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FACULTY OF MEDICINE

SECOND DEPARTMENT OF INTERNAL DISEASES

ES ENDOCRINOLOGY AND METABOLIC DISEASES

NON-INVASIVE ASSESSMENT OF ARTERIAL STIFFNESS IN PATIENTS WITH TYPE 2 DIABETES MELLITUS - CORRELATION WITH SOME BIOMARKERS.

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ABSTRACT

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CONTENTS

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ABBREVIATIONS

AS – srterial stiffness

ACC – arteria carotis communis

DAP - diastolic blood pressure

DPNP - diabetic polyneuropathy

DNP - diabetic nephropathy

DRP - diabetic retinopathy

ET- echo-tracking

IMT- intima media thickness

BMI – body mass index

IR - insulin resistance

CAD – coronary artery disease

WC - waist circumference

IFG - impaired fasting glucose

IGT - impaired glucose tolerance

OC - osteocacin

FPG - fasting plasma glucose

PPG - postprandial glucose

PP – pulse pressure

MAP – mean arterial pressure

SBP – systolic blood pressure

CVD – cardiovascular diseases

T2DM – type 2 diabetes mellitus

AC - arterial compliance

AI – augmentation index

cOC - carboxylated osteocalcin

cf-PWV- carotid-femoral pulse wave velocity

Ep – modulus of elasticity

FRS – Framingham risk score

GLP-1- glucagon-like peptide -1

HBA1c - glycated hemoglobin

HOMA-IR – homeostasis model for the assessment of insulin resistance

PWV- pulse wave velocity

PWVβ – one-point pulse wave velocity

tOC - total osteocalcin

ucOC - uncarboxylated osteocalcin

VSMCs – vascular smoothmuscle cells

I. INTRODUCTION

Macrovascular complications of type 2 diabetes mellitus (T2DM) include vascular calcification and arterial stiffness (AS). The process of calcification occurring in the medial vascular layer are considered to be one of the reasons for the development of stiffness. Once AS has occurred, it is considered a non-traditional cardiovascular risk factor. It occurs in parallel with endothelial dysfunction in the course of the development of the atherosclerotic process, and there is ambiguity regarding the causal relationship between the two processes. Measurement of arterial wall rigidity is currently of scientific interest due to its independent predictive value for cardiovascular events, especially in high-risk patients, such as those with T2DM. Advances in diagnostic ultrasonography technologies have allowed the introduction in recent years of new methods for early detection of vascular changes, namely assessed by measuring stiffness. Preclinical detection of these changes would allow delay of cardiovascular complications in patients with T2DM. Such a new technique is the one-point echotracking system giving information about the local AS of the common carotid artery. Studies have shown that local carotid stiffness correlates with aortic stiffness and therefore with the risk of developing cardiovascular morbidity and mortality. Reference values have been developed for local AS measured on the carotid artery in healthy individuals, but data on stiffness in patients with T2DM are currently scarce. The accumulation of AS data in patients with T2DM would allow better management of cardiovascular complications and therapeutic intervention in the subclinical atherosclerosis phase.

The last decade has revealed the hormonal nature of the bone protein osteocalcin (OC) and its involvement in carbohydrate, energy and lipid metabolism. It exists in two forms differing in the degree of carboxylation, and the key enzyme responsible for its transition from uncarboxylated to carboxylated form is vitamin K-dependent. Thus, the levels of vitamin K in the body directly affect the degree of carboxylating of the OC. It is known in the literature that the carboxylated OC participates in the processes of bone formation. The uncarboxylated form of OC, which is thought to have hormonal activity, is associated with increased pancreatic insulin secretion and increase serum adiponectin, and thus leads to improved insulin sensitivity. Conversely, low concentrations of OC are associated with an increased risk of developing T2DM. The connection of OC with diabetes, which is one of the leading risk factors for the development and acceleration of the processes of atherosclerosis, generates the hypothesis of participation of OC in the processes of vascular calcification and arterial stiffness . Osteocalcin is currently considered to be one of the inhibitors of vascular calcification.

In parallel with the development of knowledge about the pathophysiological mechanisms leading to vascular damage and the discovery of new vascular biomarkers, new therapeutic strategies are being sought. Of interest in this regard is vitamin K2 and its function as a γ-carboxylase cofactor converting uncarboxylated OC to carboxylated. The role of vitamin K2 in the complex treatment of osteoporosis is known and confirmed. It is also supposed to be involved in vascular calcification and arterial stiffness as a cofactor of

γ-carboxylase activating a number of proteins which are calcification inhibitors. Currently, data in the literature on the effects of vitamin K2 on vascular biology are insufficient. Additional interventional studies are needed to clarify the role of this vitamin in vascular changes occurring in T2DM.

II. AIM AND TASKS OF THE DISSERTATION

Aim:

To obtain data on the local AS of the carotid arteries in patients with T2DM without macrovascular complications by echotracking technique. To look for a connection between the parameters of AS with glucometabolic, lipid, hemodynamic parameters and serum osteocalcin levels, as well as to evaluate the effect of vitamin K2 supplementation in some patients with diabetes.

Tasks

1. To measure the local AS of the two carotid arteries, using echo-tracking technique, in patients with T2DM without established macrovascular complications and to compare with that in controls.
2. To look for a connection between the ET parameters and the age of the patients with T2DM and anthropometric, glucose and lipid levels.
3. To analyze whether there is a relationship between the indicators of AS and hemodynamic parameters (CASP, SBP, DBP, PP, MAP) in persons with T2DM.
4. To determine the serum concentrations of ucOC and cOC in some patients with T2DM and to compare with those of healthy controls.
5. Look for a link between AS in individuals with T2DM and serum osteocalcin levels.
6. To look for a change in the values of AS and serum levels of OC in patients undergoing vitamin K2 supplementation.

III MATERIALS AND METODS

1. Facilities

The present study was conducted in the following structures:

- Clinic of internal medicine, MHAT „St. Marina“- Varna
- Clinic of Endocrinology and metabolic deseases, MHAT „ St. Marina“- Varna,
- Medical center „ St. Marina“- Varna,
- Central clinical laboratory, MHAT „ St. Marina“- Varna
- Department of Pharmacology and Clinical Pharmacology and Therapy, MU-Varna
- Department of Social Medicine and Health Care Organisation, MU-Varna

2. Patient population included in the study

The study was conducted in the period October 2018 - September 2019, and included a total of 100 patients (52 female and 48 male) with type 2 diabetes and 30 healthy controls (15 female and 15 male). Patients with diabetes mellitus without a history of proven macrovascular complications, over 18 years of age, able to read, understand and sign self-informed consent was enrolled. The patients are hospitalized in the Clinic of Internal Medicine and the Clinic of Endocrinology or are a contingent of the examinations of the diagnostic-consulting rooms at the clinics. The control group will be formed by patients who have passed through the relevant clinical centers, in whom no T2DM has been proven and atherosclerotic cardiovascular disease or the reason for the examination was another disease outside the exclusion criteria. Part of the group of patients with T2DM, after written consent, received supplementation with vitamin K2 (Quinone) 75 mg / day for a period of 4 weeks (a total of 20 patients). Two of the patients receiving supplementation were not followed. A total of 18 patients (11 female and 7 male) underwent supplementation and follow-up blood and ultrasound examinations at the end of the one-month period. The study was performed after receiving a protocol for approval from the Commission on Ethics of Research at the Medical University - Varna.

3. Inclusion criteria

- patients with type 2 diabetes mellitus without a history of macrovascular complications
- over 18 years of age
- hospitalized patients in the Clinic of Internal Medicine and in the Clinic of Endocrinology and Metabolic Diseases
- persons able to read, understand and personally sign the informed consent

4. Exclusion criteria

- persons under 18 years of age
- persons who cannot read, understand and hand-sign the informed consent
- type 1 diabetes mellitus
- pregnancy
- patients with macrovascular complications, such as coronary artery disease, cerebrovascular disease, peripheral arterial disease, abdominal aneurysm
- patients with uncontrolled hypertension
- atrial fibrillation
- treatment with vitamin K antagonists
- patients with type 2 diabetes mellitus on hemodialysis
- patients taking vitamin K2 and / or vitamin D as a dietary supplement
- patients with osteoporosis or undergoing anti-osteoporosis therapy
- patients on systemic glucocorticoid therapy
- patients with cancer

5. Methods

- **Questionnaire** – all participants were interviewed on age, the duration of diabetes, the type of antidiabetic treatment, the presence of complications of diabetes. Data on antihypertensive therapy and symptoms of atherosclerotic cardiovascular disease.
- **Anthropometry** – height (m), weight (kg), waist circumference (cm) were measured. Calculate the body mass index according to the formula BMI=weight(kg)/height²(m) and waist / height ratio.

• Laboratory methods

1. Biochemical research

The study participants (patients and controls) venous blood by venipuncture of a cubital vein or other suitable peripheral vein after at least 12 hours of fasting was taken. The plasma glucose test was performed by the hexokinase method. The test for total cholesterol, HDL, and triglycerides was performed by an enzymatic method, with LDL calculated by a formula. Glycated hemoglobin was examined by immunoturbidimetric analysis. The equipment used is Olympus AU400. The laboratory biochemical tests were performed in the clinical laboratory of the University Hospital "St. Marina ", Varna.

2. Hormonal analysis

Osteocalcin - serum samples for ucOC and cOC were taken in the morning on fasting state by venipuncture. After centrifugation the serum was frozen at -80 ° C until analysis. Both forms of osteocalcin were tested by enzyme-linked immunosorbent assay (ELISA) with individual kits for human carboxylated (gla-) osteocalcin and human uncarboxylated (glu-) osteocalcin (Takara Bio, Inc., Japan), following the manufacturer's instructions. Serum osteocalcin levels were reported in ng/ml. The study was performed on an ELISA reader LKB 5060-006 (LKB Instruments, Australia) at the Department of Pharmacology, Clinical Pharmacology and Therapy, MU-Varna.

• Instrumental methods

1. Measurement of brachial pressure (AH) and central aortic systolic pressure (CASP).

The measurement was performed immediately before the ultrasound examination with a portable device for measuring central aortic systolic pressure - Caspal A-pulse CASP-AL (HealthSTATS Int.). The device is on the FDA approved list and uses a modified tonometric technique. During the measurement, a cuff is placed on the forearm in the area of the brachial artery and three consecutive measurements are registered with an interval between them of 1 min on the SBP, DBP and HR. Then the device automatically calculates their average values. The second step is to place a sensor at the site of palpation of the radial artery in the wrist, with which the device registers the pulse wave and calculates the CASP.

2. Calculation of pulse pressure (PP) according to the formula:

PP (mmHg) = SBP (mmHg) - DBP(mmHg).

3. Calculation of mean blood pressure by the formula:

MAP (mmHg) = DBP + (SBP – DBP)/3

4. Ultrasound examination

Ultrasound examination of the common carotid artery was performed bilaterally with an ultrasound machine Aloka Hitachi Prosound α7 equipped with a high-frequency linear transducer. The patient assumes a supine position with neck extension and slight rotation of 30 ° contralateral to the study area. The measurement is performed 2 cm proximal to the carotid bifurcation, with the aim of visualizing the common carotid artery at its greatest width in a longitudinal section. This ensures accurate tracking of the change in vascular diameter. Then it is necessary to turn on the echo-tracking function from the control panel. Two markers appear with the help of which we mark the boundaries of the examined vessel. The markers are placed on the near and far walls of the artery at the border between tunica media and tunica adventitia. Peripheral electrodes are placed on the patient beforehand. Thus, we observe a simultaneous ECG recording and accurately record the onset of systole and diastole of the heart contraction. At least six cardiac cycles are recorded, during which time the patient has held his breath to improve image quality and stop the image. The input of data from the previously measured SBP and DBP is also required and the automatic calculation of the indicators of AS by means of mathematical algorithms is started. The change in the diameter of the vessel is processed in groups for all pulse waves and an average value of the parameters is obtained: β-stiffness индекс, AC, PWVβ, AI and Ep.

- **Statistical methods**

The data were processed through a specialized statistical package for a personal computer SPSS Windows, version 25. The graphic representation is done with Excel and GraphPad Prism 8. The following statistical methods were used:

1. Descriptive statistical methods - the quantitative variables were described with an arithmetic mean, standard deviation, maximum and minimum value. Qualitative variables were described by: n (number of observations) and relative frequency distribution in percentages. Nonparametric tests, such as cross-tabulation and chi-square, were used to look for significant differences in the frequency representation of categorical values. To determine the type of distribution of the studied data, the Kolmogorov-Smirnov test was performed.

2. Analytical statistical methods - an indipendant t-test was used to compare the mean values of the parameters in the two studied groups. A paired t-test is used to assess the significance of the change in the studied variables before and after the supplementation. In cases where a lack of normal distribution of the data is found, the non-parametric Mann-

Whitney test is used to assess the significance of the differences. When comparing more than two groups, ANOVA and, in certain cases, post-hot analysis of Tukey HSD were used. A correlation analysis was performed to study the dependences between the different studied clinical, ultrasound and laboratory parameters and the strength of their influence. The estimation of the correlation between the variables is based on the value of the Pearson coefficient (r) or the Spearman coefficient. These coefficients determine the direction and strength of the relationship between two studied variables. In all analyzes, statistical significance of the differences was assumed to be $p \leq 0.05$

I V. RESULTS

1. Descriptive statistics of the studied groups

The study included 100 patients with T2DM with a mean age of 57.7 ± 7.48 years and 30 subjects without a history of diabetes mellitus with a mean age of 56.7 ± 6.98 years, who formed the control group. The sex distribution in the study group was 52% female and 48% male, and the control group included 50% female and 50% male. According to the type of antidiabetic treatment, the study group had the following distribution: insulin treatment 39% (N=39), noninsulin treatment 61% (N=61).

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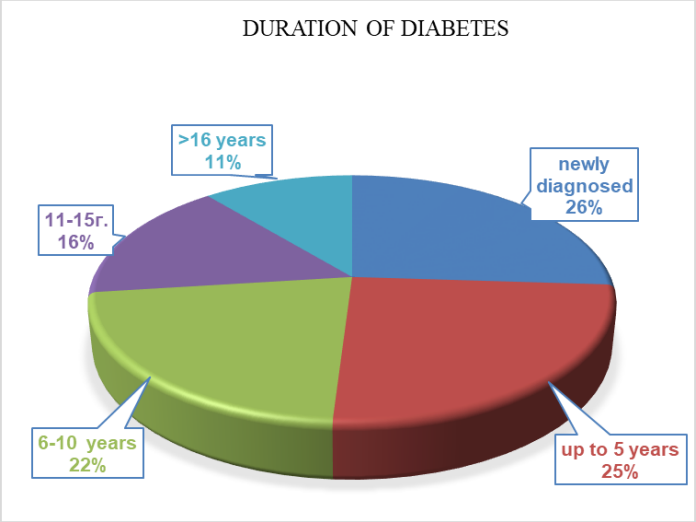
Half of the total number of participants did not have a history of any complications. Diabetic polyneuropathy was most commonly reported in patients (DPNP) (n=47), followed by diabetic retinopathy (DRP) (n=17) and diabetic nephropathy (DNP) (n=5). Patients with a history of macrovascular complications, which are part of the exclusion criteria, were not included in our study.

Figure 1. Durtion of diabetes of the studed group



Baseline anthropometric, biochemical and hemodynamic parameters in the study and control group are shown in Table 1.

Table 1. Comparison between the baseline characteristics and their significance of the differences of the two studied groups:

Parameters	T2DM	Controls	p
	mean± SD	mean± SD	
Age (years)	57,70 ± 7.48	56.76± 6.98	0.54
Duration of DM (years)	6.75±6.37		
Height (cm)	166,70± 10.11	168.28± 9.14	0.47
Weight (kg)	90,67± 20.84	84.08± 20.34	0.13
BMI (κg/m²)	32,41± 6.61	29.67± 6.08	0.05*
Waist (cm)	108,69± 14.92	100.85± 16.24	0.01*
Waist/height	0.65±0.09	0.6±0.08	0.004*
Blood glucose (mmol/l)	7,62± 2.51	5.68±0.67	0.001*
HBA1c	9,17± 2.44	5.55±0.43	0.001*
Chol (mmol/l)	5,18± 1.32	5.63± 1.49	0.15
TG (mmol/l)	2,30± 1.68	2.26± 1.76	0.81
LDL(mmol/l)	3,00± 1.12	3.30± 1.12	0.27
HDL (mmol/l)	1,18± 0.39	1.33± 0.33	0.11
Creatinin (μmol/l)	78.9± 21.14	73.89± 14.28	0.29
MDRD (ml/min/1.73 m2)	89.85± 21.1	85.21± 14.67	0.6
Hemoglobin (g/l)	140,78± 16.67	141.45± 13.13	0.85
CAPS (mmHg)	126,40± 13.79	125.76± 12.62	0.84
SBP (mmHg)	134,01± 14.63	132.64± 13.1	0.13
DBP (mmHg)	85,83± 10.26	87.50± 9.83	0.48
Pulse pressure(mmHg)	48.08± 11.53	45.14± 9.83	0.39
Mean pressure(mmHg)	101,82± 10.6	101.97± 10.28	0.62
HR (beats/min)	75,12± 11.11	73.17± 10.31	0.41

The measured anthropometric parameters in both groups are height, weight, BMI, waist circumference (WC). Compared to controls, the study group showed higher mean BMI values ($t = 1.968$; $p = 0.05$) and WC ($t = 2.407$; $p = 0.018$).

The mean values and standard deviations of the measured fasting plasma glucose in patients with T2DM were 7.62 ± 2.51 mmol / l, and in the control group, respectively, 5.68 ± 0.67 mmol / l, $p = 0.001$. The mean value of glycated hemoglobin in the group of patients with T2DM was 9.17% ± 2.44, and in the control group 5.55% ± 0.43, $p = 0.001$, respectively (Table 2).

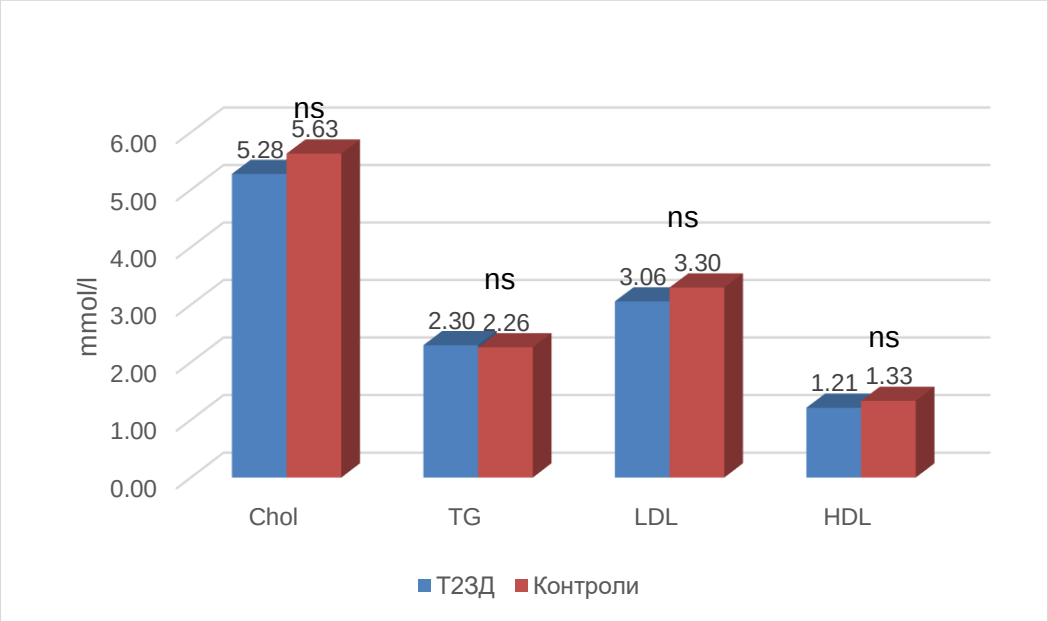
Table 2. Differences between fasting plasma glucose (FPG) and glycated hemoglobin determined by t-test.

	T2DM	**Controls**	**P**
FPG, mmol/l (mean±SD)	7.62±2.51	5.68±0.67	0.001
HBA1c, % (mean±SD)	9.1±2.44	5.5±0.43	0.001

We analyzed the lipid parameters of the two groups - total cholesterol, triglycerides, HDL-C and LDL-C. We checked the normality of the data distribution with the Kolmogorov-Smirnov test. In this test, values of $p < 0.05$ indicate an asymmetric distribution, and at $p > 0.05$ a symmetric distribution of data. Normal distribution was found in the levels of three of the indicators - total cholesterol, LDL-C and HDL-C. Therefore, we used independent t-test to compare them. It showed no significant difference between the study and control groups - total cholesterol ($t = -1.43$; $p = 0.15$), LDL ($t = -1.09$; $p = 0.27$) and HDL ($t = -1.59$; $p = 0.11$) (diagram 2). TG levels showed an asymmetric distribution. In their comparison, we used the non-parametric Mann-Whitney test as the mean TG ranks in the study group showed higher values, but without reaching a statistically significant difference with the control group ($U = 1105.5$; $p = 0.819$) (Figure 2).

We additionally looked for differences in the study and control groups regarding the smoking. The incidence of smoking in the T2DM group was 46% and in the control group 40%. The significance of the differences in these category values was assessed by the nonparametric analysis of cross-tabulation and chi-square, which showed a lack of significance ($X) = 0.33$; $p = 0.56$) (Table 1).

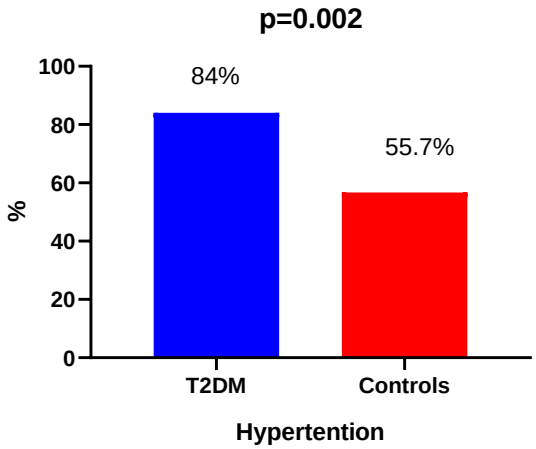
Diagram 2. Non-significant differences in the mean values of total cholesterol, TG, LDL-C and HDL-C in the two groups.



2. Analysis of hemodynamic parameters.

To compare the presence of concomitant hypertension in patients with T2DM and controls, we used the nonparametric test crosstabulation and chi-square, comparing the frequency representation of categorical values. Hypertension is more likely to be present in the T2DM group than in the control group (Figure 3).

Diagram 3. Distribution of hypertension in the study and control group.



Because systolic blood pressure (SBP), pulse pressure (PP), mean arterial pressure (MAP), central aortic systolic pressure (CASP), and heart rate (HR) are variables showing

a normal distribution, compared to their mean values with those of the controls we used t-test. Diastolic blood pressure (DBP) showed an asymmetric distribution of data, which is why the significance of the difference in its values between the two groups was assessed using the Mann-Whitney test. The differences did not reach statistical significance when comparing the individual ranks (U = 1280; p = 0.489) (Table 3).

Table 3. Values of hemodynamic parameters in the study and control group (t-test).

	Group	mean±SD	95% CI, borders		p
SBP, mmHg	T2DM	134.01 ± 14.63	-4.69	7.43	0.66
	Controls	132.64 ± 13.11	-5.40	6.67	
CASP, mmHg	T2DM	126.40 ± 13.8	-3.10	7.67	0.84
	Controls	125.76 ± 12.62	-2.69	6.57	
HR, bt/min	T2DM	75.12 ± 11.11	-8.56	5.25	0.41
	Controls	73.17 ± 10.31	-4.69	7.43	
PP, mmHg	T2DM	48.08 ± 11.53	-5.40	6.67	0.39
	Controls	45.14 ± 9.83	-3.10	7.67	
MAP, mmHg	T2DM	101.82 ± 10.6	-2.69	6.57	0.62
	Controls	102.51± 10.02	-8.56	5.25	

3. Results from the measurement of ET parameters of AS of the carotid arteries.

In the analysis of the results of the ultrasound examination of the carotid arteries in the group of patients with T2DM, the mean value of PWVβ in the left carotid artery is 7.37 ± 1.32 m / sec and respectively in the right is 7.42 ± 1.33 m / sec. In the control group PWVβ measured on the left carotid artery was 6.37 ± 0.94 m / sec, and on the right was 6.43 ± 1.11 m / sec (Table 4). Following the same statistical methods mentioned above, to determine whether this difference is significant between patients with T2DM and healthy controls, we performed a Kolmogorov-Smirnov test, which showed normal distribution of the data. Initially, it was seen that the differences in PWVβ between the two groups, expressed as a mean, were 1m / s for the left ACC and 0.99 m / sec for the right ACC. The performed t-test showed statistical significance of the higher PWVβ (left and right) in the study group compared to the control group (t = 3.764; p = 0.001 and t = 3.561; p = 0.001)

(Table 4). With "R" we marked the indicators measured on the right, and with "L" on the left common carotid artery.

Table 4. Mean values and standard deviation of PWVβ (L) and PWVβ (R) in the two study groups and performed t-test.

ET parameters	T2DM (n=100)	Controls (n=30)	p	95% CI, borders	
PWV, L (mean ± SD)	7.37±1.32	6.37±0.94	0.001	0.48	1.54
PWV, R (mean ± SD)	7.42±1.33	6.43±1.11	0.001	0.44	1.53

*n= number of subjects

In addition to identifying significant differences in PWVβ between T2DM individuals and healthy controls, we sought an answer to the question of whether there were differences in PWVβ, depending on whether the measurement was performed on the left or right carotid artery. In the T2DM group, PWV (L) was 7.37 ± 1.32 m / sec and PWV (R) was 7.42 ± 1.33 m / sec. The performed t-test showed insignificance of these differences (t = 0.56; p = 0.57) (diagram 4). For the control group, respectively, the values measured are PWV (L) 6.37 ± 0.94 m / sec and PWV (R) 6.43 ± 1.11 m / sec (t = 0.24; p = 0.8) (diagram 5).

Diagram 4. Paired t-test not detecting significant differences between PWV (L) and PWV (R) of the participants in the studied group of T2DM.

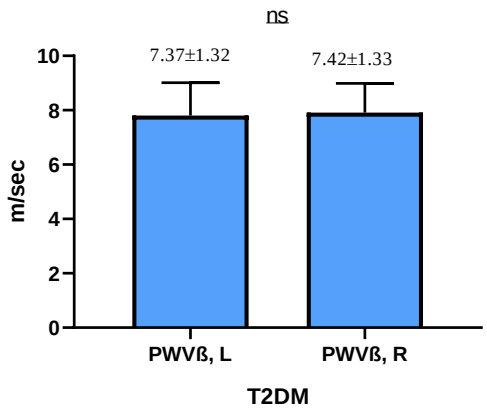
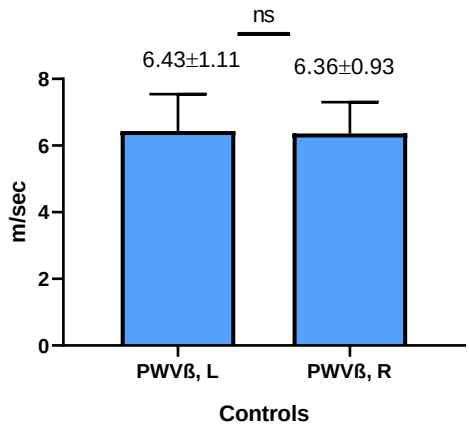


Diagram 5. Paired t-test not detecting significant differences between PWV (L) and PWV (R) of the participants in the control group.



The ultrasound parameters of AS - β-stiffness index, modulus of elasticity (Ep), arterial compliance (AC), augmentation index (AI) showed a normal distribution of data. Table 5 shows the mean values, standard deviations of the two groups and the significance of the differences between them (t-test). The results of the t-test show significant differences in all measured ultrasound parameters of AS, except AI (t = -1,408; p = 0.162) of the right carotid artery. In contrast, AI measured on the left carotid artery showed a significant difference with controls (t = -2.651, p = 0.009) (Table 5).

Table 5. Mean values and standard deviation of ET parameters with normal data distribution**.**

Descriptive statistic			t-test, p	95% CI, borders	
ET parameters	Group	Mean ± SD		lower	uper
β-stiffnes sindex, R	T2DM (n=100)	10.48±4.08	0.001	1.07	4.31
	Controls (n=30)	7.79± 2.6			
AI, R	T2DM(n=100)	13.36±10.73	0.009	-11.37	-1.65
	Controls (n=30)	19.87±13.67			
Ep, L	T2DM (n=100)	149.25±58.09	0.001	17.85	63.35
	Controls (n=30)	108.66±33.23			
AC, L	T2DM (n=100)	0.76±0.32	0.001	-0.39	-0.10
	Controls (n=30)	1.00±0.44			
AI, L	T2DM (n=100)	12.87±11.94	0.16	-8.36	1.41
	Controls (n=30)	16.35±9.89			

*n= number of subjects

The Kolmogorov-Smirnov test showed an asymmetric distribution of the data obtained for Ep (R), AC (R), β-stiffness index (L). With the non-parametric Mann-Whitney test we tested for significance the differences in the two groups, these three ultrasound parameters (Table 6). Significance of the differences was found in two of the parameters: Ep (R) (U = 771.5; p = 0.001) and β-stiffness index (L) (U = 731.5; p = 0.001). AC

values measured on the right carotid artery did not reach statistical significance (U = 1090.5; p = 0.113).

Table 6. Mann-Whitney test for the significance of the differences between the ultrasound parameters of AS, showing uneven distribution - Ep (R), AC (R), and β-stiffness index (L).

ET parameters	Ep, R	AC, R	β-stiffness index, L
Mann-Whitney, U	771.5	1090.5	731.5
Wilcoxon W	1177.5	5843.5	1137.5
Z	-3.473	-1.584	-3.807
p	0.001	0.113	0.001

Diagram 6. Statistically significant differences between the mean values of the β-stiffness index measured on the left and right carotid artery in patients with T2DM and controls. For both arteries, p <0.001.

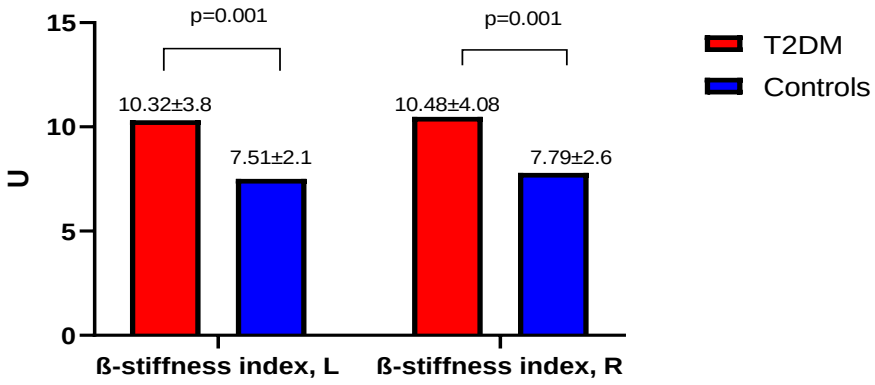


Diagram 7. Statistically significant differences between the mean values of Ep (L) and Ep (R) in patients with T2DM and controls. For both arteries p <0.001

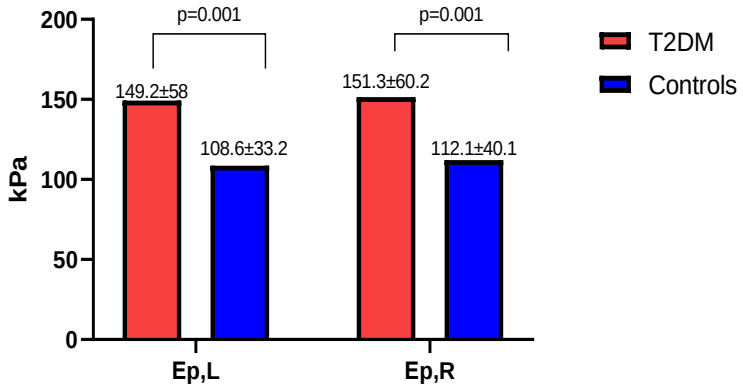


Diagram 8. Differences between mean AC (L) and AC (R) values in patients with T2DM and controls For the left ACC the difference is statistically significant (t = -3.26; p <0.001), while for the right ACC it nonsignificance (U = 1090.5; p = 0.11).

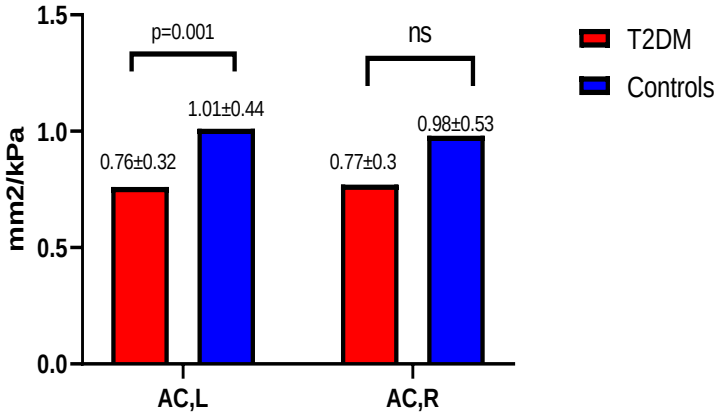
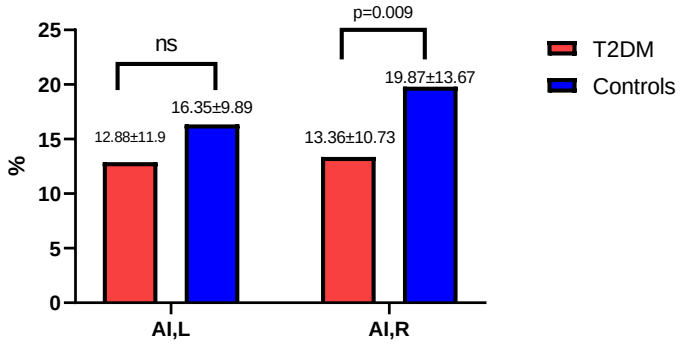


Diagram 9. Differences between mean AI (L) and AI (R) values in patients with T2DM and controls. In the right ACC the difference is statistically significant (t = -2.65; p 0.009), while in the left ACC it does not reach significance (t = -1.4; p = 0.16).



4. Results of the study of serum osteocalcin levels.

We studied the serum levels of ucOC, cOC, and tOC in 47 patients in the T2DM and 18 subjects in the control group. The mean values and standard deviation of serum cOC levels in patients with T2DM were 7.84 ± 2.41 ng / ml and were lower than those in the 10.05 ± 4.37ng / ml controls. The ucOC / tOC ratio also showed lower mean values of 0.28 ± 0.15 in patients with T2DM, compared to controls of 0.34 ± 0.17 (Table 8). The conducted Kolmogorov-Smirnov test reveals a uniform distribution of the data, both for the measured values of cOC and for the ratio ucOC / tOC. The independent t- test showed statistical significance of lower serum cOC concentrations in patients with T2DM compared to controls (t = -2,604; p = 0.011) (Table 7).

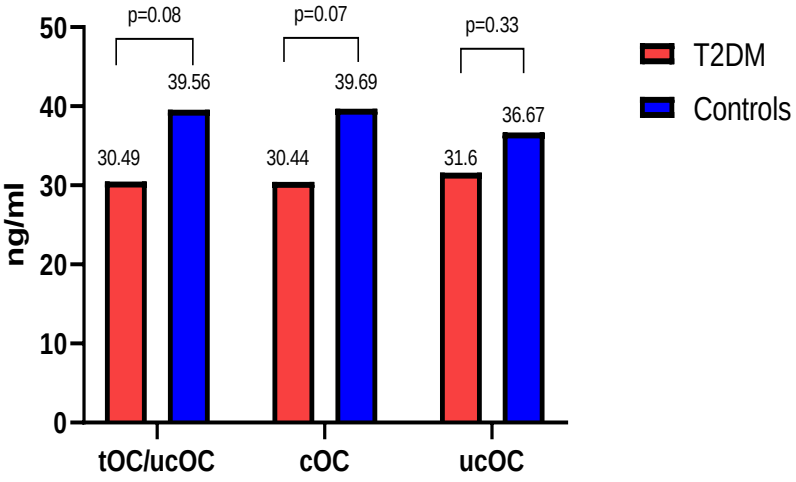
Table 7. Comparison of mean and standard deviations of serum cOC levels and ucOC / tOC ratio in the two study groups**.**

Descriptive statistic			t-test, p	95% CI, borders	
	Group	Mean ± SD		lower	upper
cOC, ng/ml	T2DM(n=47)	7.84 ± 2.41	0.01	-3.91	-0.51
	Controls (n=18)	10.05 ± 4.37			
ucOC/tOC	T2DM(n=47)	0.28 ± 0.15	0.22	-0.14	0.03
	Controls (n=18)	0.33 ± 0.17			

*n= number of subjects

The observed serum concentrations of ucOC in the group of patients with T2DM showed lower mean values of 3.81 ± 5.12 ng / ml compared to those in the healthy controls of 4.37 ± 3.0 ng / ml. This trend was maintained when comparing serum tOC concentrations in the group of patients with T2DM 11.64 ± 5.24 ng / ml and controls 14.08 ± 6.07 ng / ml. Due to the ubnormal distribution of the data, we performed a nonparametric analysis (Mann-Whitney), which revealed a lack of statistical significance of the lower concentrations of ucOC (U = 305; p = 0.08) and tOC (U = 302.5; p = 0.07) in the two studied groups (diagram 10). Due to the lower levels of the two forms of osteocalcin found in patients with T2DM, their ratio also showed lower values, again not reaching statistical significance ucOC / cOC (U = 357; p = 0.33) (Figure 10).

Diagram 10. Comparison of mean scores lacking statistical significance of differences in serum ucOC and tOC levels, as well as ucOC / cOC ratio in patients with T2DM and controls (Mann-Whitney).



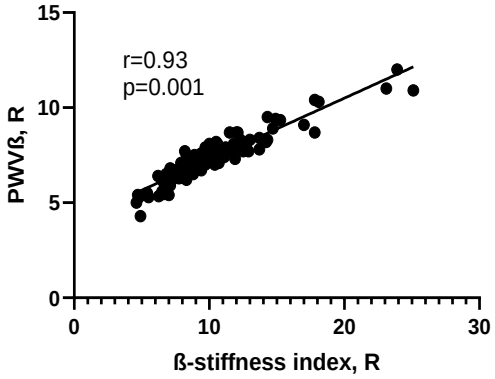
5. Correlation in the group of patients with T2DM.

5.1. Correlation dependences of ET parameters of AS

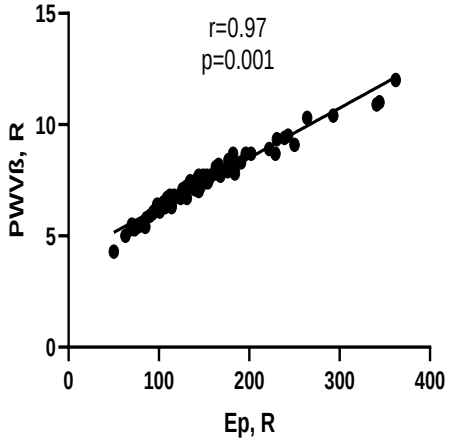
5.1.1. Correlations between the parameters of AS: PWVβ, Ep, AC, AI and β-stiff.index

The estimation of the strength of the correlation dependence is based on the Pearson coefficient (r), and statistical significance is assumed at a value of $p \leq 0.05$. We sought an answer to the question of whether there is a correlation of the main parameter PWVβ with the other ultrasound measured parameters of AS. Such a very strong positive correlation was found in the measurements of the right carotid artery between PWVβ (R) and β-stiffness index (R) ($r = 0.93$; $p = 0.001$) (Graph 1) and with Ep (R) ($r = 0.97$; $p = 0.001$) (Figure 2). A negative inverse correlation was found between PWVβ (R) and AC (R) ($r = -0.64$; $p = 0.001$) (Graph 3).

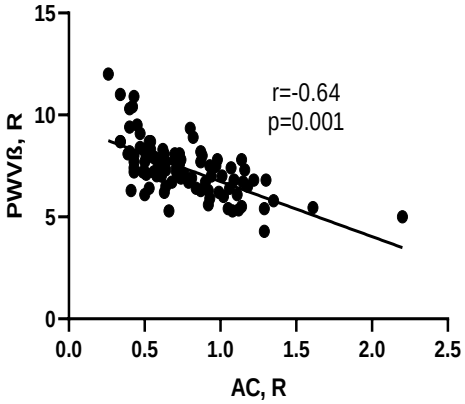
Graph 1. Correlation between PWVβ (R) and β-stiffness index (R)



Graph 2. Correlation between PWVβ (R) and Ep (R)



Graph 3. Correlation between PWVβ (R) and AC (R)



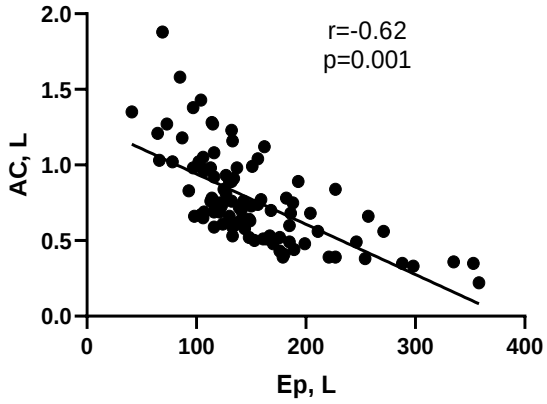
In addition to between PWVβ and AC, we also found correlations of AC with Ep and β-stiffness index of right ACC (Table 8).

Table 8. Significant correlations between ET parameters of right ACC in patients with T2DM.

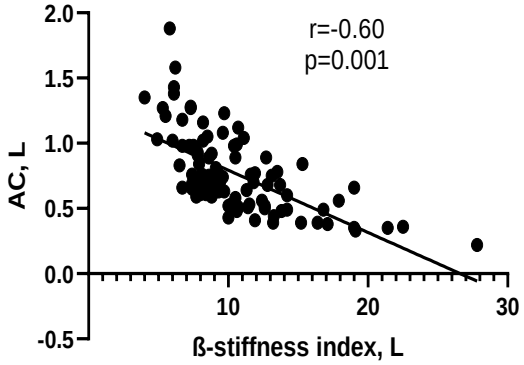
Correlation		Ep (R)	β-stiff. index (R)
AC, mm²/kPa	coefficient of Pearson, r	-0.66	-0.64
	significance, p	0.001	0.001

On the left ACC in patients with T2DM, we found a significant negative correlation of AC (L) with Ep (L) (r = -0.62; p = 0.001), and with the β-stiffness index (L) (r = -0.60; p = 0.001) (Graph. 4 and Graph 5).

Graph 4. Negative correlation of AC (L) with Ep (L)



Graph 5. Negative correlation dependence of AC (L) with β-stiffness index (L**)**



AI measured on the left carotid artery in patients with T2DM showed a weak correlation with PWVβ (L) (r = 0.19; p = 0.05) and β-stiffness index (L) (r = 0.24; p = 0.01) (Table 9).

Table 9. Correlation dependences of AI with PWVβ (L) and β-stiffness index (L)

Correlation		**PWVβ (L)**	**β-stiffness index (L)**
AI, %	coefficient of Pearson, r	0.19	0.24
	significance, p	0.05	0.01

5.1.2. Correlations of AS parameters with anthropometric, lipid and glucose indicators.

- Anthropometric indicators

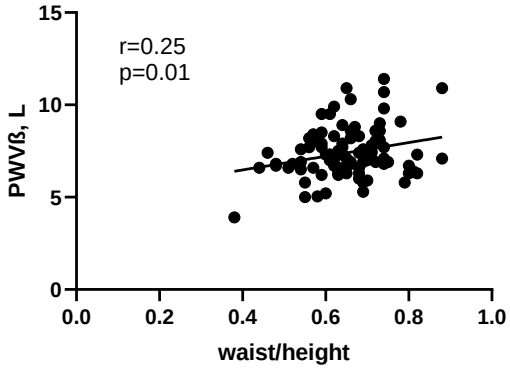
  Numerous correlations between waist circumference and ET parameters of AS were revealed. From the ultrasound parameters measured on the left ACC, the waist was moderately correlated with Ep (L) (r = 0.27; p = 0.007). In measurements performed on the right ACC, we observed a positive correlation of waist circumference with PWVβ (R) (r = 0.24; p = 0.01), with Ep (R) (r = 0.23; p = 0.02), and with β-stiffness index (R) (r = 0.22; p = 0.02). A negative significant correlation was revealed between waist and AI (R) (r = -0.21; p = 0.04) (Table 10).

Table 10. Correlations of the parameters of AS with the waist circumference in the group of patients with T2DM.

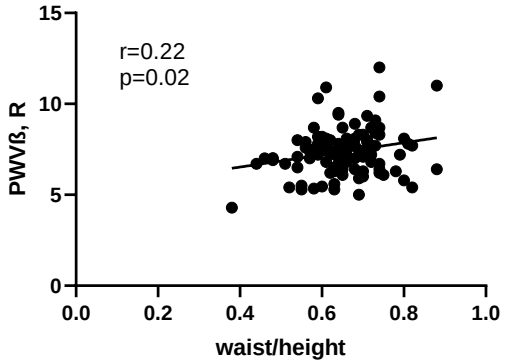
Correlation		Ep(L)	PWV (R)	Ep (R)	β-stiff. Index (R)	AI (R)
Waist circumference	coefficient of Pearson, r	0.27	0.24	0.23	0.22	-0.21
	significance, p	0.007	0.01	0.02	0.02	0.04

A correlation between the indicators of AS was also established with the waist / height ratio in the studied group. The performed correlation analysis revealed a significant positive relationship with PWV (L) (r = 0.25; p = 0.01) and PWV (R) (r = 0.22; p = 0.02) (Graph and Graph 7).

Graph 6. Positive correlation of PWV (L) with the waist / height ratio in patients with T2DM.



Graph 7. Positive correlation of PWV (R) with the waist / height ratio in patients with T2DM

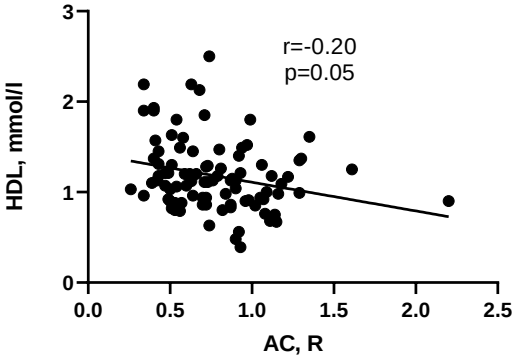


No relationships were found between the measured ET parameters and BMI in the studied patients with T2DMJI

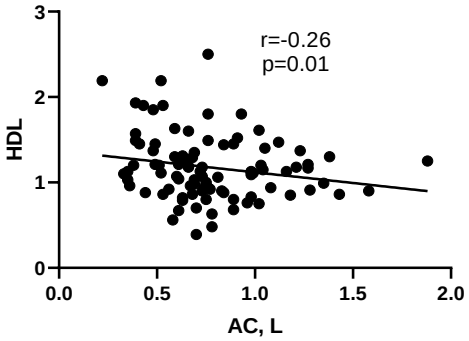
- lipid parameters.

  In studied group, no correlations were found between the AS parameters with total cholesterol, triglycerides and LDL-C. Significant negative correlation was found between HDL-C and AC (L) ($r = -0.26$; $p = 0.01$) and AC (R) ($r = -0.020$; $p = 0.05$) in the group of patients with T2DM. Such a correlation of HDL-C was not found in the control group, as well as in the analysis of the study group in combination with healthy controls.

Graph 8. Negative correlation between HDL-C and AC (R) in the group of patients with T2DM



Graph 9. Negative correlation between HDL-C and AC (R) in the group of patients with T2DM.



➢ Glucometabolic parameters.

We did not find correlations between ET parameters and fasting plasma glucose (FPG) in the group of patients and T2DM. In contrast to FPG, significant correlations were found between HBA1c and PWVβ in both left ACC and right ACC when analyzing the results in the two study groups (total for T2DM and controls) (Table 11). When the analysis was performed only in the group of individuals with T2DM, such a correlation dependence was not observed.

Table 11. Correlation analysis showing a significant association of HBA1c with PWV (L) and PWV (R) in patients with T2DM and controls

Correlation		**PWV(L) (n=130)**	**PWV (R) (n=130)**
HBA1c,%	coefficient of Pearson, r	0.23	0.21
	significance, p	0.01	0.01

*n= number of subjects

➢ Correlations with smoking in the group of patients with T2DM.

Table 12. Correlation analysis showing a significant association of smoking with PWV (L) and PWV (R) in patients with T2DM and controls.

Correlation	PWVβ (L)		PWVβ (R)	
	r	p	r	p
Smocking	-0.26	0,008	-0.27	0.006

5.1.3. Correlations of AS parameters with hemodynamic parameters.

We measured the arterial pressure of brachial artery and CASP using a Caspal A-pulse device in 82 of the patients in the T2DM group. We found correlations between the majority of hemodynamic and ultrasound parameters in the group of patients with T2DM. We observed a positive correlation in SBP with all AS parameters measured on the right ACC ($p \leq 0.05$), and with Ep and AC measured on the left ACC ($p = 0.001$). For DBP, we observed a positive correlation with Ep (L) ($p = 0.001$) and a negative correlation with the β-stiffness index (L) ($p = 0.02$). Strong correlations were found in PP with all ultrasound measured AS parameters of the two carotid arteries, except AI. These correlations were positive for PWV, Ep, β-stiff. index, and negative at AC. In contrast to PP, in MAP we found a positive correlation with PWVβ (L) ($p = 0.004$) and PWVβ (R) ($p = 0.004$), as well as Ep (L) ($p = 0.04$) and Ep (R) ($p = 0.004$). Central aortic systolic pressure showed positive correlations with PWVβ (L) ($p = 0.04$) and PWVβ (R) ($p = 0.01$), with Ep (R) ($p = 0.01$) and AI (R) ($p = 0.02$). An inverse significant correlation was observed in CASP with AC (L) ($p = 0.007$) and AC (R) ($p = 0.05$). In the values of HR of patients with T2DM, we found a negative correlation with AI measured in both carotid arteries AI (L) ($p = 0.007$) and AI (R) ($p = 0.006$) (Table 13 and Table 14).

Table 13. Correlation analysis of the parameters of AS of the left ACC and hemodynamic parameters in the group of patients with T2DM

		CASP	SBP	DBP	HR	PP	MAP
Numbers of observations	n	82	100	100	100	100	100
PWV (L), m/sec	r	0,222*	0,16	0,06	0.14	0.43**	0.28**
	p	0,045	0,10	0,52	0,15	0.0001	0.004
Ep (L), kPa	r	0,009	0,347**	0,443**	0,114	0.52**	0.2
	p	0,926	0,001	0,0001	0,259	0.001	0.04
AC (L), mm²/kPa	r	-,295**	-,341**	-,082	-,208*	-0.28	-0.18
	p	0,007	0,001	0,418	0,039	0.004	0.07
β-stiffness index (L)	r	0.15	-0.107	-0.23	0.1	0.44**	-0.08
	p	0.18	0.29	0.02	0.326	0.001	0.41
AI (L), %	r	0,075	0,057	-,045	-,278**	0.08	0.19
	p	0,504	0,578	0,660	0,005	0.39	0.05

Table 14. Correlation analysis of the parameters of AS of the left ACC and hemodynamic parameters in the group of patients with T2DM

		CASP	SBP	DBP	HR	PP	MAP
Numbers of observations	n	82	100	100	100	100	100
PWV (R), m/sec	r	.286*	.427**	0.14	0.08	0.38**	0.35**
	p	0.011	0.001	0.18	0.44	0.001	0.004
Ep (R), kPa	r	.273*	.419**	0.02	0.05	0.52**	0.28**
	p	0.015	0.001	0.88	0.61	0.001	0.004
AC (R), mm²/kPa	r	-0.21	-.291**	-0.08	-0.18	-0.33	-0.15
	p	0.05	0.004	0.43	0.08	0.0007	0.11
β-stiffness Index (R)	r	0.08	-.195*	-0.10	0.05	0.39**	0.03
	p	0.48	0.06	0.29	0.62	0.001	0.71
AI (R) %	r	.256*	0.209*	0.17	-.279**	0.06	-0.02
	p	0.02	0.04	0.10	0.006	0.52	0.84

Measurements of hemodynamic parameters in the control group similarly showed correlations with hemodynamic parameters (Table 15).

Table 15. Correlation analysis of PWVβ of left and right ACC with hemodynamic parameters in the control group

		CASP	SBP	DBP	HR	MAP
	n	26	26	26	26	26
PWV (L), m/sec	r		0.48		0.56	
	p	ns	0.008	ns	0.001	ns
PWV (R), m/sec	r	0.4	0.4		0.39	
	p	0.04	0.03	ns	0.03	ns

***n**- number of observations, ns- insignificance

5.1.4. Correlation of PWVβ with age in the group of patients with T2DM.

A positive moderate age correlation was found with Ep (R) (r = 0.358; p = 0.001) and Ep (L) (r = 0.291; p = 0.003), as well as for PWVβ (R) (r = 0.317; p). = 0.002) (Table 16).

Table 16. Correlation between age of the patients in the group of T2DM and the AS parameters**.**

		Ep (R)	AC (R)	AI (R)	PWV (R)	Ep (L)	AC (L)	AI (L)	PWV (L)
AGE	n	100	100	100	100	100	100	100	100
	coefficient of Pearson, r	0.358**	-0.151	0.126	0.317**	0.291**	-0.012	0.188	0.01
	p	0.001	0.139	0.22	0.002	0.003	0.216	0.062	0.921

We analyzed the relationship of PWVβ with the age of the studied patients by decades, divided into three groups. Group 1 - patients aged 39-50 years, group 2 - from 51 to 61 years of age; Group 3 - over 61 years. We compared the groups using analysis of variance - ANOVA (Table 17).

Table 17. Mean and standard deviation of PWVβ in the three age groups

	PWVβ (L)			PWVβ (R)		
	Mean±SD	95% CI, borders		Mean±SD	95% CI, borders	
		lower	upper		lower	upper
Group 1	6.72±1.15	6.22	7.24	6.64±0.83	6.26	7.02
Group 2	7.0±1.28	6.73	7.39	7.09±1.26	6.77	7.42
Group 3	7.49±1.38	7.07	7.90	7.6±1.54	7.13	8.07

We performed an ANOVA, which revealed a statistically significant difference in the values of PWVβ (R) (p = 0.01), which is common for the three age groups. In left ACC, an increase in PWVβ (L) was also observed with age, although not significant (p = 0.06).

To detect the presence of intragroup differences, we used ANOVA - Tukey's post hot analysis. We found a statistically significant difference in the values of PWVβ (R) between group 1 and group 3 (p = 0.02), therefore compared to patients aged 39-50 years (group 1), those over 61 years (group 3) have a significantly higher PWVβ (p = 0.02) (Table 18).

Table 18. ANOVA-post hot Tukey HSD analysis of variance revealing significant differences in PWVβ (R) between different age groups**.**

ANOVA- post hot-Tukey HSD						
dependent variable: PWVβ (R)						
(I) decades	(J) decades total	mean differences	Stand. Error	p	95% CI, bonders	
					lower	upper
Group 1	group 2	-0.45	0.33	0.37	-1.24	0.34
	group 3	-0.96	0.35	0.02	-1.78	-0.13
Group 2	group1	0.45	0.33	0.37	-0.34	1.24
	group3	-0.51	0.26	0.13	-1.12	0.11
Group 3	group3	0.95*	0.35	0.02	0.13	1.78
	group2	0.51	0.26	0.13	-0.11	1.12

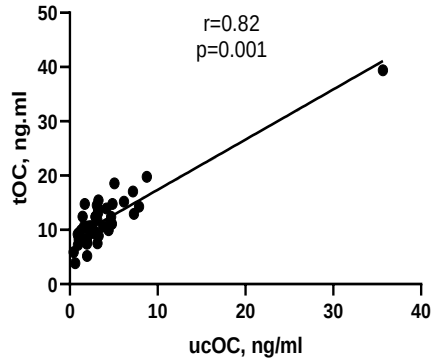
The same analysis performed for PWVβ (L) also revealed a large difference between group 1 and group 3, without reaching significance (p = 0.07)

5.2. Correlation dependences of serum osteoclacin levels.

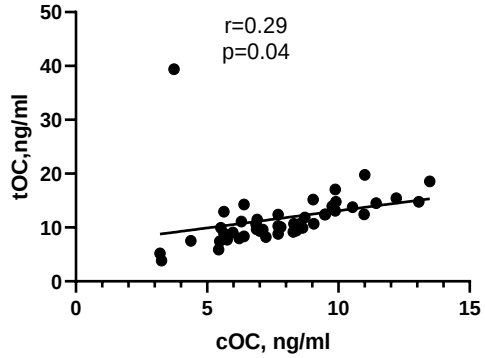
5.2.1. Correlation between different forms of osteocalcin

We used correlation analysis to investigate the relationship between different forms of osteoclacin and to assess the strength of their influence. In scientific publications, tOC is more often studied and reported, without distinguishing its carboxylated and non-carboxylated form. Therefore, we examined whether there was a relationship between the different forms of OC in the group of patients with T2DM. We found a positive correlation between tOC on both ucOC levels and cOC levels. Between ucOC and tOC, this relationship was strong and significant (r = 0.824; p = 0.001) (Graph 10). Serum cOC concentrations showed a weaker but also positive correlation with tOC (r = 0.299; p = 0.041) (Graph 11).

Graph 10. Correlation dependence of serum tOC and ucOC concentrations in the group of patients with T2DM



Graph 11. Correlation dependence of serum tOC and cOC concentrations in the group of patients with T2DM



5.2.2. Correlation dependences of OC with anthropometric, lipid and glucose parameters.

- Anthropometric indicators

We looked for correlations between osteocalcin and BMI, waist circumference, and waist / height ratio. Statistically significant correlations with anthropometric measurements were found for serum concentrations of cOC and tOC, but not for serum ucOC levels when data were included for both study groups (n = 65). Correlation analysis of anthropometric indicators in the group of patients with T2DM (n = 47) did not reveal a relationship between them and OC.

Table 19. Correlations of cOC with anthropometric parameters in patients with T2DM and controls.

parameter	**cOC**, ng/ml	
	r	p
BMI (kg/m²) n=65	-0.32	0.007
Waist circumference (cm) n=65	-0.4	0.0009
waist/height n=65	-0.39	0.005

*n = number of observations; r- coefficient of Pearson

Table 20. Correlation of tOC with waist circumference in patients with T2DM and controls

Correlation		**tOC, ng/ml**
Waist (cm) n=65	r	-0.27
	p	0.02

*n = number of observations; r- coefficient of Spearman

- Lipids parameters

We looked for correlations of OC with lipid parameters in 65 individuals from the study and control groups. We found these between all forms of OC and HDL-C. Thus, a positive correlation was revealed for ucOC and HDL-C (r = 0.25; p = 0.05), for cOC and HDL-C (r = 0.3; p = 0.01) and for tOC and HDL-C (r = 0.33; p = 0.008) (Table 21). When the same analysis was performed only in the group of patients with T2DM (n = 47), we observed dependence with even higher correlation coefficient with ucOC (r = 0.42; p = 0.004) and with tOC (r = 0.48; p = 0.001) (table 22). We also found a significant correlation between total cholesterol and tOC (r = 0.3; p = 0.04) only in the group of individuals with T2DM.

Table 21. Correlation analysis between HDL-C and osteocalcin in the group of patients with T2DM and controls (n = 65)

Correlation	**ucOC, ng/ml**		**cOC, ng/ml**		**tOC, ng/ml**	
	r	p	r	p	r	p
HDL-C, mmol/l	0.25	0.05	0.3	0.01	0.33	0.008

Table 22. Correlation analysis between HDL-C and osteocalcin within the group of patients with T2HD (n = 47).

Correlation	ucOC, ng/ml		cOC, ng/ml		tOC, ng/ml	
	r	p	r	p	r	p
HDL-C, mmol/l	0.42	0.004	0.16	0.27	0.48	0.001

We found no dependence of OC with FPG and glycated hemoglobin, both in the group of patients with T2DM and in the two study groups.

5.2.3. Correlation dependences of OC with AS parameters.

We sought a relationship between serum OC levels and ultrasound-measured AS parameters. Significant positive correlation was found for β-stiffness index (r = 0.317; p = 0.03) and Ep (r = 0.291; p = 0.05) of the right carotid artery (Table 23). In the left ACC measurements, we observed only a weak negative correlation between ucOC / tOC and AI (L) (r = -0.30; p = 0.02) in the group of patients with T2Dm (Table 24).

Table 23. Correlations of ET parameters measured on the right ACC with the different forms of OC in the group of patients with T2DM

	β-stiff. index, R	PWV, R	Ep, R	AC, R	AI, R
ucOC, ng/ml	r=-0.038 p=0.803	r=0.016 p=0.915	r= - 0.02 p=0.852	r= - 0.13 p=0.371	r=0.094 p=0.535
cOC, ng/ml	r=0.317 p=0.032	r=0.230 p=0.124	r=0.291 p=0.05	r= - 0.25 p=0.082	r=0.097 p=0.521
tOC, ng/ml	r=0.108 p=0.476	r=0.120 p=0.428	r=0.105 p=0.487	r= - 0.24 p=0.097	r=0.134 p=0.373
ucOC/tOC	r=-0.125 p=0.408	r= - 0.06 p=0.068	r=-0.112 p=0.457	r=-0.03 p=0.83	r=-0.061 p=0.686
ucOC/cOC	r=-0.52 p=0.731	r=0.000 p=0.999	r=-0.04 p=0.78	r=-0.103 p=0.496	r=0.089 p=0.554

Table 24. Correlations of ET parameters measured on the left ACC with the different forms of OC in the group of patients with T2DM

	β-stiff. index, L	PWV, L	Ep, L	AC, L	AI, L
ucOC, ng/ml	r=-0.173	r=-0.156	r=-0.158	r=-0.047	r=-0.245
	p=0.245	p=0.295	p=0.289	p=0.756	p=0.097
cOC, ng/ml	r=0.154	r=0.091	r=0.134	r=-0.183	r=0.243
	p=0.3	p=0.543	p=0.370	p=0.219	p=0.100
tOC, ng/ml	r=-0.097	r=-0.109	r=-0.092	r=-0.128	r=-0.127
	p=0.516	p=0.464	p=0.539	p=0.991	p=0.395
ucOC/tOC	r=-0.180	r=-0.164	r=-0.165	r=0.47	r=-0.30
	p=0.227	p=0.271	p=0.269	p=0.753	p=0.024
ucOC/cOC	r=-0.166	r=-0.145	r=-0.151	r=-0.043	r=-0.230
	p=0.264	p=0.331	p=0.310	p=0.774	p=0.119

6. Multivariable regression analysis

We used multivariate regression analysis to construct models based on all variables that significantly correlated with PWVβ and β-stiffness index in the performed correlation analyzes. When we used PWV (L) as a dependent variable, CASP, PP, MAP, and smoking were independently correlated with carotid stiffness (Table 25). When we used PWVβ (R) for the independent variable - SBP, PP, β-stiffness index, Ep and AC were independently correlated with carotid siffness (table 26).

Table 25. Multivariable regression analysis with dependent variable PWV (L) in the group of patients with T2DM

Model		Coefficient Beta	p	95.0% CI, Beta	
				lower	upper
Model 1	CASP	-0.14	0.07	-0.09	0.001
	PP	0.01	0.0002	0.03	0.1
	MAP	-0.29	0.03	0.004	0.11
Model 2	CASP	-0.23	0.14	-0.09	0.01
	PP	0.4	0.001	0.02	0.1
	MAP	-0.11	0.08	-0.007	0.1
	Waist	-0.48	0.22	-0.007	0.03
Model 3	CASP	-0.21	0.16	-0.009	0.01
	PP	0.14	0.001	0.02	0.1
	MAP	-0.11	0.09	-0.008	0.1
	Waist	-0.48	0.28	-0.009	0.03
	AI (L)	-0.07	0.79	-0.03	0.02
	Smoking	-0.15	0.005	-1.28	-0.22

Table 26. Multivariable regression analysis with dependent variable PWV (R) in the group of patients with T2DM

Model		Coefficient Beta	p	95.0% CI, Beta	
		Beta		lower	upper
	HBA1c	-0.07	0.35	-0.02	-0.009
Model 2	CAPS	0.024	0.27	-0.004	0.01
	SBP	-0.047	0.52	-1.22	2.38
	PP	0.047	0.51	-1.6	0.8
	MAP	0.041	0.54	-2.36	1.24
	β-stiffness ind	-0.816	0.02	0.02	0.27
	Ep, R	0.821	0.01	0.002	0.01
	AC, R	-0.211	0.0062	-0.47	-0.08
	Waist	-0.053	0.32	-0.001	0.005
	Age	-0.323	0.45	-0.004	0.009
Model 2	HBA1c	1.548	0.13	-0.05	0.007
	CAPS	1.349	0.19	-0.004	0.023
	SBP	3.196	0.0034	0.012	0.054
	PP	5.691	<0.0001	-0.044	-0.02
	β-stiffness inc	3.742	0.0008	0.14	0.48
	Ep, R	0.1461	0.88	-0.01	0.01
	AC, R	1.91	0.07	-0.522	0.01
	Waist	0.2055	0.84	-0.006	0.005
	Age	0.2875	0.78	-0.013	0.01
Model 3	HBA1c	0.8213	0.41	-0.025	0.01
	CAPS	1.242	0.22	-0.003	0.01
	SBP	3.216	0.002	0.009	0.04
	PP	7.022	<0.0001	-0.04	-0.02
	β-stiffness ind	2.754	0.007	0.046	0.29
	Ep, R	2.328	0.02	0.001	0.01
	AC, R	2.712	0.008	-0.417	-0.06
	Age	0.4455	0.66	-0.004	0.007
Model 4	HBA1c	0.795	0.43	-0.02	0.01
	CAPS	1.307	0.20	-0.003	0.01
	SBP	3.206	0.002	0.0092	0.04
	PP	7.09	<0.0001	-0.03	-0.02
	β-stiffness ind	2.768	0.007	0.047	0.29
	Ep, R	2.36	0.02	0.001	0.01
	AC, R	2.706	0.008	-0.413	-0.06
Model 5	CAPS	0.4577	0.64	-0.008	0.01
	SBP	1.578	0.11	-0.003	0.02
	PP	5.124	<0.0001	-0.031	-0.01
	β-stiffness ind	0.6581	0.51	-0.08	0.15
	Ep, R	4.603	<0.0001	0.01	0.027
	AC, R	3.224	0.001	-0.502	-0.11
Model 6	SBP	2.97	0.003	0.005	0.02
	PP	5.748	<0.0001	-0.029	-0.01
	β-stiffness ind	0.8681	0.38	-0.062	0.15
	Ep, R	4.853	<0.0001	0.011	0.02
	AC, R	2.864	0.005	-0.423	-0.07
	smoking	-1.48	0.14	-0.15	0.02

The dependent variable β-stiffness index (L) significantly correlated with the independent variables AC, DBP and PP in the group of patients with T2DM (Table 27).

Table 27. Multivariable regression analysis with dependent variable β-stiffness index (L) in the group of patients with T2DM

Model	Coefficient Beta	p	95.0% CI, Beta	
	Beta		lower	upper
AC	-0.44	<0.0001	-9.95	-6.05
AI	0.05	0.11	-0.09	0.01
DBP	-0.84	0.006	-0.13	-0.02
PP	-0.56	0.0002	0.05	0.15

The dependent variable β-stiffness index (R) significantly correlates with the independent variables SBP, PP, Ep and cOC (Table 28).

Table 28. Multivariable regression analysis with dependent variable β-stiffness index (R)

Model		Coefficient Beta	p	95.0% CI, Beta	
		Beta		lower	upper
Model 1	PWV	-0.84	0.38	-0.21	0.54
	Ep	0.8	0.0001	0.05	0.07
	AC	-0.51	0.2	-0.54	0.11
	SBP	0.05	<0.0001	-0.09	-0.07
	PP	-0.41	<0.0001	0.03	0.06
Model 2	PWV	-0.82	0.37	-0.21	0.56
	Ep	0.79	<0.0001	0.05	0.07
	AC	-0.47	0.27	-0.55	0.15
	SBP	0.05	<0.0001	-0.09	-0.07
	PP	-0.4	<0.0001	0.03	0.06
	Waist	-0.02	0.8	-0.006	0.005
Model 3	PWV	-0.82	0.03	0.04	1.23
	Ep	0.8	<0.0001	0.03	0.06
	AC	-0.58	0.32	-0.79	0.27
	SBP	0.047	<0.0001	-0.1	-0.07
	PP	-0.36	<0.0001	0.04	0.07
	Waist	0.027	0.53	-0.006	0.01
	cOC	-0.34	0.04	0.0005	0.1

7. Results of vitamin K2 supplementation.

In addition, we performed vitamin K2 supplementation for a period of 4 weeks in 18 randomly selected patients from the T2DM group who gave informed consent and appeared for a follow-up examination. Vitamin K2 was administered in the form of tablets containing menaquinone-7 (Kinon®) at a dose of 75µg, taken once a day. The aim of supplementation was to identify changes in cOC, ucOC levels and AP parameters.

Control ultrasound to determine the parametersrs of AS was performed after 4 weeks of supplementation, in the morning on an empty stomach, and at the same time serum samples were taken for levels of COC and uCOC. Baseline characteristics of patients with T2DM who underwent supplementation are shown in Table 29.

Table 29. Baseline characteristics of patients with T2DM underwent vitamin K2 supplementation**.**

Parameters	Mean.±SD	Parameters	Mean.±SD
Age (years)	59.09±8.5	AC, L	0.85±0.28
female (male) (%)	61,2 (38.8)	PWVβ, R	7.01±1.22
Duration of diabetes (years)	5,1±6.6	β-stiffness index, R	10.07±3.41
Smokers (%)	50	Ep,R	138.02±54.45
BMI (kg/m²)	31.5±7.5	AC,R	0.86±0.25
Waist(см)	105.2±17.8	PWVβ, L	7.0±1.43
Plasma glucose, mmol/l	8.0±2.6	CASP, mmHg	119.8±12.19
HBA1c, %	8.1±1.9	SBP, mmHg	127.3±13.2
Chol, mmol/l	4.98±1.34	DBP, mmHg	78.5±8.7
TG, mmol/l	2.17±2.19	PP, mmHg	48.8±11.9
HDL-C, mmol/l	1.18±0.52	MAP,mmHg	94.7±8.0
LDL-C, mmol/l	2.83±1.67	ucOC, ng/ml	4.2±2.4
β-stiffness index, L	9.97±4.17	cOC, ng/ml	8.08±2.06
Ep,L	136.1±53.03	tOC, ng/ml	12.2±3.2

- Comparative analysis of changes in osteocalcin levels after vitamin K2 supplementation.

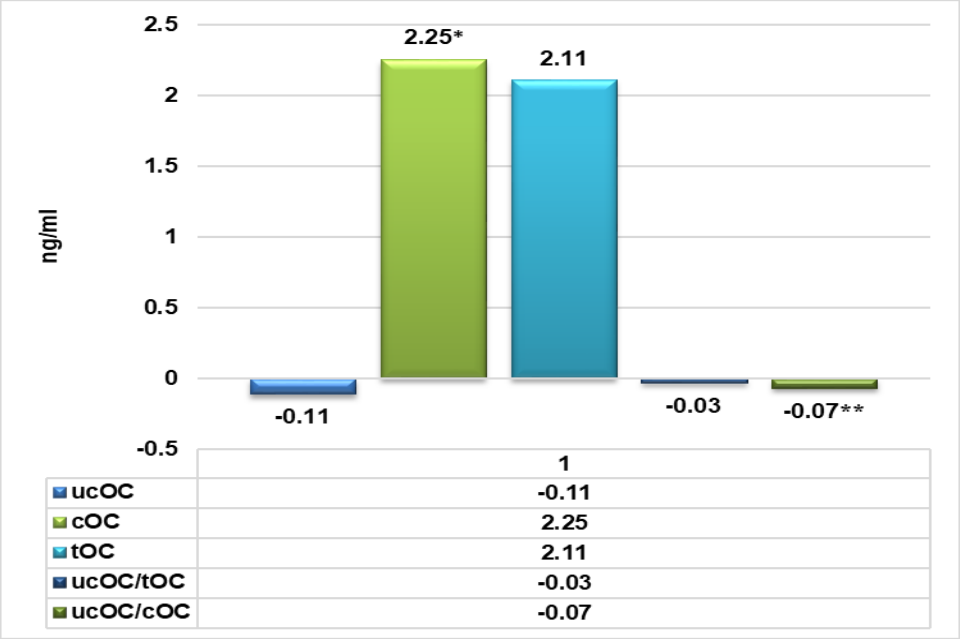
Comparison of the results showed that the mean values of cOC were higher after one month of vitamin K2 supplementation, increasing from 8.16 ± 2.11 ng / ml to 10.34 ± 3.05 ng / ml. For the ucOC / tOC ratio, we found a decrease from 0.33 ± 0.14 ng / ml to 0.29 ± 0.22 ng / ml. The significance of the differences in their serum levels was assessed by paired t-test. The results show a significant change in the direction of increase in serum concentrations of cOC (t = -2.53; p = 0.02). Although a slightly decrease is observed in ucOC / tOC levels after supplementation, which is theoretically expected to occur, but in this case does not reach statistical significance (p = 0.26).

Table 30. Comparative analysis of the mean values of cOC and ucOC / tOC before and after the performed supplementation (paired t-test)**.**

Before/After supplementation		**Mean ± SD**	**p**	**95% CI, граници**	
				lower	**upper**
cOC, ng/ml	before vit.K2	8.08±2.06	0.02	-4.02	-0.33
	after vit. K2	10.3±2.9			
ucOC/tOC	before vit.K2	0.33±0.14	0.26	-0.04	0.13
	after vit. K2	0.28±0.21			

Changes in serum concentrations of other forms of osteocalcin were assessed by the Man-Whitney test. We observed a decrease in ucOC levels after supplementation. The difference in the mean ranks at ucOC and tOC were statistically insignificant (p = 0.82, respectively p = 0.08). For the ucOC / cOC ratio, we obtained statistical significance of the change after supplementation (p = 0.05) (Diagram 11).

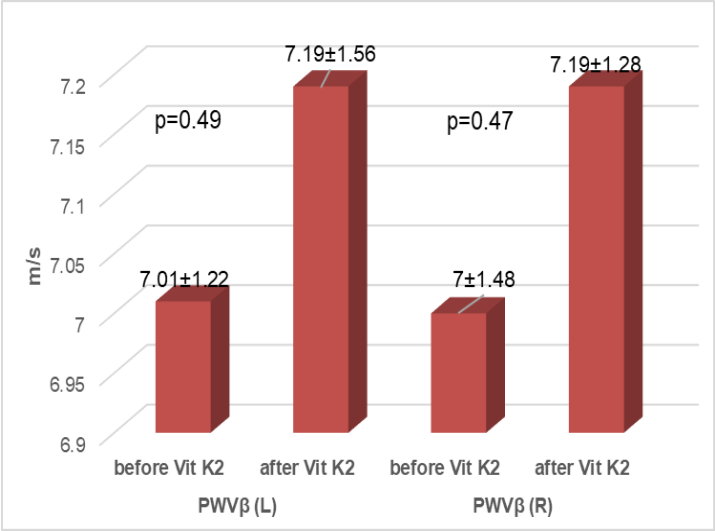
Diagram 11. Representation of the change in serum osteoclacin levels after vitamin K2 supplementation (* p = 0.02; ** p = 0.05)



- Comparative presentation of ET parameters before and after vitamin K2 supplementation**.**

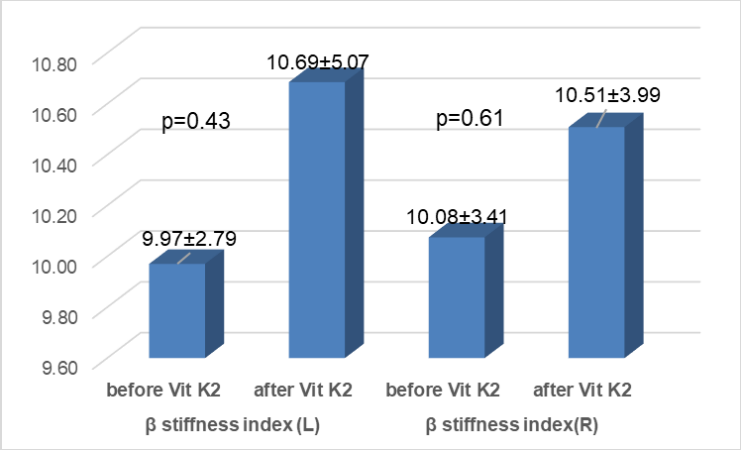
We observed an increase in the mean values of PWVβ (L) from 7.01 to 7.19 m / sec, as well as PWVβ (R) from 7.00 to 7.19 m / sec. The paired t-test did not reveal any significant differences after vitamin K2 supplementation (Diagram12).

Diagram 12. Comparative presentation of PWVβ (R) and (L) values before and after vitamin K2 supplementation in patients with T2DM (paired t-test).



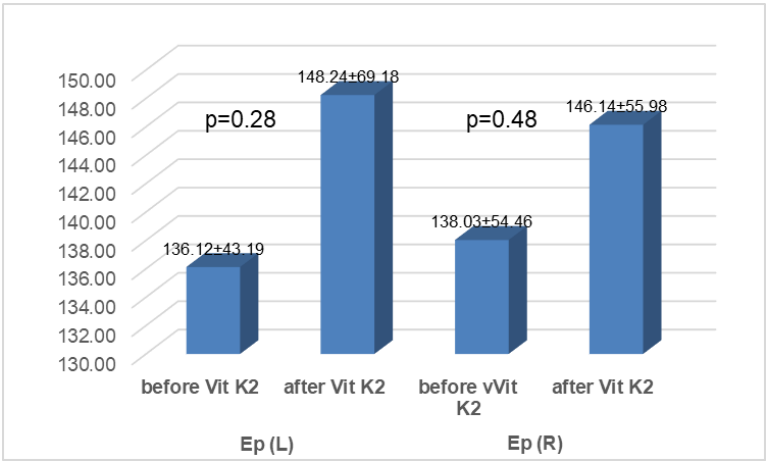
The changes in the mean values of the β-stiffness index follow the same direction of slight increase in the left and right ACC (Diagram 13).

Diagram 13. Comparative presentation of β-stiffness index (R) and (L) values before and after vitamin K2 supplementation in patients with T2DM.



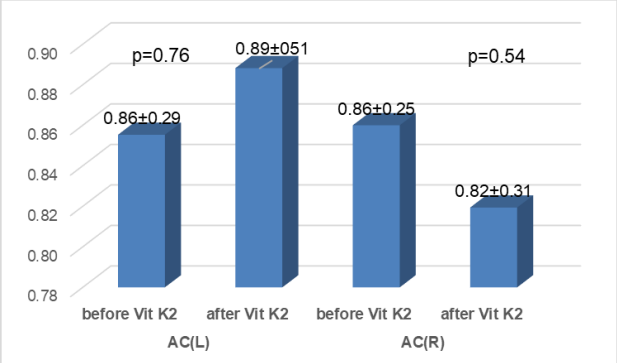
We also find an increase in Ep on the left and right ACC. However, after the paired t-test, the differences in the ultrasound parameters thus found did not reach statistical significance (Diagram 14).

Diagram 14. Comparative presentation of the values of Ep (R) and (L) before and after vitamin K2 supplementation in patients with T2DM**.**



AC measurements showed divergent values, with an increase in AC measured on the left ACC and a decrease in AC measured on the right ACC, again without reaching the significance of the change (Diagram 15).

Diagram 15. Comparative presentation of AC (R) and (L) values before and after vitamin K2 supplementation in patients with T2DM (paired t-test).



We looked for a change in the values of hemodynamic parameters before and after vitamin K2 supplementation, due to their theoretical influence on AS parameters. All showed a slight upward trend, with the largest finding in CASP from 119 to 126 mmHg, which reached statistical significance (t = -2.7; p = 0.01). Heart rate after one month of supplementation showed lower values, but without them reaching significance (Table 31).

Table 31. Comparative analysis of hemodynamic parameters of patients with T2DM performed vitamin K2 supplementation (paired t-test).

Before/after supplementation with Vit K2		**Mean ± SD**	**p**	**95% CI, border**	
				lower	**upper**
CASP	Before	119.85±12.19	0.02	-12.62	-1.38
	After	126.84±11.42			
SBP	Before	127.55±13.1	0.10	-9.27	0.93
	After	131.72±12.9			
DBP	Before	78.55±8.7	0.19	-7.57	1.68
	After	81.5±7.4			
PP	Before	48.83±11.9	0.62	-7.33	4.55
	After	50.22±13.1			
MAP	Before	94.78±8.8	0.08	-7.30	0.44
	After	98.21±7.37			
HR	Before	74.06±11.2	0.11	-1.00	8.78
	After	70.16±8.7			

VI DISCUSSION

1. Analysis of the characteristics of the studied group.

The idea for the present study arose from the existing scientific interest in the discovery and validation of new vascular biomarkers in the diagnosis of subclinical atherosclerosis. Together with the already known traditional risk factors, they would help to more accurately determine the individual cardiovascular risk in patients with T2DM. Another aspect is the interest in the biological action of vitamin K2 and more precisely vitamin K-dependent proteins, among which is osteocalcin. Recent scientific data define it as a hormone acting at the level of bone, glucose regulation and vascular homeostasis.

In our study, we included patients with T2DM who had no history of symptomatic or documented atherosclerotic vascular disease, with a mean age of 57.7 ± 7.4 years, with almost equal sex distribution (52% female and 48% male). The mean duration of diabetes in the study group was 6.75 ± 6.3 years, with 26% of patients having newly diagnosed disease. Half of the patients reported no complications of diabetes by the time of enrollment. The mean age of the subjects in the study was similar to the reported PWV measurements in patients with T2DM. According to anamnestic data, females with T2DM have menopause and do not undergo hormone replacement therapy. Atherosclerotic changes in the vessels begin at an early age and become symptomatic after the 5th decade. Estrogen protection is not observed when the females patients has T2DM.

The measured anthropometric indicators in the study group showed, as expected, higher values of BMI and WC compared to controls ($p = 0.05$, $p = 0.01$), which is part of the concomitant diabetes obesity. The International Group on Obesity (IOTF) defines the presence of overweight in the Caucasian at BMI> 25 kg / m², and obesity at BMI> 30 kg / m². Abdominal obesity for Caucasians is considered to be ≥ 94 cm for men and ≥80 cm for women (IDF, 2005). Worldwide data report 85.2% incidence of overweight and obesity in people with T2DM (183). Our results show a higher incidence of overweight and obesity, determined by BMI - 90%. The incidence of abdominal obesity in the study group reached even higher values - 93%. In comparison with the data for the general population of the European Association for the Study of Obesity (EASO, 2014), the incidence of overweight and obesity among the Bulgarian population amounts to 58.2%.

In the analysis we did not find a relationship between BMI and carotid artery stiffness. Correlation, however, was observed between the measured WC and PWVβ (R) ($r = 0.25$; $p = 0.01$). Of the other ET indicators, significant positive correlations with WC were found in Ep (R) ($r = 0.23$; $p = 0.02$) and β-stiffness index (R) ($r = 0.22$; $p = 0.02$) and negative correlation with AI (R) ($r = -0.21$; $p = 0.04$). In the left ACC, we found only a correlation with Ep (L) ($r = 0.27$; $p = 0.007$). We looked for a link between abdominal obesity and carotid artery stiffness in patients with T2DM when using the waist / height ratio. Such a significant relationship was revealed for both PWVβ (R) ($r = 0.22$; $p = 0.02$) and PWVβ (L) ($r = 0.25$; $p = 0.01$). A number of authors believe that the waist / height ratio has a better predictive value for cardiovascular events compared to WC, as it overcomes the disadvantage of the latter associated with the influence of height. The value of the ratio> 0.5 is defined as the limit, above which there is an increase in metabolic and cardiovascular risk. Analyzing the data from our cohort, it is seen that 94% of patients with T2DM have abdominal obesity, detected by increased waist / height ratio> 0.5, which correlates with increased PWVβ values in both arteries, and therefore with increased local carotid stiffness.

The study group shows mean values and standard deviation of glycated hemoglobin - 9.1 ± 2.4%. We associate this poor glycemic control with the fact that patients are hospitalized for T2DM or have another acute or chronic disease that has led to decompensation. We found a correlation between glycated hemoglobin and the values of PWVβ (R) and PWVβ (L) in an analysis performed for the group of patients with T2DM and controls. Ferreira et al. investigated the role of glycemic control over cf-PWV in patients with T2DM and found that HBA1c > 7.5% were associated with a twice as high risk of elevated cf-PWV. According to Ferreira, good glycemic control, reduction of blood pressure and heart rate are among the most important determinants for reducing the progression of aortic stiffness in papients with T2DM.

We did not establish a relationship between the mean value of FPG and echotracking measured parameters of AS. Our data are similar to those reported by some authors and in contrast to others. Fang et al. report a lack of relationship between FPG and AS scores measured by ba-PWV. However, a number of publications indicate a link between plasma glucose and AS. Davies et al. investigated the influence of glucose metabolism parameters and found a significant relationship between IFG, IGT and T2DM with AS, when they are measured by cf-PWV. The accumulated scientific information covers the measurement of AS by different techniques and in different arterial areas, and there is no data for correlations with the indicators of carbohydrate metabolism and echotracking-based measurement of PWVβ. Our study for the first time in Bulgaria reports results for

AS in T2DM. The role of hyperglycemia in the development of diabetic macrovascular disease is currently considered by most authors to be controversial.

The levels of lipid parameters - total cholesterol, triglycerides, HDL-C, LDL-C between the two groups did not differ significantly. Probably the reason for the lack of such a difference is the standard statin treatment performed in patients with T2DM. We observed higher LDL values than recommended in patients with T2DM and constellation of elevated triglycerides and low HDL-C.

The available data in the literature on the relationship between lipids, obesity and AS are inconclusive. Our study, like others, did not find a relationship between lipid parameters and PWVβ measured by ET. We found a weak negative correlation of HDL-C with AC on the right ($r = -0.2$; $p = 0.05$) and AC on the left ($r = -0.25$; $p = 0.01$). A possible explanation for this is the statin therapy performed in the group of patients with T2DM, which would affect the HDL-C values in an upward direction. A brazilian study found a link between cf-PWV and triglyceride levels. Another study found a correlation between PWVβ and HDL-C in both sexes, with LDL-C in males and TG in females. These and our data suggest a greater participation of HDL-C compared to LDL-C in the development of AS in individuals with T2DM.

Smoking is known to be a traditional cardiovascular risk factor related to vascular health. In the study group, 46% were smoking patients in contrast with control group where they are 40%. This difference turned out to be insignificant in the performed nonparametric analysis ($X^2 = 0.33$, $p = 0.56$). We found a weak correlation of smoking with PWVβ measured on both carotid arteries in subjects from both groups. In multivariate regression analysis, smoking significantly and independently correlated with PWVβ (L). Similar data are reported by other authors. Zhang et al. found a significant increase in AS and a decrease in PWV, Ep, β-stiffness index two years after smoking cessation.

Arterial hypertension is among the traditional cardiovascular risk factors and is more common in patients with T2DM, as its incidence increases with age. In a systematic review in the european population, Colosia et al. reported an incidence of concomitant hypertension in patients with T2DM in the range of 51.4-95% (187). In our study, the incidence of hypertension in patients with T2DM is 84% , and in controls respectively 56.7%. The X^2-square test showed a higher probability of the presence of hypertension in the group of patients with T2DM ($X^2 = 9.98$, $p = 0.002$).

2. Analysis of the results of echotracking measured AS of carotid arteries.

Arterial stiffness is a physical quantity that directly depends on the components of blood pressure. Therefore, patients with uncontrolled blood pressure

were not included in our study. The measured SBP, DBP, PP, MAP, CASP and HR showed higher values in the group of T2DM, but the differences with the controls did not reach statistical significance. This made the measurement of AS for both groups under equal conditions. The measurements were performed bilaterally, on both carotid arteries, due to the theoretical possibility for different results.

This is due to the fact that the two arteries are not anatomically identical. These assumptions are also confirmed by published data on higher IMT values measured on the right ACC compared to the left. Dzeko et al. also reported that cf-PWV measured by ShygmoCor showed higher values when measured on the right ACC. In our study, similar to the data above, we measured higher PWVβ on the right carotid artery 7.42 ± 1.33 m / s compared to PWVβ on the left carotid artery 7.37 ± 1.32 m / s. In the control group we observed the same tendency of a higher value of PWVβ (R) 6.43 ± 1.11m / s, compared to PWVβ (L) 6.37 ± 0.94 m / s. Published data on the direct comparison of PWVβ, measured by ET bilaterally in the carotid areas, are not known at this stage. The differences of PWVβ found in us on the left and right ACC did not reach statistical significance in both groups ($p = 0.57$, respectively $p = 0.8$). Subsequent correlation analyzes revealed more numerous relationships of PWVβ measured on the right ACC with ultrasound and hemodynamic parameters compared to those measured on the left ACC.

The pulse wave velocity is the main echotracking parameter, which at cut-off> 6.65 m / s is a marker for increased AS. Our data on the mean values of PWVβ in patients with T2DM showed statistically significant differences for both PWVβ (R) ($p = 0.001$) and PWVβ (L) ($p = 0.001$), compared to healthy controls. Alvim et al. in a study conducted in the Brazilian population found higher values of cf-PWV in individuals with T2DM, regardless of the presence or absence of hypertension. Numerous studies have examined cf-PWV in a variety of high-risk cohorts, such as those with chronic renal failure, coronary artery disease, and only a fraction of subjects were with concomitant T2DM. Diabetes mellitus is unequivocally associated with accelerated vascular damage and as such is excluded from most analyzes. In contrast to the measurement of AS with applanation tonometry (cf-PWV), data obtained by the ET method (PWVβ) are scarce in individuals with T2DM. Our study, in addition to PWVβ, found significant differences between T2DM and healthy controls in all carotid stiffness parameters measured by ET: Ep, β-stiffness, AC (L), AI (R). Exceptions are only AC (R) and AI (L) for which has been observed nonsignificance.

Increased values of the echotracking parameters provide information about increased AS in the group of patients with T2DM. These patients are at increased risk of developing subsequent macrovascular conditions.

Correlation analysis revealed a very strong positive relationship between PWVβ (R) and Ep ($r = 0.97$; $p = 0.001$) and between PWVβ (R) and β-stiffness index ($r = 0.93$; $p = 0.001$) and a strong negative correlation between PWVβ (R) and AC ($r = -0.71$; $p = 0.001$). No such dependence was found on the left ACC.

The parameter β-stiffness index is the natural logarithm of the systolic / diastolic pressure ratio related to the relative change in vascular diameter. It is independent of blood pressure in individuals without hypertension. In our study group, the mean β-stiffness index (R) was statistically significantly higher than the controls ($p = 0.001$). Correlation analysis showed a positive relationship of β-stiffness index (L) with AI ($r = 0.24$; $p = 0.01$) and a strong negative relationship with arterial compliance AC ($r = -0.60$; $p = 0.001$), measured on the same artery.

The β-stiffness index measured by ET of the right ACC showed some differences. We found a very strong correlation with PWVβ ($r = 0.93$; $p = 0.001$) and Ep ($r = 0.71$; $p = 0.001$). Like the left ACC, there is an inverse correlation between the β-stiffness index and the AC ($r = -0.64$; $p = 0.001$).

In our study, we also found a number of correlations between hemodynamic and ultrasound parameters. SBP showed a significant positive correlation with Ep and a negative correlation with AC. Only for right ACC, a significant correlation of SBP was found with PWVβ ($p = 0.001$) and AI ($p = 0.04$). Indeed, on the one hand, increased SBP leads to greater stress on the vessel wall and contributes to the development of AS, and on the other hand AS leads to increased PWV and earlier return of the reflected wave to the ascending aorta and thus increases SBP and decreases DBP. In contrast with SBP, DBP does not show a relationship to the ultrasound parameters measured in the right ACC. For the left, we found a significant negative relationship between DBP and the β-stiffness index ($p = 0.02$). These results are in agreement with other authors who report an association between low DBP values and the development of AS in adult patients.

We found significant correlations between PP and all echo-tracking parameters for both arteries, with the exception of AI. For PWV, Ep, β-stiffness index they are positive (for all $p = 0.001$), and for AC they are negative ($p = 0.0007$). Pulse pressure and AS are known to increase with age. This remarkable association of PP with AS can be explained by the fact that with increasing arterial stiffness, the reflected wave returns to the aorta earlier in the onset of systole, causing isolated systolic hypertension and an increase in PP. There are number of reports for the role of PP as an independent predictor of CVD and cardiovascular mortality. In the Hoorn study, PP in patients with T2DM was associated with a high cardiovascular risk. Any increase in PP by 10 mmHg increases the relative risk (RR 1.27), even after stratification of other risk factors. In our study, after multivariate regression analysis,

only PP showed an independent correlation with PWVβ (L), PWVβ (R), β-stiffness index (L) and β-stiffness index (R).

The elastic modulus (Ep) is sensitive to the effect of pulse pressure.The beta-stiffness index, as the logarithm of the systolic / diastolic pressure ratio, is a relatively pressure-independent quantity. This explains why the MAP significantly correlates with PWV and Ep, but not with β-stiffness. In the T2DM group we studied, the MAP showed significant correlations with PWVβ and Ep bilaterally, but not with the β-stiffness index.

Heart rate is a routine indicator of clinical practice, and its association with AS has been reported in some studies. In our sample, we found an inverse correlation between HR and AI (L and R) and AC (L). The relationship between AS and HR may be due to a decrease in the elastin content in the vessel wall over time, with an increased number of heart contractions to which the vascular bed is exposed. On the other hand, the increase in heart rate is associated with increased activity of the sympathetic nervous system, which by various mechanisms could induce an increase stiffness.

CASP is known to be more strongly associated with atherosclerosis than brachial pressure. CASP is physiologically higher than SBP. In our study, CASP showed the same correlations with ET parameters as SBP for right ACC. Thus, CASP significantly positively correlated with PWVβ ($p = 0.01$), Ep ($p = 0.01$), AI ($p = 0.02$) and significantly negatively with AC ($p = 0.05$). For the left ACC, we found a positive correlation of CASP with PWVβ ($p = 0.04$) and negative with AC ($p = 0.007$). Theilade recently report an association of CASP with CVD and the development of microalbuminuria in patients with T1DM. The risk is gradual increase with increasing CASP.

Abdominal obesity is known to be associated with disorders of carbohydrate metabolism. With an increase in waist circumference and the waist / height ratio of patients in the T2DM, we also observed a significant increase in PWVβ (R) ($p = 0.01$), β-stiffness index (R) ($p = 0.02$), Ep (R) ($p = 0.02$), Ep (L) ($p = 0.007$) and AI (R) ($p = 0.04$). Relationship between abdominal obesity and carotid stiffness was not observed in the control group.

Age is one of the main physiological determinants for increasing arterial wall stiffness in healthy individuals. In our cohort of individuals with T2DM, we observed an age-related correlation with PWVβ (R) ($p = 0.002$), but not for PWVβ (L). Subanalysis, in which patients with T2DM were divided into three age groups, revealed differences between them. Thus, a statistically significant difference in PWVβ values, again measured on the right ACC, was found between the youngest and the oldest group ($p = 0.02$). Age also showed an association with Ep (R) ($p =$

0.001) and Ep (L) (p = 0.003). Many studies report a nonlinear increase in PWV with age, with acceleration observed after the 5th decade.

The Framingham Heart Study also shows an increase in AS with age. The increase in PWV also leads to an increase in CV risk and this is observed in both hypertensive and non-hypertensive individuals. The data from our study obtained using the echotracking technique confirm that AS increases with age in patients with T2DM, similar to the data in the literature obtained using other methods, such as applanation tonometry.

Based on the many statistically significant correlations of the AS parameters with the different above indicators, we performed a multivariable regression analysis. It showed an independent correlation of carotid stiffness in patients with T2DM with SBP, PP, β-stiffness index, Ep and AC when PWVβ (R) was used as the dependent variable. CASP, PP, MAP and smoking had a significant independent correlation with the values of PWVβ (L). When we used the β-stiffness index as a dependent variable, significant correlations were observed for SBP, PP, Ep, as well as with the levels of carboxylated osteocalcin. Thus, we can summarize that SBP, pp, MAP and smoking, as well as with cOC are among the most important determinants leading to increased carotid stiffness in patients with T2DM.

3. Discussion of the results of the studied serum levels of osteocalcin.

The data on serum levels of OC obtained in our study showed lower mean values for its carboxylated form - cOC 7.84 ± 2.41 ng / ml in patients with T2DM, compared with those in controls 10.05 ± 4.37 ng / ml, and this difference was statistically significant (p = 0.01). The same trend for lower values in individuals with T2DM was observed for both ucOC 3.81 ± 5.2 ng / ml (p = 0.08) and tOC 11.64 ± 5.24 ng / ml (p = 0.07), but without reaching significance compare to controls. This could be due to the small number of patients studied (n = 47) and with the increase in the number of subjects, ucOC and tOC would also become significant. Our data correspond to Dawod et al., who report lower levels of ucOC and tOC in patients with T2DM.

It is important to note that most human OC studies are not performed by ELISA. Therefore, they do not distinguish the carboxylated forms, but provide summary information on tOC levels. Data from various studies indicate low tOC levels as a risk factor for the development of insulin resistance, metabolic syndrome and T2DM. Several studies have linked low levels of tOC and ucOC to an increased risk

of developing diabetes mellitus. Kanazawa et al. have found an increase levels of tOC in patients with known diabetes after one month of optimization of antidiabetic therapy.

In a correlation analysis, no relationship was found between serum ucOC and cOC levels. Separately, however, both forms correlated with tOC. For ucOC this correlation is very strong ($r = 0.82$; $p = 0.001$), while for cOC it is rather weak ($r = 0.29$; $p = 0.04$). Booth et al. found a strong correlation between the absolute values of the concentrations of ucOC and tOC, while expressed in percentages such a correlation was not observed.

The role of ucOC remains controversial with respect to carbohydrate metabolism. Our study did not find correlations of FPG, HBA1c with different forms of OC. Reported data on the association of ucOC with plasma glucose, HBA1c and fat mass by some authors have not been confirmed by others. Sanchez-Enriquez et al. found lower ucOC levels in T2DM compared to healthy controls, as well as a negative correlation with BMI, a positive correlation with DBP. A study of 2966 men without osteoporosis found that higher ucOC values were associated with a reduced risk of diabetes (OR 0.64).

A study of women with T2DM showed statistically significant lower cOC values compared to control women ($p = 0.02$). A possible reason for this difference is the loss of the effect on estrogen and the increase in bone turnover in menopausal women, such as our study group. Patients have no established osteoporosis and do not receive hormone replacement therapy. There is a gender difference in the metabolic effect of OC in humans. Low circulating levels of OC are likely to be associated with hyperglycemia and hyperinsulinemia in women other than men .

As a hormone, OC, affects carbohydrate, lipid and energy metabolism. In this regard, we looked for correlations with total cholesterol, HDL-C, LDL-C and TG. The strongest positive correlation HDL-C had with tOC ($p = 0.008$) and ucOC ($p = 0.05$). A correlation between HDL-C and cOC was also observed in the two study groups ($r = 0.3$; $p = 0.01$). Total cholesterol was found to correlate only with tOC ($r = 0.3$; $p = 0.04$) in the group of individuals with T2DM.

In contrast to animal model, which show the involvement of ucOC in lipid metabolism, human data are very scarce. Alfadda et al. found a significant positive correlation between ucOC and HDL and a negative one between tOC and TG.

Osteocalcin is known to increase adiponectin secretion. Patients with T2DM are characterized by abdominal obesity and low adiponectin levels. In our study group, we found negative correlations of BMI with cOC ($r = -0.32$; $p = 0.007$), waist

circumference (r = -0.4; p = 0.0009) and waist / height ratio (r = -0.39; p = 0.005). Negative correlation with waist circumference also showed tOC (r = -0.27; p = 0.02).

The hypothesis of the connection of OC with atherosclerosis and the development of AS has been the subject of scientific interest in recent years. Millar et al. published a meta-analysis of studies looking for a link between OC and atherosclerosis, the results of which showed great diversity (163). Our study aimed to find a connection between the levels of the OC and the parameters of the AS. Such a significant positive correlation was observed only between the cOC and β-stiffness index (R) (r = 0.31; p = 0.03) and between it and Ep (R) (r = 0.29; p = 0.05). After multivariable regression analysis, this relationship between β-stiffness index (R) and cOC remained significant and independent (B = -0.32; p = 0.04). In the left ACC, an inverse correlation was found between the ucOC / cOC ratio and AI (p = 0.02). Data on the association of OC with cf-PWV as a major parameter of AS are very limited, mainly in patients on chroniodialysis. Csiky et al. in their study in chroniodialysis patients, found a significant negative correlation of OC with cf-PWV and augmentation index (AI).

Studies involving the investigation of the relationship of OC with PWVβ, measured by the ET method to our knowledge, are currently unknown

4. Discussion of the results of the performed supplementation with vitamin K2.

In our study after vitamin K2 supplementation, we observed a change in mean ucOC values in the direction of a decrease from 4.2 to 4.09 ng / ml (p = 0.26). Serum cOC levels showed a reverse upward trend from baseline before vitamin K2 (MK-7) intake from 8.08 ng / ml to 10.33 ng / ml, which reached statistical significance (p = 0.02). Total osteocalcin also increased its levels from 12.2 to 14.4 ng / ml, but this change did not reach significance (p = 0.08). The results obtained by us are in accordance with the biological action of vitamin K2. It acts as a cofactor of carboxylase, which converts the non-carboxylated form of osteocalcin (ucOC) to carboxylated (cOC). Thus ucOC increases with vitamin K2 deficiency and decreases with vitamin K2 supplementation. In general, studies have shown mixed data on the increase, decrease or neutrality of tOC after vitamin K2 intake.

The increase in serum tOC levels under the influence of vitamin K2 could be explained by an increase in osteoblast activity. In this regard, in our study group, the ratios showed a decrease in their values after supplementation. For ucOC / tOC this change was not significant (p = 0.26), while for ucOC / cOC a significance was reached (p = 0.05).

As noted above, ucOC correlates with tOC. However, the expression of ucOC as a percentage of tOC, according to some authors, may more adequately reflect vitamin K status compared to the absolute value of ucOC. Thus ucOC> 20% is considered an indicator of vitamin K2 deficiency. In our study group, there is initially a deficiency of vitamin K2 - ucOC is 34.1% of total osteocalcin. After supplementation, we report that the deficit has decreased to 28%, but still the percentage of ucOC has not reached normal. As a reason for this we consider the short period of the implemented supplementation. Studies involving the assessment of OC after vitamin K2 supplementation are available mainly for patients on chroniodialysis due to their known tendency to vascular calcification. Most of them have a high percentage of ucOC, therefore vitamin K deficiency.

Non-carboxylated osteocalcin is considered a hormone. It acts at the level of the beta cell of the pancreas and stimulates insulin secretion. In addition, it increases adiponectin secreted by adipose tissue and thus improves insulin sensitivity. After administration of vitamin K2 and subsequent reduction of ucOC levels, we theoretically expect a deterioration in carbohydrate metabolism. Such results have not been observed in practice. Yoshima et al. apply vitamin K supplementation for a period of three years and thus reduce ucOC levels. They also report, a protective effect against the progression of IR in men. Choi et al. conducts supplementation with vitamin K2 (MK-4) 30 mg for 4 weeks reporting a decrease in ucOC, without change in glucose levels. Vitamin K2 supplementation (MK-4) - 45mg for a period of 36 months, found a decrease in ucOC levels without a change in glucose metabolism.

After we found changes in the levels of the forms of the OC, as a result of the supplementation we looked for differences in the ultrasound parameters of the AS. An increase in tOC is known to be associated with positive effects on the arterial wall. In a recent study of postmenopausal women, low tOC levels were associated with a risk of developing future T2DM and were negatively correlated with ba-PWV and carotid IMT. Fulton et al. publish results for vitamin K2 supplementation in the form of MK-7 at a dose of 100 μg daily for 6 months in adult patients with a history of CVD. They found an improvement in endothelial function, assessed by flow-mediated vasodilation of the brachial artery and ba-PWV, although the differences did not reach significance. Kurnatowska et al. performed supplementation with MK-7 with 90 μg for a period of 9 months in patients with CKD 3-5 degrees and found significantly lower carotid IMT and a significant decrease in tOC.

Knapen et al. performed supplementation with vitamin K2 (MK-7) 180 μg daily for 36 months in healthy postmenopausal women and reported improvement in AS scores measured by cf-PWV and by local PWV. The elastic properties of the

carotid artery in response to MK-7 are improved, but only in those women who initially had a high β-stiffness index of the carotid artery. In our study group of patients with T2DM who underwent supplementation with 75 μg daily vitamin K2 (MK-7) we report an increase in the mean values of all ET indicators measured on the left ACC. Thus PWVβ increased from 7.01 to 7.19 m / s, and β-stiffness index from 9.97 to 10.69. The largest increase was found in Ep from 136.12 to 148.2, followed by AI from 9.82 to 12.65. After the paired t-test, these differences were not significant. The right ACC showed a similar upward trend in AS. Again, in the paired t-test, we found no significance for these differences.

These results are in contrast to those reported by Knapen et al. Supplementation in their study was for a significantly longer period and nevertheless found significant differences only in the β-stiffness index. Data from a recent systematic analysis by Lees et al., published in 2018 on the effect of vitamin K2 supplementation on AS, show a significant reduction in ucOC, while changes in AS do not reach significance. At this stage, there are no data from interventional studies to assess the effect of vitamin K2 on vascular endpoints. There are also very few studies that use cf-PWV or local PWV to evaluate the effects of vitamin K2.

One of the possible reasons for the increase of ET parameters of AS in our study is the short period of supplementation, during which it is possible that there have been no functional changes in the vessels. Another reason we looked in the hemodynamic parameters. After analysis, we found an increase in the mean values of CASP (+ 5mmHg), SBP (+ 4.1mmHg), DBP (+ 2.9mmHg), PP (+ 1.4mmHg) and MAP (+ 3.4mmHg) after supplementation. A statistically significant increment occurred only at CASP ($p = 0.01$). Thus, the deteriorated control of the blood pressure could explain the increase of the AS.

At this stage, the reasons that could lead to poorer control of blood pressure, as well as whether they are related to vitamin K2 supplementation, are not very clear.

Streit et al. studied patients with hypertension, some of whom received warfarin (a γ-carboxylase antagonist that reduces the effect of vitamin K) and found that patients on anticoagulant have lower SBP and DBP. Lim followed 116 diabetic patients treated with warfarin for 36 months and found no change in blood pressure. Some polymorphisms of the vitamin K epoxide reductase complex subunit 1 gene (VKCOR1) have been found in hypertensive patients and are associated with an increased risk of developing essential hypertension. Epoxide reductase is involved in the vitamin K cycle and leads to its recycling and thus increases its concentration. As another reason for the worsening of blood pressure and the tendency to higher values of ET-parameters, worsened compliance of patients with regard to antihypertensive therapy after discharge could be discussed.

VII. CONSEQUENCES

1. Patients with T2DM have significantly increased local AS of the carotid arteries, expressed by higher values of PWVβ, Ep and β-stiffness index and lower AC and AI, compared to healthy controls.

2. Arterial stiffness in patients with T2DM increases with age.

3. With the increase in waist circumference, in patients with T2DM, a significant increase in the values of PWVβ, Ep and β-stiffness index was observed. Their higher waist / height ratio is associated with increased carotid PWVβ.

4. HDL levels showed an inverse correlation with arterial compliance, and a positive relationship with serum ucOC and tOC levels in the T2HD group.

5. Higher glycated hemoglobin values were associated with significantly higher carotid PWVβ in the study groups.

6. CASP, PP, MAP and smoking independently correlated with PWVβ (L), and SBP and PP independently correlated with PWVβ (R) in patients with T2DM.

7. Pulse pressure in patients with T2DM significantly and independently correlates with PWVβ (R), PWVβ (L), β-stiffness index (R) and β-stiffness index (L).
8. Carboxylated osteocalcin positively and independently correlates the β-stiffness index (R).

9. Serum concentrations of cOC, ucOC and tOC in patients with T2DM are lower than controls, and for cOC this difference is significant.

10. Carboxylated osteocalcin significantly increases its serum concentrations after four weeks of vitamin K2 supplementation.

11. In patients with T2DM who have undergone vitamin K2 supplementation, there is a tendency to increase the hemodynamic parameters and ET parameters of AS. Significance of these changes was reached in CASP.

VIII. CONTRIBUTION

1. Scientific-theoretical contributions

- For the first time in Bulgaria arterial stiiffness is measured in patients with T2DM using echo-tracking method
- Osteocalcin is studied for the first time in Bulgaria as a marker for arterial stiffness in patients with T2DM.
- For the first time in Bulgaria an intervention study is conducted looking for a change in carotid siffness after vitamin K2 supplementation in patients with T2DM.

2. Scientific- practical contributions

- The mesurement of increased carotid stiffness by echotracking method in patients with T2DM is non-invasive, fast and suitable for clinical practice.
- Early diagnosis at the stage of subclinical vascular damage would allow therapeutic decisions to be made in order to reduce cardiovascular complications in patients with T2DM.
- Although there are no statistically significant differences in PWVβ measured on the left and right ACC, a higher number of correlations was found in the right ACC, which makes it more suitable for the echo-tracking.

3. Confirmatory contributions

- PWV increases with age
- Total and non-carboxylated osteocalcin are in lower concentrations in patients with T2DM.

IX. CONCLUSION

The study found elevated PWVβ in the patients with T2DM, without evidence of atherosclerotic vascular disease compared to healthy controls. Reveals increased values of β-stiffness index, elastic modulus and decreased augmentation index and arterial compliance. It is known that AS increases in healthy individuals with ages, and this trend is also observed in our patients with T2DM. Significant correlations are found between the different ET indicators, but the main parameters for stiffness remain PWV.

Differences in PWV measurements performed on the left and right carotid arteries of the same patient were insignificant. There is a greater number of correlations with

right ACC and this should be taken into account for clinical practice. There is a relationship between HDL values and increased carotid stiffness. With the increase in the waist and the waist / height ratio, the PWV of the two carotid arteries also increases, and therefore the cardiovascular risk in the subjects.

There is a significant correlation between the values of glycated hemoglobin and PWVβ. No correlation was found with fasting plasma glucose levels, which is consistent with the well-known fact that macrovascular complications begin before the diagnosis of diabetes and before the development of hyperglycemia.

When CASP, SBP, DBP, PP increases, PWV, Ep, β-stiffness index also increase, but arterial compliance and AI decreases. Heart rate shows an inverse correlation with augmentation index. These data support the importance of correction and targeted control not only of blood sugar but also of blood pressure, lipid profile and abdominal obesity in the individual approach associated with patients with T2DM.

Osteocalcin, as a hormone involved in the regulation of carbohydrate homeostasis, showed lower values of all its forms in patients with T2HD, with significance for cOC levels. In terms of vascular function, a positive correlation again was discovered for cOC and β-stiffness index (R) and Ep (R). Although one month of vitamin K2 supplementation led to a significant increase in serum cOC concentrations, this did not have a positive effect on ET parameters and hemodynamic parameters, which showed a slight tendency to increase. This could be due to genetic and molecular mechanisms in which vitamin K2 is involved, related to blood pressure, or to a deteriorating patient compliance, which once again supports the importance of prevention. Prevention at the level of risk factors, early vascular damage, and support of the patient's active participation in reducing his cardiovascular risk. Once diagnosed the elevated AS is defined as a non-traditional risk factor independent of other CV risk factors.

Diabetes mellitus is one of the socially significant diseases, and the occurrence of its complications leads to premature death or deteriorating quality of life.

It is known that the macrovascular complications of diabetes begin very early and preventive strategies are of particular importance. One of these strategies is to measure arterial stiffness by echotracking.

X. LIST OF PUBLICATIONS AND PARTICIPATIONS RELATED TO THE DISSERTATION.

E. Marinova, M.Boyadzhieva, B. Kanazirev. Arterial stiffness in type 2 diabetes mellitus. Science cardiology; 2020 (3), 5-9

Marinova E., Boyadzhieva M., Hvarchanova N., Kanazirev B. Type 2 diabetes mellitus is associated with increased arterial stiffness measured by echo-tracking method. Scripta Scientifica Medica, 2020 Vol 52 Online first

Marinova E. Ultrasound measurement of local arterial stiffness through one-point echo-tracking technique. Varna Medical Forum. Vol 9. 2020, 41-45

Vitamin K2 and its role in diabetic macrovascular complications. E. Marinova, B. Kanazirev, M. Boyadzhieva, Y. Bocheva. 29th Annual Assembly of international medical association Bulgaria 9-12 may 2019 Resort Golden Sands

Biological effects of vitamin K2. E. Marinova, B. Kanazirev, M. Boyadzhieva, L. Stoyanova. Poster. 29th Annual Assembly of international medical association Bulgaria 9-12 may 2019 Resort Golden Sands

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