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"PROF. DR. PARASKEV STOYANOV" – VARNA

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DEPARTMENT OF CLINICAL LABORATORY

PROSPERITAS VESTRA FINIS NOSTRA!

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LABORATORY CARDIOVASCULAR RISK ASSESSMENT IN INDIVIDUALS WITH LONG-STANDING TYPE I DIABETES MELLITUS – ADIPOKINES, OSTEOPROTEGERIN, ASYMMETRIC DIMETHYL-ARGININE

AUTHOR'S SUMMARY

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The dissertation paper contains a total of 175 pages, illustrated with 56 tables and 52 figures. The bibliography contains 307 literary sources, of which 14 in Cyrillic and 293 in Latin script.

The study and laboratory analysis have been carried out at the Clinical Laboratory, a medico-diagnostic laboratory (MDL) at University Multiprofil Hospital for Active Treatment (UMHAT) "Saint Marina"-Varna.

The PhD student works at the Clinical Laboratory MDL at UMHAT "Saint Marina"- Varna and holds the position of Assistant at the Department of Clinical Laboratory at the Medical University "Prof. Dr. Paraskev Stoyanov"-Varna.

The dissertation was discussed, accepted and scheduled for defense before a degree committee from the Department Council No 21/05.12.22, Department of Clinical Laboratory at the Medical University "Prof. Dr. Paraskev Stoyanov" – Varna, on 05.12.2022.

The defense of the dissertation thesis will take place on 24.02.2022.

The materials on the defense are available at the Scientific Department of MU – Varna and are published on the website of MU "Prof. Dr. Paraskev Stoyanov" – Varna.

TABLE OF CONTENTS

ABBREVIATIONS USED

DKD - diabetic kidney disease

ED - endothelial dysfunction

DM – diabetes mellitus

IHD - ischemic heart disease

BMI – body mass index

CAD - coronary artery disease

MS – metabolic syndrome

CVD – cardiovascular diseases

CVR – cardiovascular risk

CVE - cardiovascular event

CVC – cardiovascular complication

T1D – type 1 diabetes mellitus

T2D – type 2 diabetes mellitus

CKD - chronic kidney disease

ADMA - asymmetric dimethyl-arginine

ADNC - adiponectin

AlbU – albuminuria

CRP – C-reactive protein

ESC – European Society of Cardiology

HbA1C - glycated hemoglobin

Lep - leptin

NOS – nitric oxide synthetase

OPG - osteoprotegerin

ROS - reactive oxygen species

ST1RE - Steno Type 1 Risk Engine

I. INTRODUCTION

Diabetes mellitus (DM) is a chronic, socially significant disease with progressively growing incidence, both globally and in Bulgaria. With increased life expectancy, cardio-vascular diseases (CVD) are becoming the main cause of mortality with Type 1 DM (T1D). Cardiovascular risk (CVR) remains high even in patients with good metabolic control, which respectively means that therapy and diagnosis approaches in this area are insufcient. To-date, risk stratification and screening strategies for the assessment of CVR in T1D have been primarily extrapolated either from studies of patients with Type 2 DM (T2D), or from the total population. This approach is unsatisfactory, due to substantial differences in pathophysiological mechanisms for CVD development between T1D and T2D.

Laboratory evaluation of CVR through the introduction of innovative biomarkers would allow optimization of diagnostic and prognostic accuracy and therapeutic approaches. This will lead to a lower frequency of annual hospitalizations for CVCs and respectively lower financial costs. The capacity of the units of grass-root organization and their national / European convertibility will be increased. A major challenge in validating laboratory biomarkers is the development of a personalized approach, as well as active collaboration between laboratory specialists and clinicians. Biomarkers can play a critical role in determining the category of CVR in patients with T1D. Their application in the clinical practice will improve the qualification of specialists and achieve continuity in diabetes care. The scientific study will make it possible to establish whether the quantification of serum biomarkers – asymmetric dimethyl-arginine (ADMA), osteoprotegerin (OPG), adiponectin (ADNC) and leptin (Lep) is of high informative value for clinical practice in terms of stratification of CVR in order to achieve a better quality of life and survival rate of patients with T1D. The present study also analyzed the interactions between hematomorphologic parameters and proposed biomarkers in the context of high CVR in individuals with long-standing T1D.

The facts referred to above render the thesis as topical and useful for clinical practice. The expected results are important for the development of science and practical behaviour for the prevention of CVD in persons with long-standing T1D with certain fundamental aspects. The topicality and significance of the problem, as well as the limited number of developments and published materials in the specialized literature, have triggered our research interest and have determined our choosing of the dissertation topic.

II. GOAL AND OBJECTIVES

GOAL

To analyze the prognostic values of: ADMA, OPG, ADNC and Lep against specific CVR assessment tools - ST1RE and ESC-2019 and against haematological parameters in persons with long-standing T1D.

OBJECTIVES

1. To assess a prognostic value of ADMA against T1D-specific CVR assessment tools.
2. To assess a prognostic value of OPG against T1D-specific CVR assessment tools.
3. To assess a prognostic value of ADNC against T1D-specific CVR assessment tools.
4. To assess a prognostic value of Lep against T1D-specific CVR assessment tools.
5. To analyze relationships between serum levels of ADMA, OPG and haematological parameters in T1D.
6. To analyze relationships between serum levels of ADNC, Lep and haematological parameters in T1D.

III. MATERIALS AND METHODS

1. Materials

The dissertation paper was prepared as part of a scientific project, entitled: *Cardiovascular and metabolic risk associated with visceral fat tissue in patients with Type 1 diabetes mellitus*, supported by the Bulgarian National Science Fund at the Ministry of Education and Science (contract DN 13/3 of 14.12.2017).

The study was conducted at the UMHAT "Saint Marina" - Varna during the period 2018 – 2020 with the participation of the: Department of Pediatrics, First Department of Internal Diseases, Department of Diagnostic Imaging, Second Department of Internal Diseases, Department of Clinical Laboratory, Department of Social Medicine and Organization of Health and Department of Hygiene and Epidemiology. The research protocol was approved by the Research Ethics Committee at MU-Varna – Decision No 72, Meeting 01.03.2018. All participants received and completed an informed consent statement.

1.1. Research object: 59 healthy volunteers and 124 patients with T1D.

1.2. Research subject: analysis of prognostic value of ADMA, OPG, ADNC and Lep against T1D-specific tools for assessment of CVR and dependencies of biomarkers with hematological indicators.

1.3. Place of scientific research: laboratory tests were carried out at MDL Clinical Laboratory at the Sveta Marina UMBAL - Varna.

1.4. Nature of the observation: comparison type case - control.

1.5. Study design: A total of 183 participants were involved in the study, with no targeted selection, and participation entirely at their request. The study took place in two stages within a week. At the first visit of the patients at the Sveta Marina UMBAL - Varna anthropometric, clinical, laboratory and imaging tests were conducted. During the second visit, an interview was conducted by a trained researcher in order to clarify ambiguities and supplement missing information.

1.5.1. Inclusion criteria: patients with more than 15 years of T1D duration; healthy volunteers of the same gender and age and with a similar BMI; willingness to participate, certified by written informed consent after the appropriate permission from the Research Ethics Committee.

1.5.2. Exclusion criteria: participation in clinical trials; significant mental injury or other type of interdiction for an independent decision to participate; significant disability and/or immobilisation; over 3% change in body

weight in the last 3 months; experienced stroke or other vascular incident; acute illness/condition during the study (excluding diabetic keto-acidosis and hopoglycaemia); pregnancy; in participants with diabetes – experienced severe hypoglycemia or diabetic keto-acidosis in the last three months and/or the presence of severe documented microvascular diabetic complications.

1.6. Biological material: for the studied indicators in the present study blood was collected under standardized conditions, i.e. in the morning before meals (after a 12-hour fasting period); first morning urine samples were also collected.

1.6.1. Full blood obtained in vacutainer with anticoagulant K_2EDTA from which full blood count and HbA1C were tested.

1.6.2. Serum obtained in a vacutainer with gel separator, centrifuged for 15 minutes at 2500 G (~8 ml). From serum, CRP, ADMA, OPG, ADNC and Lep were examined.

1.6.3. First morning urine – in the amount of 20 ml. The biological material was centrifuged for 15 minutes at 2500 G. Albumin (AlbU) concentration was studied from the supernatant.

1.6.4. The samples were examined up to two hours after receiving the biological material. The serumits for ADMA, OPG, ADNC and Lep were pipetted into additive-free vacutainers with subsequent storage of the material at -70°C until the analysis was carried out at one instant.

2. Methods

2.1. Questionnaire method.

2.2. Anthropometric indicators. The body mass index (BMI) is calculated by the formula = weight in kg/height in meters^2.

2.3. Laboratory methods:

2.3.1. Blood count indices were determined on a 5-diff Sysmex XN 1000 hematology analyzer by the method of fluorescence flow cytometry using semiconductor laser and hydrodynamic focusing.

2.3.2. Routine biochemical indicators and relevant analytical methods for quantification are presented in table 1.

Table 1. Analytical methods for the determination of CRP, HbA1C and AlbU.

Parameter	Laboratory method	Analyst	LOD	Linearity	Reference values
CRP (mg/l)	Immuno-turbidimetric analysis	ADVIA chemistry 1800	0,04 mg/l	0,12-164mg/l	0 – 5mg/l
HbA1C (%)	Immuno-inhibition analysis*	Advia chemistry 1800	2.0%	2.0 – 16%	< 6%
AlbU (mg/l)	Immuno-turbidi-metric assay	Olimpus AU600	0,25 mg/l	1,55-500mg/l	< 20 mg/24h

**HbA* 1 C *was determined by a method standardized according to DCCT/NGSP and the results are presented in percentage calculated from the ratio HbA1C/Total Hemoglobin (THb). THb was measured by a colorimetrie method.*

2.3.3. ADMA, OPG, ADNC and Lep were measured by ELISA-method (enzyme-linked immunosorbent assays) with ready-made test sets. Serum OPG levels were recorded in pmol/l, of ADMA- in μmol/l, of ADNC – in μg/ml, of Lep – in ng/ml (Table 2).

Table 2. Analytical characteristics of ELISA-methods for the quantification of ADMA, OPG, ADNC and Lep.

Parameter	Manufacturer	LOD	Cross reactivity	Linearity	Reproducibility CV%	Reference values
ADMA (μmol/l)	ADMA Fast ELISA, DLD Dianostika GMBH, Germany	0,03 μmol/l	SDMA: 0,05% NMMA: 1,93% Homoarginine: <0.01% Arginine: 0.03%	0,23 – 1,53 μmol/l	In a series: 0.58 - 1.04 μmol/l; 4.9-5.4% Between series: 0.57-1.34μmol/l; 4,3-9,6%	0,40 – 0,75 μmol/l
OPG (pmol/l)	Human Osteo-protegerin ELISA,BioVendor, Czech Republic	0,03 pmol/l	sRANKL: no TRAIL: no CD40; TNF RI; TNF RII:<0,06%	1,5 - 60 pmol/l	In a series: 4.82 - 15.28 μmol/l; 2.5-4.9% Between series: 4.83-14.33μmol/L; 1,7-9,0%	4,1 ± 2,3 pmol/l
ADNC (μg/ml)	Human Adiponectin ELISA, BioVendor, Czech Republic	0,026 μg/ml	Lep: no LEPR: no Resistin: no	0,1 – 10 μg/ml	In a series: 11.71 - 12.28 μg/ml; 3.9-5.9% Between series: 8.23-19.86μg/ml; 6,3-7,0%	Men:BMI (kg/m 2)<25: 10.9±4μg/ml;25-30: 8.8±4 μg/ml;>30-23±2.8 μg/ml. Women:BMI (kg/m^2)<25: 13.6±5.4μg/ml;25-30: 13.9±8.6 μg/ml;>30-11.4±3.8 μg/ml.
Lep (ng/ml)	Leptin-ELISA Kit, DIAsource, Belgium	0,04 ng/ml	IL-1α; IL-1β; IL-4,IL-6,IL-8,IL-10;IL-15,TNF-α; TNF-β;IFN-γ; IGF-1; insulin; glucagon- no.	0,5 – 60 ng/ml	In a series: 1.5 – 43.4 ng/ml; 3.5-13.3% Between series: 5.9-18.9 ng/ml; 10,2-12,7%	Women:BMI (kg/m^2):14-18:0,5-0,7ng/ml;18-24: 0,5-7,9 n g/ml;25-29:4,1-14,5;30-56:5,5-40,4 ng/ml. Men:BMI (kg/m 2):18-24:0,5-3,2 n g/ml; 25-29:0,5-14,6;30-56:2,5-42,1 ng/ml.

2.4. CVR evaluation calculators.

2.4.1. STENO Type 1 Risk Engine (ST1RE): and a 10-year risk of non-fatal and fatal CVD (IHD, stroke, peripheral vascular disease) is involved. The calculator includes gender, age, duration of diabetes, previous CVD, systolic blood pressure, albouminuria, HbA1C, eGFR, LDL cholesterol; smoking; physical activity. The following risk categories were observed: low (< 10%); moderate (10-20%) and high CVR (≥ 20%). Calculator link attached: http://www.sdcc.dk/T1riskengine

2.4.2. ESC Guideline from 2019 - table for systematic assessment of 10-year CVR based on the following RFs: age, gender, smoking, systolic blood pressure and total cholesterol. Source: doi:10.1093/eurheartj/ehz455

The following CVR categories were adopted: moderate risk (young patients with T1D below 35 years, with a DM duration of more than 10 years, no other risk factors. Calculated SCORE ≥ 1 % and < 5% for a 10 year risk of fatal CVD); high risk (Patients with DM without damage to the target organs, with a duration of diabetes over 10 years or another RF. Calculated SCORE ≥ 5% and <10% for a 10-year risk of fatal CVD); very high CVR (DM with damage to target organs or the presence of three major risk factors or early onset of T1D with a long duration (over 20 years). Calculated SCORE ≥ 10% for a 10-year risk of fatal CVD.

2.4.3. RiskFactor3. A model was construed – a combination of RFs established in clinical practice for the development of CVD in the presence of DM: HbA1C, CRP and AlbU. The following categories were adopted: to differentiate individuals with moderate from high CVR – CRP below and above 3 mg/l; for differentiation of patients with good from ones with poor glycaemic control - HbA1C below and above 7%; for differentiation of persons with normoalbuminuria - AlbU below 30 mg/l and microalbuminuria – AlbU between 30 and 300 mg/l, from macroalbumiuria – AlbU above 300 mg/l. Patients are grouped into the following groups: group 0 – without the presence of RFs from those included in the proposed model; group 1 – with the presence of 1 RF; group 2 – with the presence of 2 RFs; and group 3 – with the presence of 3 RFs.

2.5. Statistical methods (the statistical package SPSS 19):

2.5.1. Descriptive statistics. Determination of measures of central tendency, measures of dissipation of the distribution.

2.5.2. Correlation analysis. Pearson's correlation coefficient (r) to estimate a linear relationship and a Spearman’s Rho rank correlation coefficient.

2.5.3. Linear regression analysis. A single linear regression to analyse the relationship between one independent variable, X, and one dependent variable, Y. Multiple linear regression analysis in order to analyze both separately and jointly the influence of two or more independent variables on a dependent variable.

2.5.4. Factor analysis. Independent-samples T-test for determining a statistically significant difference between unknown arithmetic means for two samples. One-way analysis of variance (ANOVA) to test the hypothesis that the arithmetic mean values for two or more groups are equal.

2.5.5. Nonparametric statistical methods for nominal data. Chi-square 2 χ test of independence or Chi-square χ^2 test of association.

2.5.6. Binary logistic regression.

2.5.7. Receiver – Operating Characteristic (ROC) analysis and area under the curve (AUC) to assess sensitivity and specificity of loboratory indicators and to derive cut-off values.

2.5.8. A critical significance level $\alpha = 0.05$ was used, with the null hypothesis rejected at a value of p less than α ($p < 0.05$).

2.5.9. Tabulated and graphical method for presenting the data – simple and multidimensional tables; linear, pie charts and bar charts.

IV. RESULTS

1. Clinical - laboratory characteristics of the studied persons.

The present study included 59 healthy subjects and 124 patients with T1D. The men in the control group were 33 (55.9%) and with T1D were 66 (53.2%) (Table 3). The women in the control group were 26 (44.1%) and in the T1D group - 58 (46.8%). The marginal incidence for the gender variable was 99 males and 84 females. No significative difference in the frequency distribution of the gender variable was found in both groups, with $\chi 2 = 0.118$, $p = 0.731$.

Table 3. Distribution by number and gender of participants

Participant group		Gender		Total	χ^2	P value
		Men	Women			
Control group	No's	33	26	59	0.118	0.731
	%	55.9%	44.1%	100.0%		
Group with T1D	No's	66	58	124		
	%	53.2%	46.8%	100.0%		
Total	No's	99	84	183		
	%	54.1%	45.9%	100.0%		

Table 4 compares the average age of the study participants. In the control group, it was 45.14 ± 9.168 years, and in subjects with T1D - 42.68 ± 10.404 years. The difference of 2.458 years found is not significant, $t = 1.550$, $p = 0.123$.

Table 4. Distribution by age of study participants.

Participant group		N	mean	SD	t	P value	MD
Age (yr)	Control group	59	45.14	9.168	1.550	0.123	2.458
	Patients with T1D	124	42.68	10.404			

N – number, mean – mean, *SD – standard deviation, MD – mean difference*

There was no significant age difference between the genders in the two study groups, with men in the control group having a mean age of 46.42 ± 9.906 years and women - 43.50 ± 8.026 years. In patients with T1D, the average age of both genders is almost identical: for men – 42.89 ± 10.459 years, for women - 42.43 ± 10.426 years. There is no significant difference between the cases thus investigated - $p > 0.05$.

The arithmetic mean of T1D duration was 25.31 ± 8.224 years, and the median - 24 years. The gender distribution was as follows: for men it was 24.50 ± 7.455 years, and for women it was 26.24 ± 8.996 years. The difference found in the duration of diabetes was MD = 1.741 years, $t = 1.78$, $p = 0.241$. Significant intergroup difference is not found when comparing the following variables: BMI, CRP, ADMA, OPG and Lep. The average value of HbA1C% in T1D patients was

significantly higher than that of controls: 8.47 ± 1.62% vs. 5.4% ± 0.39%, F = 205.7, p < 0.0001. A significant difference in favour of T1D patients was found between AlbU mean values - 63.723 mg/L ± 147.378 mg/L vs 12.217 ± 19.878 mg/L, F = 7.004, p = 0.009 in controls. The difference, which also deserves attention, is at the ADNC level, as for T1D subjects it was 14.718 ± 10.254 µg/ml, and for controls – 10.569 ± 12.283 µg/ml. The difference reported was significant, F = 5.210, p = 0.024 (Table 5).

Table 5. Clinico - laboratory characterization of the contingent.

Parameter	**Group with T1D**			**Control group**			**F**	**P value**
	N	**mean**	**SD**	**N**	**mean**	**SD**		
BMI (kg/m2)	91	25,708	4,110	33	24,879	3,805	1,025	0,313
CRP (mg/l)	123	3,561	9,304	59	2,572	3,761	0,617	0,433
Glycated haemoglobin (HBA1C%)	124	8,472	1,619	59	5,403	0,385	205,696	**0,000**
Albuminuria (AlbU) (mg/L)	123	63,723	147,378	58	12,217	19,878	7,004	**0,009**
ADMA (µmol/l)	114	0,527	0,256	54	0,572	0,186	1,340	0,249
Osteoprotegerin (OPG) (pmol/l)	113	5,528	1,545	54	5,568	2,073	0,019	0,891
Adiponectin (ADNC) (µg/ml)	111	14,718	10,254	54	10,569	12,283	5,210	**0,024**
Leptin (Lep) (ng/ml)	109	5,463	5,754	51	4,544	3,472	1,108	0,294

Figure 1 demonstrates the percentage distribution of patients over CVR categories according to the ESC-2019 guideline: 30.6% of patients had high CVR and 69.4% had very high CVR (Fig.1). No significant difference between genders was found for ESC-2019- χ^2 = 0.480, p = 0.489.

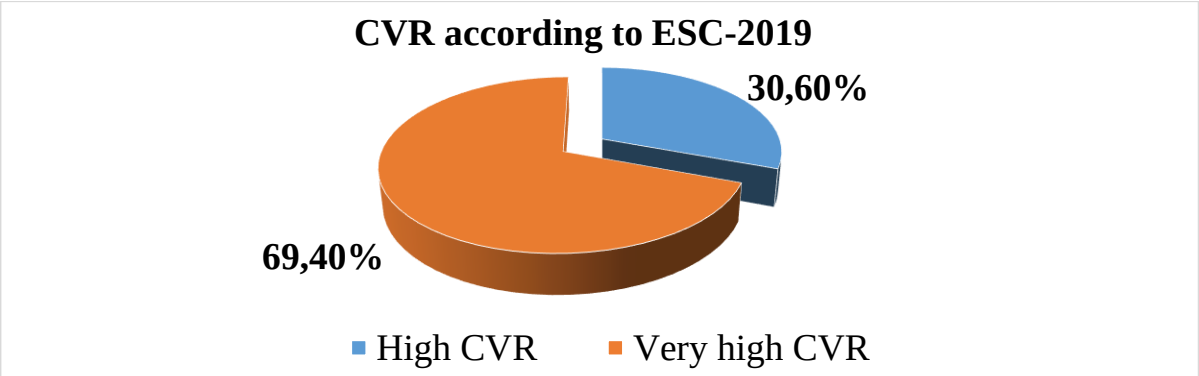

Figure 11. Percentage distribution of patients with T1D according to ESC-2019.

Figure 2 demonstrates the percentage distribution of patients relative to the calculator-defined ST1RE category of CVR: 38.7% of T1D subjects have low CVR; 28.2% moderate and 33.1% high CVR. It was a significant difference between genders - χ^2 = 5.943, p = 0.051: 39.6% of patients with low CVR were men and 60.4% were women. In the high CVR group, 60% were male and 40% female, and in the very high CVR group, 63.4% were male and 36.6% were female (Fig.2).

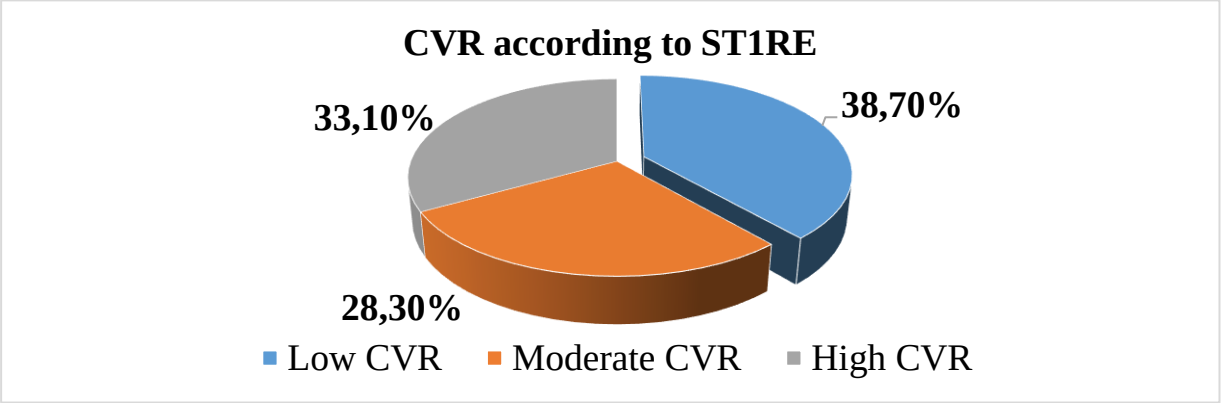

Figure 2. Percentage distribution of CVR in persons with T1D according to ST1RE.

2. Prognostic value of ADMA for estimation of CVR in patients with T1D.

2.1. ADMA – influence of gender, age and duration of diabetes.

Of the mean ADMA values, higher were reported in women in both study groups, but the differences found were not significant: 0.5401 ± 0.244 μmol/l for women with T1D vs 0.5158 ± 0.2687 μmol/l for men with T1D, MD = 0.0243, p = 0.614 and 0.6148 ± 0.1435 μmol/l for healthy women vs 0.5368 ± 0.2687 μmol/l for healthy men, MD = 0.0779 μmol/l, p = 0.126. In controls, a significant negative correlation between age and ADMA concentration was recorded, r = - 0.329, p = 0.015 (Fig.3). In the group of persons with long-standing T1D, this dependence is retained as a direction, but loses its significance: r = - 0.0016, p = 0.865 (Table 9).

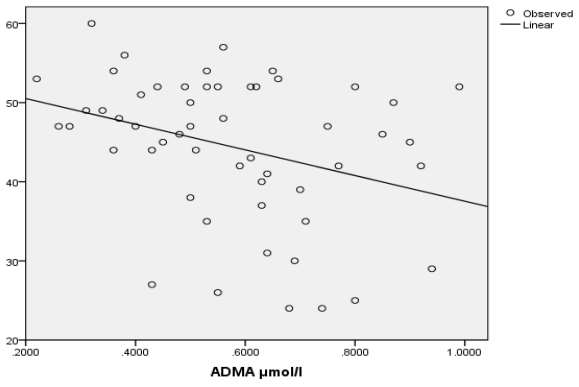

Figure 3. Correlation dependency between age and ADMA in controls.

When comparing the ADMA biomarker, the cases with a longer T1D duration had a lower level: 0.497 ± 0.239 μmol/l, and with a T1D duration below 24 years, the average value of the marker was higher - 0.561 ± 0.271 μmol/l, MD = 0.064 μmol/l, p = 0.184. No significant correlation was found between T1D duration and ADMA (Table 9).

2.2. ADMA – links with ST1RE and ESC-2019

After Tukey HDS posthoc test, a significant difference in mean ADMA levels was found between the groups with moderate CVR and high CVR according to ST1RE, but at 90% authenticity of results: MD = 0.1315 μmol/l, p = 0.078. No significant difference between the average levels of ADMA according to ESC-2019 was reported. (t = 0.985, p = 0.327). No significant differences between genders were found in mean ADMA levels: F = 1.90, p = 0.159 for males and F = 0.826, p = 0.443 for females at ST1RE and t = 1.019, p = 0.209 for males and t = 0.352, p = 0.685 for women in ESC-2019 (Fig.4).

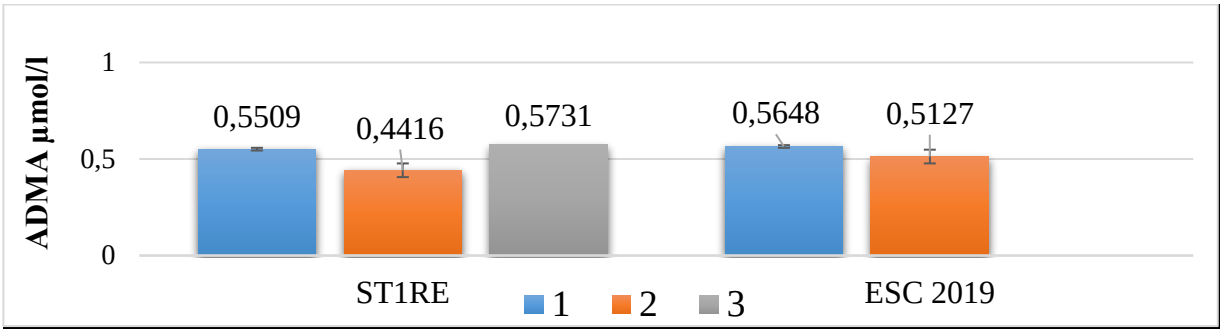

Figure 4. Mean ADMA values dependant on the CVR category. ST1RE: 1 – low risk; 2 – moderate risk; 3 – high risk. ESC 2019: 1 – high risk; 2 – very high risk

2.3. ADMA – links to AlbU, CRP, HbA1C and to RiskFactor3.

Figure 5 presents the average levels of the variable studied, relative to HbA1C below and above 7%; compared to CRP below and above 3 mg/l and to AlbU below 30 mg/l, between 30 and 300 mg/l and above 300 mg/l. A significant difference was found versus the AlbU value, F = 9.193, p = 0.000.

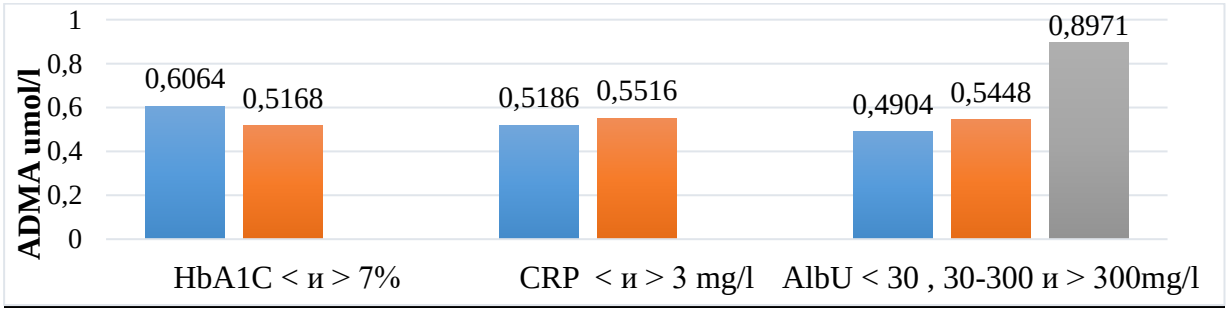

Figure 5. Mean levels of ADMA versus HbA1C, CRP and AlbU in T1D group.

A posthoc analysis of Tukey HDS demonstrated significant differences between those identified with AlbU below 30 mg/l and AlbU above 300 mg (MD = 0.407 μmol/l, p= 0.000) and between cases with AlbU from 30 to 300 mg/l compared to AlbU cases above 300 mg/l (MD = 0.352 μmol/l, p = 0.003). A predominant proportion of patients with poor glycaemic control was reported: 84.67% (105) versus 15.32% (19) with good glycaemic control. In the control group cases, no subjects with HbA1C above 7% were registered (Fig.6).

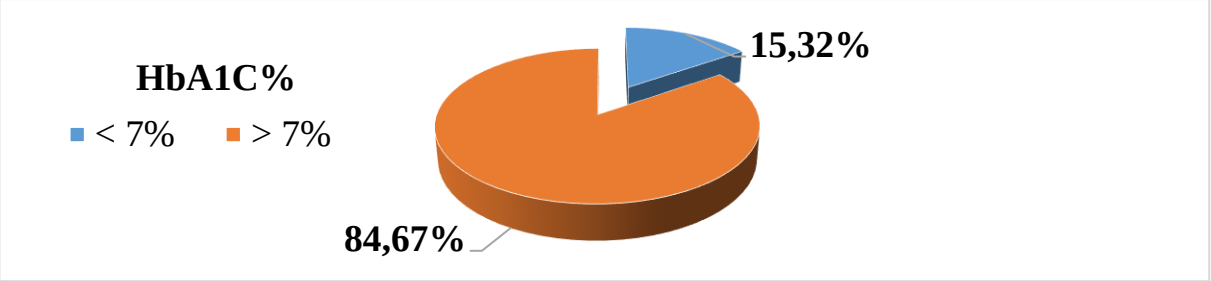

Figure 6. Distribution of patients with T1D according to HbA1C - < and ≥ 7%.

Normoalbumnuria was found in almost all controls – 91.4%, and in T1D subjects – 73.4%. Patients with microalbumnuria were 20.2% vs. 8.6% in the control group. Macroalbuminuria was recorded only in patients with T1D - 6.5%. In this comparison of relative shares, a significant difference was reported - χ^2 = 8.552, p = 0.014. In the group of controls, a sifgnificant relationship between HbA1C and CRP was established (r = 0.310, p = 0.014). In patients with long-standing T1D, ADMA was positively associated with AlbU: r = 0.371, p = 0.000. The reported dependence between ADMA and CRP is insignificant, and between ADMA and HbA1C is negative in direction and weak in force, but again does not reach significant significance: r = - 0.150, p = 0.111. A statistically significant combination of HbA1C and AlbU variables is recorded to predict the ADMA value in T1D subjects, F = 7.584, p = 0.000 (Fig.7).

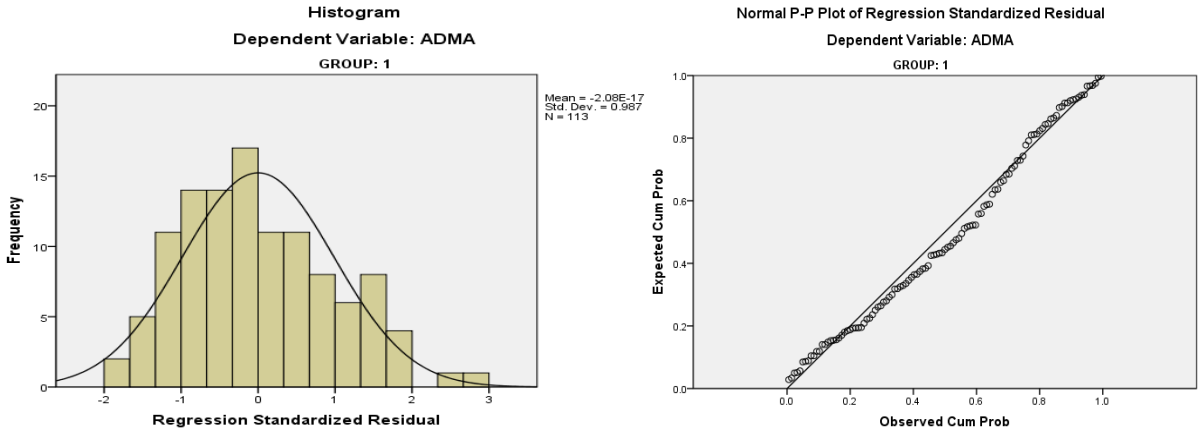

Figure 7. Charts of normal distribution: histogram and P – P Plot.

It was found that 15% of the changes in ADMA value can be explained by the presented regression model. The equation derived for the relationship between the variables is:

ADMA(µmol/l) = 0,739 + 0,387. AlbU(mg/l) – 0,186. HbA1C(%)

Patients with long-standing T1D were further distributed into subgroups according to RiskFactor3. In group 0, the relative share of patients with T1D was 10.48% in ADMA - 0.476 ± 0.18 µmol/l. The largest share was recorded in group 1 – 47.58% and mean ADMA - 0.509 ± 0.241 µmol/l. In group 2 - 34.68% of the total number of patients in ADMA - 0.535 ± 0.276 µmol/l, and in group 3 – 7.26% in ADMA - 0.67 ± 0.306 µmol/l. No significant difference in mean ADMA levels was reported, F = 1.168, p = 0.325 (Fig.8,9).

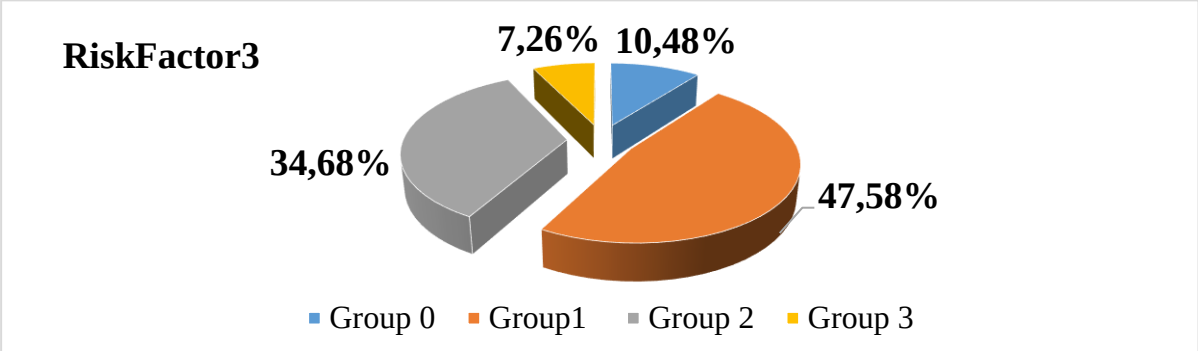

Figure 8. Distribution of patients with T1D according to Riskfactor3.

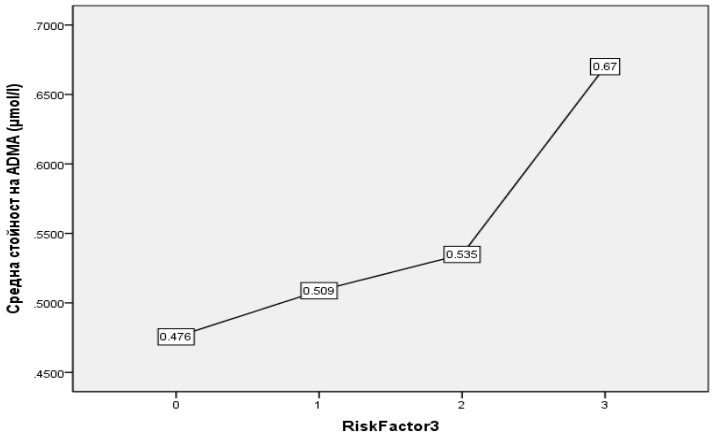

Figure 9. Mean ADMA values according to Riskfactor3.

A strong correlation was reported between ST1RE and ESC-2019 with r = 0.349, p = 0.000, followed by that between ST1RE and RiskFactor3 with r = 0.254, p = 0.004. The correlation between ESC-2019 and RiskFactor3 was significant but weaker in force with r = 0.224, p = 0.012. ADMA was associated stronger with Riskfactor3, but without statistical significance, r = 0.159, p = 0.092 (Table 6).

Table 6. Correlation dependencies in persons with T1D .

Parameter		RiskFakctor3	ESC-2019	ST1RE
ADMA (µmol/l)	Pearson Correlation P value Count	0,159 **0,092** 114	-0,093 0,327 114	0,031 0,741 114
RiskFakctor3	Pearson Correlation P value Count	/ / /	0,224* **0,012** 124	0,254** **0,004** 124
ESC-2019	Pearson Correlation P value Count	/ / /	/ / /	0,349** **0,000** 124
**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).				

2.4. ROC analyses to derive threshold ADMA values.

No good prognostic ADMA value was found relative to ST1RE: AUC-ROC = 0.564, p = 0.265, and in ESC-2019: AUC-ROC = 0.410, p = 0.131. At RiskFactor3, AUC-ROC = 0.543, p = 0.451. A higher AUC-ROC value in women compared to RiskFactor3 was found: AUC = 0.653, p = 0.067 (Fig.10).

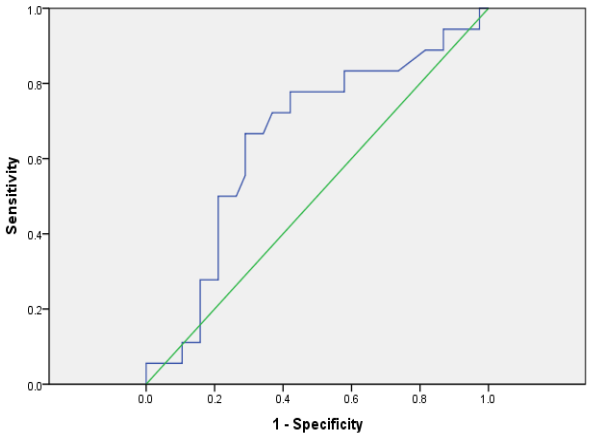

Figure 10. AUC-ROC curve for estimating a prognostic ADMA value.

A cut-off value 0.535 µmol/l at diagnostic sensitivity (Se) 72.2% and specificity (Sp) 63.2% was derived. LR+ = 1.96 and LR- = 0.51, DOR = 3.84, Youden index = 1.09 and diagnostic effectiveness (DE) = 66% were determined (Table 7).

Table 7. Diagnostic reliability of ADMA versus RiskFactor3.

Indicator	Criterion	cut-off	N	TP	TN	FP	FN	Se	Sp	DE
ADMA- women	RiskFactor3 ≥ 2 RFs	**0,535**	56	13	24	14	5	72,2%	63,2%	66%

TP - true positive, TN - true negative, FP - false positive, FN - false negative

3. Prognostic value of OPG for estimation of CVR in patients with T1D.

3.1. OPG - influence of gender, age and duration of diabetes.

In both study groups, a statistically significant difference in favor of women was found. In patients with T1D, MD was 0.3844 pmol/l, p = 0.055, and in controls - MD = 1.1077 pmol/l, p = 0.049 (Table 8).

Table 8. Mean values of OPG relative to gender.

Group		Gender	N	mean	SD	t	P value	MD
Group with T1D	**OPG** (pmol/l)	Men	58	5.341	1.199	-1.326	**0.055**	-0.384
		Women	55	5.726	1.832			
Control group	**OPG** (pmol/l)	Men	29	5.056	1.645	-2.013	**0.049**	-1.108
		Women	25	6.163	2.377			

N – number; mean – *mean value; SD – standard deviation.*

When comparing the average levels of OPG against the median of duration of T1D, higher values were reported for longer duration of T1D (5.723 ± 1.506 pmol/l) compared to a duration below 24 years - 5.317 ± 1.574 pmol/l, MD = 0.406 pmol/l, t = - 1,400, p = 0.164.

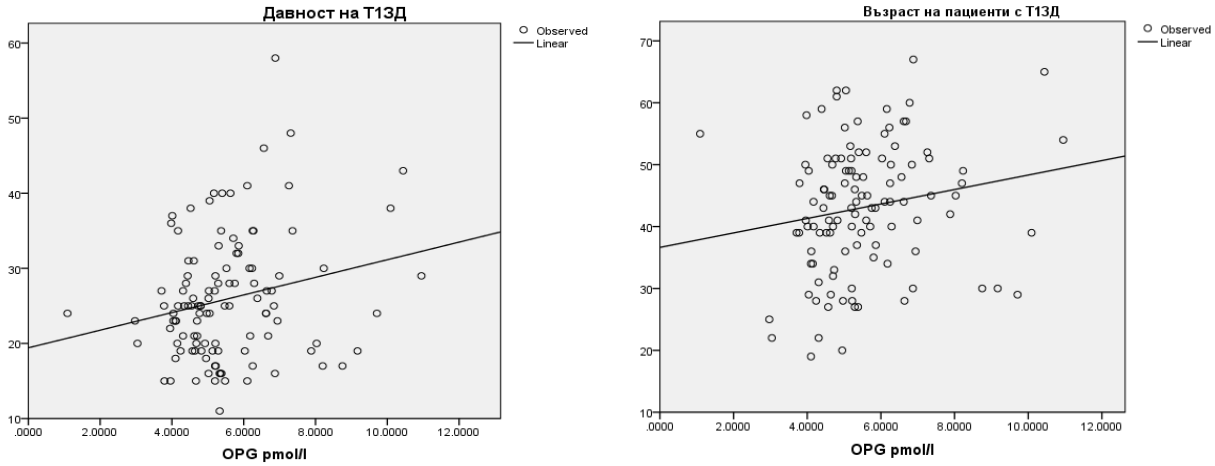

Figure 11. Dependencies between OPG and: age and duration of T1D.

In controls, between OPG and age, a negative and a weak correlation (r = - 0.284, p = 0.038) was reported, which in further gender examination was found to be significant only in men (r = - 0.565, p = 0.001). With T1D, the analyzed dependence was positive and less pronounced: r = 0.173, p = 0.067. The age of the patients was associated positively with the T1D duration, the correlation being regular: r = 0.435, p = 0.000 (Table 9). The reported coefficients of determination are: R^2 = 0.030, p = 0.06 versus age and R^2 = 0.048, p = 0.019 according to duration

of T1D (Fig. 11). There was also a significant positive correlation between OPG and ADMA in the two study groups. In controls, it was mean in force r = 0.362, p = 0.007. In individuals with long-standing T1D - weaker in force and significance: r = 0.175, p = 0.064 (Table 9).

Table 9. Correlations between OPG and: ADMA, age and T1D duration.

Group	Parameter		ADMA (µmol/l)	Age (yr)	T1D duration (yr)
Group with T1D	**OPG (pmol/l)**	Pearson Correlation P value Count	0,175 **0,064** 113	0,173 **0,067** 113	0,220 **0,019** 113
Control group	**OPG (pmol/l)**	Pearson Correlation P value Count	0,362 **0,007** 54	-0,284 **0,038** 54	/ / /
**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).					

3.2. OPG – links with ST1RE and ESC-2019.

OPG was positively associated with ST1RE, the correlation found being significant but weak in force – r = 0.183, p = 0.053. Analogic correlation was found between OPG and Riskfactor3: r = 0.194, p = 0.039. No significant relationship was reported between OPG and ESC-2019. No significant intergroup difference was reported in mean OPG levels relative to ST1RE (F = 2.322, p = 0.103) and to ESC-2019. t = - 1,003, p = 0,318 (Fig.12).

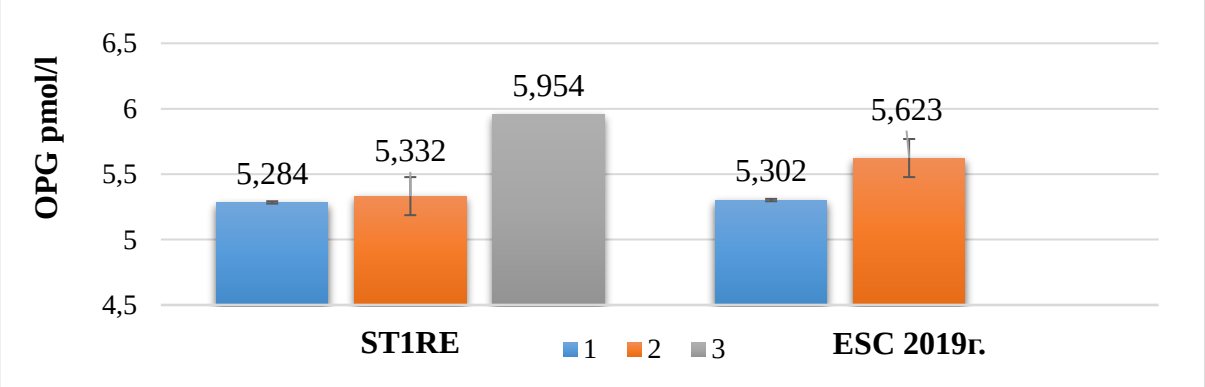

Figure 12. Average OPG levels (pmol/l) vs ST1RE and ESC-2019

The median and interquartile range of OPG in men with low CVR according to ST1RE criteria were 4.95 (4.31 – 5.86) pmol/l; in men with moderate CVR were 4.92 (4.04 – 5.37) pmol/l; in men at high risk were 5,775 (4,855 - 6,87) pmol/l; in women with low CVR were 5.73 (4.24 - 5.74) pmol/l; in moderate CVR were 5.60 (4.91 - 6.325) pmol/l; in high CVR were 6.25 (5.05 - 6.78) pmol/l (Fig.13).

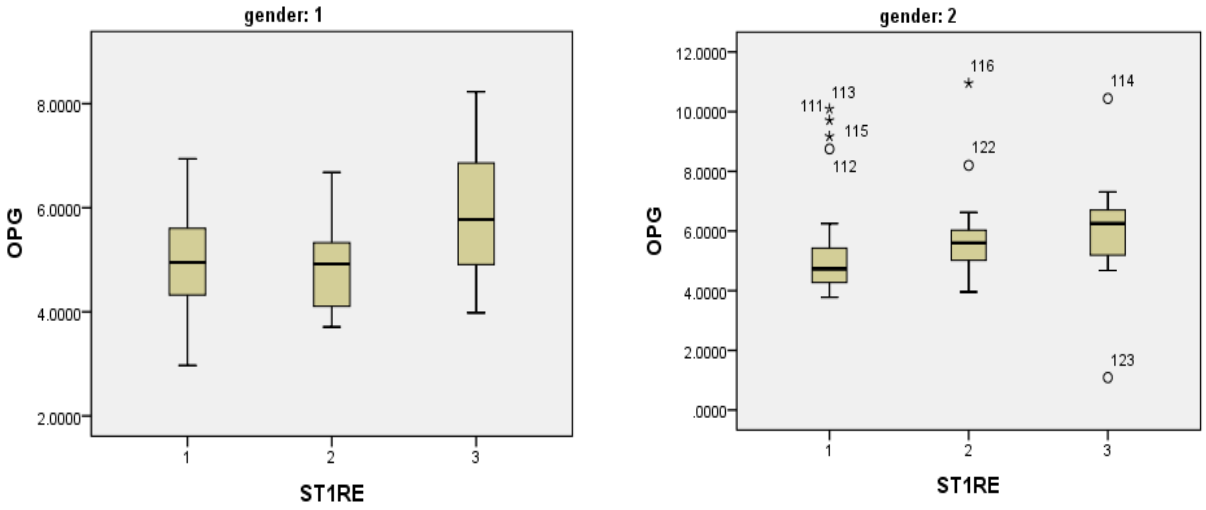

Figure 13. OPG in males and females according to ST1RE. *Gender 1 - men, 2 - women*

The medians and interquartile ranges of OPG in men according to ESC 2019 were: 5.12 (4.265 - 6.215) pmol/l for high and 5.20 (4.56 - 6.218) pmol/l for very high CVR. In women, they were the following: 4.82 (4.24 - 5.60) pmol/l for high and 5.425 (4.708 – 6.605) pmol/l for a very high CVR (Fig.14).

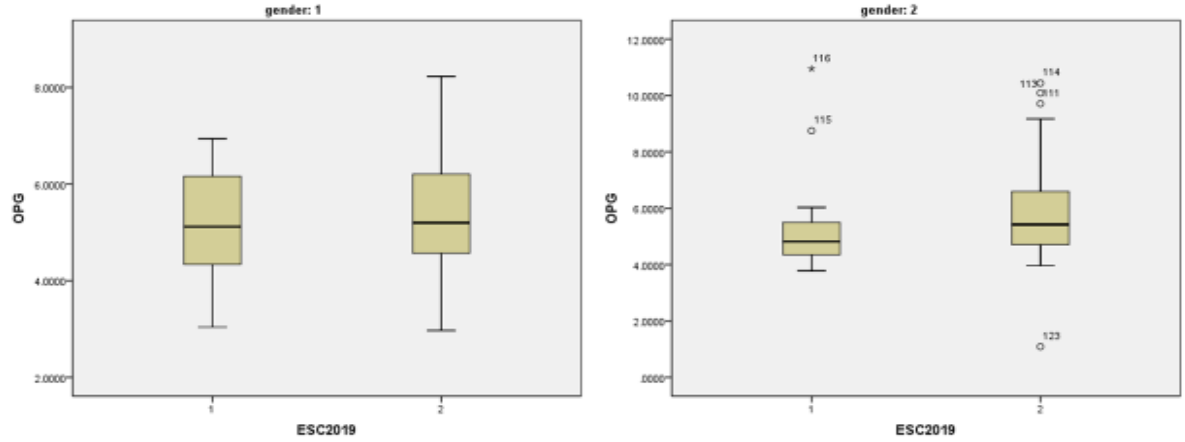

Figure 14. OPG in T1D compared to ESC 2019 *Gender 1 – men, 2 – women.*

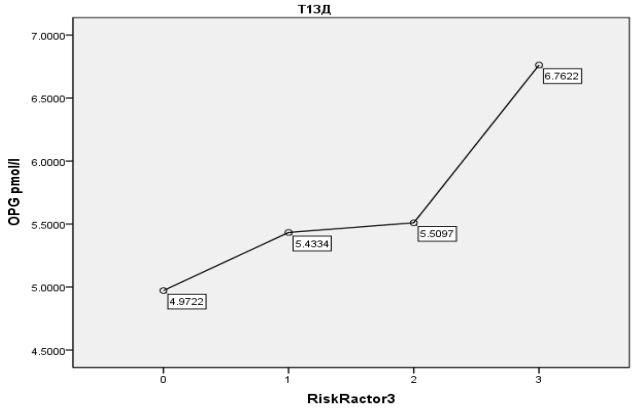

Figure 15. Mean OPG values (pmol/l) versus RiskFactor3.

In group 0, the mean OPG level was 4.972 ± 0.489 pmol/l; in group 1- 5,433 ± 1.51 pmol/l; in group 2 - 5.51 ± 1.69 pmol/l; in group 3 - 6.76 ± 1.35 pmol/l. F = 2.466, p = 0.066 (Fig. 15).

3.3. OPG - links with AlbU, CRP, HbA1C and with RiskFactor3.

A one-way analysis of variance against RiskFactor3 demonstrated a significant difference in males, but at 90% confidence interval (4.93 ± 0.391 pmol/l for group 0; 5.092 ± 1.05 pmol/l for group 1; 5.565 ± 1.392 pmol/l for group 2 and 6.823 ± 0.567 pmol/l for group 3, F = 2.550, p = 0.065). The reported mean levels for OPG in females were: 5.004 ± 0.600 pmol/l for group 0; 5.828 ± 1.855 pmol/l for group 1 ; 5.445 ± 2.024 pmol/l for group 2 and 6.732 ± 1.663 pmol/ l for group 3, F = 1.031, p = 0.387 (Fig.16).

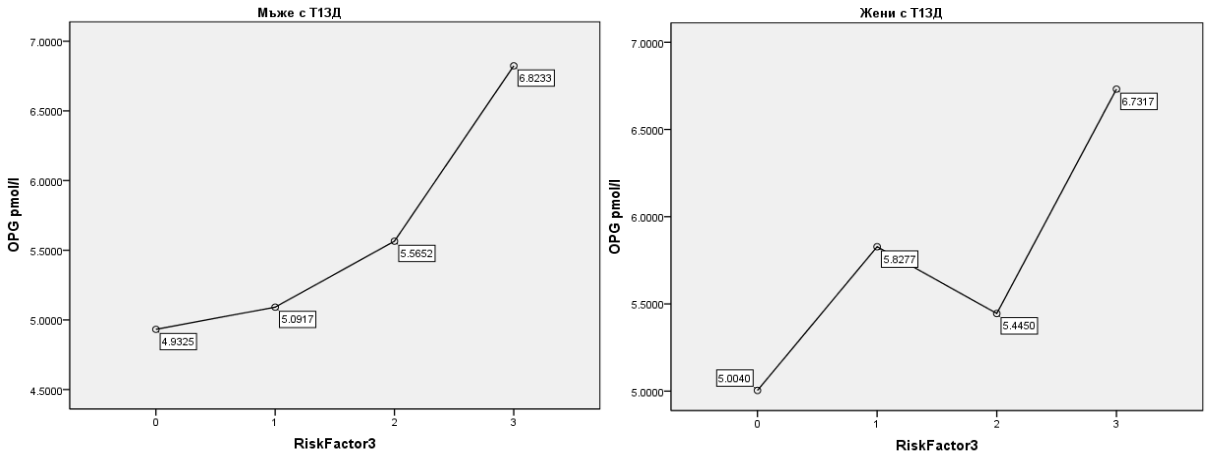

Figure 16. OPG (pmol/l) in men and women with T1D versus RiskFactor3.

Medians and interquartile ranges of OPG in males from the different RiskFactor3 groups were: 5.00 (4.53 – 5.267) pmol/l for group 0; 4.845 (4.275 - 6.165) pmol/l for group 1; 5.35 (4.76 - 6.47) pmol/l for group 2; 6.88 (6.23 -*) pmol/L for group 3. The women were: 5.30 (4.495 – 5.365) pmol/l for group 0; 5.275 (4.608 - 6.343) pmol/l for group 1; 4.86 (4.293 - 6.358) pmol/l for group 2; 6.44 (5.30 - 7.873) pmol/l for group 3.

In individuals with long-standing T1D, average OPG levels compared to HbA1C below and above 7% (5.446 ± 1.4691 pmol/l and 5.541 ± 1.563 pmol/l); compared to CRP below and above 3 mg/l (5.383 ± 1.557 pmol/l and 5.885 ± 1.499 pmol/l) and to the AlbU below 30 mg/l, between 30 and 300 mg/l and above 300 mg /l (5.407 ± 1.423 pmol/l; 5.491 ± 1.848 pmol/l and 6.937 ± 1,609 pmol/l). A significant difference was found in mean OPG levels between the groups so matched versus AlbU, F = 3.263, p = 0.042. There were significant differences in

OPG values between AlbU patients below 30 mg/l and AlbU above 300 mg/l (MD = 1.53 pmol/l, p = 0.032) and between AlbU patients from 30 to 300 mg/l and AlbU above 300 mg/l, MD = 1.45 pmol/l, p = 0.080 (Fig.17).

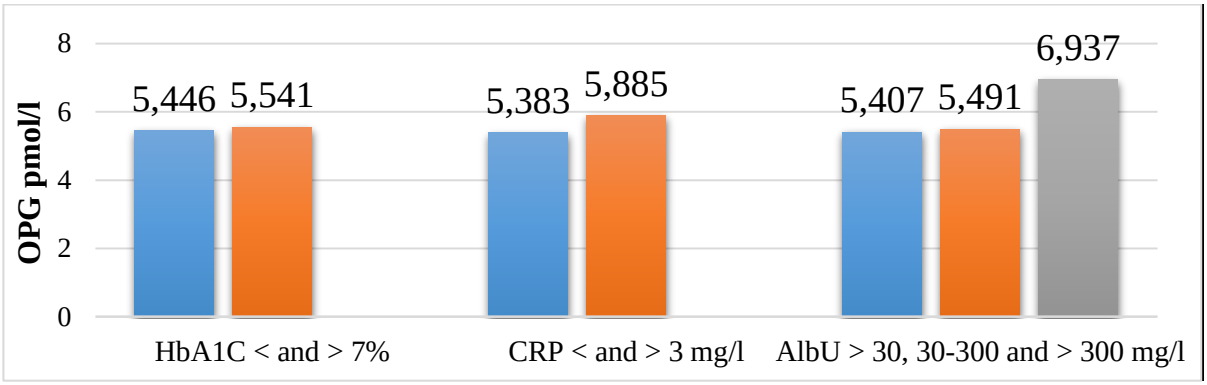

Figure 17. Average levels of OPG compared to: HbA1C, CRP and AlbU in individuals with long-standing T1D.

In the control group, the differences found to HbA1C, CRP and AlbU were insignificant. In the T1D group, a significant correlation was found between OPG and AlbU (r = 0.218, p = 0.021). In the controls, there were no notable correlations. In order to establish the linear combination between ALbU and OPG values in T1D subjects, simple linear regression analysis (F = 5.521, p = 0.021) was applied. The adjusted R^2 was 0.039, indicating that 4% of the changes in OPG concentration could be explained by the regression model presented:

OPG (pmol/l) = 5.381 + 0.218. AlbU (mg/l)

3.4. ROC analyses to derive threshold values of OPG with T1D.

In ST1RE, AUC- ROC was 0.687, p = 0.001, and in ESC-2019 - 0.589, p = 0.138. There was no good prognostic value of OPG versus RiskFactor3 reported, with AUC being 0.520, p = 0.734. For distinguishing patients with high CVR according to ST1RE a cut-off value was derived for OPG 5.315 pmol/l, with Se 64.1% and Sp 63.5%. Determined were: LR+ = 1.75 and LR- = 0.57, DOR = 3.07, Youden index = 1,006 and DE = 63,7% (Table 10, Fig.18).

Table 10. Diagnostic reliability for OPG versus ST1RE.

Indicator	Criterion	cut-off	N	TP	TN	FP	FN	Se	Sp	DE
OPG (pmol/l)	ST1RE - high CVR	**5,315**	113	25	47	27	14	64,1%	63,5%	63,7%

TP - true positive, TN - true negative, FP - false positive, FN - false negative

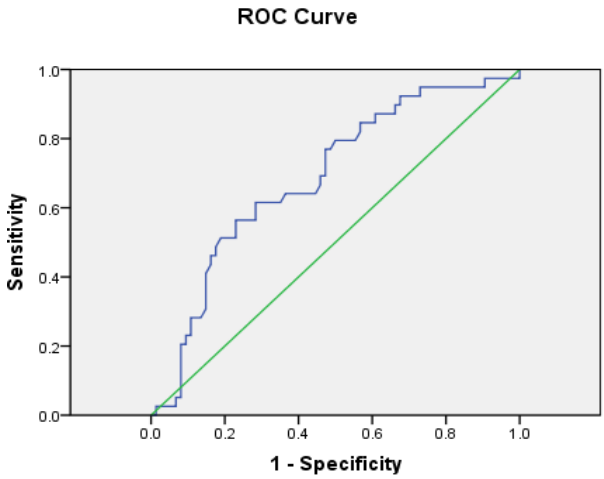

Figure 18. AUC-ROC: prognostic value of OPG with T1D versus ST1RE.

At ST1RE for males, AUC was 0.716, p = 0.005 and AUC = 0.683, p = 0.039 for females. At ESC 2019 – AUC = 0.537, p = 0.650 for males and AUC = 0.644, p = 0.102 for females. In RiskFactor3 – AUC = 0.565, p = 0.418 for males and AUC = 0.471, p = 0.727 for females. Cut-off value of OPG derived for women with a very high CVR according to the ESC-2019 – 5.025 pmol/l, at Se 70% and Sp 60%. The following were determined: LR+ = 1.75, LR - = 0.571, DOR = 3.064, Youden index = 1.1 and DE = 67.2 % (Fig. 19).

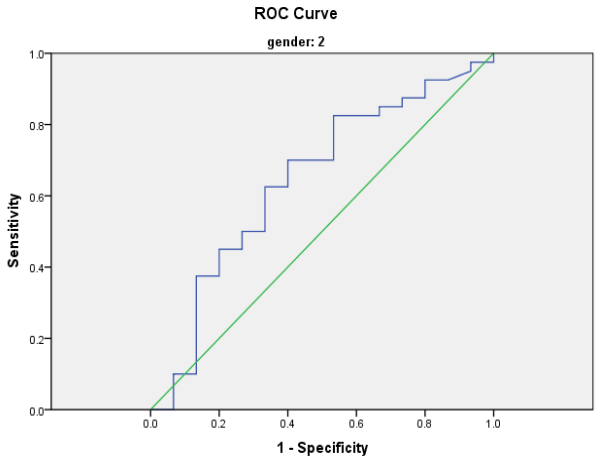

Figure 19. AUC-ROC curve, women: prognostic value of OPG vs ESC-2019.

OPG cut-off values for differentiation of persons with high CVR according to ST1RE: 5.075 pmol/l with Se 70.8% and Sp 55.9% for men and 5.355 pmol/l for women with Se 66.7% and Sp 60% were derived. The following were determined: LR+ = 1.605, LR - = 0.623, DOR = 2.576, Youden index = 1.149 and DE = 62% for men and LR+ = 1.668, LR - = 0.599, DOR = 2.784, Youden index = 1.067 and DE = 61.8% for women (Table 11, Fig. 20).

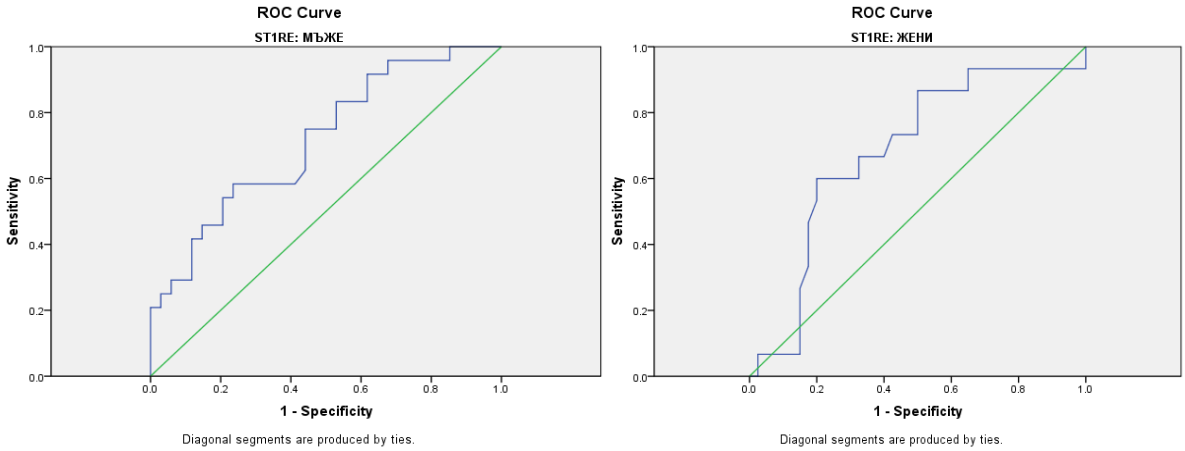

Figure 20. AUC-ROC curve to estimate a prognostic value of OPG in men and women with long-standing T1D versus ST1RE.

Table 11. Diagnostic reliability of OPG versus ST1RE and ESC 2019.

Indicator	Criterion	Cut-off	N	TP	TN	FP	FN	Se	Sp	DE
OPG(pmol/l) Men	St1RE - high CVR	**5.075**	58	17	19	15	7	70.8%	55.9%	62%
OPG(pmol/l) Women	St1RE - high CVR	**5.355**	55	10	24	16	5	66.7%	60%	61.8%
OPG (pmol/l) Women	ESC-2019 - very high CVR	**5.025**	55	28	9	6	12	70%	60%	67.2%

4. Prognostic value of ADNC for estimation of CVR in patients with T1D.

4.1. ADNC – influence of gender, age and duration of diabetes.

The mean ADNC value was significantly higher in women in the two study groups, in MD = 8.182 μg/ml, t = - 2.566, p = 0.013 for healthy individuals and MD = 7.157 μg/ml, t = -3.906, p = 0.000 for patients with T1D (Table 12).

Table 12. Mean ADNC values according to gender.

Participant group		Gender	N	mean	SD	t	P value	MD
Control group	**ADNC** (μg/ml)	Men	29	6.781	2.414	-2.566	**0.013**	-8.182
		Women	25	14.96	16.99			
Patients with T1D	**ADNC** (μg/ml)	Men	57	11.23	5.666	-3.906	**0.000**	-7.157
		Women	54	18.39	12.55			

Men with longer duartion of T1D had higher ADNC levels (12.327 ± 5.662 μg/ml) than those tested with duration below 24 years – 9.625 ± 5.394 μg/ml, MD = 2.702 μg/ml, t = 1.801, p = 0.077. In women, no significant difference was recorded (t = - 0.056, p = 0.960). A moderately positive correlation was reported in men between the T1D and ADNC durations, r = 0.273, p = 0.040. In females,

the study relationship was insignificant, r = 0.042, p = 0.761. No significant association was reported between age and ADNC. There is a significant relationship between BMI and ADNC in men with T1D, and it has the opposite direction and is large in force: r = - 0.466, p = 0.000. In women with T1D, the reported relationship was again negative, but was significant at 90% confidence of the results: r = -0.241, p = 0.080. In the group of male controls, a positive correlation was recorded between ADNC and ADMA (r = 0.430, p = 0.020). In the female controls, the dependence found was not significant: r = 0.127, p = 0.545. In the T1D group, ADNC did not significantly correlate with ADMA (r = 0.066, p = 0.625 for males and r = 0.147, p = 0.289 for females). No significant associations were found between serum ADMA and OPG levels in the two study groups (Table 13).

Table 13. Correlations between ADNC and: age, duration of T1D, ADMA and OPG.

Gender	Group			Age (years)	T1D duration (years)	BMI (kg/m^2)	ADMA (µmol/l)	OPG (pmol/l)
Men	**Group with T1D**	ADNC (µg/ml)	Pearson Correlation	0.032	0.273*	-0.466**	0.066	0.173
			P value	0.815	**0.040**	**0.000**	0.625	0.199
			Count	57	57	57	57	57
	Control-group	ADNC (µg/ml)	Pearson Correlation	-0.067	/	0/006	0.430*	0.095
			P value	0.730	/	0.977	**0.020**	0.625
			Count	29		29	29	29
Women	**Group with T1D**	ADNC (µg/ml)	Pearson Correlation	0,098	0.042	-0.241	0/147	-0.198
			P value	0.482	0.761	**0.080**	0.289	0.151
			Count	54	54	54	54	54
	Control-group	ADNC (µg/ml)	Pearson Correlation	-0.220	/	-0.193	0.127	-0.106
			P value	0.291	/	0.355	0.545	0.616
			Count	25		25	25	25

*. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed).

4.2. ADNC – links with ST1RE, ESC-2019and Riskfactor3.

The Spearman rho rank correlation coefficient between ADNC and RiskFactor3 e : *rho* = - 0.23, p = 0.015. Approx. 5% of the variance in ADNC concentrations in T1D subjects is associated with the variance in RiskFactor3. The sign of the correlation was negative and maintained for the reported relationships between ADNC and: ESC for 2019 and ST1RE, but was not statistically significant (Table 14).

Table 14. Correlations between ADNC and: ST1RE, ESC 2019 and RiskFactor3.

			ADNC (µg/ml)	**RiskFactor3**	**ESC-2019**	**ST1RE**
Spearman's rho	**ADNC** (µg/ml)	Correlation Coefficient	1.000	-0.231*	-0.074	-0.028
		P value	/	**0.015**	0.443	0.768
		Count	111	111	111	111

*. Correlation is significant at the 0.05 level (2-tailed). **. Correlation is significant at the 0.01 level (2-tailed).

There was no statistically significant difference in the arithmetic mean levels of ADNC versus ST1RE. The median and interquartile ranges of ADNC in men with low CVR were 9.19 (7.19 – 12.24) μg/ml; with moderate risk – 11.905 (6.528 – 16.488) μg/ml; with high risk - 10.57 (6.45 – 15.555) μg/ml; in females they were: with low risk - 15.39 (11.433 – 21.168) μg/ml; moderate risk -16.15 (11.895 – 26.54) μg/ml; and high risk - 15.39 (11.44 – 22.29) μg/ml. (Fig.21)

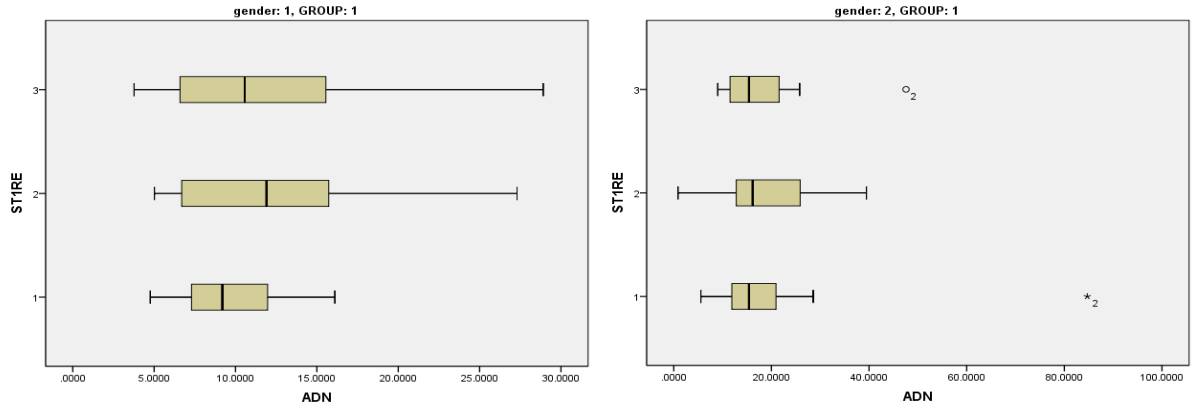

Figure 21. ADNC (μg/ml) in males and females according to ST1RE. *Gender 1–male,2-female;1–low;2–moderate,3–very high CVR.*

With ESC-2019 close to statistically significant difference was found in men: 10.913 ± 3.972 μg/ml with high and 11.386 ± 6.339 μg/ml with very high CVR, MD = - 0.4723 μg/ml, t = - 0.290, p = 0.065. In females, 20.006 ± 9.099 μg/ml with high and 17.773 μg/ml with very high CVR, MD = 2.233 μg/ml, t = 0.582, p = 0.563 (Fig.22).

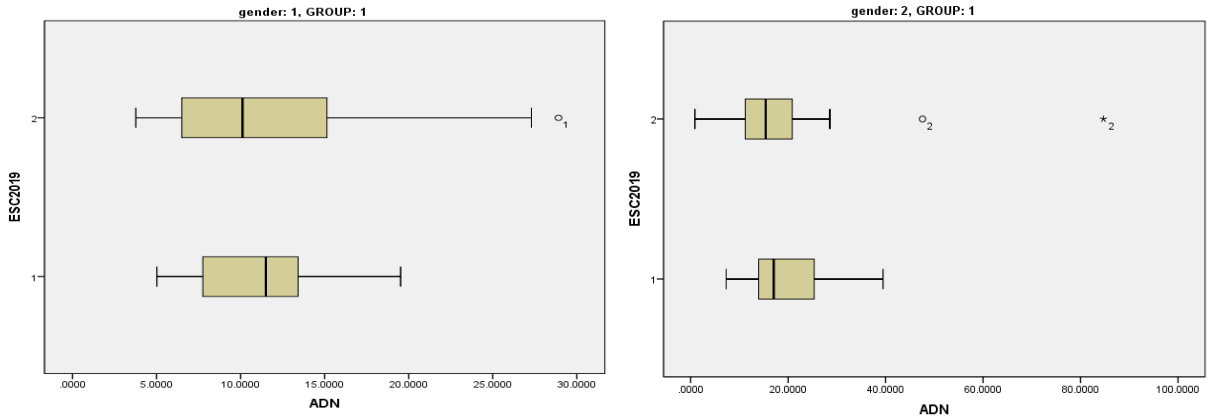

Figure 22. ADNC (μg/ml) according to ESC 2019 *(Gender 1-men,2-women)*

Medians and interquartile ranges were: 11.50 (7.665 – 13.44) μg/ml in men with high and 10.11(6.31 – 15.56) μg/ml in men at very high risk, and in women: 17.05 (13.94 – 25.46) μg/ml at high and 15.39 (10.98 – 20.85) μg/ml at very high CVR (Fig.22).

4.3. **ADNC – links** with **AlbU**, **CRP, HbA1C and with RiskFactor3.**

One-factor variance analysis versus RiskFactor3 showed a decreasing trend, but no significant difference: F = 0.485, p = 0.694 in men with T1D and F = 0.423, p = 0.738 in women with T1D (Fig.23).

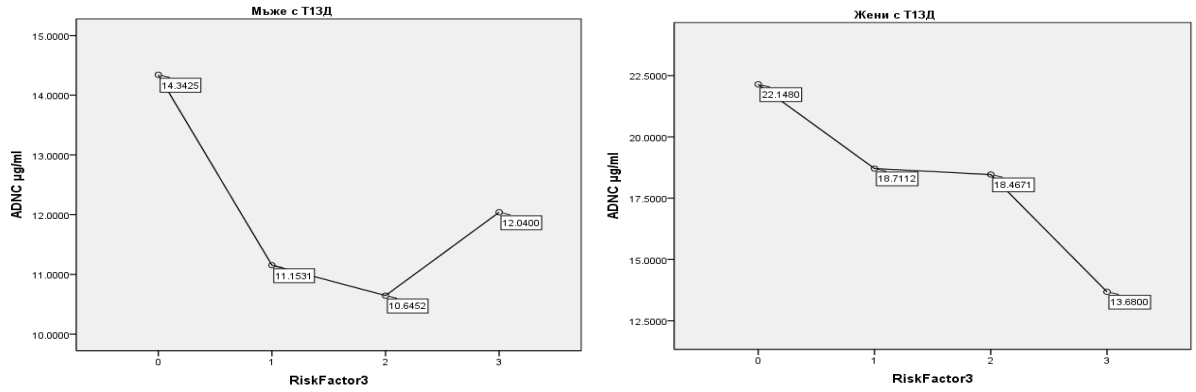

Figure 23. Mean ADNC values (µg/ml) according to RiskFactor3.

The medians and interquartile ranges of ADNC in men with T1D were: 15.405 (7.87 – 19.75) µg/ml for group 0; 10.57 (7.295 – 12.59) µg/ml for group 1; 8.04 (5.07 – 15.172) µg/ml for group 2; 10.98 (6.87 - *) µg/ml for group 3; In females, they were: 20.85 (17.935 – 27.01) µg/ml for group 0; 17.05 (13.705 – 24.795) µg/ml for group 1; 12.13 (8.735 – 19.155) µg/ml for group 2; 11.60 (9.46 – 15.74) µg/ml for group 3.

No significant differences were found in men with T1D and HbA1C below and above 7 %. The mean ADNC concentration was 13.963 ± 5.226 µg/ml and 10.916 ± 5.677 µg/ml (MD = 3.048 µg /ml, t = - 1.252, p = 0.216), respectively, and relative to CRP below and above 3 mg/l – 11.806 ± 5.775 µg/ml and 9.309 ± 5.012 µg/ml (MD = 2.497 µg/ml, t = -1.408, p = 0.165). The mean ADNC levels in men with T1D were: 10.715 ± 5.235 µg/ml at AlbU below 30 mg/l; 11.465 ± 5.318 µg/ml at AlbU between 30 and 300 mg/l and 15.11 ± 9.496 µg/ml at AlbU above 300 mg/l, F = 1.343, p = 0.270 (Fig.24).

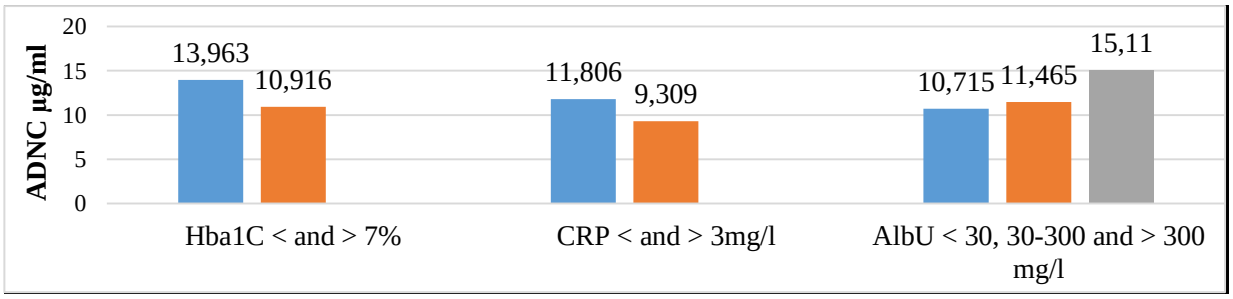

Figure 24. Mean levels of ADNC versus HbA1C, CRP and AlbU in men with T1D.

No statistically significant differences were reported between women with T1D and HbA 1 C below and above 7% (19.323 ± 5.456 μg/ml vs. 18.232 ± 13.444 μg/ml, MD = 1.09 μg/ml, t = - 0.225, p = 0.823). A significant difference in ADNC values was reported in females versus CRPs below and above 3 mg/l (21.500 ± 14.012 μg/ml and 12.054 ± 5.184 μg/ml) and it was MD = 9.447, t = - 2.683, p = 0.01. One-factor variance analysis with subsequent Tukey HDS (Tukey) posthoc test, found no statistically significant differences in ADNC concentrations in women with AlbU below 30 mg/l, between 30 and 300 mg/l and above 300 mg/l: 17.299 ± 8.016 μg/ml, 23.223 ± 23.247 μg/ml and 19.015 ± 4.632 μg/ml, F = 0.955, p = 0.392 (Fig.25).

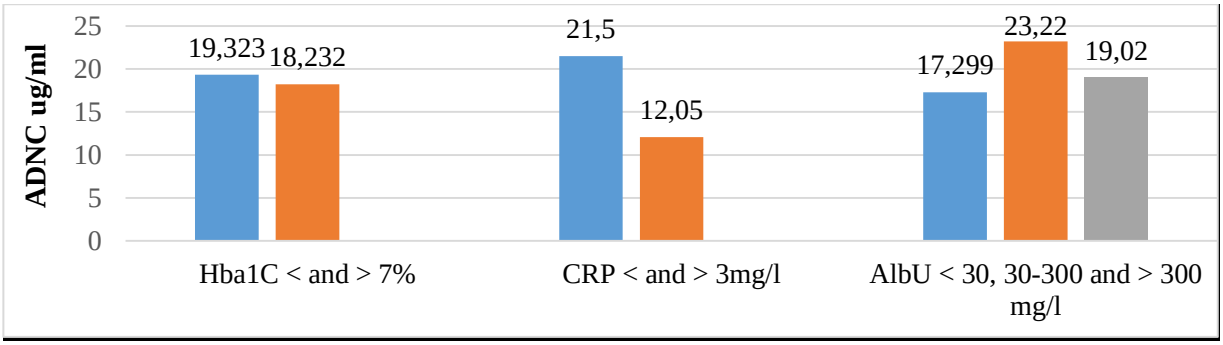

Figure 25. Mean ADNC levels versus HbA1C, CRP and AlbU in women with T1D.

For the control group, a significant difference in ADNC values was found in women with AlbU below 30 μg/ml and 30 – 300 μg/ml (12.287 ± 3.767 μg/ml versus 36.91 ± 49.81 μg/ml, F = 6.674, p = 0.017). No such difference was reported in males (6.907 ± 2.444 μg/ml vs 5.99 (N=1) μg/ml, F = 0.955, p = 0.392). Cases with AlbU above 300 mg/l and HbA1C above 7% in the control group were not recorded.

To investigate the association between the variables ADNC, CRP, HbA1C and AlbU, the Pearson's coefficient was used. In women with long-standing T1D, a close to significant negative correlation between ADNC and CRP was found (r = -0.224, p = 0.107). In the group of controls, only one significant correlation was recorded and it was between ADNC and AlbU in the women with a straight direction and great in force: r = 0.777, p = 0.000. In order to establish the linear combination between AlbU and ADNC values, a single linear regression analysis was applied. Statistical significance was registered, but only in the women's control group (F = 6.674, p = 0.017) In individuals with long-standing T1D, it loses its force and significance. The available AlbU information allowedand to predict the ADNC value in the female controls, F = 33.603, p = 0.015. The equation found for the relationship between variables was:

ADNC (µg/ml) = 7.068 + 0.477xAlbU (mg/l)

The adjusted R^2 value was 0.586, indicating that **58.6%** of the changes in ADNC concentration under physiological conditions could be explained by the regression model presented, i.e. by the AlbU value.

4.4. ROC analyses to derive ADNC threshold values against the CVR category in persons with long-standing T1D.

No good prognostic value of OPG was found against the established scales for the assessment of CVR in T1D: at ST1RE, AUC e 0.559, p = 0.476 for males and AUC = 0.578, p = 0.401 for females. Relative to the ESC-2019, AUC was 0.466, p = 0.680 for men and AUC = 0.378, p = 0.167 for women. No good prognostic value of OPG was reported and relative to RiskFactor3: AUC was 0.408, p = 0.256 for males and AUC = 0.341, p = 0.063 for females.

In summary, ROC analysis demonstrated that ADNC as a stand-alone biomarker does not have sufficient diagnostic reliability in differentiating patients with low to moderately high CVR from very high CVR according to the ST 1RE and ESC criteria of 2019. No cut-off values were therefore derived due to this insufficient reliability of the biomarker.

5. Prognostic value of Lep for estimation of CVR in T1D

5.1. Lep – influence of gender, age, duration of diabetes and BMI.

After administration of a t-test for Student's independent samples, significant differences between genders in the arithmetic mean of Lep were found in both study groups: MD = 3.109, t = - 3.540, p = 0.001 in controls and MD = -5.349, t = - 5.458, p = 0.000 in patients with T1D (Table 15).

Table 15. Average Lep values according to gender.

Participant group		Gender	N	mean	SD	t	P value	MD
Control group	Lep(ng/ml)	Men	27	3.081	1.998	-3.540	**0.001**	-3.109
		Women	24	6.191	4.045			
Patients with T1D	Lep (ng/ml)	Men	56	2.862	2.947	-5.458	**0.000**	-5.348
		Women	52	8.211	6.679			

N – number; mean – *mean value; SD – standard deviation.*

Men with T1D duration over 24 years recorded significantly higher values for Lep compared to those with a duration below 24 years in the study. (3.4685 ± 3.443 ng/ml versus 1.926 ± 1.618 ng/ml, MD = 1.54 ng/ml, t = 1.961, p = 0.048). It is

interesting to note that in women, higher values for Lep are recorded at T1D duration below 24 years (9.976 ± 8.799 ng/ml) versus those studied with a duration of more than 24 years (7.0525 ± 4.625 ng/ml, MD = 2.924, t = - 1.581, p = 0.050). The correlation reported in the two groups studied was between the men's age and the concentration of Lep (Fig.26).

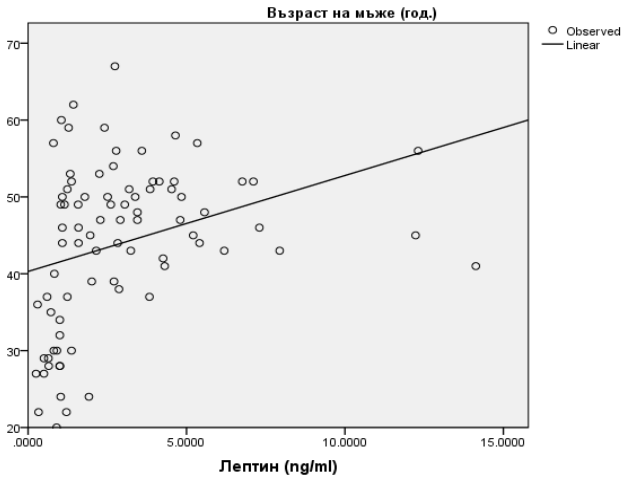

Figure 26. Correlation between Lep and age of the men studied.

In the two study groups, significant associations between BMI and serum Lep levels were found and they were straight in direction and great in gorce: r = 0.667, p = 0.000 for men with T1D and r = 0.635, p = 0.000 for women with T1D; r = 0.806, p = 0.000 for male controls and r = 0.762, p = 0.000 for female controls (Table 16).

Table 16. Correlations between: Lep, ADNC, T1D duration and age.

Group				**Age (yr)**	**Duration (yr)**	**BMI (kg/m^2)**	**ADNC (μg/ml)**
T1D	**Men**	Lep ng/ml	Pearson Correlation	0.284*	0.196	0.667**	-0.85
			P value	**0.034**	0.147	**0.000**	0.171
			Count	56	56	56	56
	Women	Lep ng/ml	Pearson Correlation	-0.024	-0.194	0.635**	-0.198
			P value	0.863	0.168	**0.000**	0.159
			Count	52	52	52	52
Control group	**Men**	Lep ng/ml	Pearson Correlation	0.425*	/	0.806	-0.067
			P value	**0.027**	/	**0.000**	0.740
			Count	27	/	27	27
	Women	Lep ng/ml	Pearson Correlation	-0.008	/	0.762	-0.052
			P value	0.971	/	**0.000**	0.810
			Count	24	/	24	24

*. Correlation is significant at the 0.05 level (2-tailed).**. Correlation is significant at the 0.01 level (2-tailed).

Using the Pearson's correlation coefficient, the linear relationship between Lep, ADMA and OPG was also evaluated. In the control group of those studied

there were no significant correlations between these variables. In the T1D group, a significant straight-direction correlation was found between Lep and OPG (r = 0.296, p = 0.027) in men. In women, no significant results are reported.

5.2. Lep – links with ST1RE, ESC-2019 RiskFactor3.

In order to investigate the relationship between the Lep concentration and the CVR category defined according to ST1RE, ESC-2019 and the proposed RiskFactor3 model, the rank correlation coefficient, Spearman's Rho was used: *rho* = 0.23, p = 0.084 between Lep and RiskFactor3; *rho* = 0.361, p = 0.00 6 between Lep and ESC 2019 and *rho* = 0.549, p = 0.000 between Lep and ST1RE. It is noteworthy that all three relationships found were significant. The sign of the correlations was positive, and the magnitude of the effect was greatest in ST1RE (Table 17).

Table 17. Correlations of Lep with ST1RE, ESC 2019 and RiskFactor3.

			Lep (ng/ml)	**RiskFakctor3**	**ESC-2019**	**ST1RE**
Spearman's rho	**Lep** (ng/ml)	Correlation Coefficient	1.000	0.233	0.361**	0.549**
		P value	/	**0.084**	**0.006**	**0.000**
Men with T1D		Count	56	56	56	56
Spearman's rho		Correlation Coefficient	1	0.437**	0.200	0.070
		P value	/	**0.001**	**0.151**	0.617
Women with T1D		Count	53	53	53	53

*. Correlation is significant at the 0.05 level (2-tailed).**. Correlation is significant at the 0.01 level (2-tailed).

The Spearman's Rho rank correlation coefficient between the variables thus studied in females was: *rho* = 0.437, p = 0.001for Lep and RiskFactor3; *rho* = 0.20, p = 0.151 for Lep and ESC2019 and *rho* = 0.070, p = 0.617 for Lep and ST1RE. Here, a significant positive correlation is found only between the Lep value and RiskFactor3 (Table 17).

Relative to ST1RE, significant difference was reported in the mean Lep levels between men with low CVR and those studied with high CVR (1.206 ± 1.026 ng/ml vs 4.354 ± 3.743 ng/ml, MD = 3.148 ng/ml, p = 0.003) and between men with moderate and those with high CVR (2.162 ± 1.561 ng/ml vs. 4.354 ± 3.743 ng/ml, MD = 2.192 ng/ml, p = 0.029), F = 7.070, p = 0.002. In women with T1D, the results of the F-test showed that the null hypothesis H_0, F = 2.029, p = 0.142 could not be categorically deviated. Median and interquartile ranges of Lep were: in males with low - 0.95 (0.458 – 1.468) ng/ml; with moderate - 1,585 (1,000 – 3,293) ng/ml; with high CVR - 3.39 (1.355 – 5.07) ng/ml; in women with low - 7.43 (3.975 – 9.103) ng/ml; with moderate - 5.06 (3.345 – 9.688) ng/ml; with a high CVR - 5.94 (4.27 – 16.11) ng/ml (Fig.27).

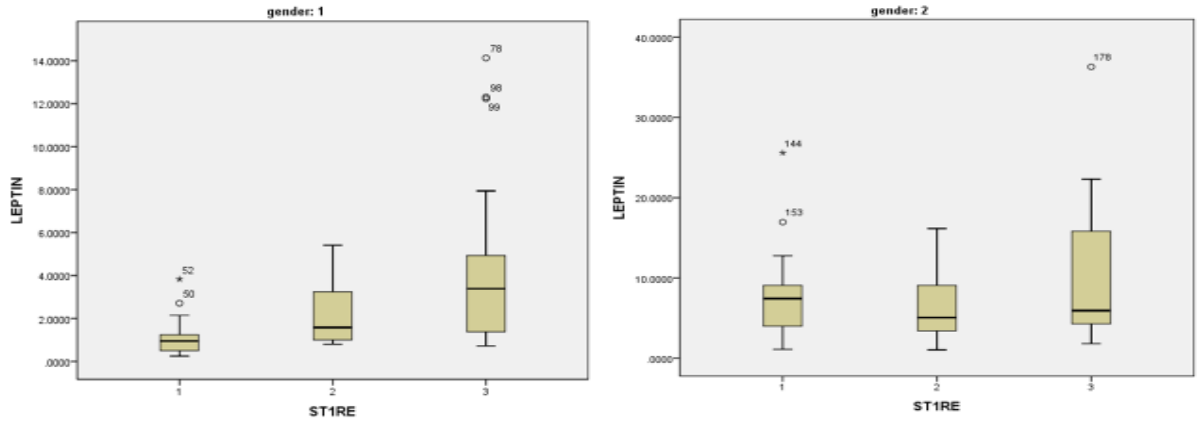

Figure 27. Lep (ng/ml) in males and females relative to ST1RE. *(Gender* 1 *– male, gender* 2 – female, *1* – low CVR, 2 – moderate CVR, *3 – high CVR)*

In terms of ESC-2019, the reported difference between men with high and very high CVR was 1.6288 ng/ml, t = - 1.982 , p = 0.053 (1.757 ± 1.664 ng/ml vs 3.386 ± 3.281 ng/ml). In females, MD was 3.188 ng/ml, t = - 2.080, p = 0.043, 5.925 ± 3.699 ng/ml vs 9.113 ± 7.387 ng/ml (Table 18).

Table 18. Arithmetic mean values for Lep in persons with T1D, grouped according to CVR assessment criteria from ESC-2019.

Gender		ESC-2019	N	mean	SD	MD	t	P value
Men	Lep (ng/ml)	High CVR	18	1.757	-1.628	1.664	-1.982	**0.053**
		Very high CVR	38	3.386	-1.628	3.281		
Women	Lep (ng/ml)	High CVR	15	5.925	-3.188	3.699	-2.080	**0.043**
		Very high CVR	38	9.113	-3.188	7.387		

Medians and interquartile ranges are: in men at high risk - 0.95 (0.6125 – 2.895) ng/ml, and in men at very high CVR - 2.21 (1.133 – 4.365) ng/ml; in women at high risk are 5.21 (2.95 – 9.33) ng/ml, and in women with very high CVR - 7,245 (4,203 – 11,205) ng/ml (Fig.28).

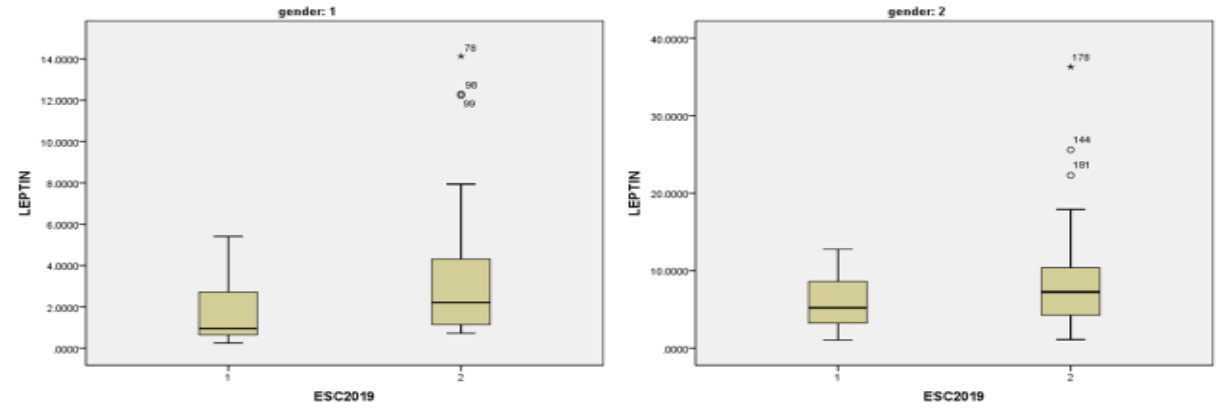

Figure 28. Lep (ng/ml) in males and females according to ESC-2019 *(1 – high CVR, 2 – very high CVR, gender 1 – men, 2 – women)*

5.3. Lep – links with AlbU, CRP, HbA1C and with RiskFactor3.

One-factor variance analysis for verification of the statistical significance of the difference between the arithmetic mean Lep values in T1D patients according to RiskFactor 3 demonstrated again a trend of proportionally significant increases in Lep values (F = 6.717, p = 0.001 in males and F = 3.666, p = 0.018 in women) (Table 19).

Table 19. Mean values for Lep in males and females according to RiskFactor3.

Gender	Group	N	Lep (Mean) ng/ml	SD	F	P value
Men with T1D	0	4	2.102	0.818	.	
	1	28	2.157	2.664		
	2	21	3.056	2.123	6.717	**0.001**
	3	3	9.093	5.502		
	Total	56	2.862	2.947		
Women with T1D	0	4	4.890	2.523		
	1	25	5.810	3.757		
	2	18	10.835	8.581	3.666	**0.018**
	3	6	12.555	7.547		
	Total	53	8.2111	6.679		

N – number; mean – *mean; SD – standard deviation.*

Medians and interquartile ranges of Lep in males of the different RiskFactor 3 categories were: 1.87 (1.47 – 2.298) ng/ml for group 0; 1.24 (0.924 – 2.485) ng/ml for group 1; 3.05 (1.015 – 4.87) ng/ml for group 2; 12.23 (2.74 – *) ng/ml for group 3. Medians and interquartile ranges of Lep in females were: 4.965 (2.457 – 7.247) ng/ml for group 0; 4.91 (3.22 – 8.745) ng/ml for group 1; 8.235 (5.758 – 14.125) ng/ml for group 2; 13.065 (4.58 – 19.01) ng/ml for group 3 (Fig. 29).

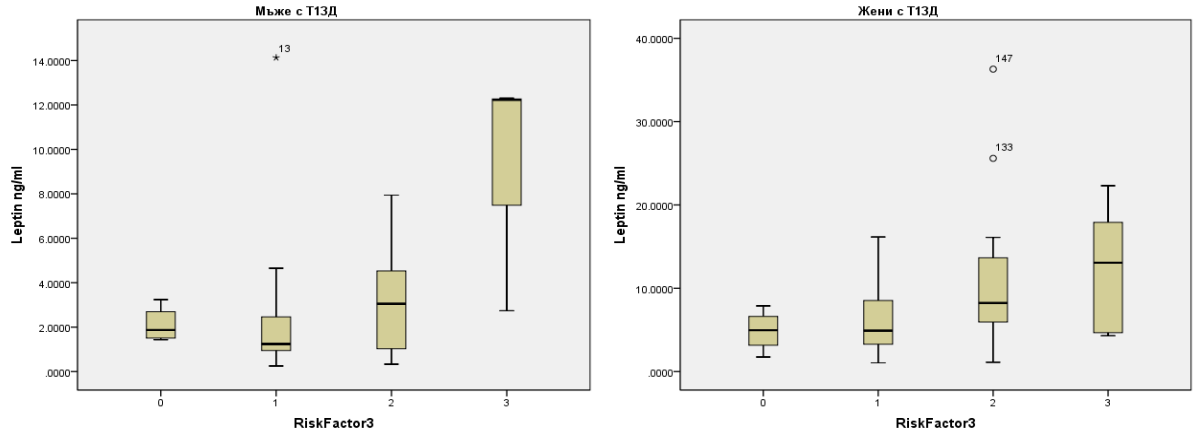

Figure 29. Lep (ng/ml) in males and females versus RiskFactor3.

No significant differences in mean Lep levels were recorded in the T1D patients studied versus HbA1C above and below 7% (2.662 ± 2.64 ng/ml at HbA1C% > 7% versus 4.532 ± 4.853 ng/ml at HbA1C% < 7%, MD = 1.869 ng/ml, t = - 1.484, p = 0.127 for men with diabetes and 8.361 ± 7.045 ng/ml at HbA1C% over 7% versus 7.226 ± 3.626 ng/ml at HbA1C% below 7%, MD = 1.135 ng/ml, t = 0.416, p = 0.249 for women with diabetes). In the two study groups, men with T1D and healthy men scored significantly higher for Lep at CRP above 3 mg/l (5.175 ± 3.685 ng/ml versus 2.163 ± 2.312 ng/ml for men with T1D, MD = 3.011 ng/ml, t = 3.552, p = 0.001 and 5.016 ± 3.685 ng/ml vs. 2.641 ± 2.312 ng/ml for male controls, MD = 2.374 ng/ml, t = 2.664, p = 0.013. A trendline is marked to show a proportional increase in Lep values (Fig.30).

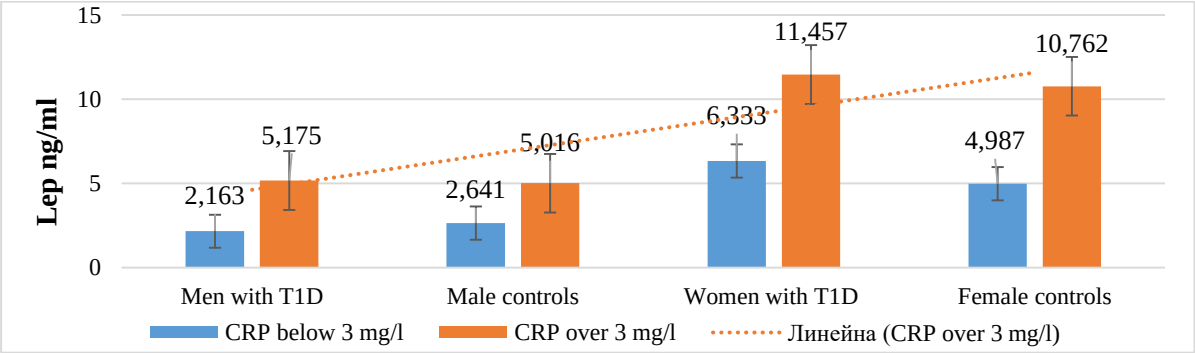

Figure 30. Mean values for Lep depending on CRP below and above 3 mg/l in males and females.

In the comparison thus made, Student's T – test for independent samples showed a significant difference in values for Lep and in females. The difference reported in controls was MD = 5.774 ng/ml, t = 3.446, p = 0.000 (4.988 ± 1.983 ng/ml at CRP below 3 mg/l compared to 10.762 ± 6.589 ng/ml at CRP above 3 mg/l) and in women with T1D, MD = 5.125 ng/ml, t = 3.446, p = 0.000 (4.987 ± 1.983 ng/ml at CRP below 3 mg/10.762 ± 6.589 ng/ml for CRPs above 3 mg/l), respectively (Fig.31).

In men with T1D, the mean values of Lep relative to AlbU below 30 mg/l, between 30 and 300 mg/l and above 300 mg/l were respectively: 2.251 ± 1.756 ng/ml, 3.070 ± 3.570 ng/ml and 5.619 ± 2.513 ng/ml, F = 8.401, p = 0.001. In females they were: 7.023 ± 4.786 ng/ml, 8.989 ± 7.221 ng/ml and 22.735 ± 19.1838 ng/ml, F = 6.713, p = 0.003. After administration of Tukey's posthoc test HDS (Tukey), these differences remained significant between men and women with AlbU above 300 mg/l and those with AlbU below 30 mg/l on the one hand and those with ALbU between 30 and 300 mg/l on the other hand (p < 0.05). A trend

line was marked, according to which there is a proportional increase in the values of the studied variable (Fig.32).

In the male control group, a similar difference was reported (F = 0.135, p = 0.717), and in women - a significant difference in Lep values was reported between AlbU cases below 30 mg/l and those with AlbU between 30 and 300 mg/l: 5.064 ± 2.213 ng/ml vs 9.93 ± 6.620 ng/ml , F = 7.180, p = 0.014 (Fig.32).

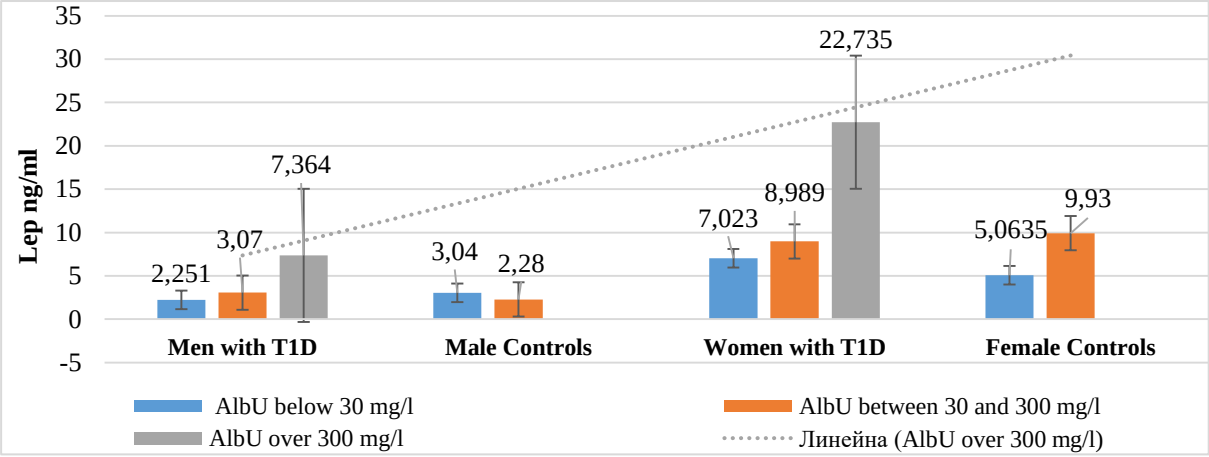

Figure 32. Average levels for Lep depending on the AlbU value.

In the T1D group, three significant correlations were found: between Lep and AlbU in men (r = 0.367, p = 0.005) and between Lep and AlbU (r = 0.413, p = 0.002) and Lep and CRP (0.378, p = 0.006) in women. All corelations listed so far were average by force and straight in direction. In control cases, the following positive correlations were recorded with Lep: with CRP in males and females (r = 0.309, p = 0.116 and r = 0.685, p = 0.000) and with HbA1C in females - r = 0.505, p = 0.012 (Table 20).

Table 20. Correlations between Lep and: AlbU, CRP and HbA1C.

Group	Gender			AlbU mg/l	CRP mg/l	HBA1C%	Lep ng/ml
Group with T1D	**Men**	Lep ng/ml	Pearson Correlation	0.367**	0.012	-0.149	1
			P value	**0.005**	0.929	0.272	
			Count	56	56	56	56
	Women	Lep ng/ml	Pearson Correlation	0.413**	0.378**	0.110	1
			P value	**0.002**	**0.006**	0.432	
			Count	53	52	53	53
Control-group	**Men**	Lep ng/ml	Pearson Correlation	0.131	0.309	0.189	1
			P value	0.516	**0.116**	0.346	
			Count	27	27	27	27
	Women	Lep ng/ml	Pearson Correlation	0.297	0.685**	0.505*	1
			P value	0.168	**0.000**	**0.012**	
			Count	23	24	24	24

**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).

The combination of variables CRP and AlbU for prediction of value for Lep was statistically significant in men and women with T1D and in women in the control group (F = 4.14, p = 0.021 in men with T1D, F = 8.955, p = 0.000 in women with T1D and F = 5.57, p = 0.012 in healthy women (Table 21).

Tablica 21. Multiple linear regression analysis with dependent variable Lep (ng/ml) and independent variables: CRP (mg/l) and AlbU (mg/l).

Group	Std. Beta	t	P value	F	P value	R	R^2
Men with T1D	Const 2.372	5.605	0.000	4.14	**0.021**	0.368	0.102
	CRP 0.018	0.142	0.888				
	AlbU 0.367	2.876	0.006				
Women with T1D	Const 5.424	5.194	0.000	8.955	**0.000**	0.517	0.238
	CRP 0.325	2.627	0.011				
	AlbU 0.358	2.892	0.006				
Healthy women	Const 3.615	4.221	0.000	5.57	**0.012**	0.598	0.294
	CRP 0.521	2.896	0.009				
	AlbU 0.345	1.916	0.070				

The adjusted R^2 value for women with T1D was 0.238 and for healthy women is 0.294. This shows that 23.8% (in women with T1D) and 29.4% (in healthy women) of the changes in Lep concentration can be explained by the regression models. The following regression equations have been derived:

Lep (ng/ml) = 5.424 + 0.325. CRP (mg/l) + 0.358. AlbU (mg/l) for women with T1D**;**

Lep (ng/ml) = 3.615 + 0.521. CRP (mg/l) + 0.345. AlbU (mg/l) for healthy women;

In men with T1D, in the course of linear regression analysis, CRP as an independent variable lost its significance, and a reduced regression model was created in which only AlbU was included: F = 8.412, p = 0.005. The value of adjusted R^2 is 0.119. This shows that 11.9% of the changes in Lep concentration can be explained with the presented regression model, which according to Cohen (Cohen, 1988) is again a small magnitude of the effect. The following regression equation is derived:

Lep (ng/ml) = 2,388 + 0,367. AlbU (mg/l)

5.4. Lep - logistic regression models and ROC analyses to derive threshold values.

With regard to ST1RE, Exp(B) showed that if the concentration of Lep increases by 1 ng/ml, then the chance of the patient falling into a category of high CVR increases by 1,693-fold for men. With ESC-2019, the chance of a patient falling into a CVR category from high to very high is 1,404 times in men, respectively. With RiskFactor 3, any increase in Lep concentration of 1 ng/ml resulted in an increase in the chance that women would have ≥ 2 RF (HbA1C ≥ 7%, CRP ≥ 3 mg/l or AlbU ≥ 30 mg/l) by 1.21-fold (Table 22).

Table 22. Prognostic value of Lep against: ST1RE, ESC-2019and RiskFactor3.

Model	Gender	OD%	95% CI	P value
ST1RE - high CVR	Men	1.693	1.169 – 2.451	**0.005**
	Women	1.087	0.990 – 1.193	0.081
ESC-2019 – very high CVR	Men	1.404	0.971 – 2.029	**0.071**
	Women	1.113	0.969 – 1.278	0.129
RiskFactor 3 ≥ 2 RFs (HbA1C ≥ 7%,CRP ≥ 3 mg/l or AlbU ≥ 30 mg/l)	Men	1.161	0.946 – 1.424	0.153
	Women	1.210	1.045 – 1.400	**0.011**

With ST1RE, the AUC for men was 0.780, p = 0.000, and for women - 0.598, p = 0.269. ROC analysis demonstrated good diagnostic accuracy of Lep in differentiating men with high CVR. At cut-off value of Lep = 2.28 ng/ml, Lep had MS 70.8% and DS 78.1 %. LR+ = 3.23 and LR- = 0.309, DOR = 10.45, Youden index = 0.927 and DE = 75% (Fig.33).

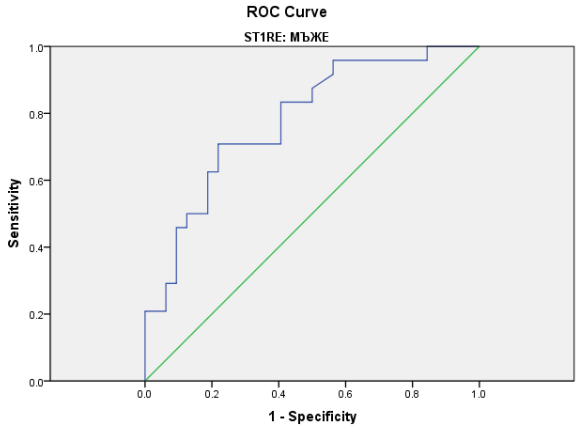

Figure 33. AUC - ROC for Lep (ng/ml) in differentiating men with high CVR, according to ST1RE.

At ESC-2019, the AUC for men was 0.723, p = 0.007, and for women it was 0.628, p = 0.462. ROC analysis here again demonstrated better diagnostic

effectiveness in men versus women with T1D. At cut-off value of Lep = 1.38 ng/ml for men, the biomarker had Se 65.8% and Sp 66.7%; at cut-off = 5.475 ng/ml for women with Se 60.5% and Sp 60% (Fig.34). The following were determined: LR+ = 1.98 and LR- = 0.506, DOR = 3.91, Youden index = 0.991 and DE = 66 % in males and LR+ = 1.51 and LR- = 0.661, DOR = 2.28, Youden index = 1.005 and DE = 71.7 %.

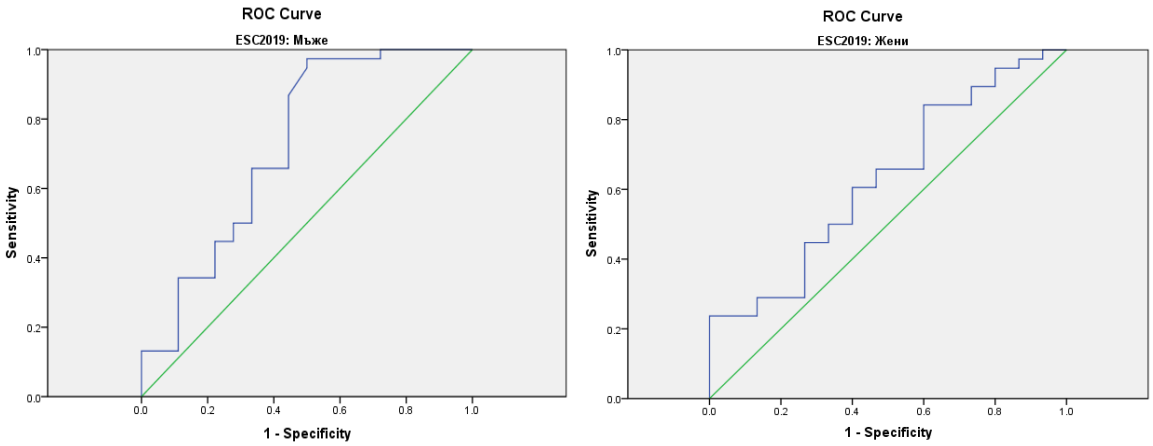

Figure 34. ROC curves for the diagnostic reliability of Lep in differentiating men and women with very high CVR according to the ESC-2019.

With RiskFactor3, the AUC for males was 0.563, p = 0.437, and for females was 0.673, p = 0.041. At a cut-off value of Lep = 5.815 ng/ml for females, the biomarker had Se 77.8% and Sp 62.9% (Fig. 35) No cut-off value of Lep for men was derived. The following were defined: LR+ = 2.097 and LR- = 0.476, DOR = 4.405, Youden index = 1.149 and DE = 67.9% for women with T1D (Table 23).

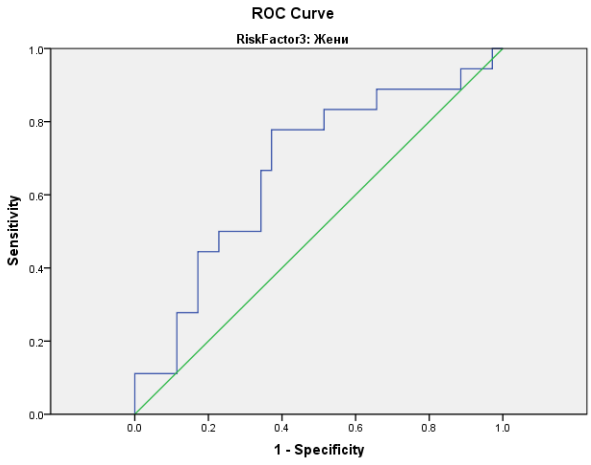

Figure 35. AUC - ROC for Lep in differentiating women from groups 0, 1, 2 versus group 3 of RiskFactor3.

Table 23. Diagnostic reliability of Lep according to ST1RE, ESC-2019 and RiskFactor3.

Indicator	Criterion	cut-off	N	TP	TN	FP	FN	Se	Sp	DE
Lep (ng/ml) Men	St1RE - high CVR	**2.28**	56	17	25	7	7	70.8%	78.1%	75%
Lep (ng/ml) Men	ESC-2019 - very high CVR	**1.38**	56	25	12	6	13	65.8%	66.7%	66%
Lep (pmol/l) Women	ESC-2019 - very high CVR	**5.475**	53	23	15	9	6	60.5%	60%	71.7%
Lep (pmol/l) Women	RiskFactor3 - presence of 2 RF	**5.815**	53	14	22	13	4	77.8%	62.9%	67.9%

TP - true positive, TP - true negative, FP - false positive, FN - false negative

From the ROC analyses of the data so derived, it can be concluded that Lep had a good prognostic value for men in differentiating high CVR versus ST1RE (approximately 80%) and ESC from 2019 (approximately 70%), and women when differentiating a very high CVR from ESC-2019 (approximately 63%) and groups 2 and 3 of RiskFactor3 (approximately 70%).

6. Hematomorphological indicators.

6.1. Comparison between patients with T1D and healthy subjects.

Student's T – test for independent samples demonstrated a statistically significant difference for the following leucocyte variables: WBC - 7.03 ± 1.91.10^9/L vs 6.42 ± 1.64.10^9/L, MD = 0.604.10^9/L, p = 0.038, t = 2.090), Neu - 4.21 ± 1.50.10^9/L vs. 3.71 ± 1.26. 10^9/L, MD = 0,502. 10^9/L, t = 2.223, p = 0.027 and Eo - 0.22 ± 0.217.10^9/L versus 0.16 ± 0.104. 10^9/L, MD = 0,053. 10^9/L, t = 2,235, p = 0,027. The following particulars were noted regarding erythrocyte variables: all reported values were lower in patients with T1D. Only RDW_CV% was higher in patients versus controls (13.03 ± 1.56% vs 12.98 ± 1.41%, MD = 0.049%, p = 0.837). Differences close to significant were recorded in MCH and MCHC. In patients with T1D, MCH level was 29.21 ± 2.17 pg, and in controls it was 30.47 ± 4.91 pg with MD = 0.952 pg, p = 0.071. In MCHC, the mean level for patients was 331.62 ± 15.73 g/L, and in controls - 334.75 ± 11.91 g/L with MD = 3.125 g/L, p = 0.096. In intergroup comparison, higher average levels of all platelet parameters were found in patients with long-standing T1D. Two of the differences found were significant with 95% authenticity of the results - PLT (280.60 ± 76.016.10^9/L for group with T1D compared to control group - 253.08 ± 47.098.10^9/L, MD = 27.520 x10^9 /L, t = 2.999, p = 0.003) and PCT - 0.299 ± 0.070% for subjects with T1D vs 0.264 ± 0.048% for controls, MD = 0.0345, t = 3.069, p = 0.003 (Table 24).

Table 24. Hematological indicators in T1D and healthy subjects.

	Participant group	N	Mean	SD	t	P value	MD
WBC 10^9/L	patients with T1D	124	7.025	1.908	2.090	**0.038**	0.604
	control group-healthy	59	6.421	1.638			
Neu 10^9/L	patients with T1D	124	4.214	1.499	2.223	**0.027**	0.502
	control group-healthy	59	3.712	1.261			
Neu %	patients with T1D	124	58.99	9.458	1.599	0.112	2.132
	control group-healthy	59	56.86	7.895			
Eo 10^9/L	patients with T1D	124	0.216	0.216	2.235	**0.027**	0.053
	control group-healthy	59	0.162	0.104			
Eo %	patients with T1D	124	2.891	2.213	1.019	0.309	0.326
	control group-healthy	59	2.566	1.533			
Ba10^9/L	patients with T1D	124	0.043	0.025	-0.052	0.959	-0.001
	control group-healthy	59	0.044	0.022			
Ba %	patients with T1D	124	0.647	0.377	-0.956	0.340	-0.056
	control group-healthy	59	0.703	0.352			
Mo 10^9/L	patients with T1D	124	0.519	0.189	1.241	0.216	0.031
	control group-healthy	59	0.488	0.138			
Mo %	patients with T1D	124	7.511	1.878	-0.806	0.422	-0.224
	control group-healthy	59	7.735	1.701			
Lym 10^9/L	patients with T1D	124	2.025	0.648	0.374	0.709	0.034
	control group-healthy	59	1.991	0.531			
Lym %	patients with T1D	124	29.49	7.641	-1.945	**0.054**	-2.234
	control group-healthy	59	31.72	7.079			
Hb g/L	patients with T1D	124	138.2	17.73	-1.605	0.111	-3.919
	control group-healthy	59	142.2	14.21			
RBC 10^12/L	patients with T1D	124	4.660	0.589	-1.291	0.198	-0.118
	control group-healthy	59	4.778	0.554			
HCT L/L	patients with T1D	124	0.413	0.051	-1.583	0.115	-0.011
	control group-healthy	59	0.424	0.038			
MCV fl	patients with T1D	124	88.96	5.419	-0.436	0.663	-0.416
	control group-healthy	59	89.37	7.133			
MCH pg	patients with T1D	124	29.51	2.173	-1.819	**0.071**	-0.952
	control group-healthy	59	30.46	4.914			
MCHC g/L	patients with T1D	124	331.6	11.73	-1.676	0.096	-3.125
	control group-healthy	59	334.8	11.91			
RDW_CV %	patients with T1D	124	13.03	1.556	0.206	0.837	0.049
	control group-healthy	59	12.98	1.408			
MPV fL	patients with T1D	124	10.56	1.172	1.846	**0.067**	0.341
	control group-healthy	59	10.22	1.161			
PDW fL	patients with T1D	109	12.93	2.188	1.993	**0.049**	0.685
	control group-healthy	47	12.25	1.868			
P-LCR %	patients with T1D	109	31.53	8.123	1.486	0.141	1.969
	control group-healthy	47	29.56	7.354			
PCT %	patients with T1D	109	0.299	0.070	3.545	**0.001**	0.035
	control group-healthy	47	0.264	0.048			
PLT x10^9/L	patients with T1D	124	280.6	76.02	2.999	**0.003**	27.52
	control group-healthy	59	253.1	47.09			

N – number; mean – *mean; SD – standard deviation.*

The differences found between MPV and PDW were at 90% authenticity of the results. The intergroup difference in MPV was 0.340 fL for the benefit of patients with T1D - 10.652 ± 1.172fL vs 10.22 ± 1.16 fL in controls, t = 1.840, p = 0.067. In PDW, the difference found was 0.685fL, t = 1.872, p = 0.063, with the

mean variable level in T1D patients being 12.924 fL ± 2.19 fL vs 12.25 ± 1.87 fL for controls. Only with P-LCR no intergroup significative difference was found, but again mean levels were higher in the T1D patients - 31.527 ± 8.123% vs 29.557 ± 7.354%, MD = 1.969, t = 1.428, p = 0.155 (Table 24).

Patients with longer duration of T1D, Eo and Neu showed a reduction. Ba, Mo and Lym increase with longer illness duration, p > 0.05. RBC showed a decrease in prolonged illness, with parallel increases registered in MCV, MCH and RDW_CV: 4.75 ± 0.54.10^12/L vs 4.56 ± 0.63. 10^12/L, MD = 0.19.10^12/L, p = 0.069 for erythrocytes; 90.42 ± 4.11 fl vs 87.55±6.15 fl, MD = 2.87fl, p = 0.003 for MCV; 29.96 ± 1.7 pg vs 29.08 ± 2.48 pg, MD = 0.88 pg, t = -2.319, p=0.022 for MCH; 12.94 ± 1.79% vs 13.13 ± 1.27%, MD = - 0.19%, t = - 0.695, p = 0.488 for RDW-CV%. With increasing duration of T1D, PLT and PCT decreased, but increases were recorded in mean MPV, PDW and P-LCR levels: 295.03 ± 72.45.10^9/L vs 265.70 ± 77.32.10^9/L, MD = 29.33.10^9/L, t = 2.180, p=0.031 for PLT; 0.307 ± 0.07% vs 0.293 ± 0.07%, MD =0.014, t = 1.047, p = 0.297 for PCT; 10.34 ± 1.26fL vs 10.79 ± 1.03fL , MD = 0.44fl, t = - 2.132, p = 0.032 for MPV; 30.15 ± 9.01%, vs 32.78 ± 7.06% , MD = 2.63%, t = -1.700, p = 0.092 for P-LCR and 12.68 ± 2.46 fL vs 13.17 ± 1.89 fL, MD = 0.49, t = - 1.173, p = 0.243 for PDW. Of the five variables analysed, significant differences are recorded for MPV and PLT (Table 25).

Table 25. Hematomorphological parameters and established differences depending on the duration of diabetes (under and over 24 years).

Parameter	T1D duration	N	Mean	SD	t	P value	MD
RBC 10^12/L	under 24 years.	63	4.754	0.537	1.833	**0.069**	0.192
	over 24 years.	61	4.562	0.627			
MCV fl	under 24 years.	63	87.55	6.149	-3.045	**0.003**	-2.869
	over 24 years.	61	90.42	4.108			
MCH pg	under 24 years.	63	29.08	2.476	-2.319	**0.022**	-0.885
	over 24 years.	61	29.96	1.715			
MPV fL	under 24 years.	63	10.34	1.264	-2.132	**0.035**	-0.442
	over 24 years.	61	10.79	1.031			
P-LCR %	under 24 years.	52	30.15	9.014	-1.700	**0.092**	-2.625
	over 24 years.	57	32.78	7.064			
PLT x10^9/L	under 24 years.	63	295.0	72.45	2.180	**0.031**	29.33
	over 24 years.	61	265.7	77.32			

N – number; mean – *mean; SD – standard deviation.*

In individuals with T1D and HbA1C above 7%, the identified value for MCV was 88.352 ± 5.436 fL vs 92.311 ± 3.999 fL at HbA1C below 7%, MD = 3.958 fL, t = - 3.025, p = 0.003. In cases with poor glycaemic control, MCH level was 29,320 ±2,256 pg, and in HbA1C below 7% - 3,589 ± 1,193 pg, MD = 1,269 pg, t = -

3,613, p = 0,001. RDW-CV% was higher at HbA1C above 7%: 13.057 ± 1.652% vs 12.895 ± 0.875%, but without significant difference. Hb (g/l) decreased at HbA1C > 7% - 137.9 ± 18.443g/L vs 139.74 ± 13.453 g/L at HbA1C < 7%, MD = 1.775 g/l, p = 0.690. Significant differences were found in PCT and PLT, and they were higher in HbA1C above 7%: 286.32 ± 78.259. 10^9/L vs 249.00 ± 53.438.10^9/L, MD = 37.324. 10^9/L, p= 0.014 for PLT. For PCT the differences found were significant, at 90% authenticity of the results: 0.304 ± 0.071% vs 0.271 ± 0.059%, MD = 0.033%, p=0.059 (Table 26).

Table 26. Significant differences depending on HbA1C% below and above 7% in individuals with long-standing T1D for MCV, MCH, PCT and PLT.

	HbA1C ≥ and < 7%	N	Mean	SD	t	P value	MD
MCV fl	Over 7%	105	88.35	5.436			
	under 7%	19	92.31	3.989	-3.025	**0.003**	-3.958
MCH pg	Over 7%	105	29.32	2.256			
	under 7%	19	30.59	1.193	-3.613	**0.001**	-1.269
PCT %	Over 7%	93	0.304	0.071			
	under 7%	16	0.271	0.059	1.982	**0.059**	0.033
PLT x10^9/L	Over 7%	105	286.3	78.26			
	under 7%	19	249.0	53.44	2.584	**0.014**	37.32

N – number; mean – *mean; SD – standard deviation.*

In the T1D group, significant differences between genders were found in all platelet indices with a predominance in women: 10.524 ± 0.999 fl vs 10.605 ± 1.35fl, p = 0.006 for MPV; 31.188 ± 6.751% vs 31.926 ± 9.549%, p = 0.026 for P-LCR%; 0.2824 ± 0.067% vs 0.319 ± 0.069%, p = 0.006 for PCT% and 268.73 ± 75.031. 10^9/L vs 294.12 ± 75,501.10^9/L, p = 0.063 for PLT (Table 27).

Table 27. Platelet variables depending on gender.

Group		Gender	N	Mean	SD	t	P value
Group with T1D	**MPV fl**	Men	66	10.52	0.999	-0.382	**0.006**
		Women	58	10.61	1.350		
	PDW fl	Men	59	12.94	1.869	0.009	**0.026**
		Women	50	12.93	2.532		
	P-LCR %	Men	59	31.19	6.751	-0.471	**0.026**
		Women	50	31.93	9.549		
	.PCT %	Men	59	0.282	0.067	-2.800	**0.006**
		Women	50	0.319	0.069		
	PLT 10^9/L	Men	66	268.7	75.03	-1.875	**0.063**
		Women	58	294.1	75.50		
Control group	**.MPV fl**	Men	33	10.04	1.261	-1.348	0.192
		Women	26	10.45	0.998		
	PDW fl	Men	24	12.23	1.877	-0.058	0.895
		Women	23	12.27	1.901		
	PLCR %	Men	24	28.85	7.459	-0.666	0.688
		Women	23	30.29	7.336		
	.PCT %	Men	24	0.247	0.043	-2.805	**0.007**
		Women	23	0.284	0.047		
	PLT 10^9/L	Men	33	244.3	47.75	-1.631	0.108
		Women	26	264.2	44.70		

N – number; mean – *mean; SD – standard deviation.*

The exception was the results for PDW, which were higher in men: 12.936 ± 1.869fl vs 12.932 ± 2.535fl, p = 0.026. In the control group, a predominance was again reported in women for the mean levels of the variables, but the differences found were not significant. The exception was the PCT results - 0.25 ± 0.04% vs 0.28 ± 0.05% (Table 27).

From a subsequent intergroup comparison of platelet variables, significant differences were found for MPV, PCT and PLT in men and for PCT and PLT in women, and they were – F = 4.282, p = 0.041, F = 5.811, p = 0.018, F = 2.893, p = 0.092 and F = 4.980, p = 0.029, F = 3.517, p = 0.064, respectively.

The cases in the two tested groups were compared according to their MPV level (limited above and below 10%) in men and women. Women in the control group with MPV above 10% were 20 (76.9%) and men were 21 (63.6%). There is a difference with a predominance of women, but it is not significant - X^2 = 1.211, p = 0, 270. According to current data, the risk of women in the control group having an MPV above 10% is nearly twice as high as men - OR= 1.905 (95% CI 0.600-6.049). In patients with T1D, there is an reverse trend for the analyzed indicators. Men have a higher level of MPV cases – over 10%, and they are 48 (72.7%). Women with have this level of MPV respectively are 30 (65.5%). The difference between the groups thus compared is non-significant - p = 0.385. The reverse trend reported here would be the starting point for hypothesizing that men with T1D respond with increasing MPV levels above 10% and women show an opposite trend versus control cases.

6.2. Links with OPG and ADMA.

Table 28 presents correlations between OPG, ADMA and hematomorphological parameters. The rank values of each correlation coefficient and the interaction direction between the variables are also presented. The absolute rank value is determined by the force of the relationship of the established correlation, which is in three degrees: up to 0.30 is of rank 1 - this is a weak force of interaction between the tested variables; from 0.30 to 0.70 is an average force of interaction and is of rank 2; above 0.70 the interaction the force is defined as strong and accordingly, the rank is 3. In front of the rank are placed the signs “+” or “-“ depending on whether the direction is positive or negative, and where there is no significant correlation, the rank is 0.

No significant correlations were found between ADMA, OPG and leucocyte variables. In erythrocyte variables, three significant correlations were reported in

the control group, which were negative and weak in force - with rank - 1: between OPG and MCHC (r = - 0.261, p = 0.057) and ADMA and: Hb (r = - 0.290, p = 0.033), MCHC (r = - 0.268, p = 0.050). In T1D cases, two significant correlations were found in OPG and they were negative and weak in force – with rank - 1: with Hb (r = - 0.207, p = 0.027), with MCHC (r = - 0.187, p = 0.048). No significant links were recorded between ADMA and erythrocyte indicators (Table 28).

Table 28. Relationships between OPG, ADMA and erythrocyte variables.

Control group		**OPG (pmol/l)**	**Rank – correlation direction**	**ADMA (µmol/l)**	**Rank – correlation direction**
Hb (g/l)	Pearson Correlation P value Count	-0.231 0.093 54	**0**	-0.290 0.033 54	**-1**
RBC 10^12/L	Pearson Correlation P value Count	-0.157 0.256 54	**0**	-0.156 0.260 54	**0**
HCT L/L	Pearson Correlation P value Count	-0.153 0.270 54	**0**	-0.220 0.110 54	**0**
MCV fl	Pearson Correlation P value Count	0.050 0.718 54	**0**	-0.025 0.858 54	**0**
MCH pg	Pearson Correlation P value Count	-0.100 0.474 54	**0**	-0.160 0.248 54	**0**
MCHC g/l	Pearson Correlation P value Count	-0.261 0.057 54	**-1**	-0.268 0.050 54	**-1**
RDW-CV%	Pearson Correlation P value Count	0.190 0.169 54	**0**	0.130 0.350 54	**0**
Group with T1D		**OPG (pmol/l)**	**Rank – correlation direction**	**ADMA (µmol/l)**	**Rank – correlation direction**
Hb (g/l)	Pearson Correlation P value Count	-0.208 0.027 113	**-1**	0.002 0.982 114	**0**
RBC 10^12/L	Pearson Correlation P value Count	-0.158 0.096 113	**0**	0.007 0.943 114	**0**
HCT L/L	Pearson Correlation P value Count	-0.153 0.103 113	**0**	0.045 0.631 114	**0**
MCV fl	Pearson Correlation P value Count	0.024 0.797 113	**0**	0.077 0.417 114	**0**
MCH pg	Pearson Correlation P value Count	-0.072 0.445 113	**0**	-0.050 0.597 114	**0**
MCHC g/l	Pearson Correlation P value Count	-0.187 0.048 113	**-1**	-0.012 0.900 114	**0**
RDW-CV%	Pearson Correlation P value Count	0.102 0.284 113	**0**	-0.132 0.160 114	**0**
**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).					

Table 29 presents correlations between OPG, ADMA and platelet parameters. In those examined out of the control group, only one significant correlation between OPG and PCT was found and it was positive and weak in force: r = 0.290, p = 0.056. In patients with T1D, ADMA interacted significantly with MPV, the

association being negative and weak in force: r = - 0.188, p = 0.045. The observed correlation between OPG and PCT here again was positive (r = 0.166, p = 0.101), but not significant. In ADMA, linear regression analysis with dependent variable - MPV(fl) was applied as independent variable. A regression model of statistical significance was found against ST1RE in men with very high CVR: F = 6.666, p = 0.017, in a = 11.638, p = 0.000 and b = - 1.616, p = 0.017. The equation found for the relationship betweenthe variables is:

MPV (fl) = 11,638 – 1,616.ADMA (µmol/l)

The adjusted R^2 value was 0.198, indicating that 19.8% of the changes in MPV in men with very high CVR according to ST1RE, could be explained by the presented regression model.

Table 29. The correlations between OPG, ADMA and platelet indicators

Control group		**OPG (pmol/l)**	**Rank – correlation direction**	**ADMA (µmol/l)**	**Rank – correlation direction**
MPV fL	Pearson Correlation P value Count	0.005 0.969 54	**0**	-0.114 0.413 54	**0**
PDW fL	Pearson Correlation P value Count	0.191 0.214 54	**0**	-0.046 0.765 54	**0**
P-LCR %	Pearson Correlation P value Count	0.216 0.159 54	**0**	-0.054 0.726 54	**0**
PCT %	Pearson Correlation P value Count	0.290 0.056 54	**+1**	0.164 0.287 54	**0**
PLT x10^9/L	Pearson Correlation P value Count	0.193 0.163 54	**0**	0.109 0.432 54	**0**
Group with T1D		**OPG (pmol/l)**	**Rank – correlation direction**	**ADMA (µmol/l)**	**Rank – correlation direction**
MPV fL	Pearson Correlation P value Count	-0.078 0.411 113	**0**	-0.188 0.045 114	**-1**
PDW fL	Pearson Correlation P value Count	0.078 0.440 113	**0**	-0.122 0.227 114	**0**
P-LCR %	Pearson Correlation P value Count	0.064 0.527 113	**0**	-0.127 0.208 114	**0**
PCT %	Pearson Correlation P value Count	0.166 0.101 113	**0**	-0.119 0.240 114	**0**
PLT 10^9/L	Pearson Correlation P value Count	0.102 0.283 113	**0**	-0.058 0.539 114	**0**
**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).					

6.3. Links with ST1RE, ESC-2019 and Riskfactor3.

Using the Pearson correlation coefficient the linear relationship between the studied haematological parameters and ST1RE was analysed. With regards the erythrocyte variables, the correlations found were negative. In the platelet variables

on the one hand, an inverse dependency of ST1RE with PLT was registered, and on the other – a positive one with PDW (Table 30).

Table 30. Between ST1RE and blood count parameters.

		Lym 10^9/L	RBC x10^12/L	Hb g/l	MPV fL	PDW fL	P-LCR %	PLT x10^9/L
ST1RE	Pearson Correlation	-0,153	-0,224*	-0,142	0,138	0,190	0,154	-0,129
N(124)	**P value**	0,089	**0,012**	0,116	0,128	**0,048**	0,109	0,152
**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).								

Table 31 presents the correlation coefficients of associations between hematomorphological parameters and ESC-2019. The correlations found with Hb, RBC and HCT are not significant here, but by analogy with ST1RE are again in the opposite direction.

Table 31. Correlations between ESC-2019 and blood count parameters.

		Ba x10^9/L	RBC x10^12/L	Hb g/L	HCT L/L	MPV fL
ESC-2019	Pearson Correlation	-0.174	-0.125	-0.139	-0.138	0.107
N (124)	**P value**	**0.053**	0.167	0.124	0.126	0.236
**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed)						

Table 32 presents the correlation coefficients of a number of variables of reported significance or close to significant at RiskFactor3.

Table 32. Correlations between RiskFactor3 and haematological indicators.

		WBC 10^9/L	Ba 10^9/L	Mo 10^9/L	Hb g/l	HCT L/L	MCV fl	MCH pg	MCH C g/l	RDW-CV%	PCT%	PLT x10^9/L
RF3	Pearson Correlation	0.200	0.194	0.162	-0.265	-0.145	-0.212	-0.268	-0.197	0.138	0.228	0.190
N (124)	**P value**	**0.026**	**0.031**	**0.073**	**0.003**	0.108	**0.018**	**0.003**	**0.028**	0.126	**0.017**	**0.035**
**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed)												

6.4. Anemia and CVR in individuals with long-standing T1D.

Linear graphs of the arithmetic mean Hb levels (g/l) in men and women with T1D according to ST1RE and ESC-2019 are presented (Fig.36).

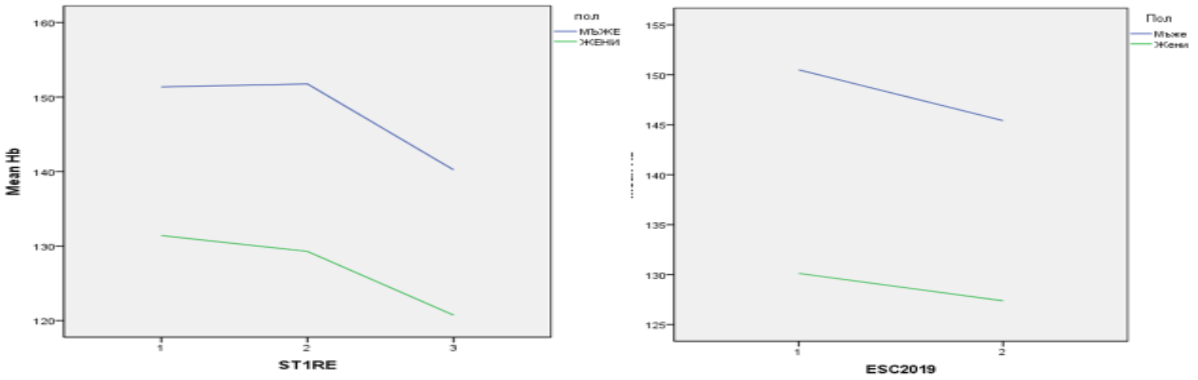

Figura 36. Anemia tendency in men and women according to ST1RE and ESC 2019.

The identified anemia tendency is best manifested in relation to RiskFactor3 (Fig. 37).

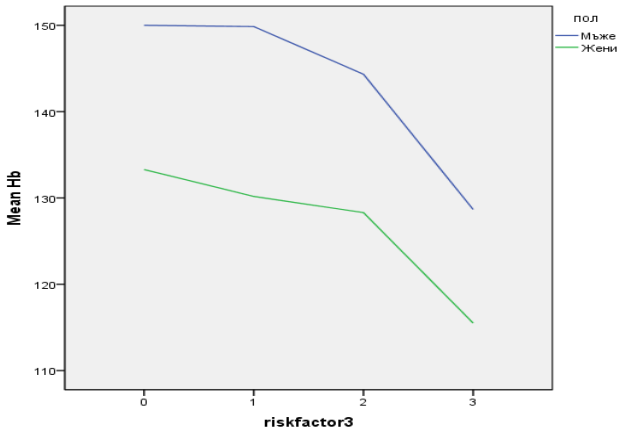

Figure 37. Tendency for anaemia (mean Hb g/l) in males and females according to Riskfactor3.

A statistically significant difference in mean Hb levels was found after administration of one-way factor variance analysis in ST1RE for men: F = 5.50, p = 0.006. After administration of Tukey's posthoc HDS test (Tukey), this difference remained significant between men with low and studied with high CVR (MD = 11.138 g/l, p = 0.023) and between men with moderate and those with high CVR (MD = 11.531 g/l, p = 0.014). In females, the difference recorded did not reach statistical significance (F = 2.48, p = 0.093). No statistical difference was found in the mean Hb levels between the ESC-2019 groups in men (t = 1.354, p = 0.181) and women (t = 0.594, p = 0.555). In Riskfactor3, a statistically significant difference was found in men: 148.25 ± 12.209 g/l for group 0.150.74 ± 14.047 g/l for group 1, 144.33 ± 14.493 g/l for group 2 and 128.67 ± 10.786 g/l for group 3 (F = 2.792, p = 0.048). After administration of Tukey's posthoc test, this difference turned out to be between significant group 1 and group 3 males (MD = 22.075, p = 0.052). In the females, the following Hb results are reported: 134.60 ± 10.213 g/l for group 0, 130.04 ± 15.392 g/l for group 1, 127.63 ± 16.067 g/l for group 2 and 115.50 ± 15.437 g/l for group 3 (F = 1.811, p = 0,156).

A regression model of statistical significance was found in men with high CVR according to ST1RE: F = 7.409, p = 0.012. 21.8% of the changes in Hb concentration in men with high CVR according to ST1RE, can be explained by the presented regression model:

Hb (g/l) = 11.673 – 0.041. OPG (pmol/l)

With ESC-2019, a pattern of statistical significance was found again in men with very high CVR: F = 7.213, p = 0.011 at a = 9.861, p = 0.000 and b = - 0.031, p = 0.011. 13.7% of changes in Hb concentration in men with very high CVR according to ESC 2019, can be explained by the presented regression model:

Hb (g/l) = 9.861 – 0.031.OPG (pmol/l)

A regression model with statistical significance against Riskfactor3 was found in group 2 men: F = 7.141, p = 0.015 at α = 12.381, p = 0.000 and b = - 0.047, p = 0.015. 23.5% of the Hb (g/l) changes in Group 2 according to Riskfactor3 can be explained by the regression model presented:

Hb (g/l) = 12.381 – 0.047. OPG (pmol/l)

In order to establish the linear combination between the serum ADMA levels and the Hb value in the different CVR categories, linear regression analysis was also applied, but no regression models of statistical significance were found.

6.5. Leucocytosis and CVR in patients with long-standing T1D.

There was no statistically significant difference between mean WBC for men and women depending on the CVR category. With ST1RE, it was F = 1.550, p = 0.220 for men and F = 0.785 p = 0.461 for women. At ESC 2019 - t = - 0.484, p = 0.157 for men and t = - 0.582, p = 0.412 for women. Riskfactor3 showed a tendency for leucocytosis in patients with T1D. Of close to significant significance, different effects were reported in women - F = 2.224, p = 0.096. No significant difference was found in males - F = 0.790, p = 0.504. The mean values for WBC were as follows: 6.513 ± 1.778. 10^9/L for group 0, 7.319 ± 1.969. 10^9/L for group 1, 6.97 ± 1.77. 10^9/L for group 2 and 8.23 ± 2.14. 10^9/L for group 3 in males and 5.748 ± 0.944. 10^9/L for group 0, 6.515 ± 1.797. 10^9/L for group 1; 7,384 ± 2,189. 10^9/L for group 2 and 8.108 ± 1.847. 10^9/L for group 3 in women (Fig. 38).

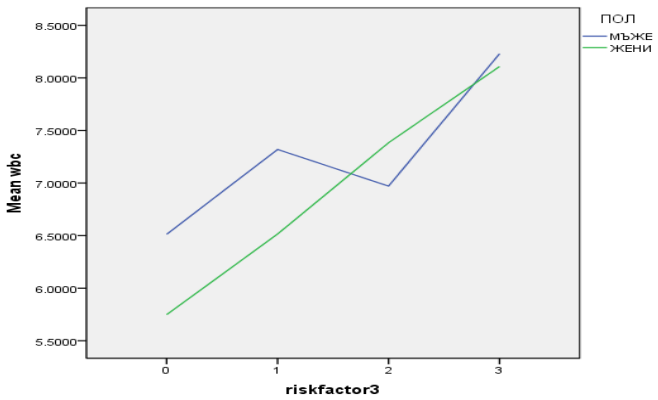

Figure 38. Arithmetic mean values for WBC according to Riskfactor3.

6.6. Links with ADNC and Lep.

Negative and mean force correlations were found in controls between ADNC and: WBC (r = - 0.309, p = 0.023); Mo (r = - 0.301, p = 0.027) and with Ly (r = - 0.330, p = 0.015). The listed correlations are of -2 rank. In patients with long-standing T1D, there was a change in correlation dependencies and, respectively, in ranks, and new correlations between Lep and: WBC (r = 0.177, p = 0.068); Mo (r = 0.260, p = 0.007) and Ly (r = 0.0180, p = 0.063). Therewere also negative associations between ADNC and: WBC, which is medium in force (r = - 0.248, p = 0.009); Neu with rank -1 (r = - 0.187, p = 0.049); Mo with rank -1 (r = - 0.247, p = 0.009); Ly with rank -1 (r = - 0.199, p = 0.036). Between the other variables, no significant ant correlations were recorded (Table 33).

Table 33. Correlations between ADNC, Lep and leucocyte variables.

Control group		ADNC (µg/ml)	Rank – correlation direction	Lep (ng/ml)	Rank – correlation direction
WBC 10^9/l	Pearson Correlation P value Count	-0,309* 0,023 54	-2	0,097 0,498 51	0
Neu 10^9/L	Pearson Correlation P value Count	-0,222 0,107 54	0	0,095 0,506 51	0
Eo 10^9/L	Pearson Correlation P value Count	-0.151 0,276 54	0	0,101 0,483 51	0
Ba 10^9/L	Pearson Correlation P value Count	0.012 0,931 54	0	0,040 0,782 51	0
Mo 10^9/L	Pearson Correlation P value Count	-0,301* 0,027 54	-2	0,049 0,733 51	0
Ly 10^9/L	Pearson Correlation P value Count	-0,330* 0,015 54	-2	0,049 0,735 51	0
Group with T1D		**ADNC (µg/ml)**	**Rank – correlation direction**	**Lep (ng/ml)**	**Rank – correlation direction**
WBC 10^9/l	Pearson Correlation P value Count	-0,248** 0,009 111	-1	0,177 0,068 108	+1
Neu 10^9/L	Pearson Correlation P value Count	-0,187* 0,049 111	-1	0,121 0,213 108	0
Eo 10^9/L	Pearson Correlation P value Count	-0,068 0,479 111	0	-0,032 0,741 108	0
Ba 10^9/L	Pearson Correlation P value Count	-0,072 0,454 111	0	-0,018 0,856 108	0
Mo 10^9/L	Pearson Correlation P value Count	-0,247** 0,009 111	-1	0,260** 0,007 108	+1
Ly 10^9/L	Pearson Correlation P value Count	-0,199* 0,036 111	-1	0,180 0,063 108	+1

**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).

In table 34 are presented correlations between erythrocyte variables and the studied adipokines. In the controls, three significant correlations with ADNC were found - negative and mean in force of interaction (r = - 0.463, p = 0.00 with Hb, r

= - 0.302, p = 0.026 with RBC and r = - 0.513, p = 0.00 with HCT). In individuals with T1D, a number of significant dependencies were found. The correlations between ADNC were with: Hb - r = - 0.423, p = 0.00; with RBC r = - 0.27, p = 0.004; with HCT – r = -0.381, p = 0.00 0; with MCV – r = -0.262, p = 0.005; with MCH – r = - 0.349, p = 0.00; with MCHC – r = -0.301, p = 0.001 and with RDW_CV r = 0.352, p = 0.00. The latter association was the only one established with a positive sign. In Lep, all the significant interactions found here were again negative. The corresponding correlations between Lep were with: Hb – r = - 0.355, p = 0.00 0; with RBC- r = - 0.257, p = 0.007; with HCT – r = - 0.325, p = 0.001; with MCHC – r = - 0.278, p = 0.004 and with MCH – r = - 0.181, p = 0.061 (Table 34).

Table 34. Correlation between adipokines and erythrocyte parameters.

Control group		**ADNC (µg/ml)**	**Rank – correlation direction**	**Lep (ng/ml)**	**Rank – correlation direction**
Hb g/l	Pearson Correlation P value Count	-0.463** 0.000 54	**-2**	-0.178 0.212 51	**0**
RBC 10^12/L	Pearson Correlation P value Count	-0.302* 0,026 54	**-2**	-0.222 0.117 51	**0**
HCT L/L	Pearson Correlation P value Count	-0.513** 0.000 54	**-2**	-0.144 0.313 51	**0**
MCV fl	Pearson Correlation P value Count	-0.165 0.233 54	**0**	0.116 0.418 51	**0**
MCH pg	Pearson Correlation P value Count	-0.127 0.360 54	**0**	-0.074 0.606 51	**0**
MCHC g/l	Pearson Correlation P value Count	-0.013 0.924 54	**0**	-0.131 0.361 51	**0**
RDW-CV%	Pearson Correlation P value Count	0.152 0.273 54	**0**	-0.149 0.297 51	**0**
Group with T1D		**ADNC (µg/ml)**	**Rank – correlation direction**	**Lep (ng/ml)**	**Rank – correlation direction**
Hb g/l	Pearson Correlation P value Count	-0.423** 0.000 111	**-2**	-0.355** 0.000 108	**-2**
RBC 10^12/L	Pearson Correlation P value Count	-0.270** 0.004 111	**-1**	-0.257** 0.007 108	**-1**
HCT L/L	Pearson Correlation P value Count	-0.381** 0.000 111	**-2**	-0.325** 0.001 108	**-2**
MCV fl	Pearson Correlation P value Count	-0.262** 0.005 111	**-1**	-0.056 0.566 108	**0**
MCH pg	Pearson Correlation P value Count	-0.349** 0,000 111	**-2**	-0.181 0.061 108	**-1**
MCHC g/l	Pearson Correlation P value Count	-0.301** 0.001 111	**-2**	-0.278** 0.004 108	**-1**
RDW-CV%	Pearson Correlation P value Count	0.352** 0.000 111	**+2**	0.153 0.114 108	**0**
**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).					

Table 35 presents correlations between the analysed adipokines and platelet indicators. No significant correlations were found in those examined by the control group. In T1D patients, two correlations were reported, weak in force and straight in direction: between Lep and PCT - r = 0.251, p = 0.014; Lep and PLT, r = 0.165, p = 0.088 (Table 35).

Table 35. Dependences between adipokines and platelet parameters.

Control group		**ADNC (µg/ml)**	**Rank – correlation direction**	**Lep (ng/ml)**	**Rank – correlation direction**
MPV fL	Pearson Correlation P value Count	-0.196 0.156 54	**0**	0.118 0.411 51	**0**
PDW fL	Pearson Correlation P value Count	0.126 0.417 54	**0**	0.045 0.780 51	**0**
P-LCR %	Pearson Correlation P value Count	0.200 0.192 54	**0**	0.074 0.646 51	**0**
PCT %	Pearson Correlation P value Count	0.126 0.415 54	**0**	0.229 0.150 51	**0**
PLT x10^9/L	Pearson Correlation P value Count	-0.216 0.118 54	**0**	0.112 0.433 51	**0**
Group with T1D		**ADNC (µg/ml)**	**Rank – correlation direction**	**Lep (ng/ml)**	**Rank – correlation direction**
MPV fL	Pearson Correlation P value Count	-0.062 0.516 111	**0**	0.061 0.528 108	**0**
PDW fL	Pearson Correlation P value Count	0.058 0.571 111	**0**	0.047 0.654 108	**0**
P-LCR %	Pearson Correlation P value Count	0.075 0.464 111	**0**	0.049 0.640 108	**0**
PCT %	Pearson Correlation P value Count	0.025 0.808 111	**0**	0.251* 0.014 108	**+1**
PLT 10^9/L	Pearson Correlation P value Count	0.062 0.518 111	**0**	0.165 0.088 108	**+1**
**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).					

6.6.1. ADNC – association with anemia and leucocytosis.

In individuals with T1D – 21% and in healthy individuals – 24% of the changes in serum ADNC levels can be explained by the presented regression models:

ADNC (µg/ml) = 54.599 – 0.230. Hb(g/l) – 1.175. WBC(10^9/L) with T1D;

ADNC (µg/ml) = 73.133 – 0.358. Hb(g/l) – 1.849. WBC(10^9/L) with controls;

Relative to **ST1RE**, statistical significance was found in men with moderate risk and women at low risk: F = 11.289, p = 0.001 and F = 6.108, p = 0.007, adjusted R^2 = 0.548 and adjusted R^2 = 0.290. With **ESC-2019** - in men at high risk (F = 3.618, p = 0.052) and at very high risk (F = 3.151, p = 0.055) and in women at very

high risk: F = 3.887, p = 0.030. The coefficients of determination found were adjusted R^2 = 0.235, adjusted R^2 = 0.102 and adjusted R^2 = 0.132, respectively. In **Riskfactor3** - in males of group 1 (F = 4.883, p = 0.016) and in females of group 0 (F = 14.690, p = 0.064) and 2 (F = 4.846, p = 0.025), in adjusted R^2 = 0.217, adjusted R^2 = 0.873 and adjusted R^2 = 0.325.

6.6.2. Lep - association with anemia, leucocytosis and thrombocytosis.

17.8% of the changes in serum Lep levels in subjects with T1D can be explained by the presented regression model:

Lep(ng/ml) = 17.157 – 0.122.Hb (g/l) + 0.852. WBC (10^9/L) – 0,043. PLT (10^9/L)

Of the four biomarkers studied, Lep was most strongly associated with platelet indices in individuals with long-standing T1D. 24.6% of the changes in Lep in women can be explained by the regression model presented:

Lep (ng/ml) = 133.8.6 – 2.923. MPV(fL) + 1.383. PDW(fL) + 2.369. PCT% - 2.224. PLT.10^9/L

A regression model with statistical significance is reported in the dependent variable - Lep and independent variables - MPV, PDW, PCT, P-LCR, PLT versus ESC-2019 in women with very high CVR (F = 3.464, p = 0.016). The value of adjusted R^2 is 0.284.

V. DISCUSSION

1. Assessment of CVR according to ESC guideline from 2019 and ST1RE with T1D.

The stratification of CVD risk in patients with T1D has an important clinical significance, which presupposes different therapeutic strategies. CVR estimation calculators use mathematical equations that include certain combinations of factors. When calculating the CVR for the general population, DM was presented as a dichotomous variable (yes/no) and therefore placed T1D patients always in a higher risk category. [10] The ADA recommends the application of algorithms that take into account T1D-specific risk factors and serve as a tool for CVD risk assessment. [28]

This interdisciplinary study is the first of its kind conducted in Bulgaria to assess the risk of CVD according to the criteria of specific scales - ST1RE and ESC-2019 in persons with long-standing T1D without registered historical CVD or a co-morbidity CVD. In the present study, it was found that 61.3% of the 124 T1D patients studied fell into the category of moderate to high CVR according to the ST1RE calculator. Given the reported average diabetes duration of 25.3 ± 8,224 years, all patients were identified as high-risk (30.6% with high CVD and 69.4% with very high CVR) according to the ESC-2019 criteria. Therefore, the frequency of high CVR found in individuals with long-standing T1D is in line with that established by a number of researchers. [49, 58, 118, 158, 169, 206, 232]

When considering gendered groups at risk, a significant difference was found only in ST1RE - $\chi 2 = 5.943$, $p = 0.051$. Subgroup analysis showed that men with T1D had a significantly higher calculated CVR than women: 71.2% of men versus 50% of women had moderate to high risk. In contrast to our results, some of the studies found in the literature found no differences between genders in stratifying the risk for T1D subjects [58], and according to others, a higher CVR has been reported in women. According to a meta-analysis of 214,114 individuals (2015), women with T1D have an approximately 40% higher risk of overall mortality and twice the risk of fatal and non-fatal vascular events than men with T1D. 112] In 2021, Colom C. et al. published results of epidemiological studies that show that the incidence of CVD is much higher in women with T1D. The relative risk of CVD in women, adjusted for age, is 4 to 10 times higher than that in men. [58] A possible explanation for the observed differences between genders in the cohort we studied compared to the data described in the literature are characteristic population

features of the sample, differences in geographical distribution, social status and methodological characteristics of the analyzed indicators.

2. Prognostic value of ADMA for estimation of CVR in patients with T1D.

2.1. ADMA – age and gender-related differences.

The present study did not find any statistically significant difference between groups in the serum ADMA levels, as cited in most literature in favor of individuals with long-standing T1D. The difference between genders we found in both study groups is unsignificant, but the reported higher values in women are noteworthy: 0.54 ± 0.24 μmol/l 0.52 ± 0.27 μmol/l with T1D versus 0.61 ± 0.14 μmol/l vs 0.54 ± 0.21 μmol/l in controls. In the study of Deneva T. et al. (2011) were derived reference range of ADMA in plasma by ELISA method among 150 healthy subjects aged 18 – 65 years. [67] In accordance with our results, the researchers found no significant differences between genders. A study by Fadhel B. et al. (2014) demonstrated higher serum levels for ADMA in healthy men versus women, and on the other hand described a significant increase in women after age 50. [78] Differences in hormonal status and onset of menopause in women are considered as an explanation for this fact. Given the average age of the cohort we studied – 46 ± 10 years, i.e. close to 50 years, we can confirm the thesis of Fadhel B. et al. (2014), Horowitz J. et al. (2007) for likely biomarker rise in females in this age group.

Statistical processing of the data showed a significant negative correlation between age and serum ADMA levels, but only in the control group ($r = -0.329$, $p = 0.015$). This dependence is consistent with that observed by other authors. [78,254] In the T1D cases, we found no significant correlation between the variables studied, as described by Ersoy B. et al. [76] When comparing patients to median diabetes duration (24 years), the results of our study demonstrated lower serum ADMA levels in subjects with longer disease duration (0.497 ± 0.239 μmol/l vs. 0.561 ± 0.271 μmol/l, $p = 0.184$). In unison with our results, Ersoy B. et al. [7 6] , Marcovecchio M. et al.[166] reported lower serum ADMA levels for patients with T1D with greater diabetes persistence and with advancing age. Huemer M. et al. [110] suggest that lower levels of ADMA in children and adolescents with T1D are an early indicator of impaired protection against oxidative stress. On the other hand, data from previous studies have reported a parallel increase in serum concentrations of ADMA relative to the duration of diabetes. According to Ersoy B. et al. [78], the increase of ADMA is a defense mechanism, both in terms of the development of subclinical atherosclerosis and in order to preserve myocardial

function. In addition, studies in non-diabetic adults have demonstrated that reported higher levels of ADMA are associated with diastolic dysfunction and cardiovascular risk profile. [209]

2.2. ADMA – links with ST1RE and ESC-2019.

In this interdisciplinary study, the relationship of the researched biomarker ADMA was evaluated for the first time on a global scale, with specific tools for the assessment of CVR in individuals with long-standing T1D. In the study of Markova Al. and co-auth. (2021) the role of ADMA and its association with CVR calculators was analyzed, but in patients with T2D. [10] The researchers found no dependence of the biomarker with any of the calculators used (UKPDS Risk engine, ADVANCE Risk Engine, SCORE and Framingham risk engine). [10]

We found a significant difference in the 90% confidence of the results for mean ADMA levels only between patients with moderate and those with high calculated CVR according to ST1RE (0.44 ± 0.21 μmol/l vs 0.57 ± 0.28 μmol/l, p = 0.078). The results obtained in our study are close to those indicated in the literature for patients with high CVR, but not identical, which could be explained by the size and characteristics of the analyzed cohort of patients with T1D and the method of analysis used. Long-term monitoring of patients is needed to make more reliable observations about ADMA levels with increasing CVD risk in T1D. According to meta-analysis, increased ADMA levels are associated with an increased risk of CVD and are an independent predictor of cardiovascular mortality in high-risk patients. [119, 281, 294] Therefore, in line with the hypothesis stated, we believe that parallel measurement of serum ADMA levels could improve the stratification of CVR in patients with T1D as a high-risk population.

2.3. ADMA – links with HbA1C, CRP, AlbU and RiskFactor3.

In the present study, the relationships between serum ADMA levels and the included variables in the proposed Riskfactor3 model (HbA1C, CRP and AlbU) were analyzed. We found no significant negative association between ADMA and HbA1C, as reported by a number of researchers in individuals with T1D. [76, 103, 110, 166, 254] On the other hand, it is important to note that we reported a decrease in mean ADMA levels in cases with poor glycemic control (HbA1C > 7%): 0.6064 ± 0.2467 μmol/l vs. 0.5168 ± 0.2568 μmol/l. The mechanisms by which high glucose levels lower blood ADMA concentration are not fully understood. The results of our study demonstrated poor glycemic control (HbA1C > 7%) in 84.6% of patients with T1D. According to most researchers, chronic hyperglycemia leads

to a decrease in plasma ADMA levels through mechanisms of hyperfiltration and accelerated renal clearance. An alternative hypothesis offers an explanation in the suppression of methylarginine synthesis or an increase in metabolism in the liver by DDAH. [254] Intensive insulin therapy in young patients with T1D has been reported to reduce the plasma concentration of ADMA by modulating DDAH activity. [254] In summary, the results of our study support in part the hypothesis that reduced NOS inactivation by ADMA acts as an important mechanism for endothelium-dependent relaxation damage in arteries exposed to high glucose levels.

When processing the data in the present study, the most significant finding in ADMA was the established relationship between serum biomarker levels and AlbU value in T1D subjects. The correlation found between ADMA and AlbU was positive and average in force: $r = 0.371$, $p = 0.000$. There was also a proportional trend observed of increasing in serum ADMA levels relative to the AlbU value (below 30mg/l; from 30 to 300 mg/l and above 300 mg/l): $F = 9.193$, $p = 0.000$. The results of the applied regression analysis confirmed the independent influence of AlbU on serum ADMA levels in individuals with long-standing T1D. In combination with a measurement of HbA1C%, AlbU was determined to predict 15% of the ADMA value in the patients studied. Therefore, our observed dependence confirms the association of methylarginine with AlbU, a routine marker for the assessment of OBD, established by a number of researchers.[149, 244, 291, 307] In the studied cohort of patients with long-standing T1D, microalbuminuria was reported in 20.2% and macroalbumnuria in 6.5% of patients with T1D. According to a study based on data and by the National Swedish Registry for Diabetic Patients (SNDR), HbA1C and AlbU are the most important predictors of mortality and CVD in T1D. [100] Poor glycaemic control was associated with a 2% higher risk of mortality (HR 1.02; 95% CI: 1.017-1.023) and micro- and macroalbuminuria with 2 to 4-fold higher CVR and T1D mortality.[100] Poor glycaemic control is a major factor in the development and progression of DN. Similar results are shared by researchers from FinnDiane (2018), DCCT/EDIC (2016) and EDC (2018).

Diabetic nephropathy (DN) has been identified as the leading cause of end-stage renal disease and is the strongest predictor of mortality in diabetes [27, 149, 291] Under pathological conditions, ADMA stimulates the development of oxidative stress and so performs a key role in the initiation and development of DN. [189] Proteinuria (AlbU) is a traditional marker for assessing the progression of renal impairment in DM. Experimental and clinical studies have reported that

elevated plasma levels of ADMA correlate with the degree of proteinuria. [189] Our results confirmed that ADMA was significantly increased in patients with micro- and macroalbuminuria relative to those tested with normoalbuminuria. Therefore, we recommend the implementing of ADMA in clinical laboratory practice and its parallel determination with routine markers (AlbU, uACR) to assess the presence of DN.

With respect to the proposed RiskFactor3 model and its defined groups, a statistical data processing showed an expected trend for proportional increases in serum ADMA levels. The found intergroup difference is non-significant, which could be explained by the presence of only one significant relationship between ADMA and the included variables in RiskFactor3 – described with AlbU. A logical significant correlation was found, but at 90% authenticity of the results between the serum levels of ADMA and Riskfactor3 ($r = 0.159$, $p = 0.092$). The distribution of studied patients with long-standing T1D according to the RiskFactor3 groups found the largest share for group 1 (47.58%), followed by group 2 (34.6 8%), group 0 (10.48%) and group 3 (7.26%). Therefore, the majority of T1D subjects have only one RF of the model included in the proposed model, which we attribute to poor glycemic control in nearly 90% of cases. The percentage share of patients with AlbU over 30 mg/ l and those with a CRP above 3 mg/l is comparable (about 30%), which explains the presence of two RFs in nearly 35% of cases. The presence of three RFs have the smallest percentage of cases and logically corresponds to the results obtained in the studied cohort.

2.4. ADMA – cut-off values compared to ST1RE, ESC-2019 and RiskFactor3.

ROC analysis demonstrated that ADMA as a stand-alone marker does not have sufficient diagnostic effectiveness in differentiating patients with low and moderate CVR from those studied with very high CVR according to the criteria of established models such as ST1RE and ESC-2019. In view of this unsatisfactory reliability, cut-off values were not derived. Sufficient diagnostic efficacy (65.3%) of ADMA is found only in women with respect to the proposed RiskFactor3 model. The derived threshold ADMA value for differentiating out groups 0 and 1 of 2 and 3 in females was 0.535 μmol/l with a sensitivity of 72.2% and a specificity of 63.2%. The specificity of the observed difference between genders in Riskfactor3 adds to the accumulating evidence of a more adverse effect of hyperglycemia on CVD risk in women than in men.[113] Previous studies have reported higher levels of coronary artery calcification and other ED indicators, as well as more extensive

atherosclerotic lesions associated with hyperglycemia in women than in men with T1D. According to Huxley and co-authors (2015), women have generally higher cumulative exposure to hyperglycemia throughout their lives due to poorer glycemic control compared to men. [113] Previous studies of individuals with T1D have shown significant differences between genders in plasma glucose and HbA1C control; young girls and women are more likely to be in permanently poor glycaemic control than young boys and men. The gender-related mismatch in glycaemic control is explained with impaired insulin sensitivity during puberty and increased tendency to eating disorders and insufficient insulin dosage in women, suffering from T1D. [113]

An alternative hypothesis explains increased CVR in T1D women with hypothalamic-pituitary-ovarian axis disorders: late age of menarche, menstrual disruptions and premature menopause. In their study, Koleva D. and co-authors (2017) analyzed results of plasma ADMA levels in 24 MS women aged 16-39 years, 38 women with PCOS aged 16-35 years, and 24 age-matched clinically healthy women.[8] The researchers reported comparable plasma levels of ADMA in women in the clinical groups and significantly higher than in healthy controls. Higher ADMA values in the women studied with MS and PCOS are an indicator of increased CVR. [8] The results in the present study partially support data from the literature that present ADMA as a biomarker for the assessment of CVR in DM. An association was found between elevated serum levels of ADMA and cardiovascular RF in T1D, such as AlbU and poor glycemic control. Nevertheless, the lack of sufficient diagnostic effectiveness of the biomarker against specific instrumental evaluation of CVR in T1D determines the need for targeted studies with a higher number of patients.

3. Prognostic value of OPG for estimation of CVR in patients with T1D.

3.1. OPG – age and gender-related differences.

The present study found a statistically significant difference between genders in OPG concentration, as is reported in most literature sources. In the two study groups, higher mean levels for OPG were found in women versus men: 5.7261 ± 1.8323 pmol/l vs 5.3417 ± 1.996 pmol/l in T1D subjects and 5.0555 ± 1.6451 pmol/l vs 6.1632 ± 2.3772 pmol/l in the control group cases, $p < 0.05$. According to a number of researchers, the difference between genders is more pronounced before menopause for women. After age 50, serum levels for OPG in men and women are described as similar, due to estrogen deficiency in women and its established influence on OPG synthesis. On the other hand, testosterone has an

inhibitory effect, which may partly explain the lower levels of OPG in young men compared to premenopausal women, as well as the negative association between OPG and testosterone in older men. [33, 129, 223] In our study, the average age in men and women is lower than 50 years and therefore suggests a difference between genders as we find.

Results of the present study confirm the quoted negative relationship between age and serum OPG levels in men. The correlation we found in the men of the control group is negative and large in force: r = - 0.565, p = 0.001. On the other hand, we confirm part of the data in the literature that age and duration of DM are significant positive determinants for serum OPG levels in patients with T1D. [25, 74, 219] In a number of studies, OPG concentrations were found to increase with age. [98, 129] The mechanisms responsible for the relationship between OPG and age are not fully understood, but changes in bone metabolism with advancing age, as well as changes related to glucose homeostasis and vascular physiology, have been suggested. The influence of the duration of DM as an independent variable on the concentration of OPG was reported higher (5%) than that of age (3%) in the cohort of patients (p = 0.05) we studied. According to a 2020 meta-analysis, the results on the relationship between the duration of the disease and serum OPG levels are contradictory: in some cases it is positive, in others it is not reported. [55] In the study of Boyadzhieva M. et al. (2013) no analogous relationship with the T2D duration has been registered. The researchers found comparable serum OPG levels between men newly discovered, and tested with known T2D.[2] All this necessitates further research in this direction.

3.2. OPG – links with ADMA.

When processing the data, the present study found a significant positive correlation between OPG and ADMA values in the two study groups ($p < 0.05$). There is a growing number of studies examining the dependence between the two markers. [54, 176, 266, 289] Tsioufis C. et al. (2011) found the indicated correlation relationship in patients without DM and with essential hypertension. According to the researchers, ADMA as a variable in linear combination with AlbU had an independent influence on serum OPG levels and predicted 11.7% of its concentration variations. [266] The mechanisms that have been proposed to explain this relationship are as follows: increased levels of OPG in response to ADMA - mediated vascular dysregulation even before the development of hypertension; increased collagen formation and end products of advanced glycation and last but not least the impact of both biomarkers on eNOS activity. ADMA and OPG are

classified as ED markers. Augoulea et al. (2012) investigated the relationship between serum OPG levels and endothelium-dependent arterial dilation in patients with newly discovered T1D. [31] The researchers found elevated OPG values significantly associated with ED markers. [31] Further studies are needed to establish the synergistic action of ADMA and OPG on vasculature and respectively development of CVD with DM.

3.3. OPG - links with ST1RE and ESC-2019.

The current interdisciplinary study assessed the association of the studied biomarker OPG with established scales for the assessment of CVR in individuals with long-standing T1D for the first time on a global scale. A proportional trend is observed of increasing serum OPG levels relative to the CVR category according to ST1RE and ESC-2019, which however did not reach statistical significance. With gender differentiation, a significant difference was recorded in the mean biomarker levels between males with low and high CVR (4.99 ± 1.229 pmol/l vs 5.914 ± 1.218 pmol/l, $p = 0.041$) and between males with moderate (4.89 ± 0.858 pmol/l) and those studied with high CVR ($p = 0.012$). The recorded correlation between serum OPG and ST1RE levels was significant and shows a straight direction ($r = 0.183$, $p = 0.053$). No significant association was found between OPG and ESC-2019. Therefore, the results of the present study confirm the association of OPG with risk of CVD development with DM with respect to ST1RE risk stratification. We see the identified correlations between the biomarker and a number of variables from the CVR assessment calculator – ST1RE (with gender, age, duration of diabetes, AlbU, glycemic control) as an explanation for the reported greater association of OPG with ST1RE compared to the ESC-2019. We can confirm the hypothesis of Boyanov M. et al., according to which risk calculators assign weights to different RFs (degree of transfer). [3] In ESC-2019, individual RFs do not receive different weight, are not quantified, and assume some subjectivity.

The ability to determine the category of CVR based on serum protein levels could provide tremendous benefit in stratifying risk, centralizing resources, and eliminating the need for further research in a large segment of patients. Browner et al. [42] and Olesen et al. [188] were some of the first researchers to demonstrate the link between serum OPG levels and the progression of diabetic complications. Gordin et al. [94] defined the biomarker as an independent predictor of CVC in DM. Subsequently, the relationship between the severity of atherosclerotic plaques, of CAD (coronary arterial disease) and serum OPG levels has been described

multiple times. [36] A number of researchers have analyzed OPG – the associated mechanisms for the development of CVD, as well as the prognostic role of the CVS biomarker in DM. [276, 31, 265, 276] As high-risk populations, patients with T1D and T2D showed increased values for OPG versus healthy controls. [55, 265] The latter were reported as significantly higher in persons surviving CVE. It is important to note that in response to intensive insulin treatment (6 months), serum OPG levels were reported to decrease significantly. [276] There is also conflicting data in the literature on the impact of statin treatment on circulating OPG in patients with very high CVR - in some cases it lowers it, in others it increases it, and there is even a variation between the impact of different drugs. [37] Among the patients studied by Boyadzhieva M. et al. (2013), those with newly discovered T2D showed similar results for serum OPG, regardless of the presence or absence of known CAD. Patients with proven CAD underwent standard statin therapy that could affect OPG levels and be a possible reason for the lack of differences between the non-CAD group.[41]

According to literature data, OPG is associated with a large number of cardiovascular RFs: age, smoking, hypertension, insulin resistance, obesity, DM, renal impairment. The relationship between OPG and CVD remains significant even after the precise control of the listed dependent RFs, suggesting additional effects of other factors. [265] Raaz-Schrauder D. et al (2017) studied 414 individuals with moderately high CVR assessed according to the Framingham Risk Score and ESC criteria and found a significant correlation between plasmic OPG levels and a number of atherogenic cytokines.[216] The role of OPG in the development of diabetic macroangiopathy is not fully understood. Although the biomarker is associated with the development of CVD, the source of expression is still questionable. Boyadzhieva M. and co-authors (2013) found a significant association between serum OPG levels and carotid IMT levels in men with newly-diagnosed T2D, which gives them reasons to assume that vascular changes are involved in the regulation of the protein or that it is an important regulatory molecule in the development of vascular dysfunction early in the course of diabetic evolution. [41]

It is interesting to note that we found significant differences between serum OPG levels depending on the categories of CVR in men only. This gives us reason to suppose that the regulation of osteoprotegerin synthesis in men and women with long-standing T1D varies in the presence of CVD. On the other hand, a possible explanation would be that the insufficient number of patients as a representative sample in the present study, which we consider as a limiting factor.

3.4. OPG – links with HbA1C, CRP, AlbU and with RiskFactor3.

In the present study, a significant positive correlation was found between serum OPG levels and the proposed RiskFactor3 model. After application of a one-factor variance analysis, a significant difference, but at 90% confidence in the results, confirmed the proportional tendency to increase in the OPG values: in group 0, the average OPG level was 4.972 ± 0.489 pmol/l; in group 1, it was 5.433 ± 1.51 pmol/l; in group 2 was 5.51 ± 1.69 pmol/l; in group 3 - 6.76 ± 1.35 pmol/l, F = 2.466, p = 0.066. With subsequent gender differentiation, a significant difference at 90% confidence of the results was found only between group 1 and group 3 males (5.113 ± 1.039 pmol/l vs 6.823 ± 0.567 pmol/l, p = 0.0 74). In women, we assume that with a larger number of studied subjects, the reported difference will reach statistical significance.

When partially analyzing the interactions between serum OPG levels with each of the variables included in RiskFactor3, a significant association with AlbU (r = 0.218, p = 0.021) was recorded. In contrast with previous studies, we found no significant positive correlations between serum OPG and HbA1 C% levels. [55, 80, 199] and CRP. [37] It is important to note that in the T1D patient group, higher OPG values were reported at HbA1C above 7% (compared to those with HbA1C below 7%) and CRP above 3mg/l (compared to those with CRP below 3mg/l), which, however, are not statistically significant. When examining a larger number of patients, the observed differences are likely to reach statistical significance.

In individuals with long-standing T1D we found a significant difference in serum OPG levels between those testing with AlbU below 30 mg/l and AlbU above 300 mg/l (MD = 1.53 pmol/l, p = 0.032) and at 90% authenticity of results - between patients with AlbU from 30 to 300 mg/l and AlbU above 300 mg/l (MD = 1.45 pmol/l, p = 0.080). The results of linear regression analysis demonstrated an independent influence of a variable AlbU in predicting the concentration of OPG in individuals with long-standing T1D, which equals 4%. In the group of controls, an analogous association is not registered. This fact gives us once again reason to confirm the thesis of differences in OPG levels under physiological and pathophysiological conditions.

The results of the current study confirmed the association of OPG with AlbU in DM found in the literature. DN is a major microvascular complication in DM, found in over 40% of patients with high duration of diabetes. [72] DN is diagnosed in routine clinical laboratory practice by means of the assessment of albuminuria (uACR, uAER) and eGFR. In the study of Perez de Cirza C. et al. (2015)

hypotheses have been presented by a number of researchers about the positive association of OPG with DN in T1D. [201] The combination of DN and poor glycaemic control, as we found in the majority of the studied cohort of patients, is considered a major prerequisite for the development of CVD. For a period of nearly 10 years, Gordin D. et al. (2013) monitored 1939 adult individuals with T1D and defined OPG as an independent predictor of CI. [94] Researchers reported a 2.7-fold higher CVD in patients with macroalbuminuria (cox regression 2.70 (1.67-4.37), $p < 0.001$). [94] According to Jorsal A. et al. (2008), plasma OPG is an independent predictor of total and cardiovascular mortality in patients with nephropathy. [121] Researchers support the hypothesis of OPG accumulation in the arterial wall, leading to generalized vascular changes and calcification in individuals with long-standing T1D. [121] Grauslund J. et al. (2010) found a significant difference in OPG levels between patients with macro-, microalbuminuria and those studied with normoalbuminuria at a mean T1D duration of 43 years. [96] Later, Elsamahy M. et al (2015), Wang Sh. et al (2013), Fekih O. et al (2016) again demonstrated significantly higher OPG scores at AlbU above 30 mg/24h versus AlbU below 30mg/24h. [74, 80, 282] In summary, the results of our study support the hypothesis that under conditions of poor glycemic control, OPG is positively associated with AlbU and respectively with the development of CI in persons with long-standing T1D.

3.5. OPG – cut-off values compared to ST1RE, ESC-2019 and RiskFactor3.

The results of the applied ROC analysis against the established scales for the assessment of CVR in T1D demonstrated that OPG has a good prognostic value (approximately 70%) for men and women relative to ST1RE. The derived OPG threshold values for males and females at differentiating low to moderately high from those with very high CVR according to ST1RE are as follows: 5.075 pmol/l with a sensitivity of 70.8% and a specificity of 55.9% for males and 5.355 pmol/l for females with a sensitivity of 66.7% and a specificity of 60%. With ESC-2019, the marker had a good prognostic value only for women - approximately 65%. The derived threshold value of OPG when differentiating high from very high CPR is 5.025 pmol/l with a sensitivity of 70% and a specificity of 60%. With regard to ST1RE, ROC The analysis eliminates the differences commented above from a correlation analysis – here the results are significant for both genders. On the other hand, it is important to focus on the applied criterion - high CVR, which in our opinion implies a better prognostic value of the marker in high-risk patients.

In conclusion, our findings confirm that OPG is a potential biomarker for the assessment of CVD in individuals with long-standing T1D. We recommend additional research with a larger number of patients and clarification of pathophysiological mechanisms in the context of CVD with T1D. Implementing OPG in routine clinical-laboratory practice is useful for better stratification of CVR and adequate treatment.

4. Prognostic value of ADNC for estimation of CVR in patients with T1D.

4.1. ADNC - age and gender-related differences.

The results of the present study are consistent with some data in the literature that report differences between genders in serum/plasma ADNC levels. Women in the two study groups scored significantly higher on adipokine compared to men: 14.964 ± 16.998 μg/ml for women versus 6.781 ± 2.415 μg/ml for men in the control group and 18.394 ± 12.551 μg/ml for women versus 11.237 ± 5.667 μg/ml for men in the T1D group, $p < 0.05$. The difference between genders is explained by differences in hormonal status and body fat distribution. [240, 260] In the study by Kaza M. et al., (2022) no difference between genders was found in children and adolescents with T1D.[126] Lausten-Thomsen U. et al. (2015) studied a total of 1,193 healthy Danish non-obese students (730 girls, 463 boys) aged 6-18 years with the aim of deriving paediatric reference values and found no significant difference between genders. [143] Some authors have suggested that an increase in androgen concentration during puberty in men is the age period from which levels begin to differ, and a proportional increase in difference between genders with advancing age has also been reported. [144] Kuo S. et al. (2011) analyzed study results in 4,852 healthy adults (between 18 and 59 years) and rejected the hypothesis of differences between genders influenced by body fat distribution and BMI. According to the researchers, reported gender-related changes in plasma ADNC levels are the result of metabolic disorders. [139]

It is important to note that in the current trial, serum ADNC levels in men and women with long-standing T1D were statistically significantly higher than those in controls ($p < 0.05$). The difference found was confirmed by a number of studies and meta-analyses. [193, 303, 211, 240] We confirm the hypothesis of the so-called "adiponectin paradox". The synthesis of ADNC as an anti-inflammatory factor is suppressed in obesity, MS, T2D and CVD. On the other hand, circulating hormone levels are increased in immune-mediated diseases such as T1D, which implies pro-inflammatory action and participation in immunity. [139, 303]

The mechanisms responsible for a significant increase in ADNC concentration in patients with T1D are not yet fully understood. Men with a longer duration of the disease showed higher levels of the hormone (12.327 ± 5.662 μg/ml) compared to those tested with a duration of less than 24 years – 9.625 ± 5.394 μg/ml. We have no explanation for the insignificant differences from the median T1D duration in women. A possible reason is an insufficient number of people with long-standing T1D, gender-conditioned peculiarities or analytical characteristics of the reagent kit used.

The results of the current study did not confirm the positive association found by a number of researchers between serum ADNC levels and the age of patients. [143,144] Regarding the duration of diabetes, we found a positive dependence, as cited in the literature [126,144,163], but only in men with long-standing T1D ($p < 0.05$). According to Lindstrom T. et al. (2006), disease duration is the strongest predictor of ADNC concentration, followed by age independent of BMI changes in T1D subjects. [148] According to the researchers, the observed association is due to age-related hormonal changes that regulate the secretion of ADNC. On the other hand, there is evidence that residual β-cell function of the pancreas, as assessed by quantification of C-peptide, correlates negatively with ADNC-levels. [148] As the duration of diabetes progresses, endogenous insulin synthesis requires more high-intensity treatment with exogenously imported insulin, which increases the expression of ADNC-gene in 3T3–L1 adipocytes. [300] In T1D, exogenous insulin is injected subcutaneously and probably potentiates the secretion of ADNC from this tissue and creates conditions for peripheral hyperinsulinemia. Such a mechanism would explain why the traditional negative association between BMI, waist circumference or insulin levels and circulating ADNC was not found in participants with T1D in a number of studies. Therefore, we can assume, that high ADNC levels in T1D patients with no evidence of micro- and macroangiopathies are probably due to the local effect of exogenously imported insulin on ADNC release from subcutaneous fat. However, this hypothesis should be supported by further studies.

In the current study, we found a significant negative dependence between serum ADNC and BMI levels in T1D subjects, as described by a number of researchers ($r = - 0.466$, $p = 0.000$ for men and $r = - 0.241$, $p = 0.080$ for women). In the control group, the described correlation relationship lost its significance.

4.2. ADNC - links with ADMA.

An interesting positive correlation linkage in the group of male controls we observed, was that between serum ADNC and ADMA levels, r = 0.430, p = 0.020. In accordance with other researchers, Koleva D. et al. (2016) merge the two variables as markers of ED in the context of an atherosclerotic process. [134] ADNC activates eNOS and respectively the synthesis of the NO vasodilator, and ADMA is a single-gene inhibitor of eNOS. Paduszyńska Al. et al. (2020) found a negative dependence between serum ADMA and ADNC levels in obese patients and a mean age of 53 years [194], Hamee E. et al. (2014) – in children with obesity and Heilman K. et al. (2009) – in children with T1D. [101,103] Eleuterio N. et al. (2022) investigated the association between ADMA and ADNC in pregnant women with preeclampsia. [73] The researchers assigned the patients to group 1 (with high serum ADMA levels) and group 2 (with low serum ADMA levels) and found significantly higher ADNC values in the first group. Higher ADMA concentrations probably interfere with physiological activation of eNOS by ADNC. Therefore, the biological mechanism behind this finding is still unknown, which limits the in-depth discussion on our part. We find no data in the literature on the observed dependence in healthy individuals.

4.3. ADNC – links with ST1RE and ESC-2019.

In the current interdisciplinary study, the association of the investigated ADNC biomarker with T1D-specific CVR assessment tools: ST1RE and ESC-2019 was assessed, as well as with the proposed RiskFactor3 model. A significant correlation relationship was observed only between serum adipokine levels and RiskFactor3, *rho* = - 0.23, p = 0.015. The negative sign of the association was also maintained in the reported connections of ADNC with ESC-2019 and ST1RE, but did not reach statstatic significance. There were statistically significant differences in serum ADNC levels in men and women of different categories of CVR. It is noteworthy that the median of ADNC results in patients with moderately high CVR is higher than that recorded in those tested with very high CVR according to the ESC-2019 guidelines and the ST1RE calculator, i.e. a decrease in the concentrations of the hormone is reported. The results found so far are consistent with the demonstrated trend by many researchers to proportionally lower serum ADNC levels with increasing CVD risk. On the other hand, median ADNC in men and women with low CVR are lower than those reported in subjects with moderately high CVR according to ST1RE. Therefore, the results of this trial do

not allow us to draw a definite conclusion about serum ADNC levels against the ESC-2019 guidelines and the CVR category according to the ST1RE calculator.

Data in the literature are contradictory regarding the relationship between ADNC and cardiometabolic characteristics in T1D subjects. According to Menzaghi Cl. et al. (2018), elevated ADNC levels are an independent predictor of total and cardiovascular mortality in adult T1D individuals. Malecha-Jedreaszek A. et al. (2012), Sheriff D. et al. (2017) reported higher serum ADNC levels in patients with chronic complications versus individuals without CI. [163, 239] Meta-analysis of early studies [170] suggested that the paradoxical relationship between increased ADNC and increased mortality was observed only in the presence of CVD. Similar independent associations were reported later in asymptomatic adult subjects of the general population and in patients affected by several diseases, including CAD, peripheral arterial diseases, CKD and cancer. It has recently been reported, that ADNC levels predict cardiovascular mortality in a gender-specific manner, with the paradoxical effect observed in men but not in women. [170] A similar sexual dimorphism is also described for the associations between ADNC and CKD and an increase in carotid IMT. [170, 205] Other authors confirm the hypothesis that reduced serum ADNC concentration is associated with an increased risk of CAD even with T1D, independent of conventional RF, markers of inflammation and insulin resistance. [60, 156] Le Caire T. et al. (2015) reported lower ADNC values in patients with T1D and present microvascular complications. [144] According to Menzaghi Cl. et al. (2018), the paradigm for the beneficial role of ADNC on metabolic, inflammatory and atherosclerosis processes has been derived mostly following pioneering studies carried out on cellular and animal models [84, 231], in which the main findings are consistent with results from early smaller studies on humans. [170] However, when considering large epidemiological and genetic studies, it becomes apparent, albeit completely unexpected, that high serum ADNC is prioritized as a marker of insulin sensitivity and glucose homeostasis and is neutral with respect to CVR, cardiovascular mortality, and overall mortality. [38, 108, 170]

4.4. ADNC – links with HbA1C, CRP, AlbU and with RiskFactor3.

The statistical data processing showed that ADNC values in males and females depending on the group defined by the proposed RiskFactor3 model were without significant differences. We observed a proportional decreasing trend in ADNC concentrations with increasing number of RFs defined as groups according

to RiskFactor3. In women, unlike men, the observed trend is more explicit, but again did not reach statistical significance.

When partially analysing the results against individual RFs (HbA1C, CRP and AlbU), a difference in ADNC values was reported in women versus CRPs below and above 3 mg/l (21,500 ± 14,012 μg/ml and 12,054 ± 5,184 μg/ml, p = 0,01). In men, with increasing CRP value, there was again a decrease in serum ADNC levels, but did not reach statistical significance. After applying a correlation analysis, in patients with T1D, the relationship between ADNC and CRP did not reach statistical significance, such that has been reported in a large number of studies. It is important to note that in women with T1D the recorded correlation between variables is negative (r = - 0.224, p = 0.107) and in men - positive (r = 0.181, p = 0.177). No significant correlation relationship was reported in the control group.

CRP is one of the most sensitive inflammation markers. In experimental and epidemiological studies, a positive association of CRP with the development of atherosclerotic process and CVD has been established. Yuan G. et al. (2007) report for the first time that CRP suppresses adiponectin gene expression and hormone secretion by 3T3-L1 adipocytes. [300] Some researchers found a greater effect of the observed reciprocal relationship in women versus men and explained it with a higher body fat percentage in women. Putri E. et al. (2021) and Abraham A. et al. (2017) assessed a pro-inflammatory status associated with metabolic control and insulin resistance in adult T1D individuals. [16, 215] Researchers confirmed the inverse relationship between ADNC and CRP, as well as with pro-inflammatory cytokines (TNF-α and IL-6) and emphasised the incidence of MS in patients with T1D, which is similar to that found in the general population. MS patients have increased abdominal fat, and visceral adipocytes have intense secretory activity and are considered equivalent to endocrine organs. The presence of MS in young individuals with T1D is associated with increased insulin resistance and a pro-inflammatory condition. This leads to a premature increase in the global CVR. [24]

The pro-inflammatory condition associated with MS in patients with T1D further impairs glycaemic control, increases the required daily dose of insulin and is closely related to the development of chronic diabetic complications. In summary, a negative correlation between ADNC and CRP favors the hypothesis of anti-inflammatory and antiatherogenic action of the hormone the only adipocytokine that enhances insulin-mediated glucose uptake.

Regarding glycaemic control, no significant differences were found in the mean ADNC levels for men and women with HbA1C above 7% compared to those

studied with HbA1C below 7%. The results of the correlation analysis of the two variables also did not reach significant importance. In the present study, we found a non-significant decrease in ADNC values in patients with HbA1C over 7% compared to those tested with HbA1C below 7% (13.963 ± 5.226 μg/ml vs. 10.916 ± 5.677 μg/ml, p = 0.216 for men and 19.323 ± 5.456 μg/ml vs. 18.232 ± 13.444 μg/ml, p = 0.823 for women). According to Pilacinski S. et al. (2016), 'hyperadiponectinemia' is associated with chronic hyperglycaemia in T1D subjects. [208] Glycosylation of lysine residues in the collagen domain of ADNC is an important mechanism of posttranslational modification. [125] A positive association between HbA1C and ADNC in T1D was noted primarily in children or adults without recorded complications, but not in all studies.[208] In addition, there is evidence that the relationship between ADNC and HbA1C could be masked by the development of DKD. [144] On the other hand, various cross-sectional studies have documented a negative association of ADNC with obesity, hypertension, dyslipidemia, fasting plasma glucose levels, and insulin resistance, which are known RFs for the subsequent development of T2DM. [193, 303]

In the present study, no statistically significant differences in ADNC levels versus AlbU rates were found in the T1D patients. It is important to note that, notwithstanding this fact, an inverse relationship between ADNC and AlbU is observed, which is to be expected according to most literature sources. Adipokine has been reported to increase with the progression of AlbU in patients with T1D, which is consistent with the described hyperadiponectinemia in patients with CKD. [167, 193, 204, 248] High plasma concentrations of ADNC decreased after kidney transplant [167, 286]. In studies of patients with T2D, paradoxical decreases in ADNC levels with parallel progression of AlbU levels were observed. [193] Patients with proteinuria had increased insulin resistance compared to patients without proteinuria, and this explained a decrease in serum adipokine levels in patients with T2D and proteinuria. [193]

ADNC is an adipokine that has been reported to have renoprotective effects via AMPK-activated pathways and thus prevents micro- and markoalbuminuria. The relationship between circulating ADNC and AlbU has been reported to indicate a two-phase pattern. There is a negative relationship in the case of normoalbuminuria, possibly reflecting increased renal clearance along with the renoprotective effects of adipokine, and a positive association with macroalbuminuria indicating the counteraction of increased ADNC synthesis as a compensatory response to renal impairment. [53]

In the present study, the fact that a very strong correlation between serum ADNC and AlbU levels was found in the female controls, but not in the T1D group, was of particular interest. After application of linear regression analysis, we found that AlbU determined 58.6% of the changes in ADNC concentration in healthy women. Therefore, based on the hypothesis that ADNC has renoprotective properties, the positive dependence found should be a physiological response to prevent further renal damage in the controls. In individuals with long-standing T1D, the hypothesis of elevated serum ADNC levels due to decreased renal clearance was not supported by our study. Elevated serum ADNC levels in the patients studied were found to be independent of renal function, assessed with the degree of AlbU. Further clinical studies are therefore needed to confirm the nephroprotective role of ADNC in DM patients.

4.5. ADNC – cut-off values compared to ST1RE, ESC-2019 and RiskFactor3.

In the current trial, ROC analysis demonstrated that ADNC as a stand-alone marker does not have sufficient diagnostic effectiveness in differentiating patients with low to moderately high CVR from patients with very high CVR according to the criteria of T1D-specific CVR assessment tools - ST1RE and ESC-2019. In view of this unsatisfactory marker reliability, cut-off values have not been derived. Therefore, our findings do not support the cause-and-effect relationship between ADNC and CVD risk in patients with long-standing T1D. Conclusions from a large-scale Mendelian-type randomization study to assess the relationship between serum ADNC and CVD risk are also supportive of our findings. Data from genome-wide study consortia were used (CARDIoGRAM - 22,233 CVD cases and 64,762 controls and CARDIoGRAMplusC4D Metabochip - 63,746 cases of CVD and 130,681 controls) with detailed phenotyping of CAD, AMI, or both. Researchers do not support the protective role of ADNC in CVD and show that the relationship between genetically elevated ADNC levels and lower CVR is mainly driven by horizontal pleiotropy. [38]

5. Prognostic value of Lep for estimation of CVR in patients with T1D.

5.1. Lep - age and gender-determined differences.

The present study examined the influence of BMI, age, gender, and duration of diabetes on serum Lep levels. An expected positive correlation between serum Lep levels and BMI was found in the two study groups: $r = 0.667$, $p = 0.000$ for men with T1D and $r = 0.635$, $p = 0.000$ for women with T1D; $r = 0.806$, $p = 0.000$ for healthy men and $r = 0.762$, $p = 0.000$ for healthy women. The correlation found

confirms the established link by a number of researchers, as well as the importance of BMI in deriving reference values.

The results of the statistical processing also demonstrated an difference between genders in serum Lep levels, which is well established by a number of researchers. [48, 117, 221] Women in both study groups had higher mean values for Lep: 6.1908 ± 4.045 ng/ml versus 3.0815 ± 1.998 ng/ml in controls and 8.211 ± 6.679 ng/ml versus 2.863 ± 2.948 ng/ml in T1D subjects. It has been suggested that higher levels of Lep in women involve the different pattern of bodily fat distribution and/or the role of sex hormones. [117] It has been shown that after menopause, women have lower serum concentrations of Lep than during the fertile period. [48, 221] It was also found that adipocytes isolated from the fatty tissue of female donors, secrete significantly higher amounts of Lep under culture conditions than those from males. [48, 117, 221] In-vitro studies have shown that estradiol enhances the release of Lep when incubating adipose tissue samples of female donors, but not of male donors. [117] Other researchers have reported that estrogen administration to postmenopausal women has no effect on circulating Lep and have ruled out that estrogens are responsible for differences between genders.[117] It is also possible that sexual dimorphism in Lep concentrations is related to male gender and due to androgens. Several crosssectional studies have reported that in males testosterone is negatively correlated with serum Lep levels, irrespective of BMI. In in-vitro studies, Wabitsch M. et al. (2017) and Funcke B. et al. (2014) demonstrated a direct long-term inhibitory effect of testosterone on the production of Lep by human adipocytes under culture conditions. [85, 279]

Patients with long-standing T1D showed higher values for Lep compared to controls. This fact is consistent with the results of a number of researchers. It is interesting to note that in women, higher values for Lep are recorded with duration of diabetes below 24 years (9,976 ± 8,799 ng/ml) compared to those studied with a duration of more than 24 years (7,0525 ± 4,625 ng/ml, $p = 0.050$), and in men, the observed trend was reversed (3.4685 ± 3.443 ng/ml at a duration over 24 years versus 1.926 ± 1.618 ng/ml at a duration below 24 years, $p = 0.048$).

A significant correlation was recorded between serum Lep levels and the age of men in the two study groups: $p = 0.034$ for men with T1D and $p = 0.027$ in healthy men. Consequently, the observed tendency to increase the concentration of Lep in healthy men with age is also maintained under pathophysiological conditions - the presence of T1D. It is a well-known fact that ageing affects body composition with a decrease in total muscle mass and an increase in fat storage

depos. It is suggested that these phenomena are associated with changes in serum Lep levels and/or its synthesis. Based on previous studies which reported that the concentration of Lep was reduced, unchanged or even increased with ageing, the relationship between age and Lep is not yet clear. In previous studies, Lep levels have been shown to increase in old and very old adult males and remain unchanged in young and postmenopausal women. [117] The reason for these conflicting results is not understood. A likely explanation is the variability in the experimental design, different ages and/or BMI of the study subjects, statistical analysis, or the fact that often the age-Lep relationship is not the main focus of most of the studies.

In conclusion, the results of this trial support the hypothesis that serum Lep levels are gender- and BMI-dependent and increase in men with advancing age, while remaining unchanged in women. With regard to the duration of diabetes, the average levels of Lep in men change naturally with age. Significant decrease is reported in women, which is probably an expression of pathophysiological mechanisms.

5.2. Lep - links with OPG.

In the current study, a significant positive correlation between serum Lep and OPG levels was found in men with long-standing T1D. In women with T1D and the controls, there was no significant correlation between these variables. The first suggestion that Lep can regulate bone resorption is by Holloway et al. [222], which prove Lep - conditioned inhibition of osteoclastogenesis in culture media of human peripheral blood mononuclear cells. Other researchers found that Lep increased OPG synthesis and decreased RANKL levels in the stromal cells of human bone marrow. [222] Therefore, the positive correlation found between OPG and Lep corresponds to the hypothesis made. As an explanation for the observed dependence in men alone, we propose the theory of gender-conditioned hormonal regulation. The inhibitory influence of testosterone on Lep and OPG simultaneously suggests legitimate changes in serum marker levels. In females, the described influence of estrogens on Lep concentration is contradictory, and is considered positive on OPG concentration and accordingly does not suggest a similar relationship between the two markers.

5.3. Lep - links with ST1RE and ESC-2019.

In the current study, the prognostic value of the Lep biomarker was assessed against the CVR category, calculated using the ST1RE calculator and according to the ESC-2019 guidelines. The results of the applied correlation analysis showed a

significant positive association of Lep with ST1RE (*rho* = 0.549, p = 0.000) as well as with ESC from 2019 d. (*rho* = 0.361, p = 0.006), but only in men with long-standing T1D. One-factor variance analysis confirmed the relationships found and proved significant intergroup differences in serum Lep levels in men vs. ST1RE (p = 0.002) as well as ESC-2019 (p = 0,053). In females, a significant difference in serum Lep levels we found between the high and very high CVR subgroups according to the ESC-2019 criteria. (p = 0,043). Therefore, the higher the CVR in patients with long-standing T1D, the higher values for Lep are reported.

Recent studies have shown that under physiological conditions, Lep is an important factor in regulating energy balance, but in pathophysiologically determined hyperleptinemia, adipokine is associated with CVD progression. [179] This effect is likely mediated by various atherogenic effects of Lep, including its effect on blood pressure, platelet aggregation, plaque instability, and inflammatory vascular response. High levels of Lep are thought to be associated with arterial rigidity, a lower index of circulatory function, and have been found to be involved in the pathogenesis of the atherosclerotic process. [179]

Azar S. et al. (2002) report that Lep levels are low in newly diagnosed patients with T1D and increase after initiation of insulin therapy, regardless of changes in body weight. [32] It is possible that this is due to the stimulating effect of insulin on Lep production. Patients with intensified insulin therapy have higher levels of Lep than patients with conventional insulin therapy. The study of Atwa H. et al. (2018) investigated the association between adipokines (Lep and ADNC) with carotid IMT in children and adolescents with T1D. [30] The researchers found a positive correlation between Lep and carotid IMT and concluded that adipokine may serve as a reliable non-invasive marker for ED and subclinical atherosclerosis in children and adolescents with T1D. The described association between Lep and IMT has also been confirmed in adults. [62]

5.4. Lep - links with AlbU, CRP, HbA1C and with Riskfactor3.

We also analyzed the relationship of the marker with the proposed RiskFactor3 model. The Spearman's Rho rank correlation coefficient between serum Lep and RiskFactor3 levels in men was again straight in direction but was significant at 90% authenticity of the results: rho = 0.23, p = 0.084 between Lep and RiskFactor3. In females, notable significance was found in 95% authenticity of the results: *rho* = 0.437, p = 0.001. One-factor variance analysis demonstrated significant differences in serum Lep levels in males and females from different groups according to RiskFactor3 ($p < 0.05$).

In subsequent partial analysis of RF, we found that serum Lep levels correlated with the AlbU value in individuals with long-standing T1D ($p < 0.05$). The observed dependence was confirmed by a number of researchers who reported that serum Lep was significantly higher in patients with micro- and macroalbuminuria compared to controls and normoalbuminuric DM patients. [109, 297]. According to Tony A. et al. (2022), serum Lep is an independent RF for the development of DKD. With regard to T1D, data in the literature is limited. [261]

The results of the statistical processing showed a significant positive relationship between serum Lep levels and CRP, as reported by a number of researchers. In our country, the indicated correlation relationship was established in the study of Lateva M. et al. (2015) in preschoolers with abdominal obesity. [9] In the present trial, we found that 23.8% of the changes in serum Lep concentration could be explained by changes in AlbU and CRP values in women with long-standing T1D and in healthy women 29.4%. In men with T1D, only AlbU was found to have an independent influence on serum Lep levels – 11.9%.

The correlation beween CRP and Lep suggests a relationship between endothelium activation and chronic inflammation. CRP is defined as a strong predictor of CVD. [24] T1D is thought to create a proinflammatory medium in which the concentration of cytokines produced by macrophages, adipose tissue and endothelium is induced by hyperglycemia. [51] Lep in turn stimulates the production of various cytokines, including IL-6. Because LEPR mediates intracellular signaling with a specificity similar to IL-6-type receptors, Lep can regulate the production of CRP not only through IL-6, but also through LEPR. An alternative explanation is that obesity enhances the production of both Lep, and of a cytokine regulating CRP synthesis without causality between Lep and CRP. However, further research is needed to determine the intracellular mechanisms by which Lep regulates CRP production. Furthermore, the role of CRP in the development of leptin resistance has also been proposed, but should be further analysed. [51] The identified differences between genders in relation to the relationship between these markers in the current study necessitate further research.

5.5. Lep – cut-off values relative to ST1RE, ESC-2019 and RiskFactor3.

In the current study, serum Lep was found to rise two- to three-fold in men with high CVR according to ST1RE. Men and women with very high CVR showed almost twice as high Lep values as those tested with high CVR according to the ESC-2019 guidelines. These findings confirm the hypothesis that *hyperleptinemia* is associated with elevated CVR. According to a meta-analysis, summary results

from 7580 participants, serum Lep levels were positively and significantly associated with the risk of increased arterial rigidity (DOR coefficient 1.04; p <0.01). [62] According to the conclusion of another meta-analysis with 1,904 randomly selected adults enrolled in the Multiethnic Atherosclerosis Study (MESA), the highest tertile of Lep scores was statistically significantly associated with 4% (1–7%) greater progression of CAC over an average of 7 years. [274] Another report reported that serum concentrations of Lep were associated with adverse cardiac remodeling in patients with CAD. [79]

In logarithmic regression analysis, we found that if the concentration of Lep increased by 1 ng/ml, then the chance of the patient falling into a high CVR category according to ST1RE increased by 1.7-fold for men (95% CI 1.17-2.45). In the ESC-2019, the chance of a patient shifting from a high to a very high CVD category was 1.404-fold in men (95% CI 0.971-2.029), respectively. In women, the results did not reach an analogous significant significance (p = 0.081). We assume that the reason for this difference is the limited number of patients with T1D or population characteristics and features studied in the representative sample.

After administration of ROC analysis, it can be summarized that Lep has better prognostic value in differentiating men with high CVR versus ST1RE (approximately 80%) and ESC-2019 (approximately 70%). The deduced threshold values were 2.28 ng/ml and 1.38 ng/ml, respectively. The difference in threshold values confirms the aforementioned not so good correspondence between the CVR assessment scales with T1D. In women, Lep has sufficient diagnostic accuracy in distinguishing high from very high CVR only according to ESC-2019. (approximately 60%) at a cutoff value of 5.475 ng/ml. Each increase in Lep concentration by 1 ng/ml resulted in an increase in the chance that the patient had ≥ 2 risk factors (HbA1C ≥ 7%, CRP ≥ 3 mg/l or AlbU ≥ 30 mg/l) by 1.21-fold in women (95% CI 1.05-1.4). ROC analysis demonstrated that in females, Lep had sufficient diagnostic accuracy in distinguishing groups 0 and 1 from groups 2 and 3 of RiskFactor3 (approximately 70%) with a cut-off value of 5.815 ng/ml. In men, no similar important significance was reported.

In summary, of the four biomarkers analysed, Lep turned out to have the best prognostic value against established scales for assessment of CVR with T1D. The diagnostic reliability of the marker in men is better than that recorded in women.

6. Hematomorphological changes and CVR with T1D.

6.1. Tendency for anemia in persons with long-standing T1D.

Data processing showed that mean levels of Hb, HCT, RBC, MCV and MCH were lower in patients with T1D compared to those reported in healthy subjects. Only RDW_CV% was higher in patients compared to controls. Although the differences found did not reach statistical significance, we could make an inference about an observed trend of microcytic, hypochromic anemia with evidence of anisocytosis in persons with long-standing T1D, which is exacerbated by poor glycaemic control. We reported significantly lower values for MCV and MCH in patients with HbA1C% above 7%. We can therefore confirm the proposition made in a large part of the studies that are found in the literature. [19, 89, 97]

In the current study, we found almost twice the proportion of men with anemia in the T1D group (16.7%) compared to those studied by the control group (9.1%). In T1D, the proportion of women with anaemia was again found to be greater than in the controls, but the results were closer (20.7 in T1D versus 15.4% in controls). Anemia is a common and underrecognized complication in patients with T1D. One in five (~20%) individuals with T1D have Hb levels below the reference range. [19, 89] Patients with DM and anaemia have an increased risk of adverse effects of diabetic retinopathy, neuropathy, nephropathy, and CVD. [89] The most discussed cause of DM anemia is reduced erythropoietin production by cortical interstitium cells, which may worsen microvascular complications. Other causes are systemic inflammation, suppression of erythropoietin release, medication, kidney damage, altered iron metabolism, and chronic hyperglycemia. [26, 89]

T1D-related anemia is primarily associated with autoimmune causes such as autoimmune gastritis and pernicious anemia. [226] Patients with autoimmune gastritis are often diagnosed with iron deficiency anemia (microcytic, hypochromic), which may precede pernicious anemia or both may coexist. Iron deficiency anemia occurs in 20-40% of patients with autoimmune gastritis, while pernicious anemia can be identified in 15–25% of patients. [226] Decreased acidity of the stomach or hypo/achlorhydria in autoimmune gastritis, due to the destruction of H+/K+ATP-ase-containing parietal cells, reduce the availability of iron for absorption and lead to the development of iron deficiency anemia. [226] Pernicious anemia, which is considered to be end-stage autoimmune gastritis, is a consequence of altered absorption of vitamin B12. In this study, we reported significantly higher values for MCH and MCV in people with diabetes duration over 24 years, which

determines a tendency for macrocytosis and is consistent with the described causes of anemia exacerbation in persons with long-standing T1D.

The higher the CVR according to ST1RE and ESC-2019, the lower the Hb concentration in individuals with long-standing T1D. In ST1RE we also reported a statistically significant difference in Hb concentration in men. The higher the number of RF (HbA1C > 7%, AlbU > 30mg/l and/or CRP > 3mg/l), the more definite the tendency for anemia. These findings are in line with those found in DM by a number of researchers. In our country, the relationship between the presence of anemia and CVD is well studied by Dimova M. et al. (2019) and Georgieva Zh. et al. (2012). [6, 88] The risk of anaemia in diabetic patients is estimated to be two to three times higher than in patients without diabetes.[226] The early evidence suggests that cases of anaemia in DM are usually associated with the presence of kidney disease. [226] The risk of developing anaemia in patients with DM associated with DKD is greater than in patients with otherwise-caused kidney disease. [226] However, the early onset of anaemia in patients without renal disease suggests the presence of other causes in such patients. [155] Patients with poor glycaemic control are at a higher risk of developing anaemia compared to patients with good glycaemic control and the risk further increases with the onset of kidney disease. [20, 226] Therefore, screening, prompt diagnosis and correction of anemia are critical to improving clinical outcomes and quality of life in T1D patients.

6.2. Tendency for leucocytosis in persons with long-standing T1D.

A statistical processing of the results showed significantly higher values for WBC, Neu and Eo in subjects with long-standing T1D versus cases from the control group ($p < 0.05$). This finding is in line with the trend of leucocytosis in DM established by a number of researchers. [18, 89] Leucocytosis is one of the main components of the inflammatory process, which contributes to atherosclerotic progression and CVD. [89] Haematological changes have been shown to significantly increase blood viscosity, adversely affecting microcirculation and leading to micro- and macroangiopathy. [89] Patients with T1D have a higher absolute number of Neu, which a number of researchers say is associated with an increased risk of vascular disease. [89] Excessive production of Neu by the bone marrow and participation of marginal cells (from a parietal pool) in the circulation reservoir explains the increase in circulating granulocytes with subsequent secretion of pro-inflammatory cytokines and adhesion molecules. In conditions of poor glycaemic control, as we found in nearly 90% of the studied cohort of patients with T1D, angiotensin and cytokines are considered to stimulate the formation of

mono- and polymorphonucleae. [89] As opposed to the observations described, in the study by Harsunen M. et al. (2013) newly diagnosed adults with T1D were studied who reported lower total WBC, Neu, Ba, Mo and Ly counts compared to control subjects. [102] According to the researchers, leucocyte changes are not a consequence of chronic hyperglycemia, but suggest a direct involvement of innate immunity in the pathogenesis of T1D even before its development with impaired "leucocyte homeostasis". We did not report significant differences for Ba, Mo and Ly between the study groups in the present study.

The observed trend of leucocytosis in persons with T1D is not maintained with regard to the duration of diabetes, which is in contrast to the findings of some authors and in congruance with others. [89, 175] According to Adane T. et al. (2021), reduction in the total number of polomophorphonuclear WBCs is expounded with functional changes in cells. [18] Furthermore, extreme cytokine production can lead to inappropriate activation, tissue damage and increased vulnerability to pathogenic microorganisms. Therefore, the increased response of Neu in DM can be considered as part of diabetic pathophysiology.

6.3. Changes in platelet parameters in persons with T1D.

The present study demonstrated significantly higher numbers of PLT and platelet indices in T1D patients than in controls. Only with P-LCR no intergroup significant difference was found, but again mean levels were higher in T1D subjects. PLT and PCT were significantly higher in patients with poor glycaemic control. This finding is consistent with that established by a number of researchers. [18, 66, 187, 269] In DM, PLTs were found to have increased baseline activation rates as well as increased activation and aggregation responses induced by different stimuli. Altered morphology and function of PLT has been reported in DM patients. [18] There is a strong association between platelet dysfunction and platelet hyperactivity in both T1D and T2D. [18, 66, 187, 269] PLTs have unregulated signaling pathways that result in an increased tendency to activate and aggregate in response to a given stimulus. [18] Hyper-reactive phenotype of PLT may be the cause of inadequate response in patients with DM to antiplatelet compared to patients without DM. [18] Due to microhemorrhages in atheromatous plaques, the bone marrow receives a signal to release spare and immature giant PLTs. A study by Eibl et al. showed that patients with DM had greater expression of markers of PLT activation compared to an age-matched non-diabetic control group.[71]

In the current study, we found that relative to median diabetes duration (24 years), platelet indices (MPV, PLC-R) increased significantly, but a decrease in the

total number of PLT was reported. MPV reflects the average size and function of PLTs. PLC-R is an index for the relationship between PLT and large cells and is inversely proportional to PLT and directly related to PDW and MPV. PLC-R% is increased by reactive thrombocytosis and decreased by thrombocytopenia. The latter is another haematological abnormality seen with DM as the duration of diabetes progresses and is associated with the risk of haematomas or bleeding during insulin injections. [89] With regard to PLT, the results of our survey and those available in the literature are contradictory, which necessitates additional research in this area.

Hyperreactivity of PLT in individuals with long-standing T1D is a significant finding in the current study. Recent research has found that MPV is a strong and independent predictor of CVD in DM. [131,241] It is considered that the higher the MPV, the more likely is thrombus formation and damage to the vascular endothelium. MPV correlated positively with PLT adhesion and aggregation. Larger PLTs were more active due to increased content of prothrombotic factors, such as thromboxane A2, thromboxane B2, platelet factor 4, serotonin and platelet-derived growth factor (PDGF). MPV reflects the reactivity of PLT, younger and physiologically more active, with greater prothrombogenic potential compared to small mature PLTs. According to recent studies, MPV is a promising biomarker for risk stratification and CVD progression. A meta-analysis of case-control and cross-sectional studies showed that high MPV was positively associated with the incidence and angiographic severity of CAD. [241]

In men with T1D, we found significantly higher MPV values versus healthy men. An interesting finding we made is that the risk of control women having an MPV above 10% is nearly twice as high as men – OR = 1.905 (95% CI: 0.600-6.049), and in the group of T1D individuals, a reverse trend is registered. The latter would be the starting point for positing the hypothesis that men with T1D react with increasing MPV levels above 10%, and women show an opposite trend to control cases. Therefore, men with long-standing T1D show higher PLT activity and respectively have a higher CVR. Platelet size differentiated during megakaryocytopoiesis and thrombopoiesis and did not always positively correlate with their age. Several studies have reported that MPV negatively correlates with age of PLT. [162] This is probably a consequence of the compensatory accelerated platelet consumption in order to maintain a constant functional activity of the platelet mass. [162] In support of the hypothesis, we also found an inverse relationship between MPV, P-LCR, PDW and platelet count, as well as between PLT and diabetes duration and the age of T1D patients. Therefore, we could assume

that with advancing age and duration of diabetes, the proportion of reactive PLT in T1D increases. The higher calculated CVR according to ST1RE significantly correlated with the relative proportion of platelet anisocytosis (PDW).

In the current study, we identified significantly higher values for all platelet indicators (MPV, PLC-R and PLT) in women versus men with T1D. The exception is the results for PDW, which are higher in men ($p < 0.05$). In the control group, we again reported a predominance in women for the mean levels of the variables, but the differences found did not reach notable significance except for PCTs. A statistical processing of the data in our trial also demonstrated significantly higher values for PCT and PLT in men and women with T1D versus men and women in the control group ($p < 0.05$). The identified differences between genders are in line with those found by other researchers. [23] In the study of Ali U. et al. (2019), 2,376 samples were analyzed for a full blood count of the Sysmex XN-10 hematology analyzer with a view to deriving gender-dependent reference intervals. [23] The researchers reported higher values for MPV, PLC-R and PCT in women, and for PDW they found no statistically significant difference in terms of gender. Contrary to our results for PDW, some of the researchers also found no significant differences in PDW values in men and women. [15, 39, 164] A likely explanation is variation in the preanalytical stage (venipuncture method, time differences between blood collection and sample analysis, sample transport and storage, etc.), heterogeneity of the studied cohorts by age and ethnicity, different ratio of men and women.

Platelet indicators (PLT, MPV, PDW, P-LCR, PCT) do not require specialized hemostasiologic equipment. They are determined with automatic hematological counters and are derived from the results for full blood count, which determines their efficiency in clinical diagnostic terms. The potential diagnostic applications of platelet indices have expanded beyond the limits of differential diagnosis of platelet disorders. Variations in PLT, MPV, PDW, PLC-R and PCT are associated with CVD, autoimmune and inflammatory diseases. Therefore, the derivation of reference values according to gender determines the need for additional multicenter and multiethnic prospective studies to confirm their use in routine clinico-laboratory practice.

6.4. Dependencies between ADMA, OPG and hematologic indicators.

In the present study, we investigated the relationship between ADMA, OPG and hematomorphological indicators. In the subjects of the control group, we found a significant negative correlation between the serum levels of the indicated

biomarkers and the mean levels of Hb and MCHC ($p < 0.05$). Under pathophysiological conditions (T1D), these dependencies persisted only with respect to OPG. In addition, we analyzed the influence of OPG as a variable on Hb concentration in patients with T1D of different categories of CVR according to ST1RE and ESC-2019. We found that 21.8% of the changes in Hb value, but only in men with very high CVR according to ST1RE could be explained with an increase in serum OPG levels, according to the ESC-2019. – 13,7%. Regarding men in group 2 according to Riskfactor3, 23.5% of the changes in Hb concentration can be explained in an analogous way. Therefore, the increase in serum levels of ADMA and OPG suggests the development of anemia, with men being more vulnerable than women with T1D. This trend is also confirmed when analyzing the correlations between erythrocyte variables and established scales for the assessment of CVR in T1D (ST1RE and ESC-2019), as well as with the proposed RiskFactor3 model.

The results of the correlation analysis between platelet indicators and serum biomarker levels (ADMA and OPG) demonstrated an interesting negative relationship between MPV and ADMA ($r = - 0.188$, $p = 0.045$). In addition, we found that 19.8% of the MPV changes in men with very high CVR according to ST1RE could be explained with variations in serum ADMA levels. We found no identical association in the literature we studied. In the study by Gawrys J. et al., 2020 the concentration of intraplatelet ADMA was determined and its association with activation and aggregation of PLT in DM was assessed. [98] The researchers believe that the pathophysiology of altered PLT function in response to impairment of glucose metabolism should be studied in detail in view of the reported high resistance to antithrombotic therapy. Gawrys J. et al. did not report a correlation between plasma ADMA levels and intraplatelet ADMA, but found a higher mean concentration of the latter in DM patients versus controls. Intraplatelet ADMA correlated with increased PLT activity assessed with ADP-induced aggregation. [98]

6.5. Relationships between ADNC, Lep and hematologic parameters.

A statistical processing of the results of the present trial demonstrated a negative correlation between serum ADNC levels and a number of leucocyte variables. Early studies have reported that ADNC is a negative regulator of hematopoiesis and immune function. [61] ADNC has been identified as a growth factor for haematopoietic stem cells, and serum levels of ADNC are inversely related to the risk of chronic lymphocytic leukaemia, acute myeloid leukaemia,

myelodysplastic syndrome and multiple myeloma. [61] ADNC is synthesized by Lym, and its receptors, AdipoR1 and AdipoR2, are expressed by precursor cells in the bone marrow. ADNC is considered to have an inhibitory effect on the formation of granulocyte-macrophage colony-stimulating factor. Our results confirmed a significant negative relationship between serum ADNC levels and total WBC, Neu, Mo and Ly ($p < 0.05$) in healthy subjects and in patients with long-standing T1D. Therefore, the functional role of ADNC as a negative regulator on leukopoiesis (anti-inflammatory effect) is also maintained under pathophysiological conditions.

In the present study, we found significant correlations between leucocyte variables (total number of WBC, Mo and Ly) and serum Lep levels, but only in the T1D group ($p < 0.05$). Unlike ADNC, in Lep the established correlations are positive and confirm the pro-inflammatory effect of the hormone. According to a number of researchers, Lep is involved in the mechanisms of leucocytosis in DM and the subsequent development of micro- and macroangiopathies. LEPRs were found in Neu, Mo and Ly. [130] Expression of LEPRs is also established in immune cells, which also explains the involvement of Lep in innate and acquired immunity. [50] Lep modulates nautrophil activation, monocyte-macrophageal cytokine production, and potentiates the cytotoxicity of NK-cells. On the other hand, the hormone stimulates B cell proliferation and reduces T- and B-cell apoptosis or in summary, Lep has a proinflammatory effect on the immune system. [50] There is only a limited number of studies that investigate the relationship of Lep with WBC in T1D.

In contrast to the opposite relationships we found in ADNC and Lep versus WBC in the present trial, the association with erythrocyte variables was negative in both biomarkers. We found that the higher the serum adipokine levels, the greater the tendency for microcytic, hypochromic anemia with anisocytosis in persons with long-standing T1D. Therefore, the negative regulatory role of ADNC on leukopoiesis is also preserved on erythropoiesis. Of particular note is the fact that in healthy individuals, serum levels of ADNC are negatively associated with RBC, Hb and HCT only, while no significant correlation is reported with Lep. Given the described observations for iron-deficiency anemia in DM and its morphological classification of microcytic, hypochromic anemia, we assume the involvement of adipokines in its development. We found that 21% of the changes in serum ADNC levels in T1D subjects and 24% in healthy subjects, respectively, could be explained by variations in Hb concentration and WBC. Subsequent regression analysis depending on the category of CVR according to ST1RE demonstrated that changes in serum ADNC levels predicted 54.8% of the variation

in Hb and WBC in males at moderately high-risk and 29% in low-risk women. According to the ESC-2019 guidelines, we found a significance of the regression model for men at high (23.5%) and very high risk (10.2%) and women at very high risk (13.2%). Therefore, the results for serum ADNC levels correspond to the described tendency for anemia and leucocytosis in persons with long-standing T1D, and again men are more vulnerable than women.

We found no significant correlations between serum ADNC levels and platelet indicators, but reported a positive association between Lep (ng/ml) and: PCT and PLT ($p < 0.05$). Classified as a cytokine, Lep complements its proinflammatory effect by influencing PLT function and exerting a prothrombogenic effect, respectively. In vitro and ex vivo studies found that Lep has been shown to potentiate ADP-stimulated platelet aggregation. [162] This dependence is in consensus with the available literature data on probable Lep-mediated thrombocytosis with hyperreactivity and subsequent thrombogenesis. The mechanisms described were initiated by binding of Lep to ObRb expressed in platelet membrane as initial stage and activation of JAK/Stat signalling system as a second intermediary. [162]

In the current study, we found that 24.6% of changes in Lep concentration in women could be attributed to changes in platelet performance (MPV, PDW, PCT and PLT), while no statistical significance was reported in men. In women with very high CVR according to ESC-2019, the reported influence is equal to 28.4%. With regard to T1D, the literature data is insufficient. We find no explanation for the lack of statistical significance in men. This fact necessitates the need for additional studies in larger cohorts to accurately analyze the observed trend. In the current study, we reported that 17.8% of the changes in: Hb (g/l), WBC and PLT predicted changes in serum Lep levels in patients with T1D and vice versa. Further processing of the data relative to ST1RE demonstrated statistical significance of the regression model in men with very high CVR, with a reported impact of 19.7%, and 32.1% for women with low CVR. With regard to the ESC-2019, this is 18.9% for men and 14.8% for women with very high CVR, respectively. In summary, while the regulatory role of ADNC on hematopoiesis was observed under physiological and pathophysiological conditions in this trial, Lep was mainly commented on in patients with T1D. This fact suggests pathologically-determined mechanisms of Lep on hematopoiesis and necessitates the need for further research.

VI. CONCLUSIONS

1. In patients with T1D, a significant positive correlation was found between the AlbU value and serum **ADMA** levels. Combined with HbA1C determination, AlbU predicted 15% of the ADMA concentration in T1D test subjects.
2. **ADMA** as a stand-alone biomarker does not have sufficient diagnostic effectiveness in differentiating patients with very high CVR according to the criteria of established models such as ST1RE and ESC-2019.
3. **OPG** has a good prognostic value (approximately 70%) for men and women, relative to ST1RE, when differentiating individuals with high CVR. With ESC-2019, OPG has a good prognostic value in differentiating women with very high CVR – approximately 65%. OPG cut-off values ≥ 5,075 pmol/l for men and ≥ 5,355 pmol/l for women vs ST1RE and ≥ 5,025 pmol/l for women compared to the ESC-2019 point to a very high CVR and require urgent preventive measures.
4. The age of the patients, the duration of diabetes and the presence of micro/macroalbuminuria were significant positive determinants for serum **OPG** levels in individuals with long-standing T1D.
5. **ADNC** as a stand-alone biomarker does not have sufficient diagnostic effectiveness in differentiating patients with very high CVD according to the criteria of T1D-specific instruments for the assessment of CVR - ST1RE and ESC-2019.
6. In women with T1D, a negative correlation relationship between **ADNC** and CRP was found, and in healthy women - 58.6% of the variance in the measured AlbU concentrations was associated with variance in the ADNC results.
7. With men from the both study groups, there was a tendency of increasing the concentration of Lep with advancing age. In men with T1D, an independent positive influence on serum **Lep** levels has an AlbU value of 11.9%.
8. In females, AlbU and CRP were significant positive determinants for serum **Lep** levels. These variables predicted 23.8% of the changes in serum Lep concentration in women with long-standing T1D and 29.4% in healthy women.
9. **Lep** has very good diagnostic effectiveness in differentiating men with very high CVR versus ST1RE (approximately 80%) and ESC-2019 (approximately 70%). The cut-off values deduced were ≥ 2.28 ng/ml and ≥ 1.38 ng/ml, respectively. Any increase in Lep concentration of 1 ng/ml resulted in an

increase in men's chance of falling into a very high CVR category by 1.7-fold according to ST1RE and by 1.404-fold compared to the ESC-2019. In women, Lep had sufficient diagnostic efficacy according to ESC-2019 (approximately 60%) at a cut-off value ≥ 5.475 ng/ml.

10. In individuals with long-standing T1D, there was a positive association between serum levels of OPG, ADNC, Lep and a tendency for microcytic, hypochromic anemia. In terms of leucocyte variables, adipokines have the opposite effect: ADNC is a negative regulator and Lep is a positive one.

11. In men with long-standing T1D and calculated very high CVR according to established ST1RE and ESC-2019 models, the risk of developing anemia is greater than that of women with T1D.

12. A significant finding in the present study was the hyperreactivity of PLT in individuals with long-standing T1D, which corresponded with serum Lep and ADMA levels.

VII. CONTRIBUTIONS

1. Contributions of an original nature

1.1. For the first time in Bulgaria, a prognostic value of ADMA, OPG, ADNC and Lep was assessed against specific tools for assessing CVR: ST1RE and ESC-2019 in persons with long-standing T1D.

1.2. For the first time in Bulgaria, the prognostic value of ADMA, OPG, ADNC, Lep and blood count parameters was evaluated against a constructed model – a combination of established in clinical practice RFs for the development of CVD in DM (RiskFactor 3: HbA1C ≥ 7%, CRP ≥ 3 mg/l and AlbU ≥ 30 mg/l).

1.3. For the first time in Bulgaria, the influence of hamatomorphological parameters on serum levels of ADMA, OPG, ADNC and Lep and their relationship with ST1RE and ESC-2019 in persons with long-standing T1D was evaluated.

1.4. For the first time in Bulgaria, hematological changes in subjects with long-standing T1D and unsatisfactory control were analyzed.

2. Contributions of a theoretical and scientifically applied nature.

2.1. The importance of OPG as a prognostic factor influencing CVD risk in individuals with long-standing T1D has been confirmed.

2.2. The importance of Lep as a prognostic factor influencing CVD risk in individuals with long-standing T1D has been confirmed.

2.3. The importance of AlbU as an independent variable on serum levels of ADMA, OPG, ADNC and Lep was confirmed.

2.4. The importance of BMI and CRP on serum adipokine levels has been confirmed.

2.5. The regulatory role of adipokines on hematopoiesis in individuals with long-standing T1D and healthy controls has been confirmed.

2.6. The need to derive gender-dependent reference values for platelet indices (MPV, PLC-R, PDW, PCT) and implement them in routine clinical laboratory practice has been confirmed.

VIII. SCIENTIFIC PUBLICATIONS RELATED TO THE DISSERTATION PAPER

1. **Popcheva G.,** Bocheva Y., Yotova V, Galcheva S., Yotov Y., Balev B., Boyadzhieva M., Usheva N., Pancheva R.. Laboratory biomarkers for cardiovascular risk assessment in patients with type 1 diabetes mellitus. Science Endocrinology, 2019, 2: 4-13
2. **Chausheva G**., ASYMMETRICAL DIMETHYLARGININE - NATURE, ANALYTICAL METHODS FOR DETERMINATION AND CLINICAL APPLICATION, Varna Medical Forum, 2022,11, issue 1.

PARTICIPATION IN SCIENTIFIC FORUMS IN CONNECTION WITH THE DISSERTATION

1. **G. Chausheva**, S. Shefket, Y. Bocheva, V. Iotova, K. Tsochev, S. Galcheva, I. Yotov, G. Valchev, N. Usheva, M. Boyadzhieva. Differences between genders in leptin and its correlation with C-reactive protein in patients with long-standing type 1 diabetes mellitus. *XXVIII Balkan Clinical Laboratory Federation Meeting and XIII National Conference of Clinical Laboratory*, Sofia, Bulgaria, Sept 8-11, 2021.

2. **G. Chausheva 1**, Y. Bocheva 1, S. Shefket 1, K. Tsochev 3, V. Iotova 3, Y. Yotov 2, T. Chalukova 2, N. Usheva 4, M. Boyadzhieva 5, G. Valchev 6, R. Pancheva 7. Correlation between osteoprotegerin, asymmetric dimethylarginine and disease duration in patients with long-term type 1 diabetes mellitus- *24th IFCC- EFLM European Congress of Clinical Laboratory Medicine,* Munich, Germany, April 10-14, 2022.

3. **G. Chausheva,** Y. Bocheva, S. Shefket, K. Tsochev, T.Chalakova, N. Usheva., M. Boyadzhieva, G. Valchev Y., V. Iotova, Changes in platelet count and platelet indices in relation to disease duration and patient age in long-term type 1 diabetes mellitus. *IFCC WorldLab Seoul Congress*, June 26-30, 2022

4. **G. Chausheva**, Y. Bocheva, S. Shefket, Y. Yotov, V. Iotova, M. Boyadzhieva, K. Tsochev, N. Usheva, Correlations between asymmetric dimethylarginine, osteoprotegerin and albuminuria in longstanding type 1 diabetic patients, *IFCC WorldLab Seoul Congress,* June 26-30, 2022.

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