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CHANGES IN PLASMA CYTOKIN AND LYMPHOCYTIC SUBPOPULATION LEVELS IN PATIENTS WITH METABOLIC SYNDROME

ABSTRACT

OF DISSERTATION WORK FOR AWARDING THE EDUCATIONAL AND SCIENTIFIC DEGREE "DOCTOR"

SCIENTIFIC SPECIALTY "Endocrinology"

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Pleven, 2022

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I.INTRODUCTION:

Over the last 30 years, the stereotype of the pathogenesis of type 2 diabetes mellitus (DMT2) has changed from a classic metabolic to a multifactorial chronic disease with low-grade chronic inflammation. Visceral obesity is not only a major factor in the development of insulin resistance (IR), but also a cause of low-grade chronic inflammation among patients with metabolic syndrome (MS) and DMT2.

The initiation of the inflammatory process in these conditions is carried out by activating and dysregulating innate immunity. Elevated serum concentrations of non-saturated fatty acids and glucose by several different mechanisms lead to intracellular activation in adipocytes, macrophages, hepatocytes, muscle, endothelial and pancreatic β-cells of the major regulatory pathways of inflammation and support the production of IL-1 inflammatory cells. (interleukin 1), IL-6 (interleukin 6), TNFα (tumor necrotic factor α) and CRP (C-reactive protein).

The chronic hyperglycaemia, in patients with DMT2 and MS, and associated glucotoxicity, along with cells and inflammatory products, cause β-cell stress and apoptosis, autoantibody production, and activation of T lymphocytes targeting pancreatic β-cell antigens. The degree of obesity and the volume of visceral adipose tissue are critical for the development of pancreatic β-cell apoptosis and they represent a pathogenetic "bridge" separating type 1 diabetes (DMT1) from DMT2. Autoimmune-mediated pancreatic β-cell destruction is found in both types of diabetes, but the speed of development is much slower in DMT2.

The present dissertation examines and analyzes the relationship between low-grade chronic inflammation, immune changes and clinical and laboratory abnormalities in MS and DMT2 by observating the levels of serum cytokines IL-1, IL-6, TNFα, hsCRP, T-lymphomas. and NK cells (natural killers) among patients with MS and DMT2.

II. AIM AND TASKS

1.AIM

The aim of the present work is to study the levels of cytokines: interleukin-1, interleukin-6, tumor-necrotic factor alpha and lymphocyte subpopulations in peripheral blood as markers for low-grade chronic inflammation and immune response among patients with metabolic syndrome and to analyze the interaction between them and the various clinical and laboratory abnormalities included as components in metabolic syndrom.

2. MAIN TASKS

To achieve this goal, the following specific tasks were set:

A: In the first part of the study (including MS patients without DMT2 and patients with MS and DMT2):

2.1. To make a comparative characteristic in terms of age, anthropometric data and general clinical and laboratory characteristics of the studied groups of persons with MS without DDT2 and with DMT2.

2.2. To compare the main parameters of carbohydrate metabolism (changes in glycemia during 75 g of OGTT) and insulin indices: Insulin sensitivity (HOMA-IR), Insulin secretion (HOMA-% β) and the triglyceride / HDL-cholesterol ratio of participants from the studied groups of persons with MS without DMT2 and persons with MS and DMT2.

2.3. To study and compare the levels of plasma cytokines - IL-1, IL-6, TNF-α and hsCRP, erythrocyte rate sedementation and all leucocyte count in blood between the studied groups of people with MS without DMT2 and people with MS and DMT2.

2.4. To study the correlations between the plasma levels of the studied cytokines and the markers of low-grade chronic inflammation (erithrocytes rate sedementation, total leukocyte count, hsCRP) with the indicators of carbohydrate metabolism, insulin indices, visceral obesity and levels of lipids in plasma.

B: In the second part of the study (including patients with MS without DMT2, with MS and DMT2 and patients with DMT1):

2.5. To perform a comparative quantitative and qualitative analysis of the total and differentiated leukocyte profile and lymphocyte subpopulations [Total non-specific T lymphocytes (CD + 3), Th (CD + 4), Ts (CD + 8), NK cells (CD3- / CD16 + (CD56 +) and B lymphocytes (CD + 19)] in peripheral blood between different groups of people [(patients with MS without DMT2, patients with MS and DMT2 and patients with type 1 diabetes mellitus); (DMT1)].

2.6. To investigate and evaluate the relationship between lymphocyte subpopulations in peripheral blood with BMI and waist circumference.

2.7. To monitor the relationship between lymphocyte subpopulations in peripheral blood and changes in glycemia, insulin secretion and sensitivity in the studied patients.

2.8 To investigate and evaluate the relationship between lymphocyte subpopulations in peripheral blood and changes in serum cholesterol, cholesterol fractions and serum triglycerides.

III. MATERIAL AND METHODS

1. DESIGN OF THE STUDY:

Two comparative case-control studies have been performed in patients with metabolic syndrome.

1.1. The first study included: Investigation of cytokine (interleukin 1, interleukin 6, tumor necrotic factor alpha) and markers of inflammation (highly sensitive C-reactive protein) among patients with metabolic syndrome and type 2 diabetes mellitus.

1.2. The second study included: Study of lymphocyte subpopulations (CD3, CD4, CD8, CD19) and Th / Ts ratio in patients with metabolic syndrome and type 2 diabetes mellitus.

2. CLINICAL CHARACTERISTICS OF THE STUDY POPULATION:

The first study included: a total of 79 patients with MS and 21 healthy individuals, divided into three groups as follows:

1) group A - 45 patients with MS without DMT2
2) group B - 34 patients with MS and DMT2
3) group C- 21 healthy individuals

The second study included: a total of 96 patients with MS, 21 patients with ADHD1 and 21 healthy individuals, divided into four groups as follows:

1) group A - 26 patients with MS without DMT2

2) group B - 70 patients with MS and DMT2
3) group D - 22 patients with DMT1
4) group C - 21 healthy individuals used as a control group.
Subjects in groups A, B and D met all inclusive and none excluded from the pre-defined criteria:

Including criteria for groups A, B and D:

A) Persons over 18 years of age diagnosed with MS and / or known DMT2 with BMI ≥ 25 kg / m2 or known DMT1 with normal body weight.
B) Persons who have signed an informed consent to participate in the investigation.

Excluding criteria for groups A, B and D:

A) Persons with acute or chronic active inflammation
B) Persons with concomitant systematic autoimmune diseases.
C) Patients receiving immunosuppressive, immunoactivating or immunomodulatory therapy.
D) Persons with DMT1 or DMT2 with acute metabolic decompensation (diabetic ketoacidosis, hyperosmoral non-ketogenic condition, hypoglycaemia) currently or ≤ 120 days before signing the informed consent.
E) Patients with an acute coronary or cerebrovascular accident, chronic arterial insufficiency of the low extremitas with revascularization of peripheral arteries or amputation.
F) Severe liver or kidney failure.
G) NYHA class III and IV heart failure, regardless of etiology.

Control group: Participants in the comparative control group (group C) in both studies were healthy, over 18 years of age individuals with a BMI ≤25 kg / m^2 who voluntarily participated in the clinical trial after signing an informed consent. To be part of this group, individuals had to have: no personal or family history of organ-specific or systematic autoimmune disease; anamnestic and clinical data on acute or chronic active inflammation, autoimmune endocrine diseases, experienced acute cardiovascular or cerebrovascular accident, chronic arterial insufficiency of the legs with revascularization of peripheral arteries or amputation, not receive immunosuppressive, immunoactivating or immunomodulatory therapy at the time of the study and / or ≤ 120 days prior to the study, other medications and / or supplements at the time of the study and or ≤ 30 days prior to the study.

Both studies were conducted over a period of 1 year. All participants from groups A, B and D were selected from hospitalized patients in the Clinic of Endocrinology, University Hospital "Dr. Georgi Stranski" EAD, Pleven with diagnoses: MS, DMT2 or DMT1.

3. METHODS:

3.1. Documentary and survey method

The questionnaire and documentary method provided information on personal history of hypertension and current treatment (if existing hypertension), diabetes, duration of diabetes mellitus (if any), accompanying chronic inflammatory and autoimmune diseases, medications with potential anti-inflammatory, immunomodulatory, immunosuppressive or immunoactivating effects, the presence of smoking, a family history of diabetes, hypertension and cardiovascular disease.

3.2 Physical status:

3.2.1 Anthropometric measurments - height (m), body weight (kg) was measured and body mass index (BMI) was calculated. Waist circumference was measured in centimeters.
3.2.2 Measurement of blood pressure by Korotkov's auscultatory method with an aneroid sphygmomanometer after 5 minutes of rest.

3.3 Laboratory tests

The studies of ESR, total and differential leukocytes count in whole blood, plasma glucose, total cholesterol, HDL-cholesterol, triglycerides, glycated hemoglobin (HbA1c) were performed in the central clinical laboratory at the University Hospital "Dr. G. Stranski.
The study of immunoreactive insulin (IRI) and the levels of IL-1, IL-6, TNF-α, hsCRP and flow cytometric analysis of lymphocyte subpopulations was conducted in the Medical Diagnostic Laboratory of Immunology of the University Hospital "Dr. G. Stranski" -Pleven.

3.3.1. Examination of **total and differential leukocyte counts** was performed with an automated hematology analyzer Micros 60 CS / CT-16 (ABX Horiba Diagnostics, France) from EDTA-venous blood.
3.3.2. Plasma glucose-hexokinase enzyme method in whole hemolyzed blood samples with GA 3 analyzer (KABE Labortechnik, Denmark).
3.3.3. Glycated hemoglobin (HbA1c). Measurement of HbA1c in percentages (DCCT / NGSP) was performed in whole blood with in vitro tests (Roche diagnostic GmbH, Mannheim, Germany).
3.3.4. Plasma triglycerides, total cholesterol and HDL-cholesterol were tested by enzyme colorimetric method with an automatic analyzer BA 400 (BioSystems S.A., Spain). LDL cholesterol values were calculated according to Friedewald's formula: LDL = total cholesterol - [HDL + (triglycerides / 2.2)].
3.3.5. Immuno-reactive serum insulin (IRI) - in serum, fasting with immunoenzymometric method (DIAsourse INS-EASIA kit; DIAsource Immuno Assays, Belgium).
A mathematical homeostasis model was used to assess and quantify insulin resistance: the **Homeostatic Model Assessment for Insulin Resistance** (HOMA-IR). HOMA-IR values from 0.7 to 2.4 were accepted as reference levels.
A mathematical homeostasis model was used to assess and quantify β-cell insulin secretory activity: **Homeostatic Model Assessment β% (HOMA-β%).**
3.3.6 Measurement of IL-1α, IL-6, TNF-α and hsCRP- by ELISA in isolated plasma from EDTA whole blood sample (Gen-Probe Diaclone SAS, France and DIAsource Immuno Assays, Belgium). Accepted reference values: for IL-6 from 0 to 2 pg / ml; for TNF-α 0-8pg / ml for hsCRP- from 0.03 to 63.68 mg / l. Il-1 has no established reference value.
3.3.7. Lymphocyte subpopulations - were analyzed using a dual laser flow cytometer FACS Calibur cytometer (Becton Dickinson, Heidelberg, Germany) and Cell Quest Pro Software (Becton Dickinson) to determine the fluorescence of specific monoclonal antibodies. Cell subpopulations CD3 + (T-lymphocytes), CD4 + (T-helpers), CD8 + (T-cytotoxic lymphocytes), CD19 + (B-lymphocytes), CD3- / CD16 + CD56 + (NK-cells) were identified as percentages of lymphocytes. To calculate the absolute number of cells of lymphocyte subpopulations in 1 microliter of blood, the formula was used: lymph. value (%) X total

absolute lymph. number X 10 (* information on total lymphocyte count was derived from the automatic analysis of complete blood count).

3.4. Functional tests

3.4. 1. Oral glucose tolerance test (OGTT) was performed in the participants from group A and group C according to the requirements of the WHO. Fasting blood glucose values ≥ 5.6 mmol / l were considered to be impaired fasting glucose, and ≥7.8 mmol / l to ≤11.0 mmol / l per 120 minutes for impaired glucose tolerance.

3.4. 2. Blood-glucose profile. A 4-point glucose profile was conducted for the participants from group B and group D.

3.5. Instrumental methods

3.5.1. Impedancometry: Bioelectrical impedance analysis (BIA) was performed with a calibrated TANITA inner scan body composition monitor biocompedance meter BC-571. The percentage of total adipose tissue and water in relation to body weight and the proportion of visceral adipose tissue (index) were determined.

3.6. Statistical methods

The data obtained in both studies were entered, grouped and processed using computer packages: MS Excel 2010, STATGRAPHICS Centurion XV.I.

The results are described by tables, graphs and numerical values (percentages, coefficients, averages, standard deviation, etc.)

Evaluation of statistical reliability in the studied groups was performed using the value of p for the found value of chi square or Fisher's criterion. The differences at level of $p < 0.05$ were considered significantly significant.

Parametric (Student's t-test, Fisher's F-test) and non-parametric methods (Pearson's χ 2 test and Kruskall-Wallis test) were used to test hypotheses.

Correlation analysis was used to study the relationship between changes in the dependent variable and the corresponding changes in the studied factors. The strength of the correlation dependence was assessed by Pearson's correlation coefficient and a special criterion for factor influence - odds ratio (OR-odds ratio). ROC curves and calculated area under the curve are constructed to determine the specificity, sensitivity and border value of some factors. A regression analysis was performed.

IV. OWN RESULTS AND DISCUSSION

IV.1 INVESTIGATION OF CYTOKINES (INTERLEUKIN 1, INTERLEUKIN 6, TUMOR NECROTIC FACTOR ALPHA) AND MARKERS OF INFLAMMATION (HIGHLY SENSITIVE C REACTIVE PROTEIN) AMONG THE PATIENTS WITH METABOLOC SYNDROME AND TYPE 2 DIABETES MELLITUS.

IV.1 .1 Characteristics of subjects with metabolic syndrome without type 2 diabetes mellitus (group A), with metabolic syndrome and type 2 diabetes mellitus (group B) and a control group of healthy individuals (group C).

Main characteristic of the examined persons

1. Gender affiliation: in both study groups (A and B) a larger share is occupied by women: 68.89% in group A and respectively 70.59% for group B. In the controls 65% of the subjects are also female (Figure 1). There were no statistically significant differences in the sex distribution both between the two studied groups (town A and town B) and between them and the controls.

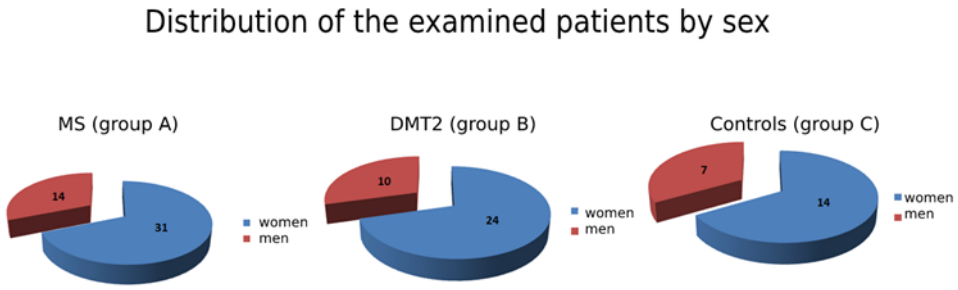


Fig.1 Distribution of the examined patients by sex

2. Age: The mean age of the patients in both groups and controls is shown in Table 1.

Table 1 Age of the studied patients with MS without ZDT2 (group A), with MS and ZDT2 (group B) and control persons (group C)

Age (years)	**Patientas wth MS group A (n=45)**	**Patients with DMT2 group B (n=34)**	**Controls group C (n=21)**	**Significance P<0,05**
Middle- age	**40,07±14,24**	**52,55±3,72**	**31,14±3,15**	p^{*}, p^{**}, p≠

legend p^{*}-p^{AB}<0,005; p^{**}- p^{BC}<0,05; p≠- p^{AC}<0,05

The mean age of patients with MS (groups A and B) was statistically higher than that of controls. Also, patients with DMT2 (group B) are one decade older than those without DMT2 (A) (Fig. 2).

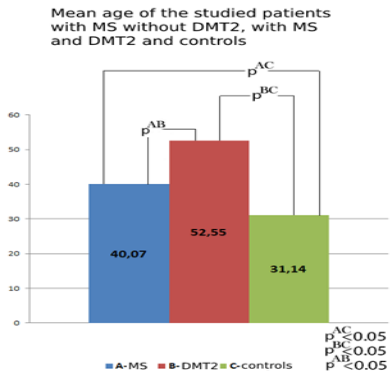


Fig. 2 Mean age of the studied patients with MS without DMT2, with MS and DMT2 and controls

3. Heredity

Heredity is an unmodifiable risk factor for the development of cardiovascular disease and was included in the analysis.

The part of subjects with a family history of ZDT2 were respectively: n = 21 (46.64%) in group A, n = 21 (64.71%) in group B and n = 10 (50%) among the controls). No statistically significant differences were found between the subjects regarding the positive anamnesis for DMT2 among their relatives.

The highest part of patients with a positive family history of cardiovascular disease was found among those with developed DMT2 (group B) - n = 22 (64%), followed by patients with MS who do not meet the criteria for DMT2 (group A) - n = 27 (60%). In healthy subjects in the control group (group C), a family history of cardiovascular disease was registered at n = 9 (45%). The observed differences did not show significance.

Clinical and metabolic characteristics

The clinical characteristics of the participants in the study, including the components of MS are reflected in Table 2.

Table. 2 Clinical characteristics of patients with MS

Indices	**MS; Group A (n=45)**	**DMT2; Group B (n=34)**	**controls; group C (n=21)**	**sifnificance p<0,05**
Atrherial hypertension % (count of patients)	77,7(35)	88,2 (30)	42,9 (9)	
Systolic blood prssure (mmHg)	130,68±15,69	134,85±16,26	116, 67±15,52	p^{AB}=NS (0,2) p^{AC} <0,05 p^{BC}<0,05
Dyastolic blood pressure (mmHg)	86,33±7,93	85,0±9,77	76,19±9,6	p^{AB} =NS(0,5) p^{AC} <0,05 p^{BC} <0,05
Fasting glucose гладно (mmol/l)	5,25 ± 0,24	9,65 ± 1,54	4,89±0,47	p^{AB} < 0,05 p^{AC} =NS(0,06) p^{BC} <0,05
НвА1с (%)	5,78±0,61	7,78±0,80	4,71±0,31	p^{AB} <0.05 p^{AC} <0,05 p^{BC} <0,05
Total cholesterol (mmol/l)	4,51±0,23	5,49±0,43	4,47±0,52	p^{AB} =NS(0,2) p^{AC} <0,05 p^{BC} <0,05
HDL-cholesterol (mmol/l)	1,03±0,09	1,12±0, 11	1,26±0,24	p^{AB} =NS(0,19) p^{AC} <0,05 p^{BC} =NS(0,08)

LDL-cholesterol (mmol/l)	3,42±0,30	3,44±0,36	2,58±0,62	p^{AB} =NS(0,94) p^{AC} <0,05 p^{BC} <0,05
Triglycerides (mmol/l)	1,59±0,22	2,33±0,49	1,06±0,33	p^{AB} <0,05 p^{AC} <0,05 p^{BC} <0,05

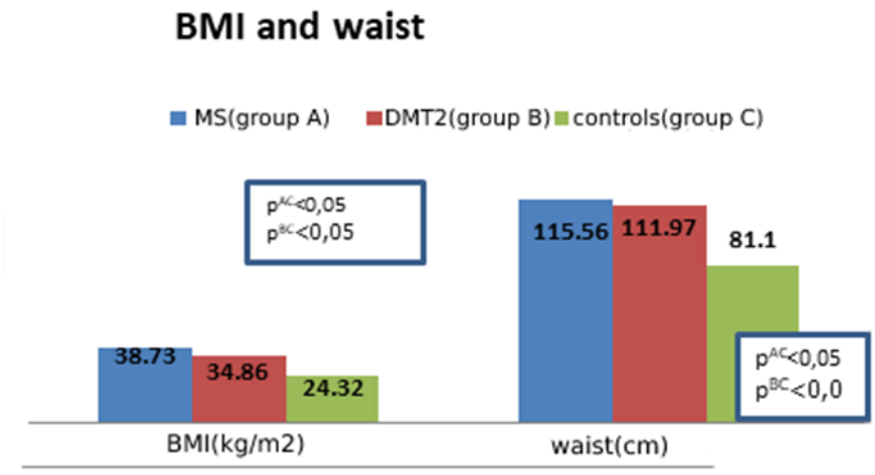
p^{AB}-MS vs. DMT2; p^{AC}- MS vs. controls; p^{BC}-DMT2 vs. controls; NS-non significance

All patients in groups A and B meet the criteria for metabolic syndrome of the International Diabetes Federation.

In all subjects with MS (groups A and B) the mean values of BMI and waist (Fig. 3), systolic and diastolic blood pressure, plasma levels of LDL cholesterol and serum triglycerides were statistically significantly higher compared to subjects in the control group (group C).

Characteristics of obesity

All participants in the study with MS (group A and group B) had average BMI and waist circumference showing obesity (Fig. 3).



P^{AC}-MS vs. controls; p^{BC} -DMT2 vs. controls; p^{AB} -MS vs. DMT2

Fig.3 BMI and waist circumference in patients with MS, DMT2 and controls

The distribution of patients with MS (group A and B) in relation to the values of the calculated BMI was shown in next figure (Fig. 4).

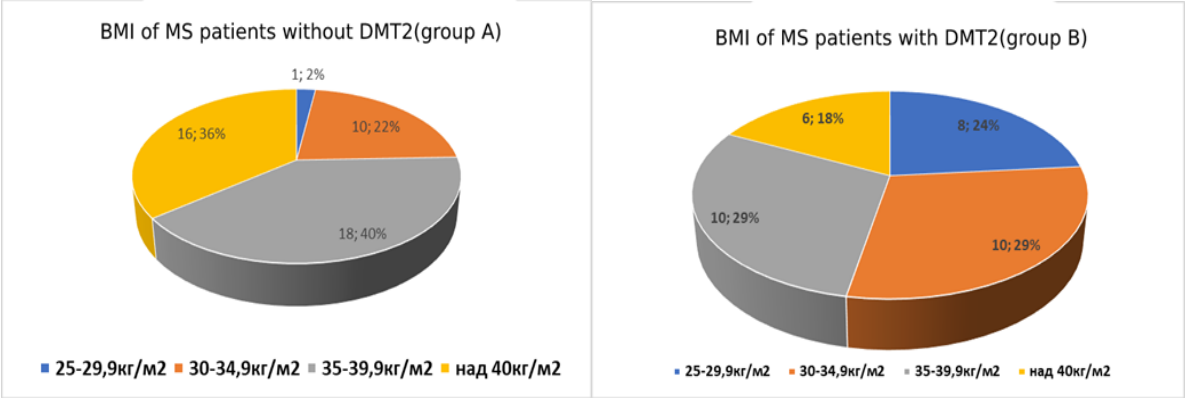


Fig.4 Distribution of patients with MS without DMT2 (gr.A) and with MS and DMT2 (gr.B) according to BMI values

Table 3 shows the general characteristics of obesity in patients with MS (groups A and B). Subjects with MS without DMT2 (group A) showed significantly higher BMI and visceral adipose tissue compared to those in the group with MS and DMT2 (group B).

Table. 3 Characteristics of obesity in patients with MS without DMT2 (group A) and with MS and DMT2 (group B)

Indices	**Group A; (n=45)**		**Group B; (n=34)**	
	Man (n=14)	Womens (n=31)	Man (n=10)	Womens (n=34)
BMI (kg/m^2)	36,93±5,1	39,54±6,43	34,54±6,43	35,00±6,16
waist (cm.)	117,79±13,13	114,55±17,13	115,00±15,2	110,71±12,14
Part of fat tissue (%)	44,14±4,85	48,48±4,39	46,0±5,66	47,08±4,14
Visceral fat tissue	28,36±10,74	24,96±7,54	21,8±3,82	21,92±6,07

IV.1.2 Main parameters of carbohydrate metabolism (changes in glycaemia during 75 g of OGTT) and insulin indices: Insulin sensitivity (HOMA-IR), Insulin secretion (HOMA-% β) and the triglyceride / HDL-cholesterol ratio of the studied groups participants with MS with DMT2 (group B) and without DMT2 (group A).

Basic parameters of carbohydrate metabolism

Glycemic levels in patients with MS (group A) and controls (group C) in the course of 75 g OGTT are shown in table 4.

Table. 4 Glucose levels during OGTT in patients with MS (group A) and controls (Group C)

glucose	Patients with MS **Group A** (**n=45)**	Controls **Group C** **(n=21)**	Significants P<0,05
0 min.	5,25±0,8	4,9±0,47	P=0,06
60 min.	8,13±2,25	6,67±1,56	p*
120 min.	6,1±1,8	5,13±1,23	p*

p*=p<0,05

Basic insulin indices

The levels of endogenous fasting insulin, HOMA-IR and HOMA% in patients with MS without diabetes (group A), MS with DMT2 (group B) and the persence from control group (group C) are presented in Table 5.

Table 5 Levels of endogenous insulin, HOMA-IR and HOMA% B in the studied patients.

Indices	**MS Group A (n=45)**	**MS Group B (n=34)**	**Controls Group C (n=21)**	Significance (p<0.05)
Endogeounos insulin (μIU/ml)	19,32±3,22	21,79±10,85	9,13±0,74	p^{AB}=0,5 P^{AC}<0,05 P^{BC}<0,05
HOMA-IR	4,62±0,8	8,06±3,16	1,97±0,16	p^{AB} <0,05 P^{AC} <0,05 $P^{BC<}$ 0,05
HOMA%B	258,77±57,76	89,97±38,42	183,31±107,92	p^{AB} <0,05 P^{AC} <0,05 $P^{BC<}$ 0,05

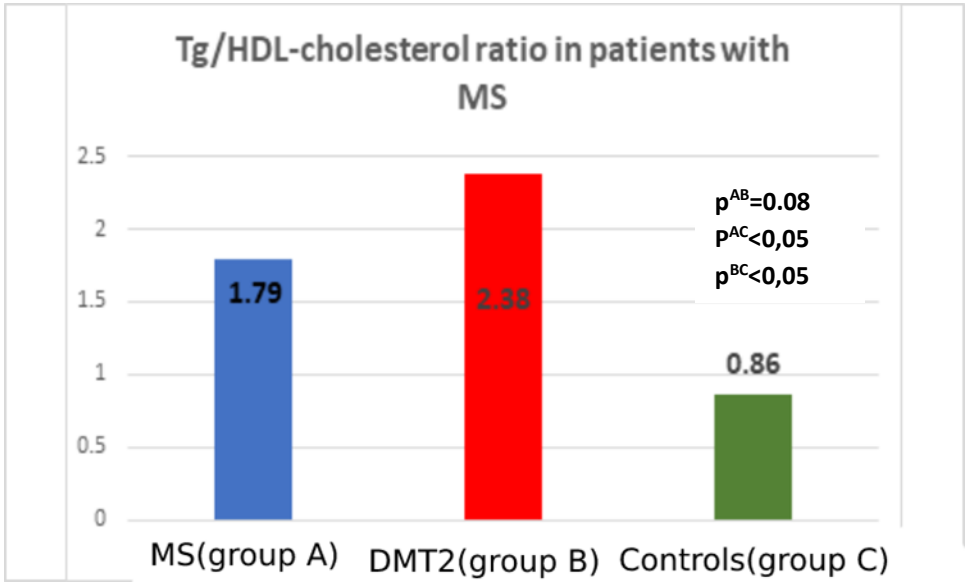
p^{AB}- MS vs. DMT2; P^{AC}-MS vs. controls; p^{BC} -DMT2 vs. controls

Basal morning fasting hyperinsulinemia and manifested insulin resistance were found in all subjects with visceral obesity and MS (groups A and B).

Tg / HDL cholesterol ratio

A comparison of the Tg / HDL cholesterol ratio as a surrogate atherogenic index and a marker of hepatic insulin resistance was done between the three groups.

The results show that the Tg / HDL ratio was increased in patients from active groups A (1.79 ± 0.91) and B (2.38 ± 1.97) compared to healthy controls (0.86 ± 0.28) (Fig. 5). The comparison of the Tg / HDL ratio between group A and group B does not establish a significant difference, while such is established when comparing them with group C.



p^{AB}- MS vs. DMT2; P^{AC}-MS vs. controls; p^{BC} -DMT2 vs. controls;

FIG. 5 Tg / HDL-cholesterol ratio in the studied patients with MS (groups A and B) and controls (group C)

In all studies with MS (group A and group B) no statistically significant correlations were found between the Tr / HDL-cholesterol ratio with BMI, waist circumference, glycemia and endogenous fasting insulin, HOMA-IR and HOVA% B.

In patients with MS without DMT2 (group A), a strong linear positive relationship was found between Tg / HDL-cholesterol ratio and plasma IL-1 levels with a Pearson correlation coefficient r = 0.5; p = 0.008.

In the group of MS with DMT2 (group B) the Tg / HDL-cholesterol ratio showed a positive linear correlation with ESR; r = 0.5; p = 0.003.

V.1. 3 Levels of plasma cytokines - IL-1, IL-6, TNF-α and hsCRP in the studied groups of persons with MS without DMT2 (group A) and with DMT2 (group B).

Plasma cytokines

The results of the studied levels of plasma cytokines (IL-1, IL-6, TNF-α) and hsCRP in patients with MS without diabetes (group A), MS with DMT2 (group B) and controls (group C) were presented by table (table 6) and graphic (fig.6).

Table. 6 Levels of plasma cytokines - IL-1, IL-6, TNF-α and hsCRP between the studied groups of individuals with MS without DMT2 (group A) and with DMT2 (group B).

Indices (referent values)	**MS without DMT2 Group A (n=45)**	**MS with DMT2 Group B (n=34)**	**Controls Group C (n=21)**	**Significance P<0,05**
IL-1	18,46±4,34	16,42±2,18	4,33±1,46	P^{ab}=**NS** (0,43) p^{AC}<0,05 p^{BC}<0,05
IL-6 (0-2pg/ml)	1,97±0,16	1,25±0,41	0,51±0,3	P^{ab} =**NS** (0,1) p^{AC}<0,05 p^{BC} <0,05
TNF-α (0-8pg/ml)	2,49±1,17	2,56±1,5	1,12±0,58	P^{AB} =**NS** (0,38) p^{AC}<0,05 p^{BC}<0,05
hsCPR (0,03-63,68mg/l)	8,24±1,48	7,53±2,22	5,9±2,36	P^{AB} = **NS** (0,5) p^{AC}<0,05 p^{BC}<0,05

p^{AB}-MS vs. DMT2; p^{AC}-MS vs. controls; p^{BC}- DMT2 vs. controls; NS-nonsignificant

Patients with MS (groups A and B) had statistically significantly higher plasma levels of IL-1, IL-6, TNF-α and hsCRP compared to controls.

When comparing the obtained results between the participants with MS without DMT2 (group A) and with MS and DMT2 (group B) no significant differences were found in the plasma levels of the studied cytokines and hsCRP (Fig. 6).

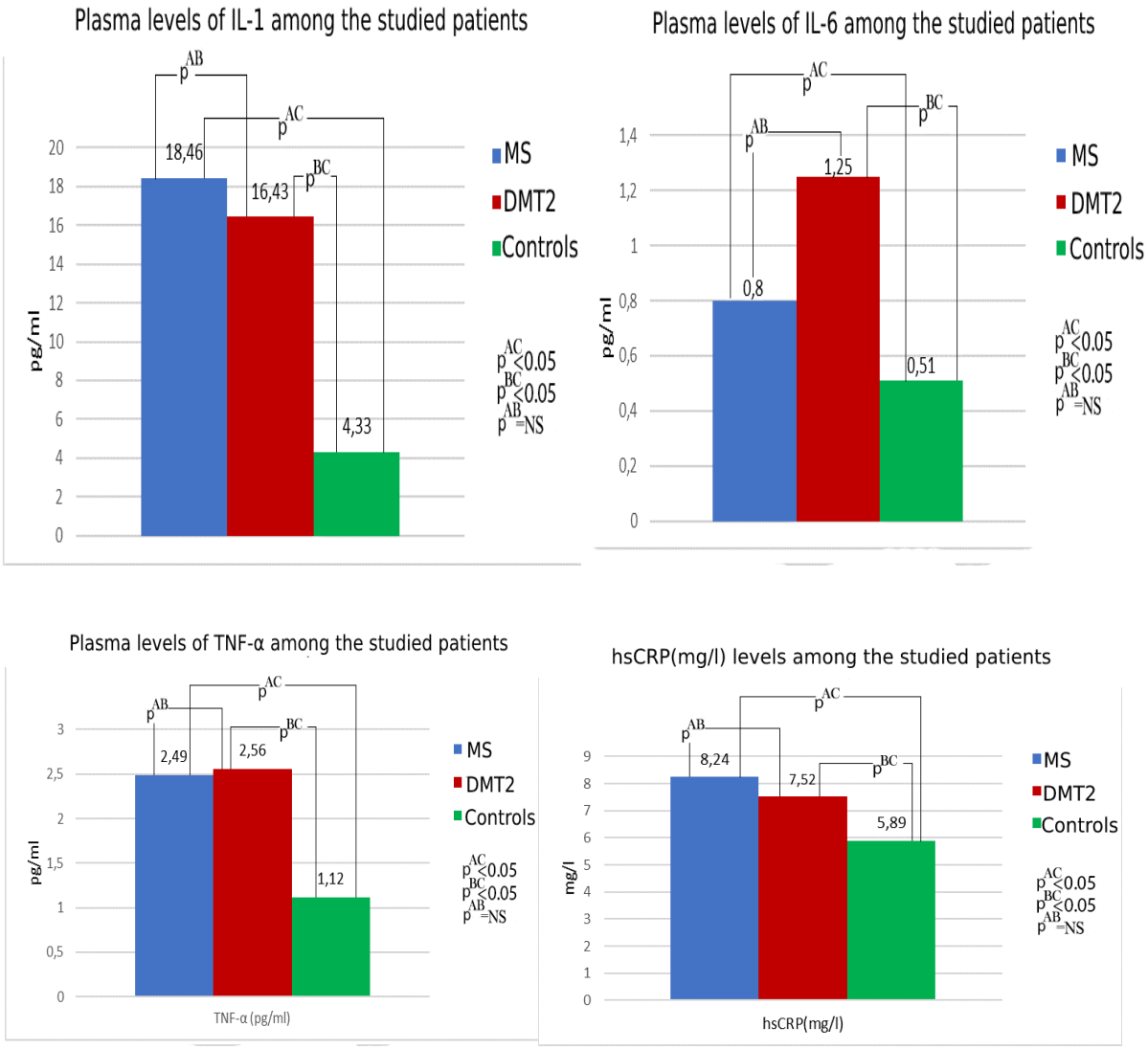


Fig.6 Plasma levels of IL-1, IL-6, TNF-α and hs CRP among the studied patients with MS without DM (group A), with MS and DMT2 (group B) and controls (group C).

Gender differences were found in plasma cytokine and hsCRP levels. The highest levels of IL-1 were reported in men with MS without DMT2 (group A), being significantly higher than in men with MS and DMT2 (group B) ($p < 0.05$) and healthy subjects (group C) ($p < 0.05$).

When comparing the plasma levels of Il-6 by sex in the different groups also in men with MS without DMT2 (group A) significantly higher levels were reported compared to men with MS and DMT2 (group B), p <0, 05) and healthy (group C) ($p < 0.05$).
The highest levels of TNFα were in women with MS and DMT2, and the differences did not show statistical significance compared with those in women in the group of MS without DMT2 (group B).
All females (groups A, B and C) have higher plasma levels of hsCRP than males.

Significance of changes in ESR and leukocyte count in the genesis of the metabolic syndrome.

There were statistically significant differences in the mean values of ESR at 1 hour between the subjects from active group B (24.27 ± 5.62) and the control subjects from group C (13.1 ± 9.39); (Fig.7).

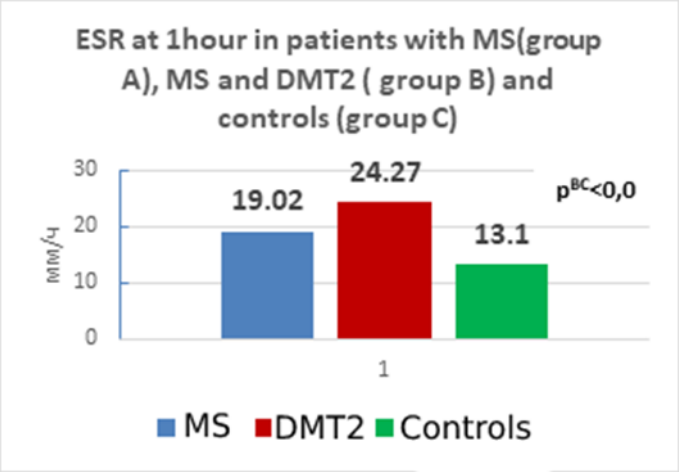


Fig.7 ESR at 1 hour in patients with MS without DMT2 (group A), with MS and DMT2 (group B) and controls.

The total leukocyte count was conducted from the peripheral blood.
In all study participants, the total leukocyte count was within the standardized reference range. The highest levels were found among patients with MS without DMT2 (group A), who showed statistically significant differences compared to the total leukocyte count in healthy individuals from the control group (group C) (Table 7).

Table 7 Total leukocyte count in peripheral blood in patients with MS without DMT2 (group A), with MS and DMT2 (group B) and controls (group C)

Indices	**MS without DMT2 Group A** (n=45)	**MS with DMT2 Group B** (n=34)	**Controls Group C** (n=21)	**Significance** (P<0,05)
All leucocyte count $x10^9/l$	8,14±0,56	7,47±0,65	6,89±0,49	p^{AB} NS(0,12) p^{AC}<0,05 p^{BC} NS(0,2)

A total leukocyte count above the upper reference range (10.10^9 / l) was found in 11 (24.4%) patients with MS without DMT2 (group A), in 6 (17.6%) patients with MS and DMT2 (group B), and respectively in 2 (9.5%) of healthy individuals in the control group (group C).

IV.1.4 Relationships between plasma levels of investigated cytokines (IL-1, IL-6, TNF-α) and low -grade chronic inflammation (hsCRP, ESR, total leukocyte count,)parameters with carbohydrate metabolism parameters, insulin indices and visceral obesity.

Relationships between the studied cytokines (IL-1, IL-6, TNF-α) and the studied markers of subclinical inflammation (hsCRP, ESR, total leukocyte count) with obesity expressed by BMI and waist circumference

In the group of patients with MS without DMT2 (group A) some dependences were found between:

- Between BMI and hsCRP levels, a moderate, statistically significant linear correlation was found with a Pearson correlation coefficient r = 0.33 and p = 0.03.
- Statistically significant, linear moderate correlation was reported between waist circumference and hsCRP levels with a Pearson correlation coefficient r = 0.32 and p = 0.04. (Fig.8)

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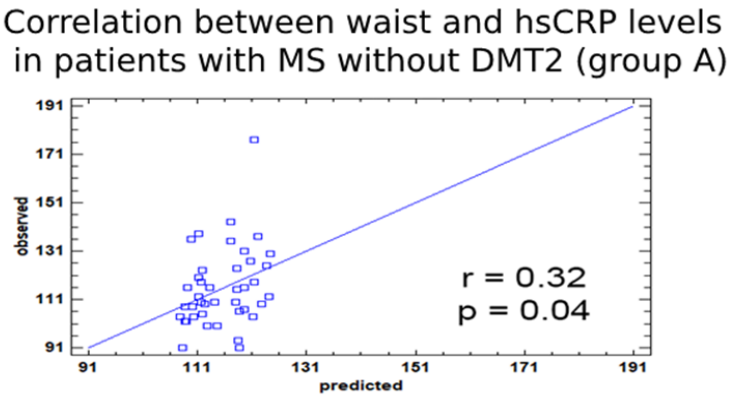

Fig.8 Correlation between waist and hsCRP levels in patients with MS without DMT2 (group A).

- Between visceral obesity described by BMI and waist circumference and plasma levels of the studied cytokines (IL-1, IL-6, TNF-α), ESR and total leukocyte count in subjects without MS without DMT2 (group A) were not statistically established. significant correlations.

Correlation dependences were also found in the subjects MS and ZDT2 (town B), which are statistically significant:

- Between waist circumference and hsCRP levels: linear, moderate dependence with Pearson correlation coefficient r = 0.45 and p = 0.02 (Fig. 9).

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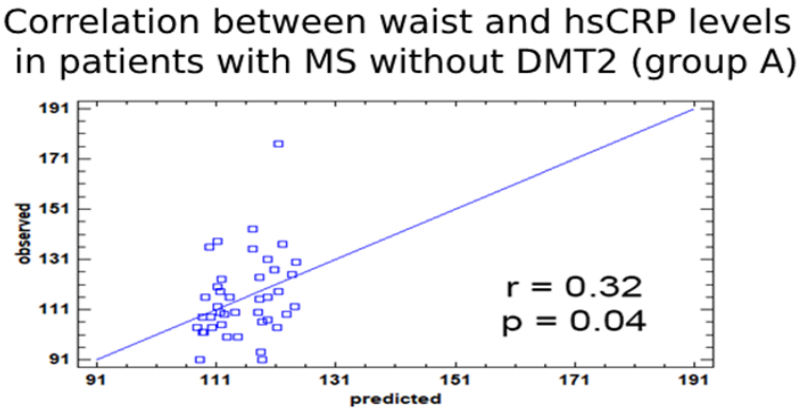

Fig.9 Correlation analysis between hsCRP levels and waist circumference in patients with MS and DMT2 (group B)

-Between waist circumference and ESR: moderate positive correlation with Pearson correlation coefficient r = 0.37 and p = 0.04.

- Between BMI and ESR: linear moderate positive relationship with Pearson correlation coefficient r = 0.37 and p = 0.03.

- Between BMI and plasma TNF-α levels: linear moderate positive correlation with Pearson correlation coefficient r = 0.48 and p = 0.004.

When analyzing the relationship between visceral obesity (described by BMI and waist circumference) with the levels of IL-1, IL-6 and total leukocyte count in individuals with MS and DMT2 (group B), no statistically significant correlations were found.

In healthy subjects used as a control group (group C), a moderate, positive linear statistically significant correlation was found only between waist circumference and ESR (r = 0.44 and p = 0.05); (table 8).

Table 8 Correlation dependences between the investigated cytokines (IL-1, IL-6, TNF-α) and markers of subclinical inflammation (hsCRP, ESR, total leukocyte count), BMI and waist circumference among patients with MS without DMT2 (gr. A), MS and DMT2 (group B)

	IL1 (gr.A)	IL6 (gr.A)	TNF-α (gr.A)	hs CRP (gr.A)	ESR (gr.A)	Leukocytes (gr.A)
BMI (gr.A)	r=-0,0863	r=0,1051	r=-0,0776	**r=0,3271**	r=0,0560	r=0,0757
	p=0,5730	p=0,4920	p=0,6124	**p=0,0345**	p=0,7214	P=0,6212
Waist (gr.A)	r=0,0466	r=0,1556	r=-0,0638	**r=0,3156**	r=0,0485	r=0,0089
	p=0,7614	p=0,3074	p=0,6773	**p=0,0417**	p=0,7577	p=0,9536
	IL1 (gr.B)	IL6 (gr.B)	TNF-α (gr.B)	hs CRP (gr.B)	ESR (gr.B)	Leukocytes (gr.B)
BMI (gr.B)	r=0,2993	r=0,1999	**r=0,4764**	**r=0,3005**	**r=0,3694**	r=0,2828
	p=0,0855	p=0,2569	**p=0,0044**	p=0,1358	**p=0,0344**	p=0,1108
Waist (gr.B)	r=0,1586	r=0,2593	r=0,1095	**r=0,4453**	**r=0,3659**	r=0,2519
	p=0,3703	p=0,1387	p=0,5374	**p=0,0226**	**p=0,0363**	p=0,1573
	IL1	IL6	TNF-α	hs CRP	ESR	Leukocytes

Relationships between the studied cytokines (IL-1, IL-6, TNF-α) and markers of subclinical inflammation (hsCRP, ESR, total leukocyte count) with glycemia and indices of insulin secretion and sensitivity (Table 9).

Table 9 Correlations between the studied cytokines (IL-1, IL-6, TNF-α) and markers of subclinical inflammation (hsCRP, ESR, total leukocyte count), and blood glucose at 0min. and 120 min from OGTT, HOMA-IR and HOMA B among patients with MS without DMT2 (group A).

Group A	K3 0 min	K3 120 min	IRI	HOMA-IR	HOMA B
IL1	r=0,0914	r=-0,1839	r=-0,0501	r-0,0585	r-0,0614
	p=0,5504	p=0,2497	p=0,7439	p=0,7025	p=0,6885
IL6	r=0,1644	**r=0,3381**	r=0,2393	r=-0,2786	r=0,1165
	p=0,3043	**p=0,0407**	p=0,1318	p=0,0777	p=0,4681
TNFα	r=-0,1301	r=0,1700	r=0,0580	r=-0,0012	r=-0,1574
	p=0,4176	p=0,3144	p=0,7189	p=0,9942	p=0,3258
hsCRP	r=-0,1692	r=0,1253	r=0,2858	r=0,1958	**r=0,4047**
	p=0,2840	p=0,4534	p=0,0665	p=0,2139	**p=0,0078**

In patients with MS without DMT2 (group A), positive correlations are found between:

1. Plasma levels of IL-6 and blood glucose at 120 minutes in the course of OGTT; moderate statistically significant linear correlation dependence with Pearson correlation coefficient r = 0, 34; p = 0.04.

2. A moderate, statistically significant positive correlation was found between the inflammatory protein (hCRP) and the insulin secretory activity of β cells (HOMA-B) (r = 0.4; p = 0.01).
3. Statistically significant positive correlation also showed ESR at 1 hour with endogenous fasting insulin (r = 0.45; p = 0.002), with levels of insulin resistance (HOMA-IR) (r = 0.43; p = 0.004) and insulin secretory activity of β cells (HOMA-B) (r = 0.35; p = 0.001).

Table 10 Correlations between plasma levels of the cytokines (IL-1, IL-6, TNF-α) and the parameters of low-grade inflammation (hsCRP, ESR, total leukocyte count) with the parameters of carbohydrate metabolism and insulin indices in patients with MS and DMT2 (gr.B).

Group B	К3 6ч	К3 12ч	К3 15ч	К3 18ч	HbA1c	IRI	HOMA IR	HOMA B
IL-1	r=-0,1037	r=-0,2507	r=-0,0430	r=0,0099	**r=0,3782**	**r=0,5376**	**r=0,4161**	**r=0,5973**
	p=0,5596	p=0,1814	p=0,8249	p=0,9592	**p=0,0431**	**p=0,0145**	**p=0,0481**	**p=0,0069**
Il-6	r=0,1144	r=-0,1711	r=0,07	r=0,08	r=0,1144	r=0,1417	r=0,108	r=0,273
	p=0,1528	p=0,36	p=0,694	p=0,554	p=0,55	p=0,548	p=0,65	p=0,287
TNFα	r=-0,0807	r=-0,1194	r=-0,0879	r=0,0337	r=-0,2330	r=-0,2330	r=0,0580	r=0,273
	p=0,6500	p=0,5297	p=0,6502	p=0,8623	p=0,2238	p=0,2238	p=0,8081	p=0,258
hsCRP	r=-0,2215	r=0,3599	r=0,3538	**r=0,4419**	**r=0,4224**	r=-0,0199	r=0,2645	r=-0,1879
	p=0,2768	p=0,0841	p=0,0977	**p=0,0347**	**p=0,0502**	p=0,9510	p=0,4060	p=0,558
ESR	r=-0,2753	r=0,1838	r=0,2024	r=0,0067	r=0,1396	r=-0,0561	r=0,1150	r=0,138
	p=0,1210	p=0,3309	p=0,2924	p=0,9724	p=0,4702	p=0,8196	p=0,6391	p=0,584
Leukocytes	r=-0,1232	r=0,1005	r=0,3001	r=0,1357	r=0,0574	r=0,2222	r=0,2384	r=0,266
	p=0,4947	p=0,5973	p=0,1138	p=0,4828	p=0,7675	p=0,3605	p=0,3257	p=0,285

*К3-blood glucose

The following correlations were found in patients with MS and DMT2 (group B):

1. Positive, strong, statistically significant correlation between plasma levels of Il-1 and endogenous fasting insulin (IRI) with a Pearson correlation coefficient r = 0.54 p = 0.01.
2. A strong, positive, statistically significant correlation was found between plasma levels of Il-1 and HOMA B (r = 0.6; p = 0.007).
3. There was also a moderate, positive correlation between serum Il-1 levels and the HOMA IR index (r = 0.42; p = 0.05).
4. HsCRP levels in these patients showed a positive, moderate correlation with pstprandial hyperglycemia at 18 h (r = 0.44; p = 0.03) of CPC and HbA1c levels (r = 0.42; p = 0.05).

Table 11 shows a moderate positive correlation between IL-1 plasma concentrations and blood glucose levels at 120 min during OGTT (r = 0.47; p = 0.03) and a negative significant

correlation between TNF -α and glycemia at 60 min in the course of OGTT (r = -0.44; p = 0.04) and between ESR and HbA1c (r = -0.52; p = 0.02) in persons from group C.

Table 11 Correlations between the plasma levels of the studied cytokines (IL-1, IL-6, TNF-α) and the parameters of low-grade inflammation (hsCRP, ESR, total leukocyte count) with the parameters of carbohydrate metabolism and insulin indices in controls (gr .C).

Group C	**Blood glucose 0 min.**	**Blood glucose 60 min.**	**Blood glucose 120 min.**	**HвA1c**	**IRI**	**HOMA-IR**	**HOMA B**
IL-1	R=0,01	R=0,38	**R=0,47**	R=-0,03	R=-0,03	R=-0,03	R=0,28
	P=0,96	P=0,09	**P=0,03**	P=0,89	P=0,90	P=0,90	P=0,22
IL-6	R-0,13	R=0,26	R=-0,19	R=0,16	R=0,02	R=-0,04	R=-0,02
	P=0,59	P=0,26	P=0,41	P=0,5	P=0,93	P=0,86	P=0,95
TNF-α	R=-0,11	**R=-0,44**	R=-0,03	R=-0,18	R=0,19	R=0,12	R=-0,06
	P=0,65	**P=0,04**	P=0,87	P=0,42	P=0,40	P=0,61	P=0,78
hsCRP	r=-0,29	r=-0,14	r=0,03	r=0,21	r=0,29	r=0,14	r=-0,11
	p=0,22	p=0,55	p=0,9	p=0,38	p=0,22	p=0,57	p=0,64
ESR	R=0,08	R=-0,23	R=0,15	**R=-0,52**	R=0,16	R=0,20	R=0,01
	P=0,73	P=0,32	P=0,51	**P=0,02**	P=0,5	P=0,39	P=0,965
leucocytes	R=0,04	R=-0,04	R=0,05	R=-0,35	R=0,33	R=0,34	R=-0,00
	P=0,86	P=0,87	P=0,85	P=0,12	P=0,15	P=0,12	P=0,99

*ERS- erythrocytes sedimentation rate

Relationships between the studied cytokines (IL-1, IL-6, TNF-α) and markers of subclinical inflammation (hsCRP, ESR, total leukocyte count) with the plasma levels of total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides.

1. No statistically significant correlations were found among the group with MS without DMT2 (group A).
2. In patients with MS and DMT2 (group B) were found:
2.1 When assessing the correlation between HDL-cholesterol levels and hsCRP, a statistically significant, strong, negative linear relationship was found with a Pearson correlation coefficient r = -0.45 and p = 0.02.
2.2 A strong, negative, statistically significant relationship was also found between HDL-cholesterol and ESR levels in the same group of patients (r = -0.5, p = 0.003).
2.3 The statistically significant linear correlation was shown by the calculated plasma levels of LDL-cholesterol and total leukocyte count (r = 0.44; p = 0.003).
3. In the control group (group C) among healthy individuals also no statistically significant correlations were found.

Logistic regression analysis was performed. The ROC curve method was applied to establish a significant threshold value.

For age, a significant threshold of 38.5 years was established with 88.24% sensitivity and 95.00% specificity (AUC 0.951, p = 0.000); (Fig. 10)

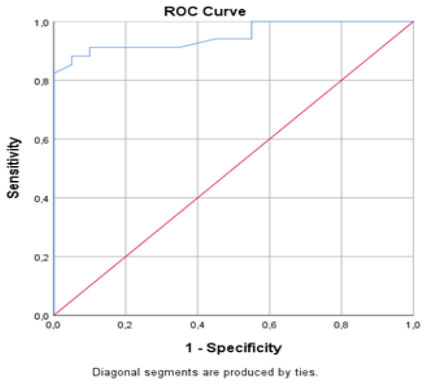

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Fig.10 ROC -age curve for determining the threshold values in differentiating patients with and without MS and DMT2.

Among the persons in our study older than or equal to 38.5 years, the risk of developing DMT2 among patients with MS is 142.5 times higher than younger (OR = 142.5, 95% CI 14, 79-1372,989; p <0.001). Our results confirm the importance of age as a non-modifiable risk factor for the development of IR and DMT2.

In the subjects that we studied with **BMI ≥26.5 kg / m^2** (cut off-26.5; 97.10% sensitivity, 95.00% specificity) the risk of developing DMT2 is 627.0 times higher than the risk in these with lower BMI (OR 627, 95% CI 37,049-10610, 933; p <0.001). Our results also confirm the leading role of obesity, described by BMI, in the development of IR, low-grade chronic inflammation, and the risk of developing DMT2 (AUC 0.970, p = 0.000); (Fig.11).

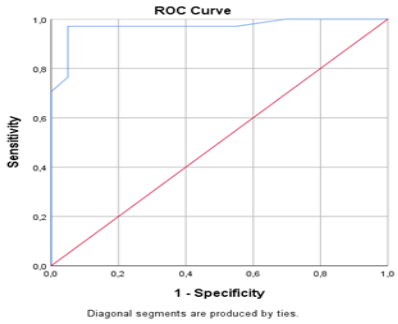

Fig.11 ROC-curve of BMI for determining the threshold values for differentiation of patients with and without MS and DMT2.

The determined threshold value of the **waist circumference ≥ of 97 cm**, which distinguishes patients with MS and ZDT2 from those without MS and ZDT2, by the method of ROC curves was with very high sensitivity (91.2%) and specificity (100.0%), p <0.001 (AUC 0.991, p = 0 000);(fig.12) This physical indicator, as a characteristic of visceral obesity in the studied patients was not used as a factor to assess the chances of developing DMT2 due to the small volume of the studied groups and the inability to apply logistic regression analysis.

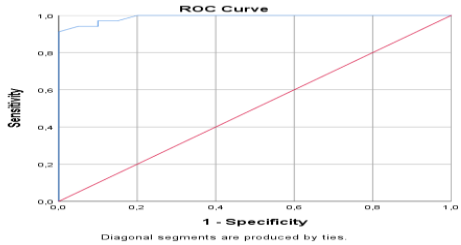

Fig. 12 ROC - waist circumference curve for determining the threshold values for differentiation of patients with and without MS and DMT2.

As a strong predictive factor for the development of MS and DMT2 stood out IR, expressed through HOMA-IR. Threshold levels of **HOMA-IR ≥ 2.39** with high sensitivity (85%) and specificity (95%) (AUC 0.948, p = 0.000) distinguish a patient with MS and DMT2 from those without DMT2. In HOMA-IR≥2,39 the risk of developing DMT2 was increased by 108 times (OR-107,667, 95% CI 10,208-1135,585, p <0,001) (Fig. 13).

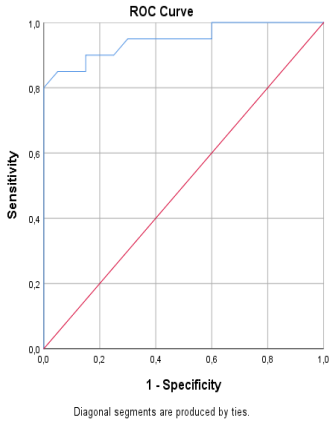

Fig.13 ROC-curve of HOMA-IR for determining the threshold values for differentiation of patients with and without MS and DMT2.

Systolic blood pressure values ≥ 122.5 mmHg with lower but acceptable sensitivity (79.4%) and specificity (75.0%) distinguish patients with and without DMT2. At the indicated threshold value of systolic blood pressure the risk of developing DMT2 is 12 times higher than that of persons with lower levels of systolic blood pressure (Fig. 14).
The established threshold value of diastolic blood pressure ≥ 82.5 mmHg by the method of ROC curves at lower sensitivity (58%) and specificity (85%) (AUC 0.807, p = 0, 000) increases the probability of developing DMT2 by 8 times (OR 8.095, 95% CI 1.987-32, 979, p = 0.004) among the study group.

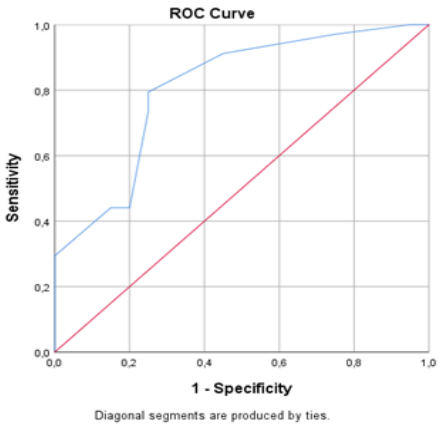

Fig.14 ROC -systolic blood pressure curve for determining the threshold values in differentiating patients with and without MS and DMT2.

The levels of total cholesterol, LDL-cholesterol and serum triglycerides were also included in the logistic regression analysis as independent variables. Table 12 shows the results obtained. No significant HDL-cholesterol thresholds were found in the ROC-curve method (p> 0.05).

Table 12 Results of logistic regression analysis in the study of serum levels of total cholesterol, triglycerides and LDL-cholesterol as independent factors for the development of DMT2.

variable	Cut-off	sensitivity	specificity	**OR**	95% CI Lower border	95% CI Upper border	Significance (P≤0.05)
Total cholesterol (mmol/l)	**≥4.975**	59.10%	90.00%	**11,848**	2,858	49,127	0.001
Triglycerides (mmol/l)	**≥1.21**	91.20%	73.70%	**28,933**	6,054	138,284	<0.001
LDL-cholesterol (mmol/l)	**≥2.925**	86.20%	77.80%	**21,875**	4,725	101,283	<0.001

A regression analysis was also performed to assess whether IL-1 could have a predictive value for the risk of developing DMT2. Levels ≥ 10.25 pg / ml (optimal crossing point) with 85.3% sensitivity and 100% specificity (p <0.001) were distinguished, distinguishing individuals with MS and DMT2 from those without DMT2 (AUC 0.95, p = 0, 000); (Fig.15).

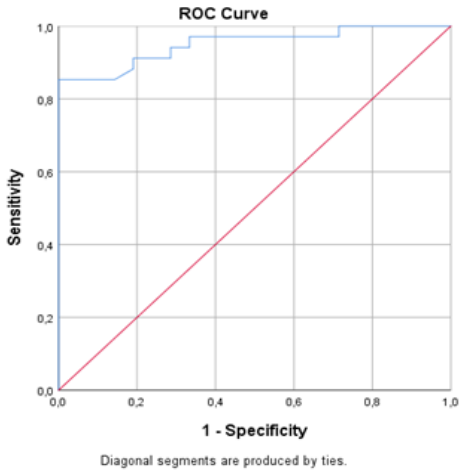

Fig. 15 ROC -curve of IL-1 for determination of threshold values in differentiation of patients with and without MS and DMT2.

However, the small number of patients studied does not allow to calculate the ratio of the chances of this factor for the development of DMT2. This result raises the question of the subsequent study of IL-1 as a predictive factor for the development of DMT2 in a larger group of patients with MS.

The levels of IL-6 ≥ 0.85 pg / ml with lower sensitivity (61%) and good specificity (81%) differentiate between individuals with and without DMT2 (AUC 0.753, p = 0.002); (Fig.16). In patients with IL-6 levels ≥ 0.85 pg / ml compared to those with lower plasma levels of this cytokine, the risk of developing DMT2 is 6.42 (OR-6, 452, 95% CI 1,796 - 23,608; p < 0.005) times higher.

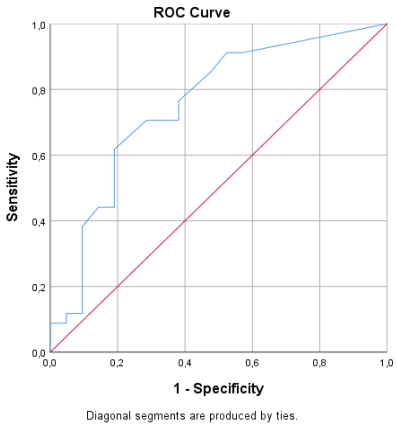

FIG. 16 ROC -curve of IL-6 for determination of threshold values in differentiation of patients with and without MS and DMT2.

For TNF-α and hsCRP, the established cut off levels did not show significance, which rejected the application of these laboratory parameters in our study as independent

informative inflammatory factors, distinguishing people with MS and DMT2 from healthy individuals.
Using a model with more than one variable in the logistic regression analysis, diastolic blood pressure (≥82.5mmHg), IL-6 levels (≥0.85pg / ml) and hsCRP (≥1.896mg / l) were found as predictive factors for the development of ZDT2 (Table 13).

Table 13. Regression logistic analysis with a model of several variables.

index	Average value	Lower range	Upper range	Significance P≤0,05	Relative risk	95% C.I. Lower border	95% C.I. Uper border
Dyastolic BP	2,590	,935	7,666	,006	**13,324**	2,131	83,319
Il-6	1,958	,816	5,764	,016	**7,088**	1,433	35,067
hsCRP	2,338	1,007	5,393	,020	**10,360**	1,440	74,529
	-2,967	1,054	7,918	,005	,051		

Using the ROC curve method, the significant threshold values of the clinical and laboratory parameters of MS and the total leukocyte count and the studied interleukins were determined (Table 14).
Table. 14 Results of logistic regression analysis in the study of clinical and laboratory indicators and inflammatory markers as independent factors for the development of MS.

Variables (factors)	Cut-off	Чувствителност	Специфичност	Сигнификантност p≤0,05
Age	**38.5**	53.30%	95.00%	0,002
BMI (кg/м^2)	**27.5**	100.00%	95.00%	<0,001
waist (cm.)	**98**	93.30%	100.00%	<0,001
Systolic BP	**117.5**	95.60%	60.00%	<0,001

(mmHg)				
Diastolic BP (mmHg)	**77.5**	93.30%	50.00%	<0,001
HOMA- ИР	**2.41**	77.80%	100.00%	0,001
HOMA B	**145.99**	77.78%	75.00%	0,005
Total cholesterol (mmol/l)	**4.975**	59.10%	90.00%	0,004
HDL- cholesterol (mmol/l)	**0.985**	61.40%	84.70%	0,001
Triglycerides (mmol/l)	**1.075**	70.50%	68.400%	0,005
LDL- cholesterol (mmol/l)	**2.905**	68.20%	72.20%	0,002
Leucocytes ($x10^9$/l)	**7.85**	55.60%	90.00%	0,004
IL-1 (pg/ml)	**10.05**	86.70%	100.00%	<0,001
IL-6 (pg/ml)	**0.85**	42.20%	81.00%	0,049
hsCRP (mg/l)	**1,699**	95.60%	38.10%	0,029

The obtained results showed that with the highest diagnostic accuracy and value in distinguishing people with MS from those without MS have BMI, waist circumference and IL-1 levels.

Analysis of the obtained data.

In all subjects with MS (groups A and B), statistically significantly increased plasma levels of the tested cytokines were reported compared to healthy subjects in the control group (C).

IL-1 levels are higher in MS without DMT2 (group A) compared to MS and DMT2 (these differences are statistically insignificant). Gender differences in plasma levels of IL-1 were found. Statistically significantly higher levels of this interleukin showed men with MS without DMT2 (group A) Only in DMT2 showed linear positive correlations between IL-1, endogenous fasting insulin (IRI), levels of insulin resistance (HOMA-IR) and insulin secretory activity (HOMA B). The levels of IL-1 ≥ 10.25pg /ml are the determined cut off value that distinguishes individuals without and with DMT2.

The highest concentrations of IL-6 were found again in patients with MS without DMT2 (group A), and the differences were not statistically significant with the reported plasma levels of this cytokine in MS and DMT2 (group B). As with IL-1, men with MS without DMT2 (group A) had significantly higher levels of IL-6. The results showed a positive linear relationship between glycemic levels at 120 minutes during OGTT and plasma IL-6 concentrations. The risk of developing DMT2 for the subjects included in our study with levels of IL-6 ≥ 0.85 pg / ml was approximately 7-fold higher compared to those in persons with lower plasma levels of this cytokine.
TNF-α levels were statistically insignificantly higher in those with MS and DMT2 (group B) than in those without MS without DMT2 (group A). In DMT2 (group B), a positive correlation was found between waist circumference and plasma concentrations of this studied cytokine.

Proven positive correlations between IL-1 levels with insulin levels and indices, IL-6 levels with glycemia, and TNF-α with waist circumference suggest the role of interleukins in maintaining low-grade chronic inflammation and their effect in activation of subsequent immunological processes in the course of MS and the development of DMT2. Among our patients, IL-6 levels with a predictive role in the risk of developing DMT2 with good diagnostic accuracy and limited diagnostic value and Il-1 levels with excellent diagnostic accuracy and value were found.

Statistically higher levels of hsCRP were found in individuals with MS (groups A and B) compared to the control group. The measured levels of hsCRP in the group with MS without DMT2 (group A) were statistically insignificantly higher compared to those in MS and DMT2 (group B). Higher hsCRP values for women were reported in all subjects (groups A, B and C). A positive significant correlation was observed between waist circumference in MS. For persons with MS without DMT2 (group A), a moderate positive relationship was observed between plasma levels of acute-phase inflammatory protein-CRP and β-secretory activity reflected by the HOMA-B index.
With the development of DMT2 (group B) in MS, there is a significant positive correlation of serum hsCRP concentration with postprandial glycemia and HbA1c. Significance was not found in the acute phase protein as a separate predictor of the development of MS and DMT2. Its presence with levels ≥1.66 mg / l in all multifactorial models defines its role as an additional marker and not as a criterion for the development of MS and DMT2.

Like other inflammatory markers, ESR per 1 hour among patients with MS (groups A and B) was increased compared to healthy subjects in the control group (group C), and the differences were statistically significant only compared with patients with MS and DMT2 (group B). In studies with MS without DMT2 (group A), the results showed significant positive relationships between reported ESR and endogenous insulin (IRI) levels, insulin resistance (HOMA-IR) and pancreatic secretory β cell activity (HOMA B). In contrast, statistically significant correlations between changes in ESR and lipid profile were reported in patients with DMT2 (group B).

The obtained results did not show deviations in the total leukocyte count among individuals with MS without DMT2 (group A) and with MS and DMT2 (group B). In different numbers of persons from the three groups deviations above the upper reference range are established, as the highest percentage is observed in the persons with MS without DMT2 (group A). A negative, statistically significant correlation was found between total leukocyte count with fasting and fasting glycemic levels with endogenous fasting insulin, calculated insulin secretion (HOMA B) and plasma LDL-cholesterol levels in the group without MS without DMT2 (group A).

Discussion:

The data obtained by us prove the existence of low-grade chronic inflammation in MS, which occurs with increased plasma concentrations of interleukins and hsCRP.

Classic non-specific laboratory markers for active inflammatory process are leukocytosis and accelerated ESR. In the conditions of low-grade chronic inflammation, which exists in MS, the total leukocyte count is within the reference range.

The measurement of total leukocyte count and changes in leukocyte populations has been identified by many authors as a useful independent marker for β cell dysfunction and insulin resistance. According to Twig and co-authors at levels above 6.91x109 / l, the increase in total leukocytes by 1.0x109 / l increases by 7.6% the risk of developing of diabetes. In our study, the total leukocyte count among patients with MS without DMT2 (gr.A) was 8.14 ± 1.87 x109 / l, with the largest proportion in this group being those with values of total leukocyte count above the reference range. In our study, levels of leukocytes in peripheral blood ≥ 7.89x109 / l were found, distinguishing people with MS from healthy ones.

No significant thresholds of total leukocyte counts were found in peripheral blood to differentiate patients with DMT2. This result could support the hypothesis that low-grade chronic inflammation occurs with the development of visceral obesity and IR in MS. For the use of this non-specific inflammatory indicator as a prognostic marker for the development of DMT2 among the Bulgarian population, it is necessary to conduct follow-up studies to cover a much larger number of populations to study.

There is not much information in the scientific literature about changes in ESR at 1 hour as a diagnostic indicator of low-grade chronic inflammation. A positive correlation was found between accelerated ESR and the number of MS components among patients with visceral obesity. In our study, a positive correlation of ESR with fasting endogenous insulin (IRI) and indices of insulin secretion and sensitivity was established among MS individuals without

DMT2 (gr.A). The most accelerated SUE was reported in the group with DM T2, where hyperglycemia was also evident. These results support current knowledge of the involvement of non-specific low-grade inflammation in visceral obesity in the onset and spread of IR in MS.

Our results show increased levels of Il-1, IL-6 and TNF-α among MS and DMT2 patients. In our study, positive correlations between IL-1 levels with insulin levels and insulin indices, IL-6 with glycemic levels, and TNF-α with waist circumference were demonstrated, testifying to the role of interleukins in the pathogenesis of MS. and their influence in the activation of subsequent immunological processes in the course of MS and the development of DMT2. These data are consistent with the results of some other studies and contrast with the reported results of other studies. Isolated IL-1 did not prove to be a significant factor that could have a predictive role in the risk of developing DMT2, which was probably determined by the insufficient number of patients included in the study.
On the other hand, the determined significant threshold value for IL-6 levels increases the risk of developing DMT2 by about 7 times compared to lower levels. However, in combination with the other variables such as age, BMI, waist circumference, arterial pressure and lipid profile included in the study of the risk for the development of DMT2, they acquire a diagnostic value. The diagnostic value for IL-1 being greater than that of IL- 6.

The relationship between increased expression of TNF-α, visceral obesity and the development of IR has been widely studied since 1993. Its modulating role regarding intracellular insulin signaling in hepatocytes and myocytes, suppressive effect on PPAR-γ activity and GLUT-4 synthesis, stimulation of serine phosphorylation of insulin receptor substrate 1 (IRS-1) is known.
Increased secretion of TNF-α leads to decreased insulin sensitivity and increases lipolysis in adipocytes. The increased levels of TNF-α and the positive correlation with BMI and waist circumference found in our study in individuals with MS without DMT2 are of a confirmatory nature compared to the existing information in the literature.

The Tg/HDL ratio was calculated and analyzed by us as a surrogate marker to assess liver IR and atherogenic risk. The healthy individuals included in the control group had a normal body weight, no evidence of IR and mixed dyslipidemia, therefore the ratio of Tg/HDL was accepted only as an atherogenic marker in the presence of anamnestic data on family history for cardiovascular diseases. All patients with MS showed IR, higher levels of serum triglycerides and lower HDL cholesterol compared to the control group. Because existing clinical-laboratory abnormalities describing MS are known cardiovascular risk factors, the Tg/HDL cholesterol ratio was accepted as a surrogate marker describing IR. Patients with MS (from both groups) showed statistically higher values of the studied atherogenic index Tg/HDL-cholesterol compared to the control group of individuals. In the MS patients with DMT2 studied by us (group B), the Tg/HDL-chol ratio is above 2.0, it is statistically

insignificantly higher than that for MS persons without DMT2 (group A) and corresponds to the elevated plasma levels of total cholesterol, LDL-cholesterol, triglycerides.

These results support the evolution of DMT2 with progression of insulin resistance, dysfunction in insulin secretion during the development of hyperglycemia. For the Tg/HDL cholesterol index, a value above 1.8 is considered an indicator of IR. Our results are consistent with those reported in the study by McLaughlin et al.

The secretion of hsCRP is part of the non-specific physiological acute-phase response to inflammatory, infectious and tissue-damaging processes and is believed not to be a diagnostic criterion by itself. As a non-specific marker of inflammation, it is influenced by many factors such as the presence of obesity or arterial hypertension, smoking, intake of estrogens or the presence of menopause in women. In the patients with MS studied by us (group A and B), elevated levels of hsCRP above 5 mg/l were observed, which also determines a high risk for cardiovascular incidents. No statistically significant differences were found in the measured plasma concentrations of the studied acute phase protein between individuals with MS without DMT2 (group A) and those with MS and DMT2 (group B). All MS subjects included in our study had visceral obesity, and an expected positive statistically significant correlation was found between BMI and waist circumference with established hsCRP levels, which would explain the lack of differences in the inflammatory protein results obtained, regardless of glycemia and the link is the presence of low-grade chronic inflammation in adipose tissue. Probably due to the fact that hsCRP levels are affected by multiple factors in MS, our study did not find significant acute-phase protein thresholds differentiating MS and DMT2 patients from healthy subjects.

The healthy subjects in the control group in our study were also found to have hsCRP levels above 3mg/l, which defines a high cardiovascular risk among them. The results obtained are due to the increased levels of hsCRP among women in this group. The Multi-Ethnic Study of Atherosclerosis (MESA) cohort of 6,814 individuals without known cardiovascular disease also found up to over 40% higher hsCRP levels among women of all ethnicities, regardless of BMI.

The obtained results for hsCRP in women from the control group indicate an increased cardiovascular risk, but it is necessary to interpret these results complexly. No other known clinical-metabolic risk markers were found in the control group, persons with hsCRP levels above 10mg/l, as well as women taking estrogen-containing oral contraceptives, were not excluded from the analysis. Probably, the genetic polymorphism in the CRP gene, which determines the differences in the production of both CRP and IL-1 and IL-6, could explain the results we obtained.

IV.II STUDY OF LYMPHOCYTE SUBPOPULATIONS IN PERIPHERAL BLOOD OF PATIENTS WITH MS WITHOUT DMT2 (group A), PATIENTS WITH MS AND DMT2 (group B), CONTROL GROUP OF HEALTHY PERSONS (group C) and PATIENTS WITH DMT1 (group D)

IV.II.1. Results of a comparative quantitative and qualitative analysis of the total and differentiated leukocyte profile and lymphocyte blood subpopulations between different groups of individuals (MS patients without diabetes, MS and DMT2 patients, and DMT1 patients).

IV.II.1.1 Investigation of total and differential leukocyte count among subjects with MS without DMT2 (group A), with MS and DMT2 (group B), with DMT1 (group D) and controls (group C).

Table 15 Total and differential leukocyte count of the examined patients with MS without DMT2 (group A), MS and DMT2 (group B), DMT1 (group D) and controls (group C).

Indices (referent ranges)	MS without DMT2 **Group A** (n=26)	MS and DMT2 **Group B** (n=70)	Controls **Group C** (n=21)	DMT1 **Group D** (n=22)	Significance (p<0.05)
All leucocyte counts (3,5-10.0 x10^9 /l)	6,78±1,8	7,28±1,85	6,89±1,07	6,91±1,32	P^{AB} NS; p^{AC} NS p^{BC} NS; P^{AD}NS P^{BD}NS; P^{CD}NS
Limphocytes (1,2-3,2 x10^9 /l)	2,55±0,79	2,34±0,647	2,04±0,64	2,35±0,69	P^{AB}NS **P^{AC}<0,05** P^{BC} NS; p^{AD} NS p^{BD} NS; p^{CD} NS
Monocytes (0,3-0,8 x10^9 /l)	0,37±0,11	0,45±0,15	0,28±0,09	0,45±0,12	**P^{AB}<0,05; P^{AC}<0,05** **p^{BC}<0,05; p^{AD}<0,05** p^{BD} NS; **p^{CD}<0,05;**
Granulocytes (1,2-6,8 x10^9 /l)	3,84±1,23	4,5±1,4	4,41±1,5	4,13±0,98	**P^{AB} <0,05** p^{AC} NS; p^{BC} NS p^{AD} NS

					p^{BD} NS; p^{CD} NS

P^{AB}-MS vs. DMT2; p^{AC}- MS vs. controls; p^{BC} -MDT2 vs. controls; p^{AD}-MS vs. DMT1; p^{BD}-DMT2 vs. DMT1; p^{DC}- DMT1 vs. controls.

In all examined patients, no deviations in the absolute leukocyte, lymphocyte and granulocyte count outside the reference range were detected.

Compared to the participants from the other groups, the healthy controls had the lowest lymphocyte count, and these differences were statistically significant compared to the MS patients without DMT2 (group A). A lower absolute monocyte count (0.28±0.09x109/l) was also observed both in relation to the lower reference limit (0.3-0.8x109/l) and in relation to all studied groups (A, B and D).

Patients with MS without DMT2 (group A) compared to those examined with MS and DMT2 (group B) showed a lower monocyte count (0.37±0.11vs. 0.45±0.15; p<0.05). The comparison of the granulocyte count between the two groups of MS with and without T2D showed a significantly lower granulocyte level in group A (3.84±1.23 vs. 4.5±1.4; p<0.05). The obtained results of total leukocyte (6.91±1.32 x109/l), absolute lymphocyte (2.35±0.69 x109/l), monocyte (0.45±0.12 x109/l) and granulocyte count (4.13±0.98 x109/l) in the subjects with DMT1 (group D) did not differ significantly from the same obtained in the patients with MS and DMT2 (group B).

Comparison of mean lymphocyte values showed that lymphocyte levels were higher in MS and DM patients (gr. A, B and gr. D) compared to the control group (gr. C). It was found that only in MS patients without DMT2 (group A) these differences were statistically significant (2.55±0.79 vs. 2.04±0.64; p<0.05) (Fig. 17).

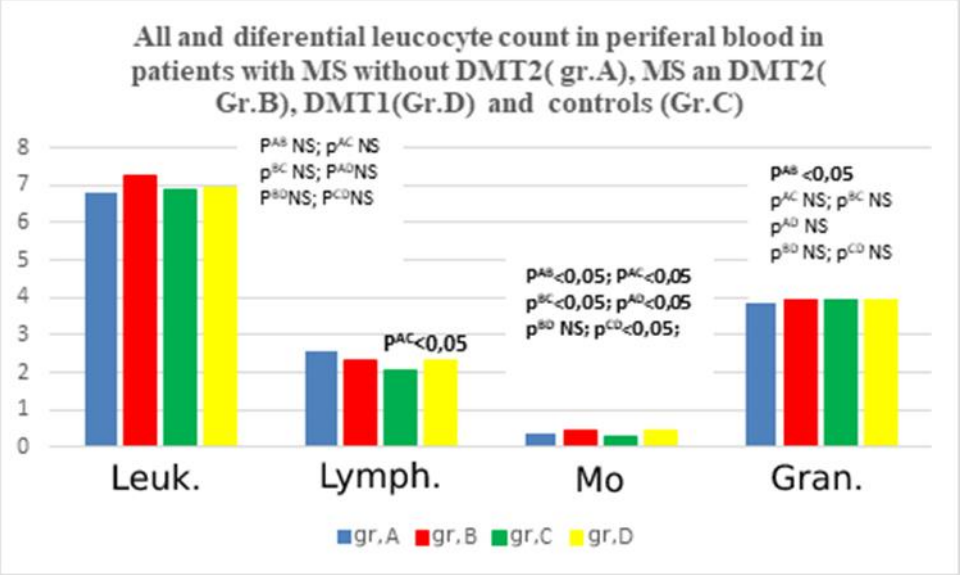

Fig. 17 Total and differential leukocyte count in peripheral blood in patients with MS without DMT2 (gr.A), DMT2 (gr.B), DMT1 (gr.D) and controls (gr.C)

The values of the total leukocyte count in the 95th percentile for MS without DMT2 (gr. A) were $10.7x10^9$/l; for MS with DMT2 (group B) - $10.9x10^9$/l, for DMT1 (group D) -$8.6x10^9$/l and 8.3x109/l for a control group of persons (group C).

When examining the total and differential leukocyte count by age group, no well-defined trend of change in these parameters with age change was found. There were also no statistically significant correlations between age and blood elements tested for study participants in all groups.
The distribution as a relative part of leukocyte subtypes, measured in percentages, is presented in tabular and graphic form (table 16, fig. 18).

Table 16 Relative part of lymphocytes, monocytes and granulocytes from the total leukocyte pool in the studied groups of patients.

Referent range	MS **Group A** (n=26)	DMT2 **Group B** (n=70)	DMT1 **Group D** (n=22)	Controls **Group C** (n=21)	Signifficance p<0,05
Lymphocytes (17-48%)	38,48±7,14	36,05±7,67	34,88±7,5	31,89±7,5	**p^{AC} < 0,05 (0,008);** p^{BC}=0,51;p^{DC}=0,31 **p^{AB}< 0,05 (0,005)** p^{AD}=0,08; p^{BD}=0,54
Monocytes (4-10%)	6,54±1,22	6,97±1,39	6,96±1,89	4,76±1,49	**p^{AC}< 0,05 (0,00007)** **p^{BC}< 0,05 (0,000000)** **p^{DC}< 0,05 (0,000000)** p^{AD}=0,08 **p^{AB}< 0,05 (0,005)**

					p^{BD}=0,54
Granulocytes (43-76%)	54,99±7,64	56,99±7,82	57,82±7,64	63,53±7,74	**p^{AC}< 0,05 (0,00002)** p^{BC}=0,08; p^{DC}=0,08 p^{AB}=0,01; p^{AD}=0,14 p^{BD}=0,61

P^{AB}-MS vs.DMT2; p^{AC}- MS vs. controls; p^{BC} -DMT2 vs. controls; p^{AD}-MS vs. DMT1; p^{BD}-DMT2 vs. DMT1; p^{DC}-DMT1 vs. controls.

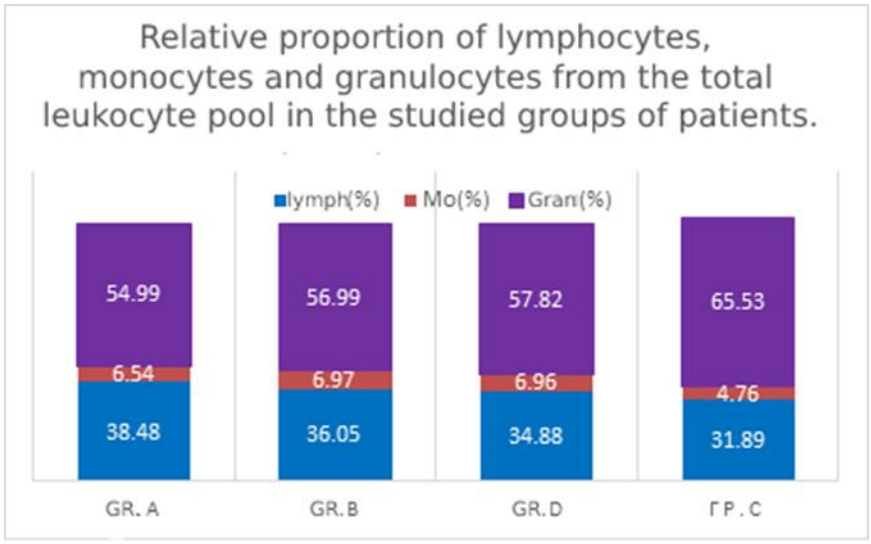

Fig. 18 Relative proportion of lymphocytes, monocytes and granulocytes from the total leukocyte pool in the studied groups of patients.

IV.1.2 Following the changes in lymphocyte subpopulations total T lymphocytes (CD3+), Th (T helpers; CD4+), Ts (T cytotoxic/suppressors; CD8+), B lymphocytes (CD19+), NK cells (CD3-/CD16+56+) among the subjects.

The results of the relative part of all lymphocyte subpopulations in percentages are presented in tabular and graphic form: total T lymphocytes (CD3+), Th (T helpers; CD4+), Ts (T cytotoxic/suppressors; CD8+), B lymphocytes (CD19+), NK cells (CD3-/CD16+56+) from the performed flow cytometric analysis of lymphocytes (Table 17, Fig. 19).

Table 17 Proportion of lymphocyte subpopulations in patients with MS without ZDT2 (group A), MS with DMT2 (group B), DMT1 (group D) and controls (group C).

Limphocytes (Reference values %)	MS **Group A** (n=26)	DMT2 **Group B** (n=70)	DMT1 **Group D** (n=22)	Controls **Group C** (n=21)	Significance (p<0.05)
T-all limphocytes (CD3+) (67-76%)	70,8±3,68	68,73±1, 86	68,98±5,15	64,72±0,62	p^{AC}-NS; p^{BC} NS p^{DC} NS; p^{AD} NS p^{BD} NS; p^{AB} NS
Th (CD4+) (36-46%)	46,08±3,63	45,63±2,75	43,78±4,10	36,74±0,23	**p^{AC}<0.05** **p^{BC}<0.05** **p^{DC}<0,05** p^{AD} NS; p^{BD} NS p^{AB} NS;
Ts (CD8+) (31-40%)	21,79±2,69	22,34±2,1	24,51±3,99	38, 72±0,77	**p^{AC}<0.05** **p^{BC}<0.05** **p^{DC}<0,05** p^{AD} NS; p^{BD} NS p^{AB} NS;
B-limphocytes. (CD19+) (11-16%)	8,73±1,82	7,36±0,97	8,91±2.37	15,09±0,38	**p^{AC}<0.05** **p^{BC}<0.05** **p^{DC}<0,05** p^{AD} NS; p^{BD} NS p^{AB} NS
NK cells (CD3-/CD16+56+) (9-15%)	18,88±6,78	21,96±8, 85	17.14±6.98	10,93±0,69	**p^{AC}<0.05** **p^{BC}<0.05** **p^{DC}<0,05** p^{AD} NS; p^{BD} NS

					p^{AB} NS

p^{AB} -MS vs. DMT2; p^{AC} – MS vs. controls; p^{BC} –DMT2 vs. controls; p^{DC}- DMT1 vs. controls; p^{AD}- MS vs. DMT1; p^{BD}- DMT2 vs. DMT1

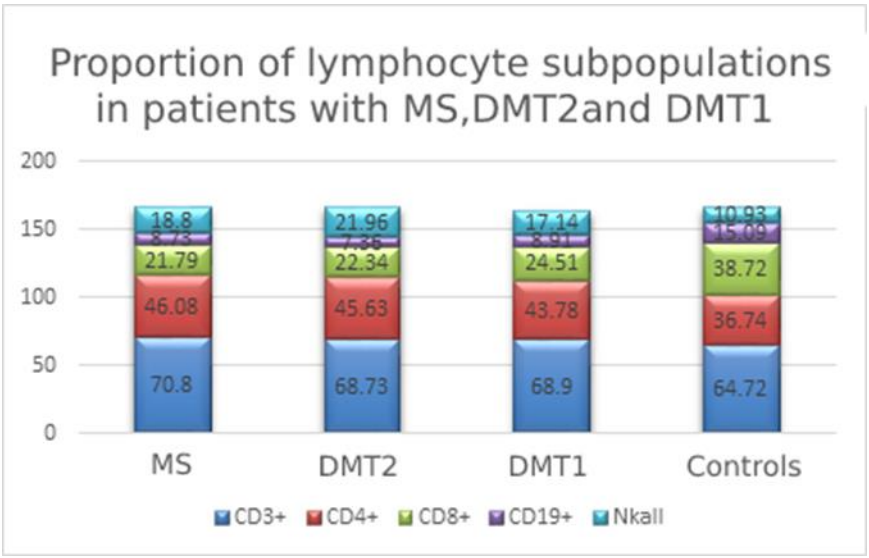

Fig. 19 Proportion of lymphocyte subpopulations in patients with MS without DMT2 (group A), MS with DMT2 (group B), DMT1 (group D) and controls (group C)
The results of the absolute number of lymphocyte subpopulations are also presented in tabular and graphic form: CD3+ (total T lymphocytes), CD4+ (Helpers; Th), CD8+ (T cytotoxic/suppressors; Ts), CD19+ (B lymphocytes), CD3-/CD16+ 56+ (NK cells) from the flow cytometric analysis (Table 18, Fig. 20).

Table 18 Absolute number of lymphocyte subtypes in patients with MS without DMT2 (group A), with MS and DMT2 (group B), controls (group C) and DMT1 (group D).

Count of lymphocytes (cells/µl)	MS with DMT2 **Group A** **(n=26)**	MS with DMT2 **Group B** **(n=70)**	DMT1 **Group D** **(n=22)**	Controls **Group C** **(n=21)**	Significance (p<0.05)
T-all lymhocytes (CD3+)	1831 ±290.18	1616.59 ±124.8	1662.55 ±302.76	1120.43 ±17.54	p^{AC}<0,05 p^{BC}<0,05

(1100-1700/ µl)					p^{DC}<0,05
Th (CD4+) (700-1100/ µl)	1193 ±178.82	1082.44 ±107.14	1041.75 ±185.69	728.81 ±17.54	p^{AC}<0.05 p^{BC}<0,05 p^{DC}<0,05
Ts (CD8+) (500-900/ µl)	578.68 ±137.53	515.10 ±67.25	604.15 ±153.47	783.48 ±36.92	P^{AC}<0.05 P^{BC}<0,05 p^{DC}<0,05
Th/Ts	2.33±0.37	2.36±0.37	2.02±0.44	1.02±0.06	p^{AC}<0.05 P^{BC}<0,05 p^{DC}<0,05
B - lymphocytes (CD19+) (200-400/ µl)	223.08 ±57.79	186.02 ±26.07	200.9 ±56.32	287.67 ±23.53	p^{AC}<0.05 P^{BC}<0,05 p^{DC}<0,05
NK cells (CD3-/CD16+56+) (200-400/ µl)	443.6 ±157.7	385.0 ±86.82	327.42 ±119.78	271.19 ±19.0	P^{AC}<0.05 P^{BC}<0,05 p^{DC}= NS (0,19)

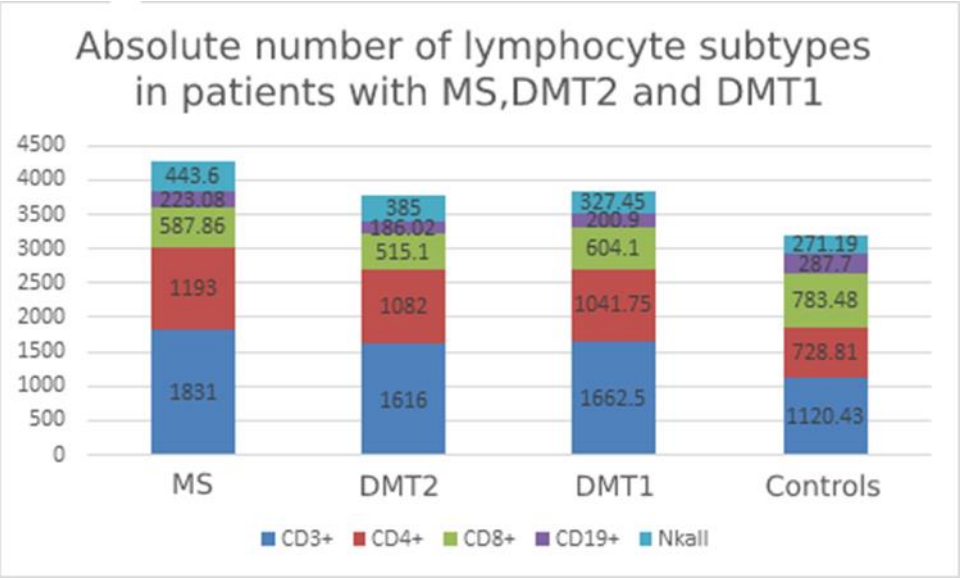

Fig.20 Absolute number of lymphocyte subtypes in patients with MS without DMT2 (gr.A), with MS and DMT2 (gr.B) and DMT1 (gr.D).

In the healthy individuals in the control group (group C), no deviations outside the reference limits were found in the absolute number of cells from the lymphocyte populations. The subjects with MS without DM (group A) and those with MS and DMT2 (group B) had significantly higher absolute numbers of non-specific T lymphocytes (CD3+), Th(CD4+) and NK (cells CD3-/CD16+56+) and statistically significantly lower proportion and absolute number of Ts (CD8+) compared to controls (group C).

A lower absolute B (CD19+) lymphocyte count was reported in the active groups (groups A, B, C), and the differences were statistically significant between individuals with MS without DMT2 (group A) and healthy (group C).
When comparing the results obtained between the two active MS groups (groups A and B), no significant differences were found in the proportion and absolute value of the analyzed lymphocyte subpopulations, although MS patients without DMT2 had a higher number of total T lymphocytes (CD3+), Th lymphocytes (CD4+), B- lymphocytes (CD19+) and NK cells per μl of peripheral blood.

All examined with DMT1 (group D) also had a statistically significantly higher proportion and absolute number of Th (CD4+) and lower proportion and absolute number of Ts (CD8+) and B (CD19+) lymphocytes compared to healthy individuals (group C) . A statistically insignificant higher proportion and number of NK cells was found in them compared to the control group.

When comparing the results of patients with DMT2 and DMT1, no significant differences were found in the results of the performed flow cytometric analysis of lymphocyte subpopulations (Fig. 21).

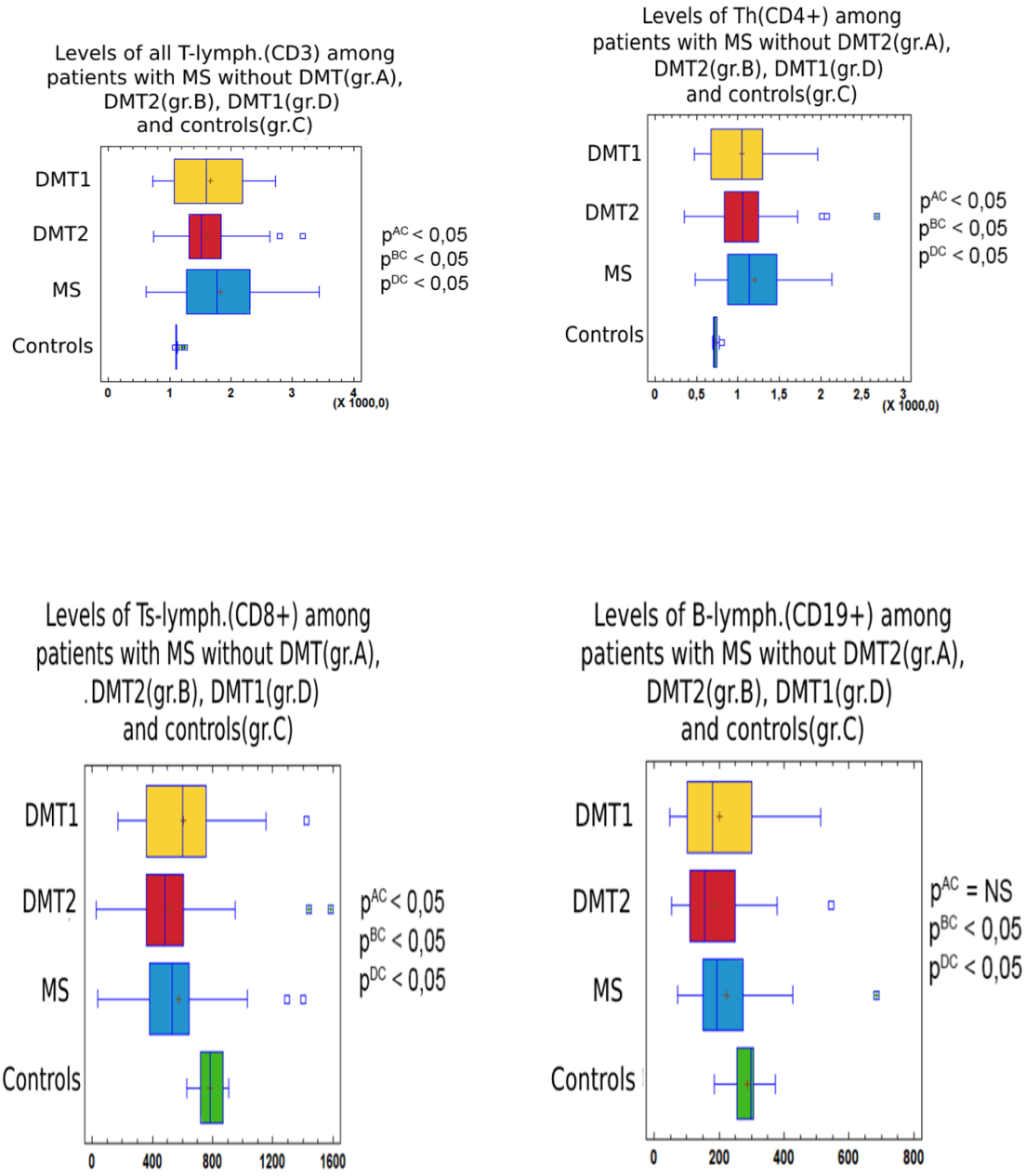

Fig. 21 Comparative analysis (mean value and median) in the levels of lymphocyte subpopulations in MS patients without DMT2 (group A), with DMT2 (group B), DMT1 (group D) and controls (group C).

The calculated ratio of Th/Ts in patients with MS (2.33±0.37), TDT2 (2.36±0.37), TDT1 (2.02±0.44) was higher compared to the reference value (from 1.0 to 1.5) and compared to that of the control group of healthy individuals (1.02±0.06).
When analyzing the results obtained for the absolute number of lymphocyte subpopulations by gender, no significant differences were registered that would indicate an influence of gender on the studied parameters

VI.II.2. Relationships between total leukocyte, monocyte, granulocyte count and lymphocyte subpopulations, BMI and waist circumference.

In order to investigate a possible relationship between visceral adiposity and changes in leukocyte and lymphocyte cell types, a correlation analysis was performed between BMI, waist circumference, leukocyte types and lymphocyte subpopulations in patients with MS without DM T2 (group A), with MS and DMT2 (group B), with DMT1 (group D) and healthy controls (table 19 and table 20).

Table 19 Correlation analysis between BMI, waist circumference and leukocyte types in patients with MS without DMT2 (group A), with MS and DMT2 (group B), with DMT1 (group D) and healthy controls (group C)

	All leukocytes	Lymphocytes	Monocytes	Granulocytes
BMI gr.A (kg/m²)	r=-0,05 p=0,81	r=-0,19 P=0,36	r=-0,14 P=0,51	r=0,05 P=0,82
Waist gr.A (cm)	r=-0,01 p=0,95	r=-0,27 p=0,18	r=-0,17 p=0,44	r=0,10 p=0,64
BMI gr.B (kg/m2)	r=0,15 p=0,21	r=-0,1 p=0,44	r=0,19 p=0,15	r=0,18 p=0,17
Waist gr.B (cm)	r=0,16 p=0,19	r=-0,04 p=0,77	r=0,19 p=0,15	r=0,16 p=0,23
BMI gr.D (kg/m2)	r=0,31 p=0,19	r=0,24 p=0,3	r=0,11 p=0,35	r=0,08 p=0,73
Waist gr.D (cm)	r=0,28 p=0,25	r=0,05 p=0,83	r=-0,03 p=0,91	r=-0,01 p=0,68
BMI gr.C (kg/m2)	r=0,16 p=0,48	r=-0,16 p=0,48	r=-0,27 p=0,24	r=-0,34 p=0,13
Waist gr.C (cm)	r=0,04 p=0,85	r=-0,17 p=0,46	r=-0,22 p=0,33	r=-0,1 p=0,67

Table 20 Correlation analysis between BMI, waist circumference and lymphocyte subpopulations in patients with MS without DMT2 (group A), with MS and DMT2 (group B), with DMT1 (group D) and healthy controls (group C)

	All T limph. (CD3)	**Th (CD4+)**	**Ts (CD8+)**	**B limph. (CD19+)**	**NK cells (CD3-/CD16 +56+)**
BMI gr. A (кg/м^2)	r=-0,19; p=0,35	r=-0,21; p=0,56	r=-0,12 p=0,56	r=-0,19 p=0,36	r=0,2 p=0,58
Waist gr. A (cm.)	r=-0,26 p=0,21	r=-0,21 p=0,31	r=-0,18 p=0,4	**r=-0,37 p=0,05**	r=0,33 p=0,36

BMI gr.B (кg/м^2)	r=-0,06 p=0,65	r=0,05 p=0,69	r=-0,15 p=0,24	r=-0,010 p=0,46	r=-0,17 p=0,4
waist gr.B (cm.)	r=-0,1 p=0,44	r=-0,05 p=0,72	r=-0,08 p=0,52	**r=-0,6** **p=0,05**	r=0,07 p=0,7
BMI gr.D (кg/м^2)	r=0,12 p=0,62	r=0,08 p=0,74	r=0,13 p=0,61	r=0,1 p=0,67	r=**0,78** p=**0,004**
Waist gr.D (cm.)	r=-0,07 p=0,79	r=0,01 p=0,95	r=-0,11 p=0,64	r=0,28 p=0,24	r=**0,44** p=**0,01**
BMI gr. C (кg/м^2)	r=-0,13 0,58	r=0,31 0,17	r=0,42 0,05	r=-0,09 0,70	r=0,1 0,66
Waist gr.C (cm.)	r=0,07 p=0,75	r=0,13 p=0,56	**r=0,56** **p=0,008**	r=0,25 p=0,27	r=-0,09 p=0,71

A strong positive linear correlation between waist circumference and the absolute number of Ts (CD8+) lymphocytes (r=0.56; p=0.008) was found in the healthy subjects used as a control group (group C).

A statistically significant negative correlation was found between waist circumference and the absolute number of B-lymphocytes (CD19+) both for those examined with MS without DMT2 (group A) (r=-0.37; p= 0.05) and for those with MS and DMT2 (group B) (r=-0.6; p= 0.05).

A positive, strong linear correlation was found between the absolute number of NK cells (CD3-/CD16+CD56+) and BMI (r=0.78; p=0.004) in participants with DMT1 (group D). A strong positive correlation also showed the absolute number of NK cells (CD3-/CD16+CD56+) with waist circumference (r=0.44; p=0.01) in the same group of subjects.

VI.II.3 Relationships between total and differential leukocyte counts, lymphocyte subpopulations, and changes in glycemia, insulin secretion, and sensitivity.

In **MS patients without DMT2 (group A)**, no statistically significant correlations were found between the total leukocyte count, the levels of monocytes, granulocytes and lymphocyte subtypes in the peripheral blood with the levels of fasting blood sugar, fasting endogenous insulin, HOMA-IR and HOMA B.

The results of the correlation analysis in the group of **MS and DMT2 (group B)** also did not show statistically significant correlation dependences between the levels of fasting blood glucose, IRI, HOMA IR, HOMA B with the total and differential leukocyte count. A weak positive linear relationship was found between the total leukocyte count and HbA1c levels among them (r=0.02; p=0.04). Also, a strong, positive statistically significant correlation was reported between HbA1c and the absolute number of NK cells in μl peripheral blood (r=0.46; p=0.04).

The results of the analysis of the relationship between the changes of leukocyte and lymphocyte subpopulations in the peripheral blood with fasting glycemia and HbA1c levels among patients **with DMT1 (group D)** did not show statistically significant significance.

Similar to the studied patients with MS (group A and B), no statistically significant correlations were found between the total and differential leukocyte count with fasting blood glucose levels, IRI, HOMA-IR and HOMA B in the **controls (group C).**

In the control group (group C), there was a moderate positive, statistically significant correlation between the absolute number of Ts (CD8+) and fasting glycemia (r=0.41; p=0.05) and a significant negative correlation between the absolute number of Ts (CD8+) with fasting endogenous insulin (r=-0.47; p=0.03) and with the index of β-cell secretory activity reflected by HOMA B (r=-0.57; p=0.006). A statistically significant strong positive correlation was also found between fasting glycemia and the number of NK cells in μl peripheral blood.

IV.II.4 Relationships between the total and differential leukocyte count, lymphocyte subpopulations and plasma levels of total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides.

In MS patients **without DMT2 (group A)**, no significant correlations were found between the investigated lipid parameters, total and differential leukocyte count.

The absolute number of Ts (CD8+) lymphocytes showed a significant linear positive correlation with the levels of total cholesterol (r=0.4; p=0.05) and serum triglycerides (r=0.36; p=0.05) and a negative correlation with the levels of LDL-cholesterol (r=-0.47; p=0.03) in the subjects with MS without ZDT2 (group A).

The results in the group of **MS and DMT2 (group B)** showed statistically significant, weak inverse correlations between the levels of HDL-cholesterol and total leukocyte (r=-0.33; p=0.004), lymphocyte (r=-0.3; p =0.02) and monocyte (r=-0.3; p=0.02) count in peripheral

blood. Again a weak, positive but statistically significant correlation was found between serum triglyceride levels with total leukocytes (r=0.36; p=0.003) (Fig. 22), with lymphocytes (r=0.3; p=0.02), monocytes (r=0.27; p=0.004) and granulocytes (r=0.28; p=0.03).

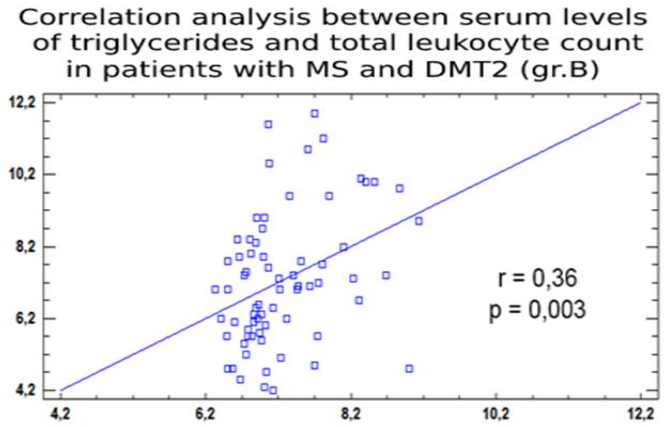

Fig. 22 Correlation analysis between serum levels of triglycerides and total leukocyte count in patients with MS and DMT2 (gr.B)

Correlation analysis between lipid levels and lymphocyte populations in DMT2 (group B) revealed a statistically significant linear correlation between serum triglyceride levels and the absolute number of total T-lymph. (CD3+) (r=0.33; p=0.001) and Th (CD4+) lymphocytes (r=0.34; p=0.009) in peripheral blood.

In the **patients with DMT1**, statistically significant weak negative correlations were found between the levels of HDL cholesterol and total leukocyte (r=-0.33; p=0.004), lymphocyte (r=-0.3; p=0.02) and monocyte (r=-0.3; p=0.02) count.

Serum triglyceride levels also showed statistically significant positive correlations with total leukocyte (r=0.36; p=0.003), monocyte (r=0.3; p=0.02), lymphocyte (r=0.3; p =0.02) and granulocyte (r=0.28; p=0.03) count in these patients.
The results of the correlation analysis between the lipid profile and the absolute number of lymphocyte subtypes in μL of peripheral blood demonstrated a moderate, negative statistically significant correlation between HDL-cholesterol levels and B-lymphocyte counts (r=-0.44; p=0.05) as well as a strong significant one between serum triglyceride levels and NK cell count (r=0.60; p=0.04).

In the healthy subjects of the **control group (group C),** the levels of LDL-cholesterol showed a significant correlation with the absolute number of lymphocytes from the automatic hematoanalyzer (r=0.47; p=0.04) and Th from the flow cytometric study (r=-0, 51; p=0.02).

A summary table (Table 21) presents the established statistically significant (strong and moderate) correlations between the clinical and laboratory parameters characterizing MS, leukocyte types and lymphocyte classes in the patients studied by us.

Table 21 Statistically significant correlation dependences were established in the studied groups

	BMI	waist	Fasting glucose	BP	Total Chol.	HDL-chol.	LDL-chol.	Triglycerides.	IRI	HOMA IR	HOMA B
MS without DMT2		**B lmph./-**			**Ts/+**		**Ts/-**	**Ts/+**			
MS and DMT2		**B limph./-**				**Leuc./-** **Limph./-** **Mono/-**		**Leuc./+** **Limph./+** **Mono/+** **Granulo./+** **Th/+**			
DMT1	**NK cells/+**	**NK cells/+**				**Leuc./-** **Limph./-** **Mono/-** **B-limph/-**		**Leuco./+** **Limph./+** **Mono./+** **Granulo./+** **NK/+**			
Controls		**Ts/+**	**Ts/+** **B/+** **NK/+**				**Limph./+** **Th/-**		**Ts/-**		**Ts/-**

Logistic regression analysis was performed to assess whether total and differential leukocyte counts and lymphocyte classes in peripheral blood could have predictive value regarding the risk of developing DMT2.

The ROC curve method was applied to establish threshold values. Significant threshold values were found for: Monocytes-0.35x109/l (54.2% sensitivity, 76.2% specificity; p<0.001), total T lymphocytes (CD3+)-1257/µl (80% sensitivity, 100% specificity; p<0.001), Th (CD4+)-811/µl (84% sensitivity, 100% specificity; p<0.001), Ts(CD8+)-621.5/µl (72% sensitivity, 100% specificity; p< 0.002), B-lymph. (CD19+)-219.5/µl (60% sensitivity, 90.5% specificity; p=0.002), NK cells (CD3-/CD16 +56+)-310/µl (70% sensitivity, 85.7% specificity; p=0.010).

The results in our study showed that in individuals with levels of Mo ≥ 0.35x109 /l in peripheral blood, the chance of developing DMT2 increases **about 4 times** (OR-3.782, 95% CI 1.045-13.680; p=0.043) compared to those examined with a lower absolute monocyte count (AUC 0.710, p=0.012); (fig. 23).

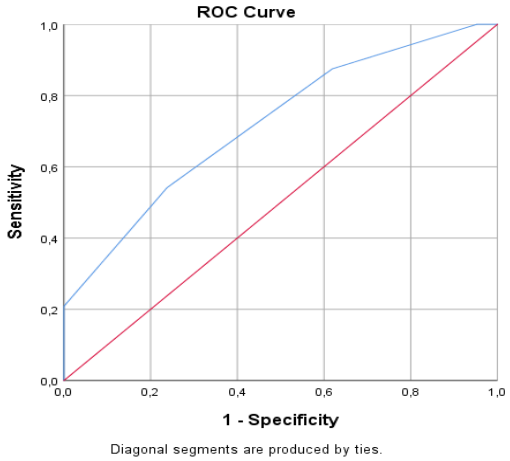

Fig. 23 ROC curve of monocytes for determining the threshold values for distinguishing between patients with and without MS and DMT2.

In patients with established levels of B (CD19+)-lymphocytes < 219.5 cells per µl of blood, the chances of developing DMT2 increased by more than 14 times compared to those with higher levels of B-lymphocytes (OR-14.25, 95% CI 2.703-75.116; p=0.02); (AUC 0.770, p=0.002) (Fig. 24).

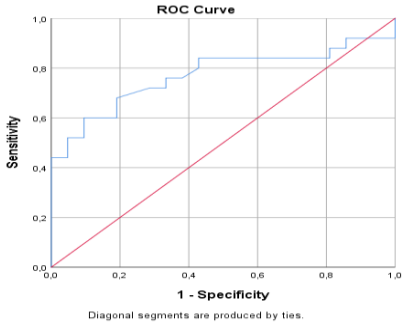

Fig. 24 ROC-curve of B (CD19+)-lymphocytes to determine the threshold values for distinguishing between patients with and without MS and DMT2.

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Also, the results of our study determined that individuals with levels of NK (CD3-/CD16 +56+) cells ≥ 310.5/µl in peripheral blood were also at a 14-fold higher risk of developing DMT2 (OR- 14, 95% CI 2.262-86.662; p=0.005); (AUC 0.790, p=0.01) (Fig. 25).

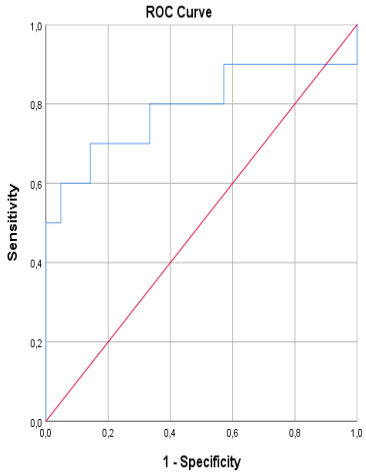

Fig. 25 ROC curve of NK (CD3-/CD16 +56+) cells for determining the threshold values for distinguishing patients with and without MS and DMT2.

Through the ROC analysis, the diagnostic accuracy and diagnostic value of the established significant variables were sought: monocytes, total T-lymphocytes, Th, Ts, B-lymphocytes and NK cells.

IV.II.5 Discussion

The tests performed regarding the total leukocyte count did not show deviations outside the accepted upper reference range among the subjects we studied. In healthy adults, the total leukocyte count depends on many different factors such as gender, age, diet, smoking, infections, trauma, stress.

Healthy individuals have lower values of total leukocyte count compared to patients with MS without DMT2 and with MS and DMT2, but without statistically significant differences in the individual groups. These data demonstrate the presence of a systemic chronic inflammatory process and activation of the immune system, which is relevant to the pathogenesis of MS and T2DM.

Both MS and T2DM are known to occur with subclinical inflammation. Patients with DMT1 have a statistically significantly higher leukocyte count in the peripheral blood, compared to healthy individuals, which testifies to the presence of a chronic ongoing immunological process in DMT1 as well.

All MS patients had elevated lymphocyte and monocyte cell counts compared to healthy subjects. The low-grade chronic inflammation and subsequent activation of the immune

system in MS originates in visceral adipose tissue. Subjects with MS without DMT2 and those with MS and DMT2 were identical in BMI and waist circumference, as markers of visceral adiposity, which is a likely reason for the identical results obtained in them.

In all study participants with MS, a statistically significantly higher absolute monocyte count was reported compared to healthy subjects. These data can be explained by the fact that, in obesity, monocytes are the blood elements that increase their absolute number and migrate to the intercellular space of adipose tissue, where they transform into macrophages.
Together with macrophages, they are the main antigen-presenting cells and stimulate Th (CD4+) lymphocytes to produce inflammatory cytokines. We established a significant threshold value of monocytes in peripheral blood above ≥ 0.35x109 /l with lower sensitivity, but good specificity, at which the chance of developing DMT2 with increases about 4 times.
A significantly higher number of monocytes was observed in DMT2, with no differences in the number of Th (CD4+) compared to MS without DMT2. One of the possible explanations for this fact is the subsequent involvement of monocyte cells in the atherosclerotic vascular process, which accelerates in the course of chronic hyperglycemia and DMT2.

Regardless of differences in age, BMI, waist circumference and disease duration, patients with DMT2 and DMT1 had identical blood pressure values, HbA1c levels, lipid profile, total and differential leukocyte counts. DMT1 is known to be an organ-specific T-cell-mediated autoimmune disease characterized by absolute insulin deficiency and immune-mediated β-cell apoptosis. The obtained similar results for DMT2 raises the question of the possible influence of hyperglycemia and dyslipidemia on changes in leukocyte count, lymphocyte populations and immune dysfunction in MS and DMT2.
In both health and disease processes, peripheral blood leukocyte levels are affected by age and a host of other endogenous and exogenous factors. It is this multifactorial influence and the small number of participants in the individual groups of our study that can be a probable reason for not establishing an age-related trend in the changes in the total and differentiated leukocyte count in all of the individuals examined by us. There was a trend for age-related differences only for individual lymphocyte subtypes only in MS patients without DMT2.

Gender differences in the levels of total leukocytes, lymphocytes, lymphocyte subpopulations, monocytes and granulocytes were found only among patients with DMT1. A higher number of analyzed blood cells was reported in women in this group. The average age of the patients with DMT1 included in the study (41.23±4.42 years) and the presumably physiologically higher estrogen levels of the women studied in this group could determine such a result. It is known that the X chromosome expresses several genes involved in immunological processes (for Toll-like receptors, cytokine receptors, genes affecting T and B cell lymphocyte activity) and estrogen levels affect the immune system through hormone receptors on immune cells.

In studies by Yang et al. involving 4579 adults over 60 years of age, higher leukocyte (5.74±1.35x109/L), lymphocyte (1.78±0.56x109/L) and monocyte (0.32±0, 12x109/l) number among adults with MS were established compared to those without MS. Gender differences were also found, despite the different racial affiliation of the individuals studied. The changes in the total and differential leukocyte count thus presented are evidence of the presence of inflammatory-immune processes in MS, but cannot describe the cause-and-effect relationship with visceral obesity and the metabolic components that characterize MS.

Non-specific T (CD3+) lymphocytes are the main cytological substrate for cell-mediated specific immunity. After antigenic stimulation, they differentiate into the main cellular modulators of the adaptive immune system: Th (CD4+) and Ts (CD8+) lymphocytes. In our study, the proportion and absolute number of non-specific total T lymphocytes (CD3+) and Th (CD4+) was significantly higher, and that of Ts (CD8+) was statistically significantly lower in persons with MS (MS without DMT2 and MS and DMT2) and DMT1 relative to the proportion and absolute number of the same lymphocyte populations in healthy individuals. There were no statistically significant differences in the absolute number of non-specific T(CD3+), Th (CD4+) and Ts (CD8+) lymphocytes between patients with MS without DMT2 and MS with DMT2. No statistical differences were found between the lymphocyte subpopulations from the flow cytometric analysis of patients with DMT1 and DMT2. These results demonstrate a systemic inflammatory process and activated cellular immunity in MS, T2DM and TDM1.
Despite the similar changes in the absolute number of peripheral blood T-lymphocytes, the pathogenetic mechanisms leading to the observed results are different in MS and DMT1.
In physiological conditions, the development of the immune response is accompanied by increased expression of insulin receptors and glucose transporters on the cell surface of activated T-lymphocytes to provide energy for their increased metabolic needs. Preexisting IR in obesity and MS may negatively influence the T cell-mediated inflammatory response.
The observed increase in the Th fraction (CD4+) is evidence of an active immune response without being able to describe whether it is a pro-inflammatory or anti-inflammatory compensatory phenomenon in the context of chronic low-grade inflammation in visceral obesity. It is known that Th (CD4+) is a heterogeneous group of Th1 and Th17 with a pro-inflammatory effect, Th2 and Treg lymphocytes with an anti-inflammatory effect.

Ts (CD8+) are thought to exert a regulatory role on macrophage activity and have a pro-inflammatory role in adipose tissue in visceral obesity. It is likely that the low absolute values of Ts(CD8+) lymphocyte populations in the peripheral blood in our MS patients is a reflection of their increased migration into visceral adipose tissue.
Our results are similar to those obtained by O'Rourke et al. when examining T-lymphocyte changes in peripheral blood of obese subjects. Probably the changes that have occurred are also irreversible. According to data in literature, the increased ratio of Th/Ts, which was also observed in our patients, is preserved in obese women and after weight reduction through bariatric surgery.

Our results also show increased Th activity and an activated autoimmune process, reflected by the increased ratio of Th/Ts in the subjects with MS without DMT2 and with DMT2.

B (CD19+) lymphocytes are the main cellular substrate of acquired humoral immunity. Along with T lymphocytes, they infiltrate visceral adipose tissue in obesity. They can modulate the immune system at multiple levels through antigen presentation, cytokine secretion, and antibody production. Multiple abnormalities in both circulating and tissue-fixed B lymphocytes have been described in visceral obesity.
In the studied individuals with MS (MS without DMT2 and MS and DMT2) and with DMT1, a statistically significantly lower number of B lymphocytes in the peripheral blood were found compared to healthy controls. In the MS group, this result is likely a consequence of an imbalance between subtype B lymphocyte populations with an increase in the number of fixed immunocompetent cells in adipose tissue involved in the immune response in chronic low-grade inflammation.
In our study, the decreased absolute number of B lymphocytes in peripheral blood was statistically significant also a factor for increasing the risk of developing CVD2 by more than 14 times. Statistically significant negative correlations with the parameters describing visceral adiposity, BMI and waist circumference were found in individuals with MS without DMT2 and those with MS and DMT2.

There are experimental studies that also describe the infiltration of adipose tissue with B lymphocytes, their involvement in the development of IR, the triggering of an inflammatory response in a high-fat diet and IR, as well as the secretion of pathological IgGs, which contribute to the spread of IR in peripheral tissues. This result obtained by us is in agreement with the study of De Furiaa et al. They also reported a low proportion and absolute number of CD19+ expressing (B lymph) lymphocytes and a negative correlation with BMI in patients with IR and visceral obesity.
Paradoxically, in our study, we did not find an increased number of B-lymphocyte cells in the peripheral blood of patients with DMT1. In the scientific literature, a predominance of infiltration with cytotoxic T lymphocytes (CD8+), less B lymphocytes and macrophages of the islet cells in insulitis has been described immunohistochemically. The production of DMT1-specific antibodies also depends on B lymphocyte activity and has a certain clinical-diagnostic characteristic related to the age of onset of the disease and changes over time.
The persons with ZDT1 included by us have a long history of the disease, which probably changes the characteristics of the ongoing immunological process, increases the influence of local metabolic and cytokine factors. In contrast to the studies of a Russian group in a similar population with DMT2 and DMT1, in our study, circulating immunoglobulin fractions and the presence of specific antibodies in the blood were not examined, which limits the interpretation of low B-lymphocytes in DMT1.
Our results show an increased number of NK cells as an element of innate immunity in peripheral blood in MS patients (without DMT2 and with DMT2) compared to healthy controls. These data confirm the information known in the literature about their involvement in the development of IR and DMT2 through the production of multiple cytokines.

An answer to the question whether the described changes in leukocyte and lymphocyte types are a cause or a consequence of the development of MS and DMT2 was sought by analyzing the relationship between these changes and the elements of MS. In 2013, researchers from Ukraine also studied the influence of obesity and BMI in MS and DMT2 on changes in lymphocyte composition. Our results are very similar to theirs.
The negative correlations found in the present study between B-lymphocyte count and waist circumference only among MS patients (without DMT2 and with DMT2) are a reflection of the inflammation occurring in adipose tissue and the inflammatory cytokines released by adipocytes. The patients included in our study in both groups were identical in terms of BMI and waist circumference. Kanneganti et al. reveal the mechanisms by which obesity disrupts normal tissue architecture, antigen presentation and leukocyte differentiation, affects the total leukocyte count and lymphocyte subtypes in peripheral blood.

Among clinically healthy individuals used as a control group, a positive correlation was observed between BMI and waist circumference with the total number of Ts in peripheral blood. Probably, with an increase in BMI and waist circumference, in conditions of low-grade inflammation, migration from peripheral blood to visceral adipose tissue and activation of various types of immunocompetent cells takes place.
The analysis of possible interdependence of the changes in the total and differential leukocyte count and lymphocyte populations with the changes in the lipid profile in the examined persons showed multiple and most pronounced interrelationships.
The presence of increased levels of lipids and non-esterified fatty acids in the plasma is characteristic of DMT2. An excess of lipid-containing nutrients can affect the function of immunocompetent cells. The positive correlations obtained of us between total cholesterol and serum triglyceride levels with total Ts lymphocytes count among MS patients without DMT2, as well as a negative correlation with HDL cholesterol levels, serve as proof of the above statement.
In the group of persons with MS and DMT2, a positive correlation was observed between serum triglycerides and total leukocyte count, lymphocytes and monocytes in peripheral blood, and a negative correlation with HDL levels. The lack of established correlations between leukocyte levels and differential lymphocyte profile with glycemia, endogenous insulin levels, and indices of IR and insulin secretory activity in all with MS likely suggests a role for lipid disorders as driving changes in immunocompetent cell types in evolution and pathogenetic mechanisms, responsible for the development of IR in conditions of low-grade chronic inflammation and visceral obesity.
On the other hand, the obtained significant threshold values of total lymphocytes, Th and Ts, distinguishing individuals with DMT2 and healthy ones with very good or good diagnostic accuracy and the impossibility to calculate the risk for developing DMT2 raises the question of conducting follow-up studies in a larger volume of population. The results of these studies would describe in more detail the changes that occur in the immune system as a pathogenetic element in the development of DMT2.

V. MAIN CONCLUSIONS:

V.1 Metabolic syndrome is a condition with low-grade chronic inflammation, in which there is increased secretion of plasma cytokines (Il-1, Il-6, TNF-α) and increased levels of hsCRP.
V.2 Interleukins, as inflammatory cytokines, are involved in the chronic inflammatory process tipycal for MS, independently of glycemic levels.
V.3 There are partial gender and age differences in the plasma concentrations of the studied cytokines and markers of low-grade chronic inflammation among patients with visceral obesity.
V.4 There are positive correlations between blood levels of inflammatory cytokines in MS, insulin levels and IR.
V. 5 Plasma cytokines IL-1 and IL-6 have a diagnostic value in studying the risk of developing DMT2.
V.6 Immune dysfunction is present in MS patients as evidenced by an increased absolute number of total T-lymph. (CD3+), Th (CD4+) and NK cells, decreased Ts (CD8+) and B (CD19+) lymphocytes in peripheral blood, increased Th/Ts ratio.
V.6. No differences were found in the type of changes of lymphocyte populations in patients with DMT2 and DMT1.
V.7. In patients with MS and DMT2, the main factors in the formation of immune dysfunction and changes in the general leukocyte and lymphocyte profile are BMI and waist circumference.
V.8. In patients with MS, reduced levels of HDL cholesterol and hypertriglyceridemia, rather than hyperglycemia, are more important for the development of immune dysfunction and changes in peripheral lymphocyte populations.

VI. CONTRIBUTIONS OF THE DISSERTATION:

VI.1. CONTRIBUTIONS OF AN ORIGINAL CHARACTER

VI.1.1 For the first time in Bulgaria, a study of lymphocyte populations by flow cytometric analysis was carried out in patients with metabolic syndrome and normoglycemia, as well as in patients with metabolic syndrome and type 2 diabetes mellitus, to prove the existence of a relationship between metabolic disorders and immune dysfunction.
VI.1.2 For the first time in Bulgaria, an attempt is made to determine a threshold value for the levels of plasma cytokines and lymphocyte populations as predictors of the risk of developing Type 2 Diabetes Mellitus.

VI.2 AFFIRMATIVE CONTRIBUTIONS

VI.2.1 The presence of low-grade chronic inflammation among patients with metabolic syndrome was confirmed regardless of the presence or absence of type 2 diabetes mellitus.
VI.2.2 Elevated levels of serum cytokines and hsCRP were confirmed as markers of low-grade chronic inflammation in patients with metabolic syndrome.
VI.2.3 The complex role of BMI, waist circumference, insulin resistance, elevated IL-6 levels and age as risk factors for the development of type 2 diabetes mellitus was confirmed.
VI.2.4 The presence of immune dysfunction reflected by a change in immunocompetent lymphocytes in metabolic syndrome was confirmed.

VI.3 CONTRIBUTIONS OF AN APPLIED NATURE

VI.3.1 A software product-calculator has been created for individual evaluation of the probability of the presence of a disease - DMT2 according to certain values of diastolic BP, levels of hsCRP and IL-6.

VII. PUBLICATIONS AND SCIENTIFIC PRESENTATIONS IN CONNECTION WITH THE DISSERTATION:

VII.1. Publications in Bulgarian journals:

1. Г. Раянова, **С. Ганева**. Метаболитен синдром. Мединфо. 2013; XIII (9): 64-66.
2. Раянова, **С. Ганева**, К. Тодорова, Цв. Луканов, Св. Гечева. Нарушения във въглехидратната обмяна при пациенти с метаболитен контрол. Ендокринология. 2014; XIX(3):139-143.
3. **С. Ганева**, К. Тодорова, Луканов Цв. Имунна дисфункция и захарен диабет тип 2. Ендокринология. 2015; XX(1): 15- 17.
4. **Ganeva Silviya**, Todorova Katya, Lukanov Tsvetan, Rayanova Ginka, Blajeva Svetla e "Levels of lymphocytes subpopulations in peripheral blood among patients with diabetes" Acta Medica Bulgarica. 2021; XLVIII (1): 75-80.
5. **Silviya S. Ganeva**, Ginka H. Rayanova, Katya N. Todorova, Tzvetan H. Lukanov and Svetla O. Blazheva. The Role of triglyceride to HDL cholesterol ratio in sera as a clinical surrogate marker for cardiovascular risk and insulin resistance in patients with metabolic syndrome. J Biomed Clin Res. 2021; 12:162-168.

VII.2. Publications in international journals:

1.**Ganeva S.**, Todorova K., Lukanov Tsv., Rayanova G., Blajeva Sv., Tsvyatkovska Tsv. B-Lymphocytes level in Peripheral Blood among Patients with Metabolic Syndrome and Diabetes Mellitus Type 2 . SMU Medical Journal. 2017; 4(1), 13-25.

2. Rayanova Ginka, **Ganeva Silviya**, Todorova Katya, Lukanov Tsvetan, Blajeva Svetla. Levels of Adipokines – Adiponectin and Resistin in Patients with Metabolic Syndrome and Newly Diagnosed Diabetes Mellitus Type 2. SMU Medical Journal. 2017; 4(2): 140-152.

VII.3. Participation in congresses and scientific conferences in connection with the dissertation in Bulgaria:

1.С. Ганева, К. Тодорова, Г. Раянова, Цв. Луканов, Св. Гечева. Нива на серумни интерлевкини (Il-1, Il-6), TNF-α и hsCRP при пациенти с метаболитен синдром и захарен диабет тип 2. X Национален конгрес по ендокринология, Сборник резюмета, Пловдив, 11-14 април 2013,103.

2.**С. Ганева,** К. Тодорова, Г. Раянова, Цв. Луканов, Св. Гечева. Нива на серумни интерлевкини (Il-1, Il-6) и TNF-α при пациенти с метаболитен синдром и захарен диабет тип 2. VIII Национален конгрес на Българската диабетна асоциация с международно участие. Слънчев бряг, 23-26 септември 2014.

3.С. Ганева, К. Тодорова, Г. Раянова, Цв. Луканов, Св. Блажева, Н. Сирачка. Нива на В-лимфоцити в периферна кръв на пациенти с метаболитен синдром. Юбилеен Национален конгрес по ендокринология, 8-11 октомври, 2015, 81.

4. **Ganeva SS**, Rayanova GH, Todorova KN, Lukanov, TH, Blajeva SO. Lymphocyte subpopulations in peripheral blood in patients with type 2 diabetes mellitus. Journal of Biomedical § Clinical Research 2019;12(1):41.

5. Rayanova GH, **Ganeva SS**, Todorova KN, Velkova AS, Lukanov, TH, Blajeva SO. The role of adiponectin and adipocytokines – interleukin-1, interleukin-6 and tumor necrotic factor-α in in the pathogenesis of metabolic syndrome. Journal of Biomedical § Clinical Research 2019;12(1):35-36. Abstracts from Jubilee Scientific Conference "45 years Medical University-Pleven", 31 Oct-2 Nov, 2019.

VII.4. Participation in congresses and scientific conferences in connection with the dissertation abroad:

1. Ganeva S., Todorova K., Rayanova G., Velkova A., Lukanov T., Blajeva S. Levels of adipokines- adiponectin and leptin and adipocytokines- interleukin- 1, Interleukin- 6, Tumor necrosis factor- α and C- reactive protein in patients with metabolic syndrome. 13th Congress of the central Europian diabetes association/33th international Dunabe symposium, 2019.

2 Ganeva S., Todorova K., Lukanov T., Rayanova G., Blajeva S. Levels of lymphocytes subpopulations in peripheral blood among patients with diabetes mellitus type 2. 22nd Europian Concress of Endocrinology, 5-9 sept 2020.

VII.5. SCIENTIFIC PROJECTS IN CONNECTION WITH THE DISSERTATION:

1. Изследователски проект №8, 2012 година, МУ- Плевен: Изследване на маркери на възпалението при пациенти с метаболитен синдром и захарен диабет тип 2.
2. Изследователски проект № 9, 2014 година, МУ Плевен: Изследване на лимфоцитни субпопулации при пациенти с метаболитен синдром и/или захарен диабет тип 2.