate the state of inflammation, which is associated with the mobilization of neutrophils and lymphocytes to the site of inflammation [22]. Taking into account the fact that our own research is a preliminary research, and the presented results, in most of the analysed cases, correlate with the study results of other researchers, a further detailed research on the use of these indicators as markers of inflammatory cell activation is required.

Summary

So far, few studies devoted to new haematological parameters in the diagnosis of inflammation have indicated the activation of immune cells [14,23,24]. The results of our study highlight a statistically significant increase in new haematological parameters in the presence of inflammation and the associated presence of activated cells in all the analysed patient groups, when compared to the control group ($p < 0.05$). They support the use of these parameters in conjunction with the biochemical markers of inflammation to assess immune cell activation. These parameters, due to the possibility of easy and quick measurement of the activation of neutrophils and lymphocytes, together with basic blood count tests, may become a significant facilitation in everyday diagnostics.

Author Contributions

All Contributors to the paper fulfil the ICMJE Criteria for Authorship. First author conceived of the presented idea. First and second authors devised the main conceptual ideas and proof outline. Third author encouraged first and second authors to investigate a specific aspect of Enhanced Inflammatory Parameters (EIP) and supervised the findings of this work. First and fourth authors wrote the manuscript. All authors discussed the results and contributed to the final version of the manuscript.

Declarations of interest:

None

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