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“Prof. PARASKEV STOYANOV” – VARNA

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DEPARTMENT OF CLINICAL LABORATORY

Dr Monika Toshkova Todorova

THE ROLE OF VITAMIN D AND VITAMIN B12 IN PREGNANT WOMEN AND NEWBORNS

DISSERTATION ABSTRACT

For awarding the educational and scientific degree

“PhD”

Scientific supervisor:

Associate professor Dr Daniela Ivanova Gerova, PhD

Varna, **2023**

The dissertation contains 187 pages, 34 figures and 59 tables. 350 literature sources are cited, of which 5 in Cyrillic, 342 in Latin and 3 websites. The numbering of figures and tables in the abstract corresponds to that of the thesis (figures and tables present in the literature review are missing). The full names of the cited references are included in the thesis.

The dissertation was discussed and referred for public defense at a meeting of the Departmental Council of the Department of Clinical Laboratory at the Medical University - Varna, held on 30.01.2023 and was referred for defense to the Scientific Jury composed of:

External members:

1. Prof. Dr. Krasimira Ilieva Ikonomova-Shakhova, PhD
2. Prof. Dr. Adelaide Lazarova Ruseva, PhD
3. Assoc. Prof. Dr. Dobrinka Dineva Savova, PhD

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4. Prof. Dr. Yana Dimitrova Bocheva, PhD
5. Prof. Dr. Margaritka Ivanova Boncheva, PhD

Reserve members:

1. Assoc. Prof. Dr. Irena Ivanova Gencheva-Angelova,PhD
2. Prof. Dr. Miglena Dimitrova Georgieva, PhD

The public defense will take place on 16.05.2023 at 14h in hall 213 of University Hospital "St. Marina" - Varna.

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

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ABBREVIATIONS USED IN ENGLISH:

1α,25(OH)2D – 1α,25-dihydroxyvitamin D

25(OH)D – 25-hydroxyvitamin D

AC – Abdominal circumference

ADA – American Diabetes Association

BMI – Body Mass Index

BPD – Biparietal diameter

CLIA – Chemiluminescent Immunoassay

CMIA – Chemiluminescent Microparticle Immunoassay

CVD – Cardio Vascular Disease

DBP – Vitamin D binding protein

DIC – Disseminated intravascular coagulation

DM – Diabetes Mellitus

EASD – European Association for the Study of Diabetes

FIGO – The International Federation of Gynecology and Obstetrics

FL – Femur length

FNB – Food and Nutrition Board

GDM – Gestational Diabetes Mellitus

GWG – Gestational weight gain

holo-TC – Holotranscobalamin

HOMA-IR – Homeostatic Model Assessment of Insulin Resistance

HPLC-UV – High Performance Liquid Chromatography with Ultraviolet detector

IADPSG – International Association of Diabetes and Pregnancy Study Groups

IF – Intrinsic factor

IR – Insulin resistance

ISSHP – International Society for the Study of Hypertension in Pregnancy

IUGR – Intrauterine Growth Restriction

LBW – Low birth weight

LC-MS/MS – Liquid Chromatography – Mass Spectrometry

LMIC – Low- and middle-income countries

MeCbl – Methylcobalamin

MMA – Methylmalonic acid

NBWI – Normal birth weight infants

NTDs – Neural tube defects

OGTT – Oral glucose tolerance test

PA – Placental abruption

PIH – Pregnancy induced hypertension

PKC - Protein kinase C

PTB – Preterm birth

PTH – Parathyroid hormone

RDA – Recommended dietary allowance

SD – Standard deviation

SGA – Small for gestational age

T2DM – Type 2 Diabetes Mellitus

tHcy – Homocysteine

VDR – Vitamin D receptor

WHO – World Health Organization

I. INTRODUCTION

Vitamin D is a fat-soluble vitamin which is obtained from food and dietary supplements or is produced in the skin when it is exposed to the sun. For centuries on end it has been considered “the bone vitamin” since its main function is associated with the control of calcium homeostasis. Over the last few years various other functions of this vitamin have been identified. It regulates the cellular differentiation and proliferation, the immune function and hormone secretion.

Vitamin D deficiency is associated with an increased risk of developing a large number of illnesses: cardiovascular, immune disorders, malignant diseases, while its occurrence on a global level is everywhere.

The condition may be detected during pregnancy as well. In multiethnic Europe the investigations on the population that study and explore the vitamin D status in this period are not sufficient. The vitamin D deficiency during pregnancy is associated with a high risk of numerous complications both for the mother and for the newborn: pregnancy induced hypertension, preeclampsia, gestational diabetes, bacterial vaginosis, premature birth, low birth weight of the newborn, bone growth disorders of the newborn, etc. A significant correlation has been found in many investigations between the vitamin D status of the pregnant woman and the vitamin concentrations in the umbilical cord blood of the newborn. The factors identified as basic to the low levels of 25(OH) D in the newborn are the low vitamin D concentrations in the mother and the absence of dietary supplements during pregnancy.

Vitamin B12 is water-soluble and is obtained mainly from meat consumption or dietary supplements. It serves as a cofactor in converting the amino acid homocysteine into methionine, as well as in forming succinyl - CoA from the L-methylmalonyl -CoA. The risk of vitamin B12 deficiency occurs as a result of insufficient intake or is due to malabsorption which tends to increase with age. The pregnant women who live in poor regions of the world are exposed to a number of micronutrient deficiencies. Their diets are often lacking in meat and pregnant women are at an increased risk of vitamin B12 deficiency. The significance of the adequate vitamin B12 status during pregnancy is of great importance since the vitamin plays a major role in the processes of neural myelinization, brain development and overall growth of the fetus. The vitamin B12 deficiency causes macrocitic anemia, neurological dysfunction and biochemical anomalies related to the accumulation of precursors - homocysteine and methylmalonic acid. Hyperhomocysteinemia is connected to a high risk of

developing some negative consequences for the pregnant woman and the newborn such as miscarriages, preeclampsia, anomalies with the fetal development, intrauterine fetal growth retardation, premature birth, low birth weight, etc.

II. AIMS AND OBJECTIVES

AIMS

The aim of the present research is to determine vitamin D and vitamin B12 status of pregnant women with normal and pathological pregnancy and to assess the role of the vitamin deficiency and/or insufficiency in the course of pregnancy and its outcomes.

OBJECTIVES

In order to achieve the aim, the cohort of pregnant women who participated in the research was subdivided into three main groups: women with normal pregnancy, women who developed GDM (Gestational Diabetes Mellitus) during pregnancy and women with preeclampsia according to the criteria established in the obstetrics and gynecology practice. Thus the following objectives have been outlined:

I. Vitamin D status:

1. To investigate the serum levels of 25 (OH) D of the pregnant women who participated in the research.
2. To determine vitamin D status and to establish the occurrence and frequency of vitamin D deficiency and/or insufficiency for the whole cohort of pregnant women as well as the ones included in the three groups mentioned above.
3. To assess the influence of the body mass index (BMI) and the season on the vitamin D status in the three examined groups of pregnant women.
4. To investigate the dependency and correlation of vitamin D status on the administration of vitamin D as a pharmaceutical product and/or dietary supplements, containing the vitamin, in the three groups of pregnant women.

II. To assess the effect of vitamin D status in relation to an unfavorable outcome of the pregnancy concerning the newborn and the occurrence of premature birth or the birth of a child with low birth weight in healthy pregnant women and those who have complications.

III. Vitamin B12 status:

1. To investigate serum levels of total vitamin B12, active vitamin B12 and methylmalonic acid in the pregnant women who participated in the investigation.

2. To determine vitamin B12 status and to establish the frequency of vitamin B12 deficiency and/or insufficiency for the whole cohort as well as in the three groups of pregnant women who were investigated.
3. To assess and evaluate the influence of the BMI on vitamin B12 status in the three groups of pregnant women who participated in the research.
4. To investigate the dependency of vitamin B12 status on the administration of vitamin B12 as a pharmaceutical product and/or dietary supplements, containing the vitamin, in the three groups of pregnant women who participated in the research.
5. To assess the effect of vitamin B12 status in relation to an unfavorable pregnancy outcome concerning the newborn and the occurrence of premature birth and the birth of a child with low birth weight in healthy pregnant women and those who have complications.

The investigations and activities thus carried out in relation to this dissertation work started after a written permission was granted by the Commission for Ethics of Research at the Medical University of Varna (protocol № 84/27.06.2019)

III. MATERIALS AND METHODS

Target population and scientific research design

The research thus carried out is prospective in character and has taken place in the Diagnostic and Consulting Centre of "St Marina" University Hospital in Varna, Acibadem City Clinic Medical Center, Varna, the Specialized Hospital for Obstetrics and Gynecology for Active Treatment "Prof. Dr D. Stamatov", Varna and General Hospital for Active Treatment 'St. Anna', from 02.07.2019 – 31.12.2021. A total of 259 pregnant women have been included in the scientific research. All participants who volunteered to take part in the investigation have received and signed an informed consent document following the requirements of the Commission for Ethics of Research at the Medical University of Varna. The personal data of the pregnant women as well as the results of their medical tests have been received, processed, saved and kept according to the National Law on Personal Data Protection and the Professional Code of Ethics.

The pregnant women that became part of the clinical trial got acquainted with the informed consent form and signed it. They also filled in a questionnaire/ survey card that contained demographic data and information connected to their style of life, the course of previous pregnancies (if there were such) and their obstetric outcomes, as well as a family history.

Pregnant women in their 24^{th} to 28^{th} gestational week (g.w.) received medical treatment on an outpatient basis. They were given the opportunity to have an oral glucose tolerance test (OGTT) with 75 grams of glucose. Just before the test started an additional, separate test-tube/vial of venous blood (5 ml) was taken for separating blood serum and investigating at a later stage vitamin D, total vitamin B12, active vitamin B12, methylmalonic acid and insulin. If the results of OGTT indicate development of GDM, the pregnant women are referred to an endocrinologist for further investigation of this condition and treatment.

The healthy pregnant women who are not at risk and are part of the investigation before the 24^{th} gestational week or after the 28^{th} gestational week were subjected to a plasma glucose test. A fasting blood glucose test was done and some venous blood was drawn, saved and kept in a serum vacuum tube (5ml), for investigating vitamin D, total and active vitamin B12, methylmalonic acid and insulin.

The pregnant women hospitalized for preeclampsia had blood tests in the process of routine laboratory investigations. As an inpatient, each one of them had 5 ml venous blood drawn and stored in an additional vacuum tube. It was used to separate serum for

determining the levels of vitamin D, total and active vitamin B12, methylmalonic acid and insulin.

Some information about the fetal development in the course of pregnancy was taken and processed from the medical documentation of the OBG Consulting centers and the hospitals and medical centers mentioned above. While some more information about the anthropometric indicators of newborns after birth /postpartum was collected from the neonatology departments, OBG specialists or from the pregnant women themselves.

1. **Criteria for investigation inclusion or exclusion:**

Criteria for investigation inclusion:

- Pregnant women at the age of 18 or above who have given their informed consent to participate in the research
- Pregnant women with single pregnancy confirmed by ultrasound up to the 12th gestational week, when the due date is known, estimated on the basis of the last menstrual period.
- Pregnant women with GDM
- Pregnant women with preeclampsia and eclampsia

Criteria for investigation exclusion:

- Pregnant women at the age of 18 and above
- Pregnant women with twins or multiple pregnancy
- Pregnant women who do not know the due date of giving birth
- Pregnant women whose pregnancy is as a result of assisted reproduction treatment (ART)
- Pregnant women with arterial hypertension, diagnosed before pregnancy
- Pregnant women with diabetes before pregnancy
- Pregnant women with chronic and system diseases, cardiovascular, renal, liver diseases, etc.

2. **Methods**

2.1. A survey/questionnaire method – all pregnant women included in the clinical trial after getting acquainted and signing the informed consent form are asked to carefully fill in a survey card, under doctor's supervision who is a participant in the survey. It contains demographic data and information related to their style of life (diet, physical activity,

administration of medicines and dietary supplements), data about previous pregnancies and their obstetric results, family history.

2.2. Anthropometric measurements.

2.2.1. Anthropometric measurements of pregnant women – after the pregnant woman has introduced herself, signed the informed consent document and filled in the survey card/questionnaire, her body weighed is measured. The aim is to calculate her GWG (gestational weight gain), while the body mass index at the moment of her getting pregnant is estimated according to a generally accepted formula: BMI=weight (kg)/[height (m)]^2 on the basis of the data in the survey card / questionnaire.

2.2.2. Anthropometric measurements of the newborn – information about the anthropometric measurements of the newborn (length and weight) as well as echocardiography data about measured biparietal diameter, abdominal circumference and femur length is available from the neonatal units in the Specialized Hospital for Obstetrics and Gynecology for Active Treatment 'Prof.Dr D. Stamatov', Varna and General Hospital for Active Treatment 'St. Anna', Varna.

2.3. Analytical methods used:

Pre analytical stage.

Venous blood was taken from all pregnant women in vacuumtainers with EDTA and without anticoagulant in the morning from 07:00 to 10:00 o'clock, after 12 hours fasting. Venepuncture was carried out with minimal risk of harm to the research participants and following all the requirements of the pre analytical stage of collecting biological material as well as complying with the conditions for sterility testing. In order to separate plasma/serum the venous blood was centrifuged at 3000 revolutions per minute for 10 minutes. Glucose concentrations are analyzed in EDTA plasma immediately after the sample is taken. The separated serum has been divided into several aliquots which are stored at temperature -20ºC until the analyses for determining the concentrations of 25(OH)D, total vitamin B12, MMA (methylmalonic acid) and insulin are carried out.

2.3.1. Analytical procedure for quantitative analysis of 25 (OH)D in blood serum.

The qualitative and quantitative analyses of the circulating form of vitamin D3 in blood serum were carried out in the Department of Biochemistry, Molecular Medicine and Nutrigenomics at the Faculty of Pharmacy, Medical University, Varna. To achieve this goal a liquid chromatographic method with mass selective and UV detection for identification and

quantitative analysis of 25-hydroxi vitamin D325 (OH)D in blood serum was developed and validated.

Pre analytical stage and preparation for sampling

25(OH)D analysis in blood serum is an analytical challenge. The lipophilic 25(OH)D is transported in the circulation tightly bonded to the vitamin D binding protein. A preliminary precipitation of the protein and a consequent extraction of the analyte from the liquid situated above are necessary for the effective separation of the analyte from the binding protein. 0,2M $ZnSO_4x7H_2O$ and methanol are used as precipitating agents. They are added to a 475µl blood serum sample in the following ratios 1:0.3 and 1:2.1 respectively. After vigorous stirring followed by centrifuging from the liquid situated above, the analyte is extracted with n-hexane in the ratio 1:3. The organic phase is separated and it evaporates under a nitrogen jet until some dry residue remains. The dry residue thus obtained dissolves in methanol with clarity for MS (mass-spectrometry), it is filtered and analyzed chromatographically.

Chromatography with UV and mass-selective detection

The chromatographic analysis was carried out with a chromatographic system Acuity UPLC QDa PDA, with a mass-selective detector and an automatic sampler produced by Waters, USA. The separation of analytes in the sample is realized with the help of a chromatographic UPLC column Acuity UPLC BEH C18 1.7µm, 2.1x50mm (Waters, USA). A gradient regime of separation is used with a mobile phase of two components. A: A: 0,1% formic acid with 5mM ammonium formate; B: methanol: water in ratio 98: 2, containing 0,1% formic acid with 5mM ammonium formate. The flow rate of the mobile phase was 0,3ml/min, the time of analysis – 20 min.

The mass spectrometry analysis was done in the regime of electrospray ionization, observing a particular characteristic ion with m/z = 383 for 25(OH) D and m/z = 389 by the internal standard deuterated hexane 25(OH)D. The conformational analysis was carried out with the help of a UV detector with a diode matrix with a wavelength of 265 nm.

Identification and quantitative analysis

The identification of 25(OH)D was made on the particular characteristic ion for 25(OH)D with m/z = 383 and by the UV spectrum of 25(OH)D. The retention time of 25(OH) D and the internal standard was 5,01 min.

The quantitative analysis was done using the method of internal calibration. Deuterated hexane 25(OH) D was used as an internal standard. Its concentration was 50 μg/mL in ethanol (D6-25-Hydroxyvitamin D3 (26, 26, 26, 27, 27, 27-D6; Merck, Germany), from which a working solution was prepared with concentration 1 μg/mL in methanol. The calibration method was realized with the help of serum calibrators on 6 levels with concentrations 9,44nmol/L; 43.2nmol/L; 77.0nmol/L; 156nmol/L; 246nmol/L; 335nmol/L, corresponding to the physiological limits of the analyte. With every series of samples, serum controls were analyzed on two levels: level 1 - 36,7nmol/L and level 2 - 135nmol/L. Matrix effect was assessed by means of an empty serum bottle (serum calibrator level 0) Calibrators and controls produced by Chromsystems Instruments & Chemicals GmbH, Germany were used.

2.3.2. Method for determining total vitamin B12:

In order to determine the serum concentrations of total vitamin B12, a Beckman Coulter (USA) – Access Vitamin B12 Reagent Pack, Cat No. 33000 was used as an automated immunology analyzer ACCESS 2. It is based on the principle of direct chemiluminescence (CLIA). The calibrating tools (Access Vitamin B12 Calibrator, Cat No. 33005) and control materials (Seronorm Immunoassay Lyo Level 1, 2, 3) recommended by the producer were used for calibrating and control of the method. The analyses were done in the Clinical laboratory at the Diagnostic and Consulting Centre of "St. Marina" University Hospital in Varna, by strictly observing the instructions of the producer and the guidelines for good laboratory practice.

2.3.3. Method for determining active vitamin B12:

In order to determine the serum concentrations of active vitamin B12, Abbott Laboratories (USA) – ARCHITECT Active-B12 (Holotranscobalamin) 3P24 trade kit was used as an automated immunology analyzer ARCHITECT. It is based on the principle of chemiluminescent microparticle immune assay (CMIA). The calibrating tools (3P24-01 ARCHITECT Active B12 Calibrators) and control materials (3P24-10 ARCHITECT Active B12 Controls) recommended by the producer were used for calibrating and control of the method. The analyses were done at the Autonomous Medical Diagnostics Laboratory Laborexpress, Varna by strictly observing the instructions of the producer and the guidelines for good laboratory practice.

2.3.4. Analytical procedure for quantitative analysis of Methylmalonic acid (MMA) in blood serum

The quantitative and qualitative analysis of MMA were carried out in the Department of Biochemistry, Molecular Medicine and Nutrigenomics at the Faculty of Pharmacy, Medical University, Varna. To achieve this goal, a liquid chromatographic method with mass selective and UV detection was used for identification and quantitative analysis of MMA in blood serum.

Pre analytical stage and preparation for sampling

The analysis of MMA in blood serum is connected to several challenges: MMA is a dicarboxylic acid with low molecular weight. MMA is a structural isomer of succinate and it has a very similar time retention, it is hydrophilic, not volatile, its concentration in the serum in healthy individuals, who are not expected to be vitamin B12 deficient, is much lower than that of the succinate, which requires a very precise, accurate and reliable chromatographic separation.

A blood serum sample (200μl) is deproteinized with a precipitating solution containing acetonitrile with 1% formic acid and 100 ng/mL internal standard 3-deuterited (d3-MMA, Ceriliant 1mg/ml, 1.5 ml; Supelco, Germany). The analyte is extracted from the liquid situated above after the separation of the proteins. This is done by solid-phase extraction on a reverse phase column Oasis Prime HLB 30mg/3ml (Waters, USA).

The eluates thus collected evaporate to leave a dry residue under a nitrogen jet. The dry residue thus obtained dissolves in H2O: acetonitrile = 98:2, containing 0,1% formic acid with clarity for MS (mass-spectrometry), it is filtered and analyzed chromatographically.

Chromatography with UV and mass-selective detection

The chromatographic analysis was carried out with the help of a chromatographic system Acuity UPLC QDa PDA, with a mass-selective detector and an automatic sampler produced by Waters, USA. The separation of the analytes in the sample was realized with the help of a chromatographic UPLC column AccQ-TAG Ultra C18 1.7μm, 2.1x100mm (Waters, USA). A gradient regime of separation was used with a mobile phase of two components:

- A: water, containing 0,5% formic acid;
- B: acetonitrile, containing 0,5% formic acid.

The flow rate of the mobile phase was 0,3ml/min, the time of analysis – 10 min.

The mass spectrometry analysis was done in the regime of electrospray negative ionization, observing a particular characteristic ion with m/z = 117,3 for MMA and m/z = 120,1 for the internal standard trideuterated MMA.

Identification and quantitative analysis

The MMA identification was carried out by means of the particular characteristic ion for MMA with m/z = 117,0. The retention time of MMA and the internal standard was 2,92 min.

The quantitative analysis was carried out following the method of internal calibration. Trideuterated MMA was used as an internal standard.

The calibration method was realized with the help of serum calibrators on 3 levels with concentrations of 219.7nmol/L; 758.8nmol/L; 1402.7nmol/L, corresponding to the physiological limits of the analyte. With every series of samples, serum controls were analyzed on two levels: level 1 - 269.5nmol/L and level 2 – 587.28nmol/L. The matrix effect was assessed by means of an empty serum bottle (serum calibrator level 0). Calibrators and controls produced by Recipe Chemicals & Instruments GmbH, Germany were used.

Method validation

The method is validated according to the requirements of the International Conference on Harmonization of Pharmaceuticals (technical requirements for registration) for Human Use (International Conference on Harmonization of Pharmaceuticals for Human Use, ICH, 2019). The following validation parameters have been defined:

- Precision – calculated by means of threefold quantification of the following concentrations of MMA in the serum:
  - 211.25nmol/L – 95,4%;
  - 338.0nmol/L – 94.88%;
  - 507.0nmol/L – 103.93%;
  - 591.0nmol/L – 103.09%;
  - 676.0nmol/L – 97.61%; (n=3)
- Linearity – the method is linear in the estimated range of concentrations with the following characteristics of the calibration curve:
  - Equation on the basis of the calibration chart: Y=0.002215*X+0.06258;
  - R^2 = 0.9946;

- Repeatability (RSD relative standard deviation %) – estimated by determining the following concentrations of MMA in the serum (n=10):
  - 6.8% RSD
- Intermediate precision – 14.52% RSD; (n=6)
- LOQ – 194.35nmol/L
- LOD – 58.3nmol/L
- Extraction effectiveness/efficiency - (n=6):
  - 211.25nmol/L – 105%;
  - 338.0nmol/L – 95%;
  - 507.0nmol/L – 109%;
  - 591.0nmol/L – 108%;
  - 676.0nmol/L – 94%;
- Matrix effect– was not detected.

2.3.5. Insulin detection method:

An insulin detection kit, Beckman Coulter (USA) – Access Insulin Reagent Pack, Cat No.33410 for automated immunology analyzer ACCESS 2 was used to determine the serum concentrations of insulin. It is based on the principle of direct chemiluminescence (CMIA)

The calibrating (Access Insulin Calibrator, Cat No.33415) and control materials (Seronorm Immunoassay Lyo Level 1, 2, 3) recommended by the producer were used for method calibration and control. The analyses were carried out at the Clinical laboratory of the Diagnostic and Consulting Centre of "St. Marina" University Hospital in Varna, by strictly observing the instructions of the producer and the guidelines for good laboratory practice.

2.3.6. Glucose detection method

Glucose levels are measured in K2EDTA human plasma that was obtained after immediate centrifuging of the venous blood drawn at 3000 revolutions per minute for the period of 10 minutes. A trade kit of the company Biosystems (Spain) – Glucose Hexokinase was used. The measurement was carried out on a Mindray biochemical analyzer at the Clinical laboratory of the Diagnostic and Consulting Centre of "St. Marina", University Hospital and Acibadem City Clinic Medical Center in Varna by fulfilling all the conditions of the producer and the guidelines for good laboratory practice.

2.4. Statistical methods for analyzing the results

An IBM SPSS, v. 25. statistical package was used for statistical processing of the data. The following statistical methods were applied:

2.4.1. Descriptive analysis – for identifying the characteristics looked for in the cross-sectional study sample.

2.4.2. Variational analysis – for presenting the mean and standard deviation of the quantitative indicators

2.4.3. Nonparametric analysis – for analyzing the categorial indications by cross tabulation and (X2) criterion.

Cross tabulation forms two way and multi - directional tables. The table structure is arranged in categories which help define the type of statistical test used. X2 is a nonparametric test, based on a frequency analysis that demonstrates the formation of some tendency in percentages and its statistical significance. The results thus obtained are considered statistically significant when their value is $p<0.05$.

2.4.4. Correlation analysis – for assessing how great the dependency between the variables is, is based on the results of the coefficient of Pearson (r) and of Spearman (p). The coefficient of Spearman estimates and assesses correlation on the basis of monotonic relationship, while that of Pearson - on the basis of linear relationship. The degree of association between the variables is defined as weak when the correlation coefficient is <0.3, moderate when the coefficient is between 0.3 – 0.5 and strong when the correlation coefficient is above 0.5 provided the level of statistical significance is $p<0.05$.

2.4.5. Parametric test – t-test of Student-Fisher, while comparing independent variables which follow a normal distribution. It estimates the differences between the values of the variables and tests whether the mean values differ from 0, i.e. there is a difference while comparing the variables and this difference is statistically significant.

2.4.6. Graphical analysis – for visualizing of the obtained results.

A critical level of significance was used $\alpha=0.05$, and the null hypothesis was rejected when the p-value was smaller than α ($p<0.05$).

IV. RESULTS

1. Clinical and laboratory characteristics of the research participants:

2.1. Clinical and demographic data about the group of pregnant women who participated in the medical investigation

Out of the total of 259 pregnant women enrolled in the research study, 210 (81.08%) were investigated as outpatients and 49 (18.91%) were hospitalized and treated as inpatients, diagnosed with preeclampsia, at the Specialized Hospital for Obstetrics and Gynecology for Active Treatment 'Prof.Dr D. Stamatov', Varna. The ratio between the outpatients and inpatients is approximately 4:1. It is demonstrated in figure 4

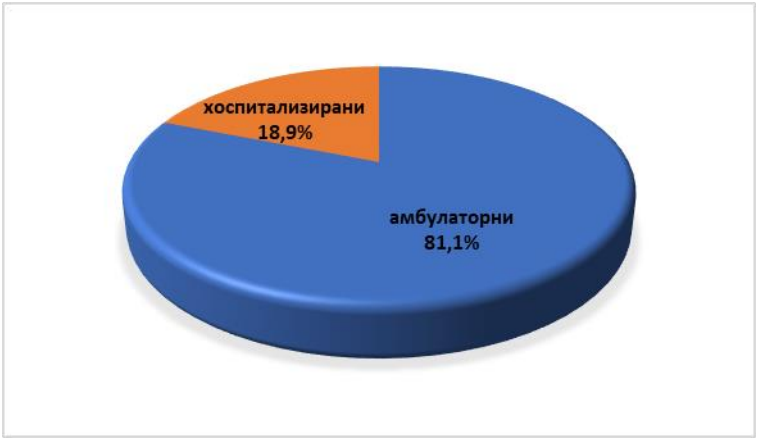

Fig.4 Distribution of pregnant women according to the way they were investigated.

The average age of the research participants (pregnant women) is 30.5 years (ranging from 19 to 49). In order to establish the influence of the age factor, the participants were subdivided into two additional subgroups: young women (age group A: up to 34 years of age inclusive) - 85.55% (n=219) and older women (age group B: ≥35 years of age) – 15.44% (n=40).

The women of Bulgarian origin prevail– 89.57% (n=232), living in a big city – 90.35% (n=234), married – 55.21% (n=143), with higher education diploma – 56.37% (n=146) and without any concomitant chronic and non systemic diseases – 94.20% (n=244). The women with concomitant diseases who do not meet the exclusion criteria (asthma, allergic rhinitis, hashimoto thyroiditis, thalassemia minor) are only 15 in number and comprise a small percentage of the research cohort (5.80%).

Depending on their health status, the pregnant women who were included in the present study are divided into three major groups:

The 1st group – healthy pregnant women, 167 (64.5%) altogether. Out of them 114 (68.3%) were in their second trimester of pregnancy, while 53 (31.7%) in their third trimester.

The 2nd group – pregnant women with GDM –43 (16.6%) altogether, out of them 26 (60.5%) in their second and 17 (39.5%) in their third trimester of pregnancy. The diagnosis was made after carrying out an OGTT with 75 grams of glucose in the period between the 24^{th} and 28^{th} gestational week inclusive or during any other time of pregnancy when there were indications of the test in compliance with the recommendations of IDF (Immune Deficiency Foundation) (2013) and WHO (2013). IADPSG criteria are applied according to which if one or more than one of the obtained results within the framework of the OGTT are abnormal, namely the plasma glucose on an empty stomach is ≥5.1 mmol/l, at the 1^{st} hour after the OGTT≥10 mmol/l, and the 2^{nd} hour after the OGTT happens to be ≥8.5 mmol/l, the pregnant woman is not considered to be healthy.

The third group of pregnant women with preeclampsia 49 (18.9%) in total, 6 (12.2%) out of whom in the second and 43 (87.8%) in their third trimester of pregnancy, were hospitalized in the Department of Pregnancy Pathology at the Specialized Hospital for Obstetrics and Gynecology for Active Treatment "Prof.Dr D. Stamatov", Varna. All diagnostic criteria were implemented to accept the preeclampsia diagnosis. They are as follows: newly detected hypertension (systolic >140 mmHg and diastolic >90 mmHg), accompanied by one or more other signs and symptoms such as proteinuria, various organ dysfunctions in the mother's body, including liver, renal, neurological or hematological damage and/or uteroplacental dysfunction with fetal growth restriction and/or abnormal Doppler ultrasound findings of the uteroplacental blood flow [48].

The distribution of the pregnant women in groups is visualized in figure 5

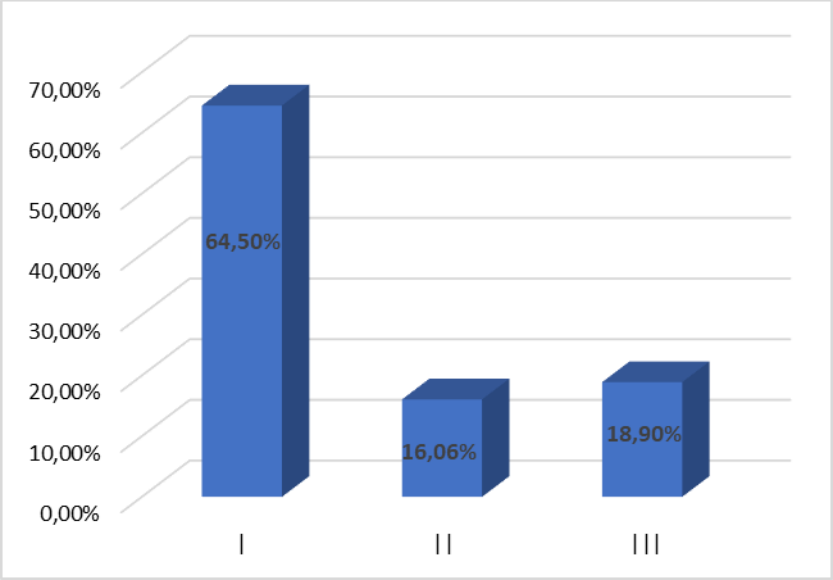

Fig.5 Distribution of the pregnant women according to their health status.
(Group I – healthy pregnant women; group II – women with GDM and group III – women with preeclampsia)

The demographic characteristics of the pregnant women in groups are presented as follows in tables 7, 8 and 9.

Table 7 Demographic characteristics of the healthy pregnant women.

Age		Ethnicity		Place of residence		Family status		Education	
<35 г.	>35 г.	Bulgarian	Other	Town	Village	Married	Single	High school	Uni-versity
56.4%	8.1%	58.7%	5.8%	61.4%	3.1%	35.9%	28.6%	28.6%	35.9%
n=146	n=21	n=152	n=15	n=159	n=8	n=93	n=74	n=74	n=93

Table 8 Demographic characteristics of pregnant women with GDM.

Age		Ethnicity		Place of residence		Family status		Education	
<35 г.	>35 г.	Bulgarian	Other	Town	Village	Married	Single	High school	Uni-versity
13.9%	2.7%	15.1%	1.5%	15.1%	1.5%	11.2%	5.4%	6.2%	10.4%
n=36	n=7	n=39	n=4	n=39	n=4	n=29	n=14	n=16	n=27

Table 9 Demographic characteristics of pregnant women with preeclampsia.

Age		Ethnicity		Place of Residence		Family status		Education	
<35 г.	>35 г.	Bulgarian	Other	Town	Village	Married	Single	High school	Uni-versity
14.3%	4.6%	15.8%	3.1%	13.9%	5.0%	8.1%	10.8%	8.9%	10.0%
n=37	n=12	n=41	n=8	n=36	n=13	n=21	n=28	n=23	n=26

The average age of the pregnant women from the 1st group is 29.84±4.79 (ranging from 19 to 43), in the 2nd group it is 31.25±4.58 (ranging from 22 to 41), while in group III , it is 32.12±7.14 (ranging from 20 to 49). While carrying out a one-way ANOVA analysis to compare the groups according to age a significant, reliable difference is established (F=4.027, p=0.019). Tukey's multiple analysis for separate comparison between the groups finds out a statistically significant difference only among the healthy pregnant women and those with preeclampsia. (figure 6).

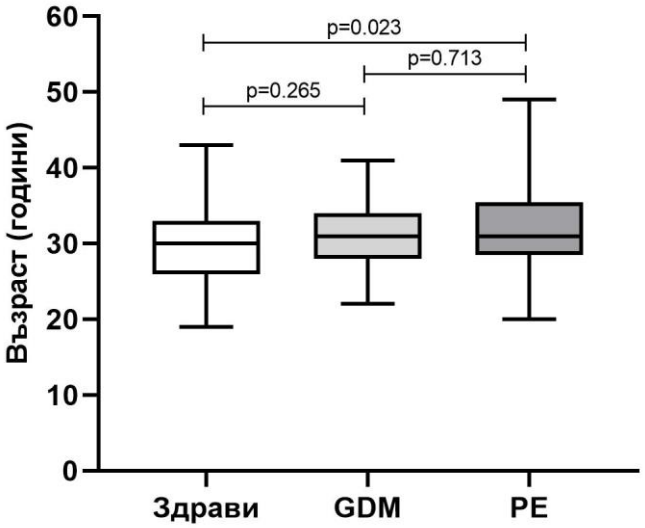

Fig.6 Comparison between the three major groups of pregnant women according to their age (PE – pregnant women with preeclampsia, GDM – pregnant women with gestational diabetes)

According to the reviewed literature, the pregnant women who participated in the research are divided into two subgroups in relation to their age – under and above 35 year-old women. Figure 7 demonstrates the frequency distribution according to the age criterion in

the three major groups of pregnant women– healthy pregnant women, the ones with gestational diabetes and those with preeclampsia.

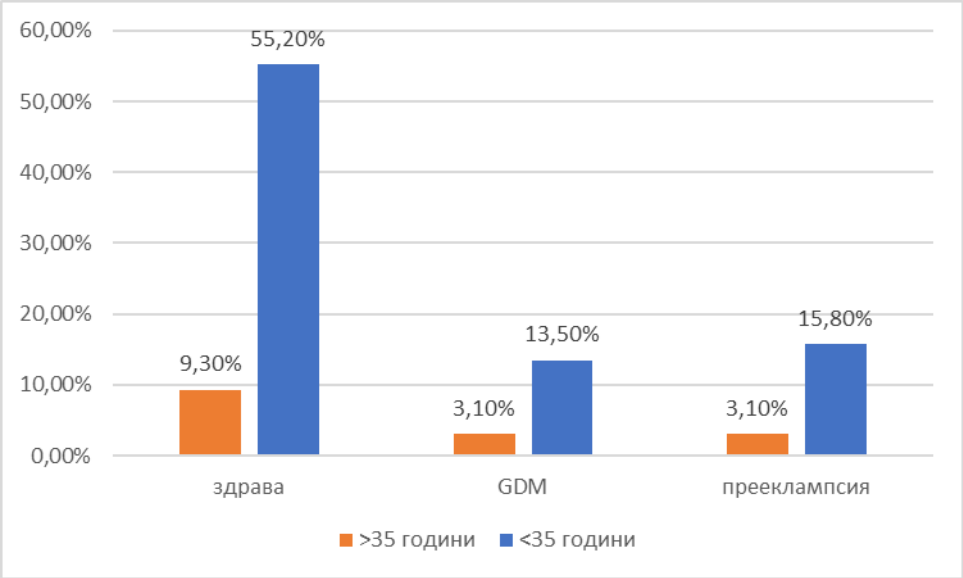

Figure 7 Frequency distribution of the women in two age categories in the three main groups of participants in the investigation

Table 10 shows detailed information about the age of the pregnant women and the occurrence of complications in the course of pregnancy (preeclampsia and GDM) with the purpose of establishing a possible risk of complications that depend on age.

Table 10 Risk of developing age and health related complications in pregnancy of the pregnant women

			Age		Total
			<35years	>35 years	
Diagnosis	Healthy pregnant women	N	24	143	167
		%	9.3%	55.2%	64.5%
	Women with pregnancy complications	N	16	76	92
		%	6.2%	29.3%	35.5%
Total		N	40	219	259
		%	15.4%	84.6%	100%

Although with the help of the correlation analysis following the method of Spearman, a statistically significant connection between the two indicators was not established

(rho=0.030, p=0.520), that was confirmed by the chi square analysis as well (X^2=0.414, p=0.677), a percentage tendency was formed and clearly detected showing that more pregnant women who were above 35 years of age (29.3%) developed pregnancy complications than those who were under 35 years of age (6.2%). Out of all the pregnant women included in the study, the highest percentage belongs to the first time mothers 47.10% (n=122), followed by 39.00% (n=101) of women with second pregnancy and 13.90% (n=36) are those with third or fourth pregnancy in a row. The distribution of pregnant women is demonstrated in figure 8 according to whether it is their first, second or third pregnancy.

Fig. 8 Percentage distribution of pregnant women according to whether it is their first, second or third pregnancy.

2.2. BMI of pregnant women, calculated at the moment of their getting pregnant and gestational weight gain in the course of pregnancy

In relation to the reviewed literature and the tendency, observed on a global level, of an increasing number of women in reproductive age who are either overweight or obese [214], BMI of the pregnant women, included in the study was calculated at the moment of getting pregnant according to the data they filled out in their survey cards about their body weight and height.

The pregnant women thus investigated were divided into three subgroups: those with normal weight (BMI<25), those who were overweight (BMI 25-30) and those who were obese (BMI>30).

Figure 9 displays the distribution of the pregnant women according to their BMI in percentages.

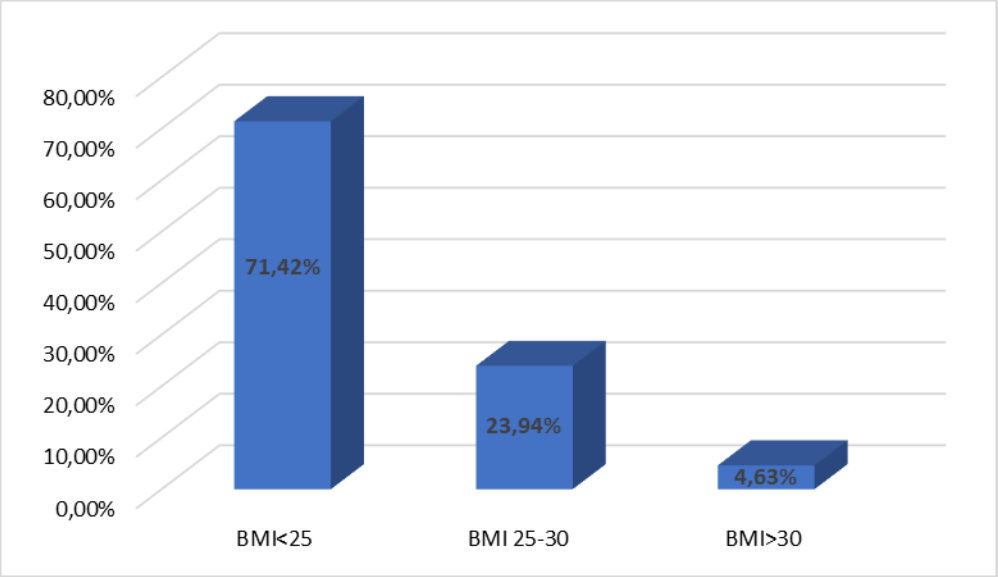

Fig.9 Percentage distribution of pregnant women according to their BMI calculated at the beginning of their pregnancy

The mean value of the estimated BMI for all participants is 23.08±3.93 (ranging from 15.24 to 44.98). In the group of healthy pregnant women the value is 22.69±3.69 (ranging from 15.24 to 37.02), the pregnant women with GDM 23.06±4.91, and the minimum (17.30) and maximum values (44.98) in this group are higher than the values of the healthy women.

The mean BMI value is highest in the women with preeclampsia 24.25±3.66 (ranging from 18.29 to 36.33). On carrying out a one-way ANOVA test to compare the groups according to their BMI, a tendency of statistical difference (F=2.752, p=0.065) was established. Tukey's multiple analysis for a separate comparison of the groups almost reaches a statistically significant difference between the healthy pregnant women and the ones with preeclampsia (figure 10).

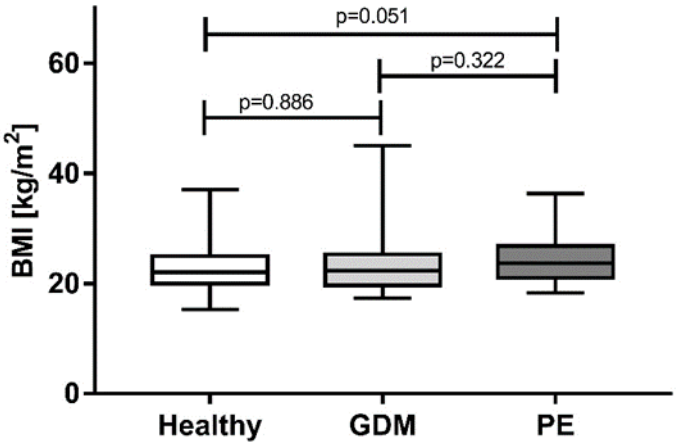

Fig. 10 Comparison between the three major groups of pregnant women according to their BMI.
(PE – pregnant women with preeclampsia, GDM – pregnant women with gestational diabetes)

Another indicator that is essential for the successful and favorable course of pregnancy and birth happens to be the gestational weight gain (GWG). Out of 259 pregnant women included in the research, 154 (59.46%) gained kilograms in accordance to the recommended gain weight for the respective gestation week (g.w.), and 40.54% (n=105) gained more weight than the acceptable weight gain. The mean GWG in the group of healthy pregnant women is 8.68±4.78 kg (ranging from 1 kg to 45 kg). 108 (64.67%) of the participants in this group gained weight within the normal range for the corresponding gestation week g.w., and 59 (35.33%) gained more weight than the acceptable. In the 2nd group of pregnant women with GDM, the mean GWG is 9.69±4.86 kg, higher than that of the first group (ranging from 2 kg to 29 kg), but in this group the pregnant women with normal weight gain for the respective gestation week g.w. also tend to prevail – acceptable weight gain is observed in 28 women (65.12%), and above the norm – in 15 women (34.88%). In the 3rd group of pregnant women with preeclampsia, the mean GWG is the highest - 13.91±4.73 kg (ranging from 5 kg to 25 kg) and in contrast to the first two major groups of pregnant women, the ratio between the women with acceptable weight compared to those with excessive GWG is just the opposite. The women whose GWG is within the boundaries of the recommended range are only 18 in number (36.73%), while those who have gained more weight than the norm are 31 in number (63.27%). The summarized data of the GWG in the three groups of pregnant women are shown in table 11.

Table 11 Gestational weight gain and percentage distribution of women with normal and abnormal weight gain in the three groups of pregnant women who participated in the research

Group I			**Group II**			**Group III**		
GWG (kg)	Normal GWG	Pathological GWG	GWG (kg)	Normal GWG	Pathological GWG	GWG (kg)	Normal GWG	Pathological GWG
Mean=8.68	n=108	n=59	Mean=9.69	n=29	n=15	Mean=13.91	n=18	n=31
SD=4.78	67.4%	35.3%	SD=4.86	65.1 %	34.9 %	SD=4.73	36.7 %	63.3 %

GWG (gestational weight gain) , Mean-average value , SD – standard deviation

By means of a nonparametric chi square analysis looking for correlation and dependencies between BMI and gestational weight gain, it was examined whether the pregnancy complications are more probable in overweight and obese women (BMI>25) before conception and GWG which is above the acceptable norm for the given g. w.

The outcomes of the frequency distribution of data demonstrate credible statistical dependencies for both indicators (BMI and GWG) concerning the risk of complications during pregnancy (X^2=6.046, p<0.049). It was established that about 43.30% of women with BMI>25 and about 35.90% of the pregnant women with excessive GWG were at risk of developing complications in the course of their pregnancy (figure 11).

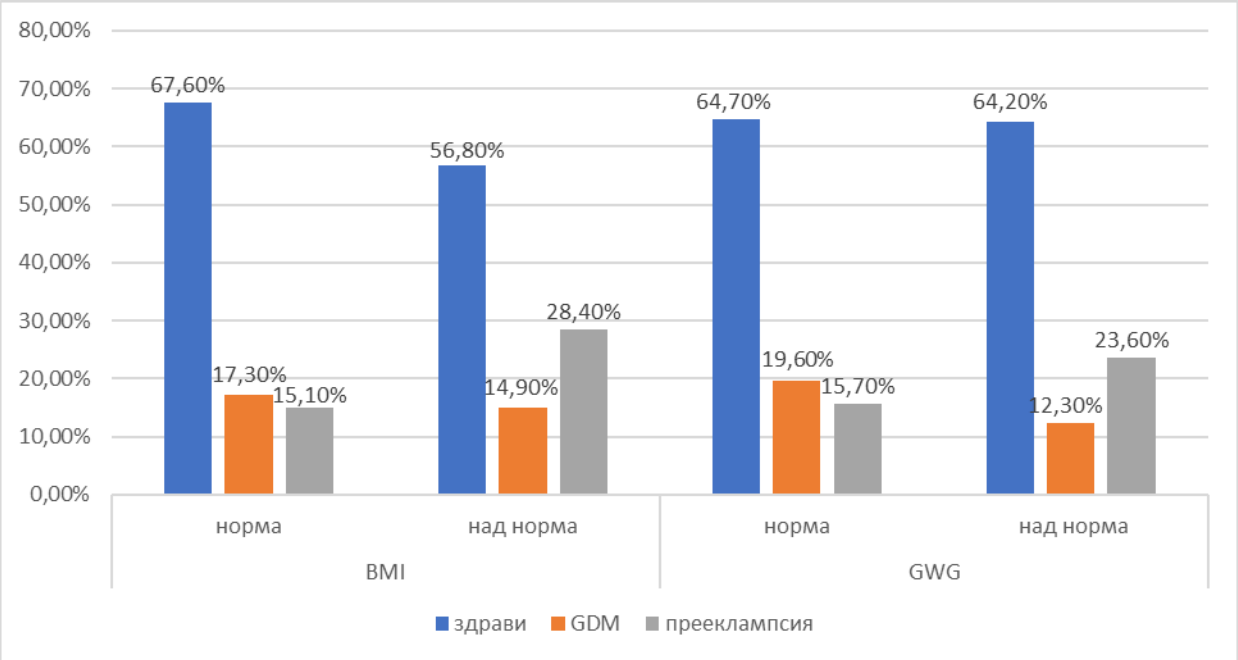

Fig.11 Risk of complications in pregnant women according to BMI and GWG.

It was checked how strong the connection between BMI and GWG indicators was and the probability of complications during pregnancy. A correlation analysis was used for this purpose, following the method of Spearman which proved that there exists a positive, proportional dependency between high BMI (>25) and the probability of developing complications such as GDM and preeclampsia (rho=0.124, p=0.046). In addition it was proved that GWG is a significant serious factor because it increases together with the increase of BMI, which results in a higher risk of complications for the pregnant woman (rho=0.134, p=0.031) (table 12).

Table 12 Probability of occurrence of a risk of complications in the pregnant woman depending on her BMI and GWG

			BMI	GWG	Complication
Spearman's rho	**BMI**	rho	1.000	0.134*	0.124*
		p	.	0.031	0.046
		N	259	259	259
	GWG	rho	0.134*	1.000	0.033
		p	0.031	.	0.601
		N	259	259	259
	Pregnancy complication	rho	0.124*	0.134*	1.000
		p	0.046	0.031	.
		N	259	259	259
*p <0.05 level of significance					

2.3. Seasonality

Since the participants were enrolled in the study for a period of 2 years and 6 months (from July 2019 to December 2021) it became possible for the pregnant women to be distributed for the sampling depending on the season. All 259 pregnant women were divided into two groups: the first group consisted of 166 participants (64.09%), whose venous blood was taken for investigation in winter and spring (from November to April) and a second group that consisted of 93 participants (35.91%), whose biological material was taken in the summer and autumn (from May to October inclusive). The pregnant women's distribution according to the month of blood sampling in each of the major groups – healthy pregnant women, pregnant women with GDM and pregnant women with preeclampsia has been displayed in table 13.

Table 13 Pregnant women's distribution according to the season of blood sampling

First group		Second group		Third group	
Winter/spring	Summer/autumn	Winter/spring	Summer/autumn	Winter/spring	Summer/autumn
n=108	n=59	n=27	n=16	n=31	n=18
64.67%	35.33%	62.79%	37.21%	63.27%	36.73%

(First group – healthy pregnant women, Second group - pregnant women with GDM, Third group - pregnant women with preeclampsia)

Out of the three major groups, approximately two thirds of the participants were enrolled in the study carried out in winter and spring. It is interesting that in the third group 63.3% (n=31) of the pregnant women developed preeclampsia during the winter and spring months. The frequency distribution of data in categories between the indicators *month of sampling* and *risk factor* for complication occurrence made by cross tabulation and nonparametric chi square analysis did not find statistical differences in relation to the occurrence of risk in the investigated sample and the season (X^2=1.152, p=0.283), in spite of the fact that about 60 % of the high risk patients were tested in the cold period of the year. The frequency distribution according to the diagnoses and the risk of complications depending on the season is demonstrated in figure 12. The additional research to detect some kind of correlation between seasonality and the probability of developing a pregnancy complication such as GDM and preeclampsia did not prove any regularity whatsoever. (Spearman rho=0.024, p=0.641)

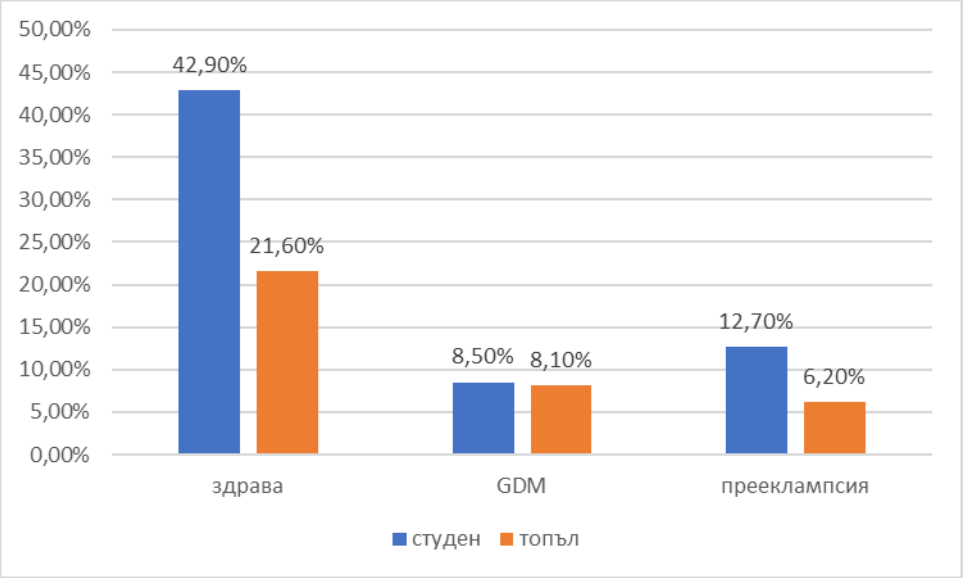

Figure 12 Risk of pregnancy complication depending on the season

2.4. Research results of indicators related to the carbohydrate exchange in pregnant women

2.4.1. OGTT

The pregnant women who were treated on an outpatient basis had an OGTT with 75 grams of glucose in the period between the 24th and 28th gestational week inclusive or during any other time of pregnancy if there were indications for doing the test (high risk patients) in compliance with the recommendations of IDF (Immune Deficiency Foundation) (2013) and WHO (2013). The diagnosis GDM was made following the IADPSG criteria according to which if one or more than one of the obtained results in the framework of the OGTT are abnormal, namely the plasma glucose on an empty stomach is ≥5.1 mmol/l, at the 1st hour after the OGTT - ≥10 mmol/l, and the 2nd hour after the OGTT the plasma glucose happens to be ≥8.5 mmol/l. In relation to the results of the OGTT women are considered either healthy pregnant women or women with GDM. In tables 14 and 15 the obtained results about the plasma glucose level in the two groups of pregnant women are presented.

Table 14 OGTT results of the healthy pregnant women

OGTT	Plasma glucose (mmol/l) Mean±SD	Plasma glucose (mmol/l) Min – Max range
0-ва min	4.41±0.88	3.8 – 5.05
60 min	6.5±4.38	3.8 – 10.0
120 min	5.21±2.98	4.0 – 8.22

Mean – average value, SD – standard deviation, Min – Max range – range from the minimum to the maximum values.

Table 15 OGTT results of the pregnant women group with GDM

OGTT	Plasma glucose (mmol/l) Mean±SD	Plasma glucose (mmol/l) Min – Max range
0 min	5.56±4.31	3.85 – 9.95
60 min	9.71 ± 5.26	6.10 – 13.55
120 min	5.97 ± 4.73	3.50 – 10.19

Mean – average value, SD – standard deviation, Min – Max range – range from the minimum to the maximum values measured.

2.4.2. Insulin

Serum concentrations of insulin were determined in 138 pregnant women, some healthy and some with pregnancy complications. The mean /average value measured of the

parameter for all women who were tested for insulin was 15.49±12.55 mIU/L (ranging from 3.18 mIU/L to 80.78 mIU/L), and for the healthy pregnant women (n=91) was 12.03±8.03 mIU/L (ranging from 3.18 mIU/L to 46.74 mIU/L), while for the pregnant women with pregnancy complications (n=47) – 22.19±16.41 mIU/L (ranging from 3.92 mIU/L to 80.78 mIU/L). The pregnant women from the two groups whose serum insulin was investigated were further divided into two additional subgroups according to their BMI estimated at the time of their getting pregnant: pregnant women with normal body weigh (BMI<25) and pregnant women who were overweight or obese (BMI≥25). The serum insulin results of the groups thus configured are presented in tables 16 and 17.

Table 16 Serum concentrations of insulin in healthy pregnant women.

N (%)	BMI	Insulin (mIU/L) Mean±SD	Insulin (mIU/L) Min – Max range
51 (56.04%)	<25	7.70±2.67	4.10 – 15.18
40 (43.96%)	≥25	17.54±9.14	3.18 – 46.74

BMI – body mass index, estimated at the time of getting pregnant, Mean – average value, SD – standard deviation, Min – Max range – range from the minimum to the maximum values measured.

Table 17 Serum concentrations of insulin in pregnant women with pregnancy complications.

N (%)	BMI	Insulin (mIU/L) Mean±SD	Insulin (mIU/L) Min – Max range
33 (70.21%)	<25	19.27±15.75	3.92 – 80.78
14 (29.79%)	≥25	29.07±15.87	7.20 – 64.59

BMI – body mass index, estimated at the time of getting pregnant, Mean – average value, SD – standard deviation, Min – Max range – range from the minimum to the maximum values measured.

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2.5. Anthropometric data about the newborn children of the investigated cohort of pregnant women.

The gestational age (g.a.) of the newborn is estimated according to the data on the last regular menstruation period filled out in the survey cards. The information about the anthropometric measurements of the newborn is received from the documentation available at the neonatal units in the Specialized Hospital for Obstetrics and Gynecology for Active Treatment "Prof.Dr D. Stamatov", Varna and the General Hospital for Active Treatment 'St. Anna', Varna, as well as from the Obstetrics and Gynecology Consulting Centers. Data were collected about the gestational age, sex, size and weight of 257 newborns out of the total of

259 monitored pregnant women. One of the pregnancies ended in abortion while another woman changed her place of residence and contact details, which was the reason why it was impossible to obtain data on the newborn. The mean gestational age of all the newborn was 37.85±2.26 g.w. (ranging from 30th to 40th g.w.). The percentage of children born on their due date 87.54% (n=225), with normal weight at birth 86.38% (n=222) and masculine gender 54.47% (n=140) is prevalent. Biparietal diameter (BPD), abdominal circumference (AC) and femur length (FL) information was measured after ultrasound and echocardiographic procedures. The summary data on the anthropometric indicators of the newborn are displayed in table 18.

Table 18 Anthropometric data of all the newborn.

Parameter	**Mean±SD**	**Min – Max range**
Weight (g)	3105.64±609.82	960 – 4590
Length (sm)	48.98±3.14	34 – 55
BPD (mm)	93.06±5.41	72 – 102
AC (mm)	315.10±31.66	214 – 370
FL (mm)	70.70±4.63	51 – 80

BPD – biparietal diameter, AC – abdominal circumference , FL – femur length , Mean – average value, SD – standard deviation, Min – Max range – range from the minimum to the maximum values measured.

Information about the anthropometric indicators was obtained from 166 out of the total of 167 newborn in the group of the healthy pregnant women. Data were not available about one of the newborns since the mother migrated to another country. In this group the prevailing newborns were male– 57.83% (n=96), and 95.7% (n=159) of them were born on their due date (after 37 g.w.), and there were 4.2% (PTB, n=7) preterm births (before 37 g.w.). The percentage of newborn with normal weight was 92.2% (n=153), and children who weighed less than 2500 grams were 7.2% (LBW, n=12) respectively. The anthropometric indicators of the newborn who were born to healthy pregnant women are displayed in table 19.

Table 19 Anthropometric data of the newborn, born to healthy pregnant women.

Parameter	Mean±SD	Min – Max range
Weight (g)	3228±525.46	1390 – 4590
Length (sm)	49.68±2.35	37 – 55
BPD (mm)	94.48±4.06	74 – 102
AC (mm)	323.14±30.42	238 – 370
FL (mm)	71.95±3.12	60 – 80

BPD – biparietal diameter, AC – abdominal circumference , FL – femur length , Mean – average value, SD – standard deviation, Min – Max range – range from the minimum to the maximum values measured.

In the group of pregnant women with GDM information was collected for all the newborn with total number of 43. The female newborn – 51.16% (n=22), and 93.02% (n=40) of the infants were born on their due date (after 37 g.w.), preterm births were 6.98% (PTB, n=3). The percentage of newborns with normal weight was 86.05% (n=37), and infants with low birth weight (LBW) – 13.95% (n=6). The anthropometric indicators of the newborn, born to pregnant women with GDM is demonstrated in table 20.

Table 20 Anthropometric data of the newborn, born to pregnant women with GDM

Parameter	Mean±SD	Min – Max range
Weight (g)	3091.39±589.22	1100 – 3880
Length (sm)	48.65±3.40	34 – 53
BPD (mm)	92.20±5.14	72 – 98
AC (mm)	316±22.18	236 – 362
FL (mm)	70.09±4.78	51 – 77

BPD – biparietal diameter, AC – abdominal circumference , FL – femur length , Mean – average value, SD – standard deviation, Min – Max range – range from the minimum to the maximum values measured.

In the group of pregnant women with preeclampsia, information was gathered for all the newborn with total number of 48. One of the pregnancies ended in abortion. Only one of the remaining 48 pregnancies, ended in stillbirth (2.08%). The distribution of the newborn according to sex is uniform (feminine gender/ female – 24, masculine gender/male – 24), and the number of infants born on their due date coincides with the number of those born before 37g.w. (PTB), n=24. The percentage of newborn children with low birth weight (LBW) is significantly higher – 33.33% (n=16), in comparison to the percentage that is established in

the first two groups of pregnant women. The anthropometric indicators of the children born to pregnant women with preeclampsia are demonstrated in table 21.

Table 21 Anthropometric data of the infants born to pregnant women with preeclampsia.

Parameter	Mean±SD	Min – Max range
Weight (g)	2693.33±720.32	960 – 3800
Length (sm)	46.83±4.20	34 – 53
BPD (mm)	88.92±7.27	72 – 100
AC (mm)	293.12±33.87	214 – 347
FL (mm)	66.92±6.48	51 – 79

BPD – biparietal diameter, AC – abdominal circumference , FL – femur length , Mean – average value, SD – standard deviation, Min – Max range – range from the minimum to the maximum values measured.

2. Vitamin D status of pregnant women.

2.1. Serum levels of 25(OH)D and determining the frequency of vitamin D deficiency or insufficiency.

Serum levels of 25(OH)D were measured in all the pregnant women included in the research by means of a validated method of liquid chromatography mass spectrometry for detection. The mean of serum concentrations of 25(OH)D is 76.26±38.27 nmol/L., while the median 107.24 nmol/L (ranging from 10.25 nmol/L to 204.23 nmol/L).

The results of serum concentrations of 25(OH)D in the pregnant women from the three major groups of participants are demonstrated in table 22.

Table 22 Serum concentrations of 25(OH) D in the three major groups of participants.

Group of pregnant women	25(OH)D (nmol/l) Mean±SD	25(OH)D (nmol/l) Min-Max range
Group I	76.72±37.14	10.25 – 204.23
Group II	73.98±37.96	17.00 – 160.43
Group III	76.85±42.92	18.08 – 194.03

Group I– healthy pregnant women, group II– pregnant women with GDM, group III- pregnant women with preeclampsia, Mean – average value, SD –standard deviation, Min – Max range – range from the minimum to the maximum values measured.

While carrying out a one-way ANOVA test, a statistically significant difference is not detected in the results of 25(OH)D between the three investigated groups (F=0.115, p=0.892). The mean value is the lowest in pregnant women with GDM (73.98 nmol/l), followed by the outcomes in the healthy (76.72 nmol/l) and then the pregnant women with preeclampsia (76.85 nmol/l). In addition, the existence of a relationship between the serum values of 25(OH)D and the belonging to a given group of the pregnant women who developed some sort of complication are checked, but the Spearman's correlation proves to be both consistent and regular (rho=0.028, p=0.661).

The detailed comparison of the average values of the measured concentrations of 25 (OH)D between the three groups of pregnant women following the method of Tukey, confirmed the absence of statistically significant difference between them (table 23).

Table 23 Comparison of 25(OH)D between the three groups of pregnant women following the method of Tukey.

Comparison between the three groups of pregnant women							
	Diagnosis	Diagnosis	Mean difference	SME	p	95% CI	
						Min	Max
25(OH)D (nmol/L)	Healthy	GDM	3.10	6.71	0.889	-12.72	18.93
		Preeclampsia	-0.13	6.53	1.000	-15.54	15.27
	GDM	Healthy	-3.10	6.71	0.889	-18.93	12.72
		Preeclampsia	-3.23	8.26	0.919	-22.73	16.26
	Preeclampsia	Healthy	0.13	6.53	1.000	-15.27	15.54
		GDM	3.23	8.266	0.919	-16.26	22.73
* The mean difference is significant at p<0.05							

SME (standard mean error), CI (confidence interval)

In order to determine the vitamin D status of the participants, the suggested Hollis scale (2011) is chosen for measuring degrees of vitamin D saturation. The distribution of all pregnant women included in the study according to this scale is illustrated in table 24.

Table 24 Vitamin D status of the whole cohort of tested pregnant women.

Vitamin D status	N (%)	25(OH)D (nmol/L) Mean±SD
Deficiency (≤25.0 nmol/L)	12 (4.63%)	18.21±4.80
Significant insufficiency (25.01 – 50.0 nmol/L)	53 (20.46%)	39.38±6.41
Deficiency (50.01 – 80.0 nmol/L)	75 (28.96%)	63.38±8.21
Sufficiency (>80.01 nmol/L)	119 (45.95%)	113.21±28.13

N – number of examined participants, Mean – average value, SD – standard deviation

In 54.05% of the investigated participants, it was established that vitamin D levels were below the commonly accepted optimal level of 80.00 nmol/L, above which it is considered that vitamin D may manifest its non-calcemic actions. Absolute deficiency was detected in 4.63% of the pregnant women, and 49.41% of the participants presented with a various degree of insufficient vitamin D saturation. A little less than half of the pregnant women (45.95%) showed optimal vitamin D levels (figure 13).

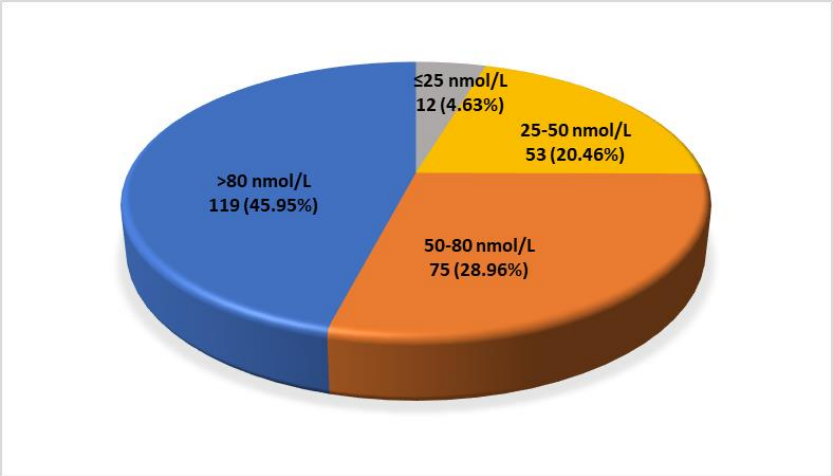


Figure 13 Distribution of the pregnant women according to their degree of saturation with vitamin D for the general group

Following Figure 14 presents the vitamin D status of pregnant women in the three main study groups. From the data obtained, it can be seen that the highest prevalence of absolute vitamin D deficiency was found in the pregnant women with pre-eclampsia (6.12%), while in the group of healthy pregnant women and in that of women with GDM, the percentage of those with absolute deficiency was approximately the same (4.79% for the healthy women and 4.65% for the women with GDM). Marked vitamin deficiency occurred with the lowest frequency in the group of healthy pregnant women (<20%), whereas in the other two groups this frequency was above 20% and was most pronounced in those with

GDM (23.26%). The overall incidence of women with 25(OH)D levels below the optimal 80 nmol/l was highest in the group with GDM (60.47%), followed by the group of women with pre-eclampsia (55.10%) and lowest in the group of healthy pregnant women (52.69%). Correspondingly, the percentage of women with optimal 25(OH)D levels >80 nmol/L was highest in the group of healthy pregnant women (47.31%), followed by the group of women with preeclampsia (44.89%), and the lowest percentage of women with optimal vitamin levels was found in the group with GDM (39.53%).

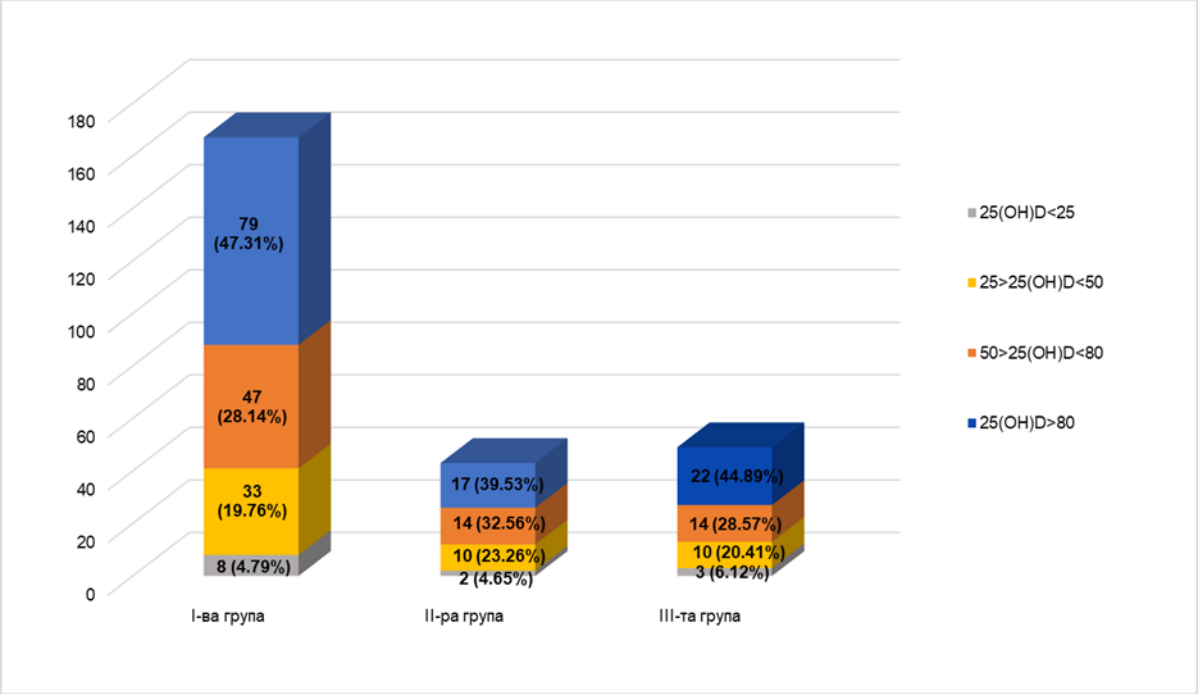


Figure 14 Distribution of the pregnant women according to their degree of vitamin D saturation for the three major investigated groups

Influence of season on vitamin D status for the whole cohort as well as in the three groups studied.

In relation to the dependence of serum 25(OH)D concentrations on the season in which sampling took place, we subdivided pregnant women into two groups according to the time of year during which their blood was taken for 25(OH)D testing (winter or summer half-year). The mean measured 25(OH)D in pregnant women sampled during the winter half-year was 71.40□36.15 nmol/l (median 64.93 nmol/l and interquartile range 46.09-91.65 nmol/l). The mean measured 25(OH)D in pregnant women tested during the summer half-year was 85.30□40.63 nmol/l (median 80.93 nmol/l and interquartile range 51.70-110.70 nmol/l). The Mann-Whitney test revealed a statistically significant difference between the serum 25(OH)D levels measured during the two semesters of the year (Figure 15).

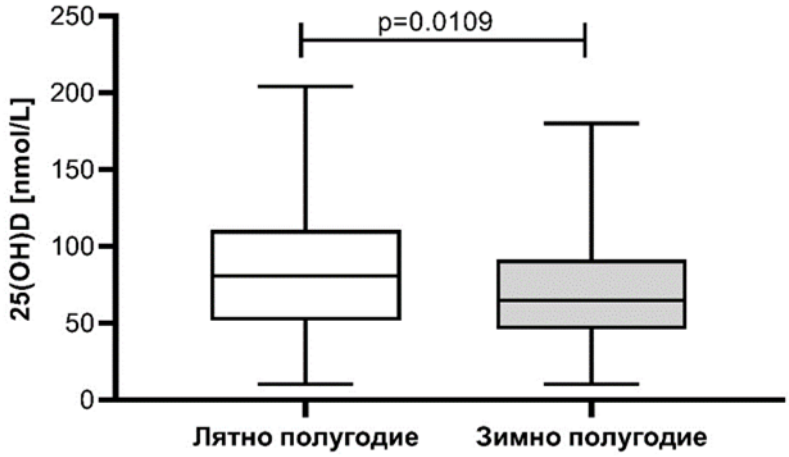


Fig15 Serum 25(OH)D levels measured during the two semesters of the year for the whole study cohort (data shown as median and interquartile range, Mann-Whitney test was used for the comparative analysis).

Data on mean serum 25(OH)D levels and medians for the two semesters of the year in the three main study groups are shown in Table 25.

Table 25 Serum 25(OH)D levels measured during the two half-years in the three main study groups.

Group	25(OH)D [nmol/l] Mean±SD	25(OH)D [nmol/l] Median (IQR)
Group I		
Winter half-year	72.95±37.04	65.87 (46.73-93.24)
Summer half-year	82.79±37.37	78.83 (51.22-109.6)
Group II		
Winter half-year	65.14±31.32	59.73 (42.48-78.34)
Summer half-year	86.76±38.20	85.74 (51.89-130.3)
Group III		
Winter half-year	68.37±34.01	66.90 (42.23-90.20)
Summer half-year	85.46±47.64	73.88 (51.43-147.5)

Group I - healthy pregnant women, Group II - pregnant women with GDM, Group III - pregnant women with pre-eclampsia, Mean - mean, SD - standard deviation, Median - median, IQR - interquartile range

The one-way ANOVA analysis performed did not demonstrate statistically significant differences between serum 25(OH)D levels among the three main groups of pregnant women studied, distributed in the two semesters of the year (F=0.4386, p=0.6457 for winter semester and F=0.07165, p=0.9309 for summer semester).

The frequency distribution of women according to their vitamin D status was examined according to the time of year when serum 25(OH)D levels were tested. In the winter half-year, 59.64% (n=99) of the pregnant women studied had 25(OH)D levels below 80 nmol/l, and the proportion of those with absolute deficiency was 6.02% (n=10). They, together with participants with serum 25(OH)D levels between 25 nmol/l and 50 nmol/l, formed a substantial proportion of the group studied in the winter half of the year - 28.31%. Participants with serum 25(OH)D levels between 50 nmol/l and 80 nmol/l accounted for 31.33% (n=52), and those with levels >80 nmol/l made up 40.36% (n=67). During the summer months of the year, the vitamin D status of pregnant women was better and suboptimal levels (80 nmol/l) accounted for 44.09% (n=41) of all 93 subjects. Expectant mothers with absolute deficiency accounted for only 2.15% (n=2). They, together with pregnant women with serum 25(OH)D levels between 25 nmol/l and 50 nmol/l (n=16) represented only 19.35% of all those studied in the summer half of the year, and about a quarter of the pregnant women in the group had suboptimal levels of the vitamin (between 50 nmol/l and 80 nmol/l). Figure 16 presents the results obtained for the frequency distribution of women according to their vitamin D status in the two half-years for the whole study cohort.

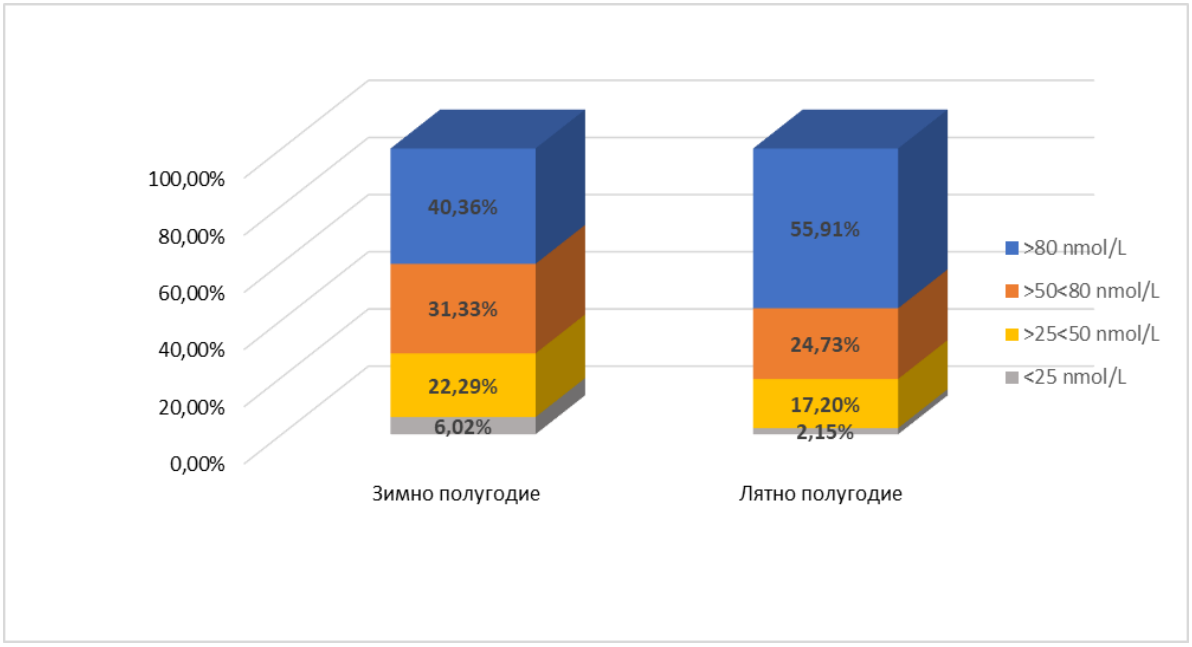


Figure 16 Vitamin D status of the pregnant women during the two halves of the year (Winter and spring season of the year Summer and autumn season of the year)

When conducting the chi-square analysis on the frequency distribution of women across the two semesters, there was a trend to reach statistical significance (chi 2=7.049, p=0.0704).

2.3. Assessment of the BMI effect on vitamin D status of the whole cohort as well as in the three investigated groups.

In order to assess and evaluate the BMI effect on serum levels of 25(OH)D, all investigated pregnant women were subdivided into two groups: those with normal body weight (BMI<25) 185 in number and those who were overweight and obese (BMI≥25) 74 in number. Table 26 illustrates the serum levels of 25(OH) D in these groups.

Table 26 Serum levels of 25(OH)D in pregnant women with normal and abnormal BMI.

BMI	N (%)	25(OH)D (nmol/L) Mean±SD
<25	185 (71.43%)	76.59±39.53
>25	74 (28.57%)	74.43±34.66

Mean – average value, SD – standard deviation

Statistically significant differences were not found in the serum levels of 25(OH) D between the group with normal body weight and that in which pregnant women were overweight (figure 17).

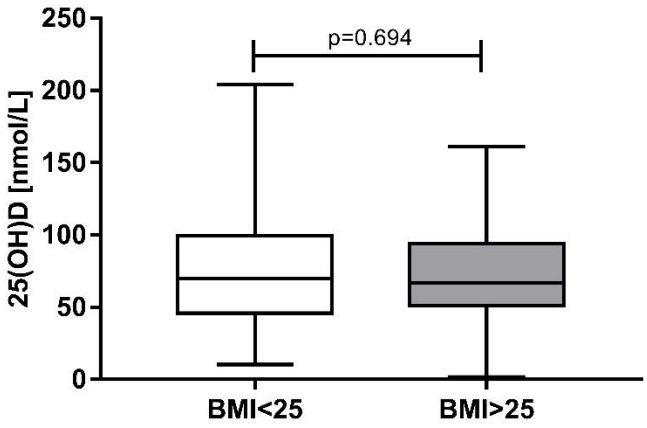


Figure 17 Comparison of serum levels of 25(OH)D between the normal body weight group of pregnant women and the overweight group.
(The comparative analysis is done with the help of an unpaired Student's *t*-test)

In table 27 the serum levels of 25(OH)D of the three major groups, subdivided according to their BMI, are displayed.

Table 27 Serum levels of 25(OH)D in healthy pregnant women and women with GDM and preeclampsia, subdivided according to their BMI

Group of pregnant women	N (%)	25(OH)D (nmol/l) Mean±SD	25(OH)D (nmol/l) Min-Max range
Group I			
BMI<25	125 (74.85%)	73.25±37.95	10.25 – 204.20
BMI>25	42 (25.15%)	85.76±34.14	24.50 – 150.80
Group II			
BMI<25	32 (74.42%)	77.77±40.43	20.95 – 160.40
BMI>25	11 (25.58%)	67.94±25.80	42.18 – 131.80
Group III			
BMI<25	28 (57.14%)	91.19±46.73	18.08 – 194.00
BMI>25	21 (42.86%)	60.47±31.85	20.50 – 161.30

Group I– healthy pregnant women, group II– pregnant women with GDM, group III - pregnant women with preeclampsia, Mean – average value, SD –standard deviation, Min – Max range – range from the minimum to the maximum values measured.

The data presented in the table show that 25(OH)D values are higher in women with normal body weight, but statistically significant differences are found for the groups of women with normal pregnancy and those with PE, while statistical significance is lost in women with GDM, probably due to the small number of women included in this group. When an oper-way ANOVA test was performed to investigate between-group differences in serum 25(OH)D levels according to BMI, statistically significant differences were found in the three study groups with BMI□25 (F=4.881, p=0.0105) but not in women with BMI<25 (F=2.048, p=0.1322).

2.4. Assessment of the dependency of the vitamin D status on the administration and intake of vitamin D as a pharmaceutical product and/or dietary supplements containing the vitamin for the entire cohort as well as in the three examined groups.

Of all pregnant women surveyed in the study, 85 (32.82%) reported no intake of vitamin D as a pharmaceutical product and/or supplements containing the vitamin. The mean measured serum 25(OH)D levels in unsupplemented women was 56.05±29.78 nmol/l (median: 48.18 nmol/l and interquartile range: 35.18 - 69.65 nmol/l). More than two-thirds of the women (n=174, 67.18%,) were supplemented with vitamin D preparations, and their mean measured serum 25(OH)D level was 86.41±38.50 nmol/l (median: 81.53 and interquartile range nmol/l (56.97 - 110.10). The Mann-Whitney test was used to demonstrate a statistically significant difference between the two groups (Figure 18).

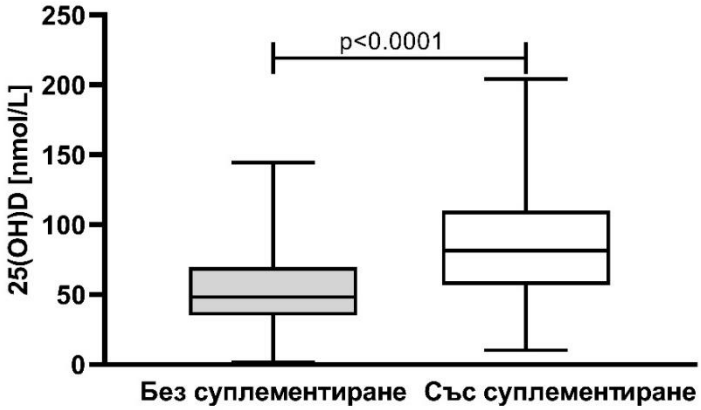


Fig. 18 Serum 25(OH)D levels in non-supplemented and vitamin D-supplemented pregnant women (data are represented by median and minimum-maximum range; Mann-Whitney test was used to demonstrate statistical difference between groups)

Of interest is whether the frequency distribution of women according to their vitamin D status differs according to their intake of vitamin D preparations. The results are shown in Table 28. It can be seen that there is a significant difference in distribution, with nearly 60% of the women who are supplemented reaching optimal serum 25(OH)D levels, whereas only 21% of the unsupplemented reached levels indicating sufficiency. Conversely, about 3% were absolutely deficient in supplemented women, and the percentage rose to 8.24% in unsupplemented women.

Table 28 Distribution of pregnant women according to their vitamin D status in relation to vitamin D supplementation

Vitamin D Status	Without Vitamin D intake - N (%)	With Vitamin D intake - N (%)
Deficiency (<25.0 nmol/L)	7 (8.24%)	5 (2.87%)
Marked insufficiency (25.01-50.0 nmol/L)	37 (43.53%)	16 (9.19%)
Insufficiency (50.01 – 80.0 nmol/L)	23 (27.06%)	52 (29.89%)
Sufficiency (>80.01 nmol/L)	18 (21.17%)	101 (58.05%)
Total	85 (100%)	174 (100%)

A Chi-square analysis showed a statistically significant difference in the distribution of pregnant women according to their vitamin D status depending on their vitamin D medication intake (chi 2 = 52.92, p<0.0001).

Table 29 presents data on mean daily intake of vitamin D preparations by pregnant women in the three main study groups.

Table 29 Average daily vitamin D supplementation in the three main groups of pregnant women.

Tested groups of pregnant women	Vitamin D intake (IU/d) Mean±SD	Vitamin D intake (IU/d) Min – Max range
Group I	540.12±706.00	0 – 3400
Group II	677.42±934.46	0 – 3400
Group III	212.24±295.54	0 – 1200

Group I– healthy pregnant women, group II– pregnant women with GDM, group III - pregnant women with preeclampsia, Mean – average value, SD –standard deviation, Min – Max range – range from the minimum to the maximum doses of daily intake of vitamin D preparations.

The table shows that the lowest mean and maximum daily intakes of vitamin D preparations (IU/day) were found in the group of pregnant women with pre-eclampsia.

According to the Institute of Medicine of the US National Academy [147] and according to the Regulation No. 1 of the Ministry of Health of the Republic of Bulgaria dated 22 January 2018 [344], the required minimum daily intake of vitamin D by pregnant women is 600 IU/day, which also served as a criterion for subdividing supplemented women into two additional groups. A dose <600 IU/day was taken by 45.56% (n=118) of the participants and only 21.62% (n=56) received vitamin D at a dose >600 IU/day. Figure 19 illustrates the frequency distribution of pregnant women according to daily vitamin D intake (IU/d) as a pharmaceutical product or as an element of multivitamin preparations and supplements.

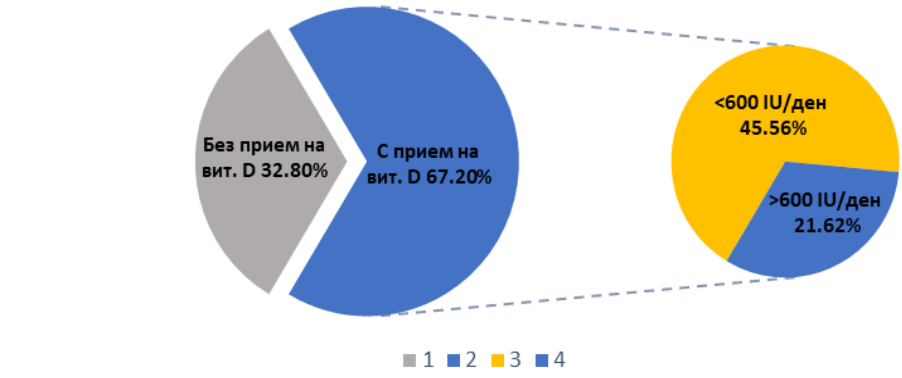


Figure 19 Distribution of the pregnant women according to their daily intake of vitamin D.
1 – pregnant women without vitamin D(n=85) supplements, 2 – pregnant women with vitamin D (n=174) supplements, 3 – supplemented with vitamin D <600 IU/d (n=118), 4 – supplemented with vitamin D >600 IU/d (n=56).

In addition to this general distribution, it is of interest whether the three main groups studied differ from each other according to their intake of vitamin D preparations. Figure 20 presents the frequency distribution of pregnant women in these groups according to their daily intake of vitamin D products. From this figure, the differences between the groups in terms of their supplementation with vitamin D preparations are clearly evident, with the high percentage of no vitamin D intake in the group of pregnant women with pre-eclampsia (57.14%) being very striking. In the other two groups of pregnant women, the proportion of non-supplemented was twofold lower (26.95% and 27.91% for healthy pregnant women and those with GDM, respectively).

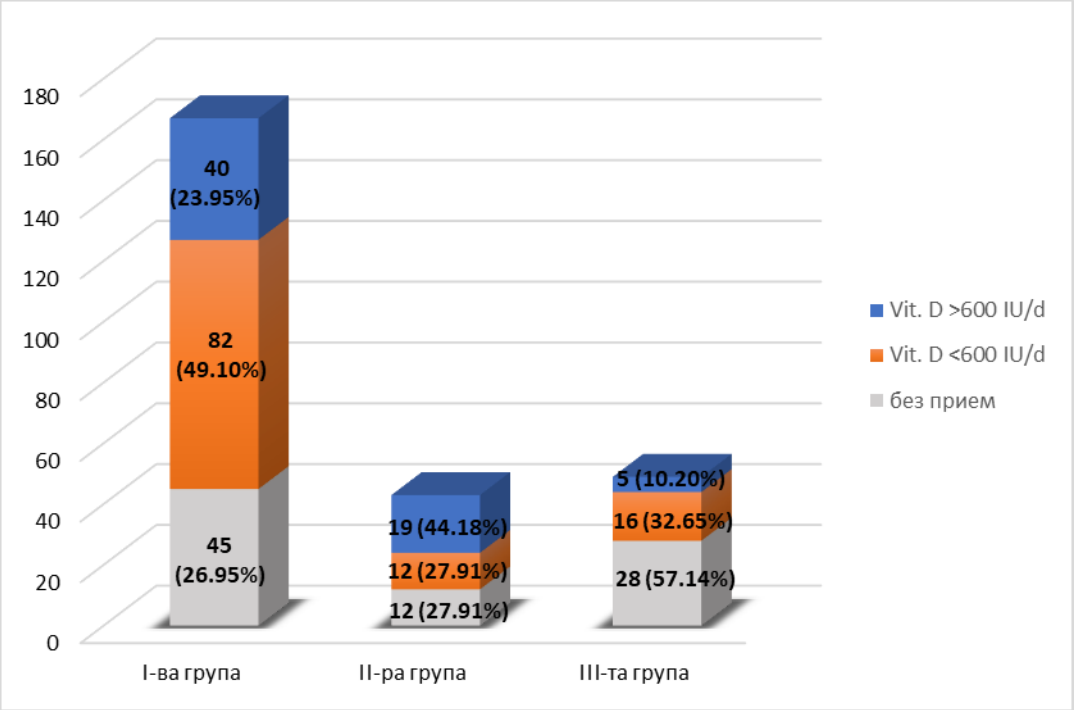


Figure 20 Distribution of the pregnant women in the three major groups according to their daily intake of vitamin D.
Group I – healthy pregnant women, Group II– pregnant women with GDM, Group III– pregnant women with preeclampsia

The differences between the groups in relation to their vitamin D supplementation become clear from the figure. The high percentage of absence of vitamin D intake in the group of pregnant women with preeclampsia (57.14%) is very impressive. In the other two groups of pregnant women, the number of unsupplemented pregnant women is twice as low (26.95% and 27,91% respectively for the healthy pregnant women and for the pregnant women with GDM). Cross tabulation and chi square statistical analysis was implemented for nonparametric distribution of the data (X^2) and a significant difference was found between the

three groups of pregnant women (X^2=7.179, p=0.028) concerning the vitamin D preparations intake during pregnancy (absent or present).

By implementing the one-way analysis of variance ANOVA, it is established that there are statistically significant differences between the groups not only in relation to vitamin D intake but also in relation to the number of units taken on a daily basis (F=4.16, p=0.017) (table 30).

Table 30 Comparative analysis in the three major groups of investigated women concerning their daily intake of vitamin D preparations

Vitamin D		N	Mean	SD	Min	Max	ANOVA F
Vitamin D intake (IU/d)	Group I	167	540.12	706.00	0	3400	F=4.16, p=0.017
	Group II	43	677.42	934.46	0	3400	
	Group III	49	212.25	295.55	0	1200	
	Total	259	580.31	161.30	0	3400	

Group I – healthy pregnant women, Group II – pregnant women with GDM, Group III – pregnant women with preeclampsia, Mean – average value, SD – standard deviation, Min – Max range – ranging from the minimum and maximum dose of daily intake of vitamin D preparations

One way ANOVA analysis performed on vitamin D intake showed statistically significant differences between healthy pregnant women and those with pre-eclampsia (540.12±706.00 vs 212.25±295.54 respectively, p=0.034), and between pregnant women with GDM and pregnant women with pre-eclampsia (677.42±934.46 vs 212.25±295.55 respectively, p=0.014).

Table 31 shows the distribution of women according to their vitamin D intake and body weight. About a quarter of pregnant women with normal BMI (n=46) took a daily optimal dose of vitamin D (≥600 IU), just under a third (n=56) did not supplement at all, and about 45% took an inadequate dose <600IU daily. As for overweight pregnant women, only 10 of them were taking a daily optimal dose of vitamin D (≥600IU), which as a proportion is almost twice the proportion of women with a normal BMI and adequately supplemented. Almost half of the overweight women (n=35) were taking insufficient amounts of the vitamin (<600IU), and 29 of them, or nearly 40%, were not supplementing at all. Although the overall impression was that women with a high BMI had a lower propensity to supplement, nonparametric chi-square analysis did not demonstrate statistically significant differences (chi 2= 4.50, p=0.1054) in the frequency distribution of.

Table 31 Vitamin D intake (IU/day) in normal and overweight women in the whole sample.

BMI (kg/m^2)	Without Vitamin D intake N (%)	With Vitamin D intake <600IU/ден N (%)	With Vitamin D intake ≥600IU/ден N (%)
<25 (n=185)	56 (30.27%)	83 (44.86%)	46 (24.87%)
≥25 (n=74)	29 (39.19%)	35 (47.30%)	10 (13.51%)
Общо (n=259)	85 (32.82%)	118 (45.56%)	56 (21.62%)

Vitamin D intake according to BMI in the three main groups of pregnant women is presented in Figure 21 When analysing the results shown in this figure, the tendency of pregnant women with a high BMI to have a lower attitude towards supplementation is again evident, most markedly in the group of women with PE.

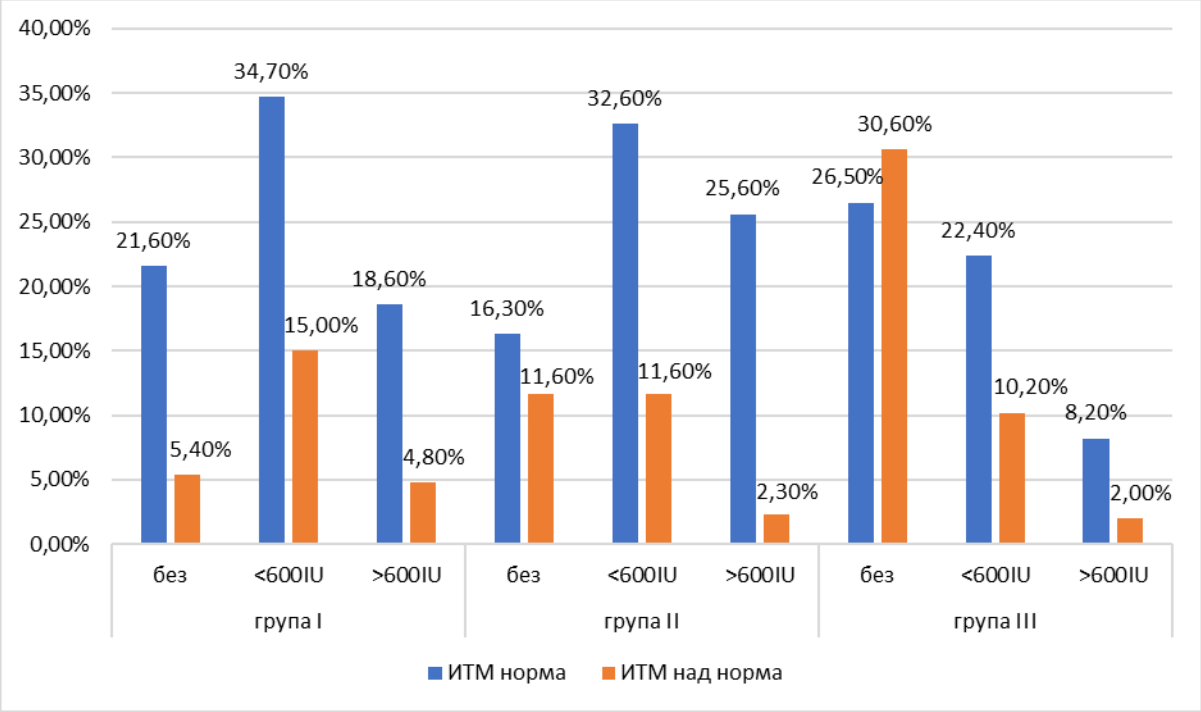


Figure 21 Vitamin D administration according to the BMI among the pregnant women in groups – healthy (I), with GDM (II), with preeclmpsia (III)

The correlation dependencies between vitamin D intake, BMI in pregnant women with normal and abnormal weight and serum levels of 25(OH) D are demonstrated in table 32.

Table 32 Correlation dependencies between vitamin D intake, BMI and serum levels of 25(OH)D

			BMI categories	Without vitamin D intake	With vitamin D intake	25(OH)D (nmol/l)
Spearman's rho	BMI categories	rho	1.000	-0.123*	-0.179*	-0.008
		p	.	0.047	0.023	0.907
	Without vitamin D intake	rho	-0.0123*	1.000	0.971**	0.512**
		p	0.047	.	0.000	0.000
	With vitamin D intake	rho	-0.179*	0.971**	1.000	0.556**
		p	0.023	0.000	.	0.000
	25(OH) D nmol/l	rho	-0.008	0.512**	0.0556**	1.000
		p	0.907	0.000	0.000	.
** The correlation is significant at 0.05 (2-tailed).						
* The correlation is significant at 0.01 (2-tailed).						

There was a significant but weak negative correlation between BMI and vitamin D intake (Spearman's rho = -0.179, p = 0.023) and a positive and significant correlation between vitamin D intake and serum 25(OH)D levels (Spearman's rho = 0.556, p < 0.0001).

2.5. Assessment of the influence of the vitamin D status concerning the unfavorable outcome of pregnancy in relation to the newborn and the occurrence of premature birth and the birth of a low-birth weight (LBW) newborn to healthy pregnant women and women with pregnancy complications

In the present study, 32 out of 257 newborns were born preterm or the incidence of pregnancies with such adverse pregnancy outcome (PTB) was 12.45%. The incidence of the complication was highest in the group of pregnant women with pre-eclampsia at 45.83% (n=22), followed by 6.98% (n=3) for pregnant women with GDM and 4.21% (n=7) for healthy controls.

To assess the role of vitamin D status on PTB, we initially examined the incidence of this complication according to vitamin intake as a pharmaceutical preparation and/or dietary supplement (absent, <600IU/day, >600IU/day) (Table 33).

Table 33 Frequency distribution of pregnant women according to the stage of pregnancy and the vitamin D preparations intake

Stages of Pregnancy/duration	Without vitamin D intake (%)	With vitamin D intake <600IU/per day n (%)	With vitamin D intake >600IU/per day n (%)	Total n (%)
>37 g.w.	73 (32.44%)	101 (44.89%)	51 (22.67%)	225 (100%)
<37 g.w.	12 (37.50%)	15 (46.88%)	5 (15.62%)	32 (100%)

The results in the table demonstrate that approximately the same percentage of the two groups of women (those who gave birth on the due date and those who had a PTB) were supplemented with vitamin D of 600 IU/per day (44.89% vs 46.88% respectively), while the number of women who did not take supplements was higher among those with PTB. But the number of women who took an adequate dose of vitamin D supplements is higher among the women who gave birth on their due date. The nonparametric chi square analysis that was carried out in order to determine credible differences between the identified categories of women does not prove the presence of statistically significant differences (X^2=0.879, p=0.645).

The vitamin D intake in women from the three major groups who experienced preterm delivery was investigated. The results are demonstrated in figure 22.

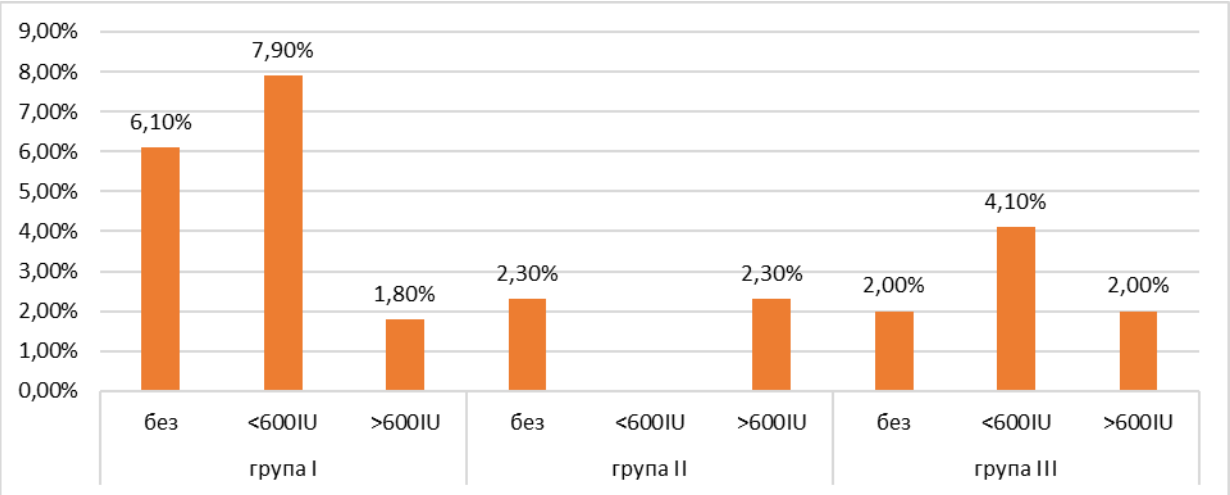


Figure 22 Vitamin D intake in pregnant women experiencing PTB.
(group I – healthy pregnant women, group II – women with GDM, group III – women with preeclampsia)

According to their frequency distribution, we can conclude that healthy pregnant women and women with pre-eclampsia are less likely to have preterm birth if they are supplemented with

an adequate dose of vitamin D (>600 IU/day) compared to those who are unsupplemented or supplemented with a low dose of vitamin D (<600 IU). No similar trend was observed in the GDM women group.

We also examined the relationship of adverse pregnancy outcome such as PTB in the three main study groups to both vitamin D intake and serum 25(OH)D concentrations by Spearman's correlation analysis. The results of the analysis are presented in Table 34 A positive and moderately strong correlation was found between serum 25(OH)D levels and duration of pregnancy in women with pre-eclampsia (rho=0. 358, p=0.016), and between serum 25(OH)D levels and vitamin D supplementation (rho=0.410, p=0.014), i.e. the possibility of preterm birth decreased with increasing vitamin D supplementation and serum 25(OH)D levels. Among healthy pregnant women, there was an increasing risk of PTV with decreasing intake of vitamin D preparations during pregnancy (rho=0.273, p=0.007), and a moderate correlation between intake of vitamin D preparations and serum 25(OH)D levels (rho=0.498, p<0.0001). In the group of pregnant women with GDM, there was a strong correlation between intake of vitamin D preparations and serum 25(OH)D levels (rho=0.848, p<0.0001), but the slightly increasing risk of PTV with decreasing vitamin D intake and serum 25(OH)D levels did not reach statistical significance (rho=0.167, p=0.416 and rho=0.138, p=0.382, respectively).

Table 34 Correlation and dependences between vitamin D intake, serum concentrations of 25(OH)D and pregnancy duration in groups.

Healthy pregnant women			Normal pregnancy duration / PTB	With vitamin D intake	25(OH)D (nmol/l)
Spearman's rho	Normal pregnancy duration / PTB	rho	1.000	**0.273****	0.052
		p	.	**0.007**	0.524
	With vitamin D intake	rho	0.273**	1.000	**0.498****
		p	0.007	.	**0.000**
	25(OH)D (nmol/l)	rho	0.052	0.498**	1.000
		p	0.524	0.000	.
Pregnant women with GDM			Norm /PTB	With vitamin D intake	25(OH)D nmol/l
Spearman's rho	Normal pregnancy duration / PTB	rho	1.000	0.167	-0.138
		p	.	0.416	0.382
	With vitamin D intake	rho	0.167	1.000	**0.848****
		p	0.416	.	**0.000**
	25(OH)D (nmol/l)	rho	-0.138	0.848**	1.000
		p	0.382	0.000	.
Pregnant women with preeclampsia			Norm /PTB	With vitamin D intake	25(OH)D nmol/l
Spearman's rho	Normal pregnancy duration / PTB	rho	1.000	0.314	**0.358***
		p	.	0.055	**0.016**
	With vitamin D intake	rho	0.314	1.000	**0.410***
		p	0.055	.	0.014
	25(OH)D nmol/l	rho	0.358*	0.410*	1.000
		p	0.016	0.014	.
* The correlation is significant at 0.05					
** The correlation is significant at 0.01					

A similar approach was implemented in the assessment and evaluation of the vitamin D status effect on pregnant women concerning yet another unfavorable pregnancy outcome, namely a newborn with low body weight (LBW).

Table 35 shows the frequency distribution of pregnant women according to intake of vitamin D preparations for the entire study cohort and the weight of their newborns.

Table 35 Frequency distribution of pregnant women according to the weight of the newborn and vitamin D preparations intake

Weight of the newborn at birth	Without vitamin D intake n (%)	With vitamin D intake <600IU/per day n (%)	With vitamin D intake >600IU/per day n (%)	Total n (%)
Normal weight (>2500g).	58 (26.00%)	113 (50.67)	52 (23.32%)	223 (100%)
Low weight <2500g)	27 (79.41%)	5 (14.70%)	2 (5.88%)	34 (100%)

The results shown in the table indicate that the percentage of non-supplemented women who gave birth to an LBW child was three times that of women who gave birth to a normal weight child (79.41% vs. 26.00% respectively). For those supplemented with vitamin D preparations <600UI, the ratio was exactly reversed, with a 3.45-fold drop in the rate of giving birth to a LBW child (50.67% vs. 14.70%), and for those adequately supplemented, there was an almost fourfold drop in the rate (23.32% vs. 5.88%). Chi-square analysis revealed statistical significance between the frequency distributions of women shown in Table 35 (χ^2=38.02, p<0.0001).

We also examined the vitamin D intake of women in the three main groups who gave birth to low birth weight children. The results are presented in figure 23.

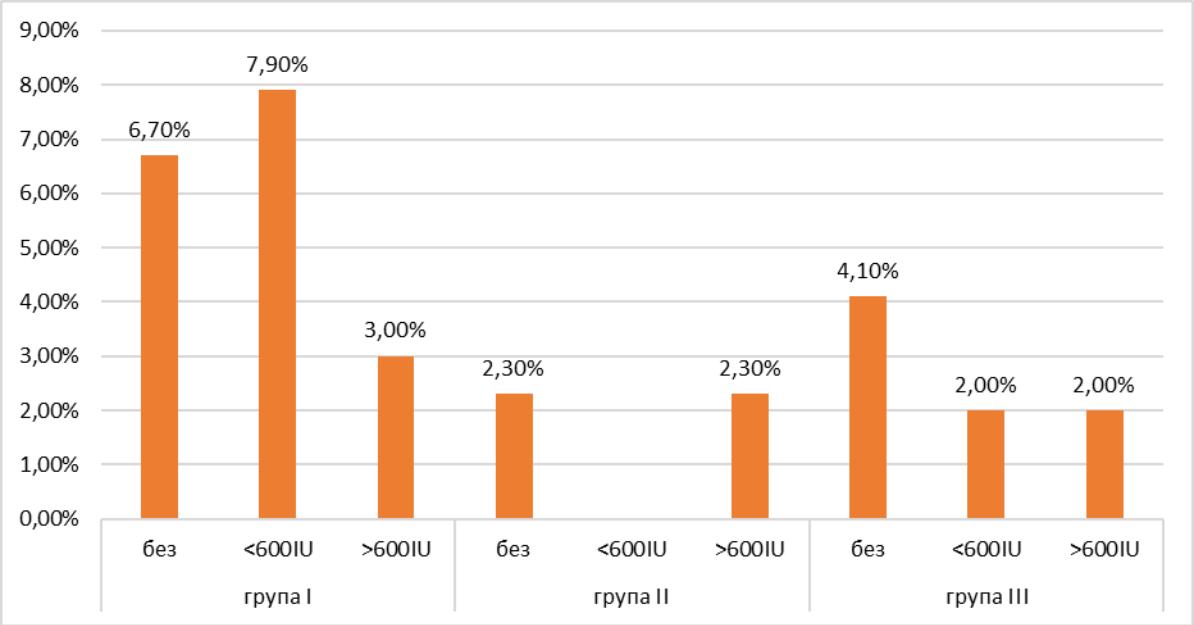


Figure 23 Vitamin D intake in pregnant women, distributed in the three major groups, who gave birth to low-birth-weight newborns.
(Group I – healthy pregnant women, Group II – pregnant women with GDM, Group III – pregnant women with preeclampsia)

In healthy pregnant women and women with preeclampsia, the probability of giving birth to a child with low-birth weight is smaller than in those who took an adequate dose of vitamin D supplements D (>600 IU/per day) in comparison to those who did not take or took a low dose of vitamin D supplements (<600 IU). A similar tendency is not observed in the group of women with GDM.

The mean values of vitamin D intake and serum levels of 25(OH)D were also compared in the newborn with normal weight in relation to those born with low-birth-weight by means of a t-test for independent samples (table 36).

Table 36 Comparative analysis concerning the mean values of vitamin D intake, serum concentrations of 25(OH)D in women who gave birth to newborns with normal weight and women who gave birth to newborns with low-birth-weight

	Weight	Mean	SD	t-test, p-value	95% CI
Vitamin D intake (IU/per day)	Normal	824.26	2167.65	t=0.667, p=0.506	[-58.7; 11.98]
	LBW	525.00	810.39		
Levels of 25(OH)D (nmol/l)	Normal	76.17	38.33	t=0.121, p=0.904	[-13.22; 14.94]
	LBW	75.31	40.40		

Mean – average value, SD – standard deviation, CI – confidence interval, LBW – newborn with low-birth-weight.

It is noteworthy that the mean intake of vitamin D was lower (525±810.39) in women with low birth weight compared to that of women with normal birth weight (824.26±2167) and was below the required minimum intake of 600 IU/day. However, the comparative analysis did not reveal statistical significance in the mean intakes between the two groups compared (t=0.667, p=0.506), probably due to the standard deviation being too large. There was also no statistically significant difference between serum 25(OH)D levels in the groups so subdivided (76.17±38.33 vs. 75.31±40.40, t=0.121, p=0.904).

We also investigated the association of low birth weight with both vitamin D intake and serum 25(OH)D concentrations in the three main study groups by Spearman correlation analysis. The results of the analysis are presented in table 37.

Table 37 Correlation relationships between serum levels of 25(OH)D, vitamin D intake and risk of giving birth to a newborn baby with LBW.

Healthy pregnant women			Normal weight/ LBW	With vitamin D intake	25(OH) nmol/l
Spearman's rho	Normal weight/ LBW	rho	1.000	0.165	0.037
		p	.	0.108	0.647
	With vitamin D intake	rho	0.165	1.000	**0.498****
		p	0.108	.	0.000
	25(OH) nmol/l	rho	0.037	0.498**	1.000
		p	0.647	0.000	.
Pregnant women with GDM			Normal weight/ LBW	With vitamin D intake	25(OH) nmol/l
Spearman's rho	Normal weight/ LBW	rho	1.000	0.167	0.138
		p	.	0.416	0.382
	With vitamin D intake	rho	0.167	1.000	**0.848****
		p	0.416	.	0.000
	25(OH) nmol/l	rho	-0.138	0.848**	1.000
		p	0.382	0.000	.
Pregnant women with preeclampsia			Normal weight/ LBW	With vitamin D intake	25(OH) nmol/l
Spearman's rho	Normal weight/ LBW	rho	1.000	0.041	0.045
		p	.	0.805	0.769
	With vitamin D intake	rho	0.041	1.000	**0.410***
		p	0.805	.	**0.014**
	25(OH)D nmol/l	rho	0.045	0.410*	1.000
		p	0.769	0.014	.
* The correlation is significant at 0.05					
** The correlation is significant at 0.01					

The analysis performed showed no correlations between either vitamin D intake or serum 25(OH)D levels with low birth weight.

Table 38 shows the mean serum 25(OH)D concentrations from the pooled results obtained in the two groups of women with an emerging complication of pregnancy (pre-eclampsia and GDM), those who delivered a normal-weight infant and those who delivered a low-weight infant.

Table 38 Serum concentrations of 25(OH) D in the group of pregnant women with complications who gave birth to an LBW newborn and to a child with normal weight.

	Weight (gr)	**Mean±SD (nmol/l)**	**SEM (nmol/l)**
25(OH)D nmol/l	Normal body weight	74.74±39.05	6.17
	LBW	51.20±22.66	16.02

Mean – average value, SD – standard deviation, SEM – standard error of mean, LBW – a low birth weight newborn.

Although there was no statistically significant difference between the measured mean serum 25(OH)D levels in the two target groups (t=0.839, p=0.407; 95% CI: -33.18 - 30.25), it is noteworthy that they were 31.09% higher in women who gave birth to a LBW child compared to those who gave birth to a LBW child.

3. Vitamin B12 status in pregnant women.

3.1. Serum levels of total vitamin B12, active vitamin B12 and methylmalonic acid and identifying the frequency of vitamin B12 deficiency or insufficiency.

According to the contemporary approach for evaluation and assessment of vitamin B12 status [54], with the aim of identifying and proving the availability of B12 deficiency or insufficiency, the pregnant women included in the research have been tested for serum concentrations of total vitamin B12, active vitamin B12 and methylmalonic acid. Since the homocysteine levels are affected not only by the cobalamin deficiency but also by the folic acid deficiency, the serum concentrations of this parameter have not been identified.

The measurement of total vitamin B12 in the pregnant women enrolled in the research has been performed by an ACCESS 2 Immunoassay System analyzer, based on the principle of direct chemiluminescence. The mean value of this parameter for the whole group of participants in the research is 177.58±90.24 pmol/l (median 429 pmol/l; range 76 – 782 pmol/l). The serum concentrations of active vitamin B12 of the pregnant women in the research have been tested by means of ARCHITECT Immunoassay analyzer, based on the principle of chemiluminescent microparticle immunoassay. The mean value of holo-TC (holotranscobalamin) for all tested participants in the research is 73.26±53.25 pmol/l

(median 273.70 pmol/l; range 16.40 – 531 pmol/l). The MMA serum concentrations have been measured by means of a selective chromatographic method, whereas the estimated mean

value of this parameter in all pregnant women is 249.53±154.73 nmol/l (median 584.34 nmol/l; range 40.94 – 1127.74 nmol/l).

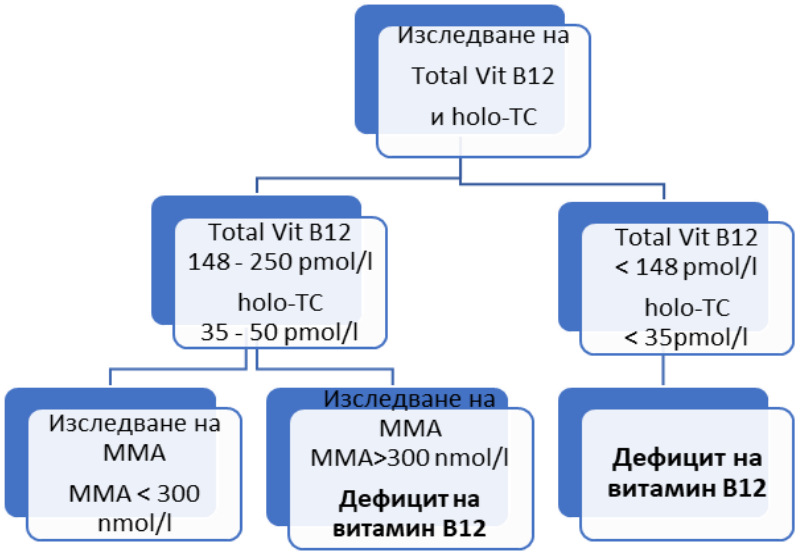
Table 39 presents data on the minimum, maximum and mean value and standard deviation for each of the indicated parameters of pregnant women in groups – healthy, with GDM and with preelampsia.

Table 39 Total Vit B12 (pmol/l), Holo-TC (pmol/l), MMA (nmol/l) – minimum, maximum, mean value and standard deviation in the three groups of pregnant women.

Laboratory parameter	Mean ± SD			Min – Max Range		
	I-гр.	II-гр.	III-гр.	I-гр.	II-гр.	III-гр.
Total Vit B12 (pmol/l)	175.43 ±87.72	206.32 ± 113.72	159.68 ± 68.97	87 – 782	89 – 588	76 – 416
Holo-TC (pmol/l)	69.92 ± 50.36	88.18 ± 61.85	71.55 ± 53.68	17.30 – 531	16.40 – 359	18 – 300
MMA (nmol/l)	250.30 ± 157.03	245.07 ± 146.47	250.55 ± 156.91	40.94 – 1127.7	82.95 – 691.35	50.01 – 594.63

Mean –average value, SD – standard deviation, Min-Max Range – reference interval

For identifying vitamin B12 status of the pregnant women who participated in the research, the algorithm suggested by Carmel (2006) was selected at the beginning. It is graphically represented in figure 24.



Figure… Algorithm for identifying vitamin B12 status in the pregnant women

In this research, the total vitamin B12<148 pmol/l (deficiency) has been estimated at 47.87% (n=124) of all examined pregnant women. Values between 148 and 250 pmol/l (insufficiency) have been found in 38.61% (n=100) of the participants. Levels of total vitamin B12 above 250 pmol/l or vitamin sufficiency was identified only in 13.51% (n=35) of the participants.

Active vitamin B12<35 pmol/l (deficiency) is measured in 13.89% (n=36) pregnant women while values between 35 and 50 pmol/l (insufficiency) have been established in 22.39% (n=58) of the participants. Women with levels of serum holo-TC above 50 pmol/l – 63.70% (n=165) tend to prevail.

In relation to the MMA metabolite parameter, serum concentrations above 300 nmol/l, that provide evidence for vitamin B12 deficiency have been examined in 31.65% (n=69) of the pregnant women participants in the research. The participants with MMA levels <300 nmol/l are 68.34% (n=149).

Table 40 illustrates the frequency of occurrence of vitamin B12 deficiency and insufficiency in the three major groups of pregnant women – healthy, with GDM and with preeclampsia.

Table 40 Frequency of occurrence of vitamin B12 deficiency and insufficiency in the three major groups of pregnant women.

Pregnant women's group	**Total Vit B12 < 148 pmol/l**	**Total Vit B12 148-250 pmol/l**	**Holo-TC < 35 pmol/l**	**Holo-TC 35 - 50 pmol/l**	**MMA > 300 nmol/l**
Group I	48.50% (n=81)	38.32% (n=64)	14.97% (n=25)	22.15% (n=37)	28.74% (n=48)
Group II	34.88% (n=15)	44.18% (n=19)	9.30% (n=4)	20.93% (n=9)	23.26% (n=10)
Group III	57.14% (n=28)	34.69% (n=17)	14.28% (n=7)	30.61% (n=15)	22.45% (n=11)

Total Vit B12 – total vitamin B12, Holo-TC – active vitamin B12, MMA – methylmalonic acid

The comparative analysis among the three major groups of pregnant women performed by means of the one-way ANOVA, contrasted the mean values of vitamin B12 indicators estimated in the sample and found that statistically significant differences could be detected concerning the total vitamin B12 parameter (F=3.24, p=0.040) (table 41).

Table 41 Comparative analysis of the estimated mean parameter values of the total vitamin B12, active vitamin B12 and MMA in the three major groups of pregnant women

		N	Mean	SD	Min	Max	ANOVA F
Total vit B12 pmol/l	Healthy	167	175.43	87.72	87.00	782.00	F=3.24, p=0.040
	GDM	43	206.32	113.72	89.00	588.00	
	Preeclampsia	49	159.68	68.97	76.00	416.00	
	Total	259	177.58	90.24	76.00	782.00	
Active vit B12 pmol/l	Healthy	167	69.92	50.36	17.30	531.00	F=2.05, p=0.130
	GDM	43	88.18	61.85	16.40	359.00	
	Preeclampsia	49	71.55	53.68	18.00	300.00	
	Total	259	73.26	53.25	16.40	531.00	
MMA nmol/l	Healthy	151	271.61	166.04	244.91	298.31	F=5.209, p=0.06
	GDM	34	197.13	89.75	165.81	228.44	
	Preeclampsia	33	202.46	131.58	155.87	249.12	
	Total	218	249.53	154.73	228.87	270.18	

Total vit B12 – total vitamin B12, Active vit B12 – active vitamin B12, MMA – methylmalonic acid, N – number, Mean – average value, Min – minimum value, Max – maximum value, SD – standard deviation

Comparable group differences in the mean values measured were analyzed for all indicators in the study groups, and significant differences were measured by Tukey's method. The results are presented in Table 42.

Table 42 Comparisons among the three major groups of pregnant women following Tukey's method.

Comparing the groups of pregnant women							
			Mean			95% CI	
	Diagnosis	Diagnosis	difference	SEM	p	Low	High
Total vit B12 pmol/l	Healthy	GDM	-30.89	15.29	0.110	-66.95	5.17
		Preeclampsia	15.74	14.53	0.525	-18.51	50.01
	GDM	Healthy	30.89	15.29	0.110	-5.17	66.95
		Preeclampsia	46.64*	18.69	0.035	2.57	90.71
	Preeclampsia	Healthy	-15.74	14.53	0.525	-50.01	18.51
		GDM	-46.64*	18.69	0.035	-90.71	-2.57
Active vit B12 pmol/l	Healthy	GDM	-18.25	9.06	0.111	-39.64	3.12
		Preeclampsia	-1.63	8.61	0.980	-21.94	18.68
	GDM	Healthy	18.25	9.06	0.111	-3.12	39.64
		Preeclampsia	16.62	11.08	0.292	-9.49	42.75
	Preeclampsia	Healthy	1.63	8.61	0.980	-18.68	21.94
		GDM	-16.62	11.08	0.292	-42.75	9.49
MMA nmol/l	Healthy	GDM	74.48*	28.81	0.028	6.47	142.49
		Preeclampsia	69.14*	29.17	0.049	0.2987	137.99
	GDM	Healthy	-74.48*	28.81	0.028	-142.49	-6.47
		Preeclampsia	-5.33	37.09	0.989	-92.89	82.22
	Preeclampsia	Healthy	-69.14*	29.17	0.049	-137.99	-0.29
		GDM	5.33	37.09	0.989	-82.22	92.89

Evidence of increased vitamin B12 deficiency among at-risk pregnant women was also demonstrated by Spearman correlation analysis (rho= -0.209, p=0.002). Higher measured values for the metabolic parameter MMA were found in pregnant women with pre-eclampsia and GDM (Table 43).

Table 43 Correlation dependencies between the estimated concentrations of total vitamin B12, active vitamin B12 and MMA in the pregnant women in their groups, following the method of Spearman..

		Diagnosis	TB12 pmo/l	AB12 pmol/l	MMA nmol/l
Spearman's rho	Diagnosis	1.000	-0.018	0.036	-0.209**
		.	0.777	0.568	0.002
	TB12 pmo/l	-0.018	1.000	0.589**	-0.057
		0.777	.	0.000	0.405
	AB12 pmol/l	0.036	0.589*	1.000	-0.019
		0.568	0.000	.	0.783
	MMA nmol/l	-0.209*	-0.057	-0.019	1.000
		0.002	0.405	0.783	.

TB12 – total vitamin B12, AB12 – active vitamin B12, MMA – methylmalonic acid

3.2. Assessment and evaluation of the effect of the season of the year on vitamin B12 status of the examined pregnant women.

Since the biological material of the pregnant women was gathered in the course of 2 years and 6 months, it was decided to report on the effect and influence of the season on the estimated serum concentrations of total, active vitamin B12 and MMA. In this research, higher serum concentrations of total and active vitamin B12 and respectively lower of MMA were detected in the serum of pregnant women whose sampling was performed in the winter and spring months / the winter and spring half of the year/.

The results of the whole examined cohort have been illustrated in table 44.

Table 44 Mean serum concentrations and standard deviation of total, active vitamin B12 and MMA, according to the month of taking the pregnant women's samples

Biological material sampling	Total Vit B12 Mean±SD (pmol/l)	Holo-TC Mean±SD (pmol/l)	MMA Mean±SD (nmol/l)
Winter/spring half of the year	186.93 ± 100.25	78.02 ± 61.77	248.89 ± 147.21
Summer/autumn time of the year	160.88 ± 64.94	64.78 ± 30.55	250.78 ± 167.69

Total Vit B12 – total vitamin B12, Holo-TC – active vitamin B12, MMA – methylmalonic acid, Mean – average value, SD – standard deviation

3.3. Evaluation and assessment of the effect of BMI on vitamin B12 status for the whole cohort, as well as the three examined groups of pregnant women.

In order to evaluate and assess the BMI effect on the vitamin B12 status, the examined pregnant women were subdivided into two major groups with normal body weight (BMI<25) – 185 in number and those who were overweight and obese (BMI ≥25) – 74 in number.

The serum levels of the parameters that reflect the vitamin B12 status in these groups are presented in table 45.

Table 45 Serum levels of total vitamin B12, active vitamin B12 and MMA in pregnant women with normal and abnormal BMI.

BMI	N (%)	Total vit B12 Mean±SD (pmol/l)	Active vit B12 Mean±SD (pmol/l)	MMA Mean±SD (nmol/l)
<25	**185 (71.43%)**	**167.74 ± 82.86**	**69.98 ± 53.62**	**266.39 ±162.76**
≥25	**74 (28.57%)**	**202.19 ± 101.88**	**83.60 ± 50.31**	**201.90 ± 115.11**

Total vit B12 – total vitamin B12, Active vit B12 – active vitamin B12, MMA – methylmalonic acid, Mean – average value, SD – standard deviation

In order to check the dependency between the serum concentrations of the parameters that reflect the vitamin B12 status and BMI of the pregnant women, a correlation analysis was performed following the method of Spearman. The results of the analysis did not find any statistically significant connection between the indicators (p>0.05) (table 46).

Table 46 Correlation dependencies between the indicators reflecting the vitamin B12 status and BMI in pregnant women with normal and abnormal weight.

Pregnant women - parameter			TB12 pmol/l	AB12 pmol/l	MMA nmol/l	BMI kg/m2
Spearman's rho	TB12 pmol/l	rho	1.000	0.589**	-0.057	0-.078
		p	.	0.000	0.405	0.210
	AB12 pmol/l	rho	0.589**	1.000	-0.019	0.035
		p	0.000	.	0.783	0.578
	MMA nmol/l	rho	-0.057	-0.019	1.000	0.084
		p	0.405	0.783	.	0.214
	BMI kg/m2	rho	-0.078	0.035	0.084	1.000
		p	0.210	0.578	0.214	.

TB12 – total vitamin B12, AB12 – active vitamin B12, MMA – methylmalonic acid

The comparative analysis with box-plot graphs confirmed the observations from the frequency distribution of the data. The pregnant women with high BMI, as well as those with normal weight, have similar mean but low levels in relation to the indicators total and active vitamin B12 (figures 25 and 26) and similar mean but higher levels concerning the metabolic parameter MMA (figure 27).

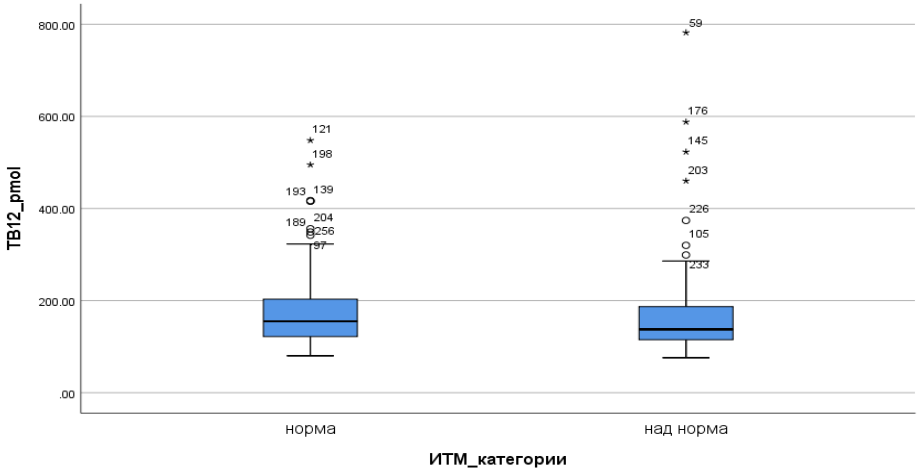


Figure 25 BMI and estimated average concentrations of total vitamin B12 pmol/l.

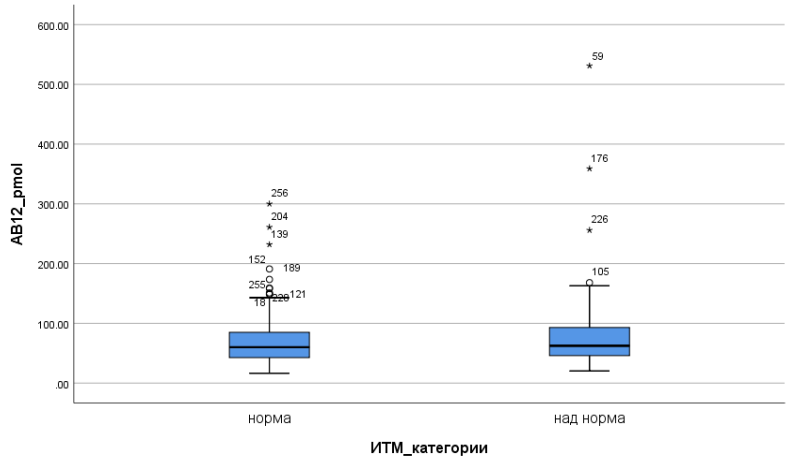


Figure 26 BMI and estimated average concentrations of active vitamin B12 pmol/l.

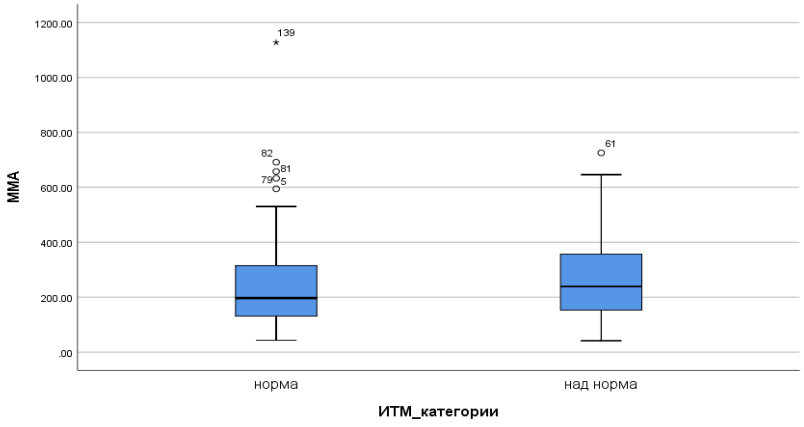


Figure 27 BMI and estimated mean serum concentrations of MMA nmol/l.

Table 47 illustrates the mean serum concentrations of the indicators of total vitamin B12, active vitamin B12 and MMA in the three major groups of pregnant women, subdivided according to their BMI.

Table 47 Serum levels of total vitamin B12, active vitamin B12 and MMA in healthy pregnant women, women with GDM and preeclampsia, subdivided according to their BMI

Groups of pregnant women	N (%)	Total Vit B12 Mean±SD (pmol/l)	Active Vit B12 Mean±SD (pmol/l)	MMA Mean±SD (nmol/l)
Group I				
BMI<25	125 (74.85%)	189.34 ± 106.31	77.81 ± 60.06	229.75 ± 149.82
BMI≥25	42 (25.15%)	193.17 ± 86.95	84.05 ± 56.00	191.15 ± 107.09
Group II				
BMI<25	32 (74.42%)	153.65 ± 47.33	62.08 ± 30.08	307.64 ± 159.29
BMI≥25	11 (25.58%)	144.33 ± 54.65	55.10 ± 25.63	317.16 ± 192.89
Group III				
BMI<25	28 (57.14%)	151.59 ± 64.91	67.81 ± 49.66	304.53 ± 154.46
BMI≥25	21 (42.86%)	165.62 ±141.93	57.81 ± 32.76	254.23 ± 141.93

Group I – healthy pregnant women, Group II – pregnant women with GDM, Group III – pregnant women with preeclampsia, Total vit B12 – total vitamin B12, Active vit B12 – active vitamin B12, MMA – methylmalonic acid, N – number, Mean – average value, SD – standard deviation

Only in the group of pregnant women with GDM, the estimated mean serum concentrations of the total and active vitamin B12 parameters are somewhat lower, and those of the metabolic parameter MMA are respectively to some extent higher in the participants who are overweight or obese (BMI≥25).

A similar correlation in the levels of the investigated parameters has not been found between the healthy control group of pregnant women and the women with preeclampsia.

3.4. Evaluation and assessment of the dependency of vitamin B12 status on the administration and intake of dietary supplements that contain vitamin B12 for the whole cohort, as well as in the three study groups.

According to the recommendations of the Ministry of Health of the Republic of Bulgaria, the recommended daily intake of vitamin B12 for the period of pregnancy is 4.5 ug/day, and for the period of lactation - 5 ug/day [344]. Of the pregnant women included in the present

study, 34.75% (n=90) reported no vitamin supplementation. The remaining participants confirmed supplementation, with 46.33% (n=120) supplementing with a dose greater than 4.5 ug/day. Figure 28 presents the use of supplements containing vitamin B12 (ug/day) in the total group of pregnant women studied.

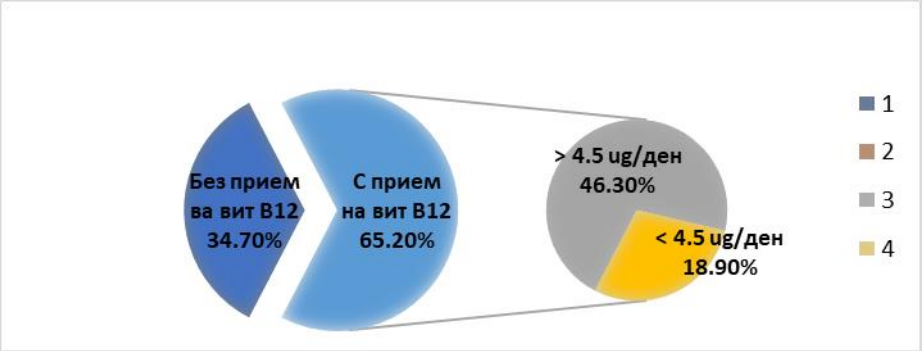


Fig. 28 Intake of food supplements containing vitamin B12 (ug /per day)

Figure 29 illustrates the frequency of supplementation with dietary supplements that contain vitamin B12 in the three major groups of pregnant women.

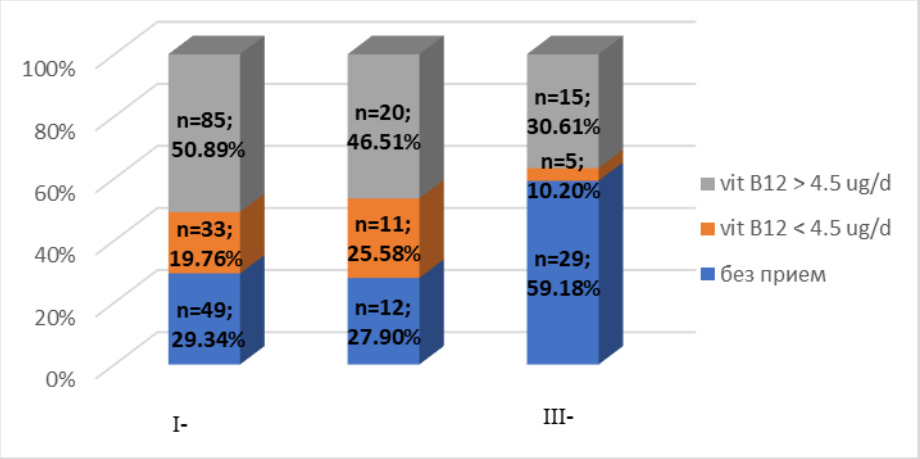


Fig. 29 Food supplements intake that contains vitamin B12 in the corresponding groups of pregnant women – healthy, with GDM and with preeclampsia.

The clear percentage differences between the three major groups of pregnant women happen to be statistically significant (X^2= 16.80, p=0.002) and they confirm the lack or insufficient intake of vitamin B12 in women who have developed GDM and preeclampsia in comparison with the healthy controls.

It was decided to investigate whether there exists a correlation between the pregnant women's group and vitamin B12 supplementation. The results of the correlation following Spearman's method showed a weak but positive relationship between the two indicators (rho=0.178, p=0.001). Data are available showing that there is increasing probability of a lower intake or lack of any vitamin B12 intake in pregnant women leading to complications during pregnancy (GDM and preeclampsia).

Table 48 represents data on the minimum, maximum and mean dose of vitamin B12 intake in the pregnant women's groups – healthy, with GDM and with preeclampsia. Among the examined major groups of pregnant women, the lowest mean and the lowest maximum daily dose of vitamin B12 intake is detected in the participants with preeclampsia.

Table 48 Vitamin B12 intake in the three groups of pregnant women – minimum, maximum, mean dose and standard deviation.

Group of pregnant women	Vitamin B12 intake Mean ± SD (ug/per day)	Vitamin B12 intake Min-Max Range (ug/per day)
Group I	11.06 ± 77.24	0 – 1000
Group II	50.53 ± 212.21	0 – 1000
Group III	4.88 ± 10.56	0 – 54

Mean – average value, SD – standard deviation, Min-Max Range – reference interval

Information on the measured serum concentrations of the parameters total, active vitamin B12 and MMA in supplemented and unsupplemented pregnant women in the three main study groups is presented in tabular form (Table 49).

Table 49 Mean serum concentrations of parameters reflecting vitamin B12 status in the three main study groups.

Group	TB12 (pmol/l)		AB12 (pmol/l)		MMA (nmol/l)	
	Without vitamin intake	With vitamin intake	Without vitamin intake	With vitamin intake	Without vitamin intake	With vitamin intake
I	165.84	179.42	59.60	74.22	244.64	252.70
II	198.16	209.48	84.43	88.73	239.13	246.91
III	153.95	168.00	68.96	73.36	273.16	218.91

Group I - healthy; Group II - women with gestational diabetes; Group III - women with pre-eclampsia; TB12 - total vitamin B12; AB12 - active vitamin B12; MMA - methylmalonic acid

We also decided to compare vitamin B12 intake from supplements in pregnant women with normal weight (BMI<25) and those with overweight and obesity (BMI≥25). We found

that 39.19% of overweight women were not supplemented, 20.27% had low levels of supplementation (<4.5ug/day) and 40.54% (less than half) were taking the vitamin at the recommended dose for the duration of pregnancy (≥4.5ug/day). The distribution was relatively similar in pregnant women with normal BMI, with 51.35% of pregnant women with no or low levels of vitamin B12 intake (<4.5ug/day) and 48.65% supplementing at a dose ≥4.5/ug/day. We investigated the prevalence of vitamin B12 intake in the sample participants using nonparametric chi-square analysis of categorical data. The reported percentage differences did not show statistical significance (X2= 1.438, p=0.487), but we found that more than half (59.46%) of overweight and obese pregnant women had insufficient vitamin B12 intake (table 50).

Table 50 Vitamin B12 supplementation in normal and overweight pregnant women in the whole sample

BMI	Without vitamin B12 intake	With itamin B12 intake < 4.5ug/ден	With vitamin B12 intake ≥ 4.5ug/ден
< 25	32.97% (n=61)	18.38% (n=34)	48.65% (n=90)
≥ 25	39.19% (n=29)	20.27% (n=15)	40.54% (n=30)
Общо	34.75% (n=90)	18.92% (n=49)	46.33% (n=120)

Additionally, we compared the frequency distribution of vitamin B12 supplementation in each of the groups of pregnant women studied (Figure 30). Although there was no statistically significant difference between the three main groups and vitamin intakes, the results clearly highlighted a picture of under-supplementation among all pregnant women.

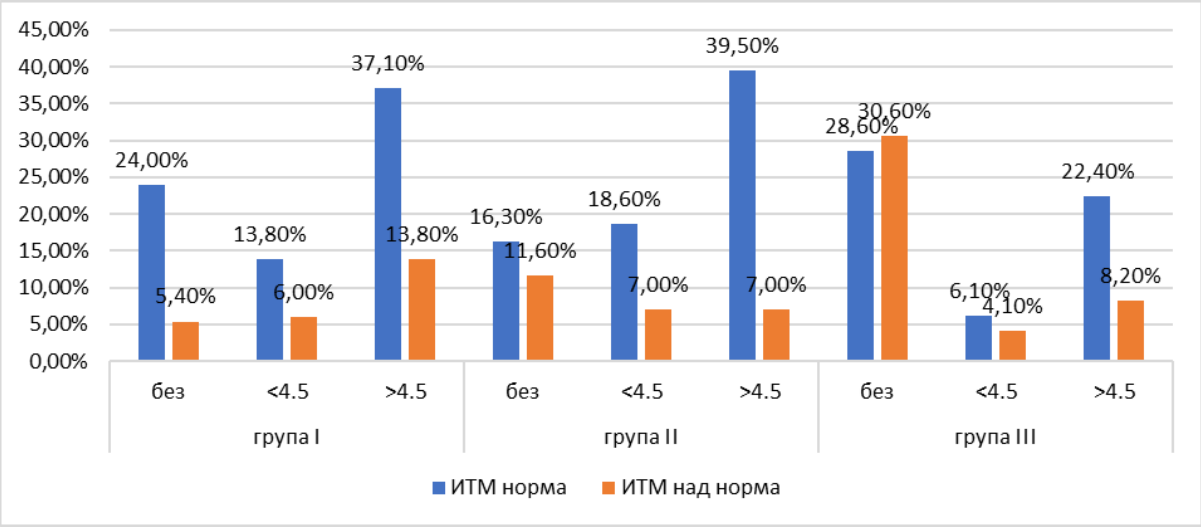


Fig. 30 Frequency distribution of vitamin B12 intake according to BMI in pregnant women by groups (group I - healthy; group II - women with GDM; group III - women with PE

3.5. Evaluation and assessment of the effect of the vitamin B12 status in relation to an adverse pregnancy outcome concerning the newborn and the occurrence of a premature birth and giving birth to a child with low birth weight in healthy pregnant women and women with pregnancy complications.

In order to evaluate the role of vitamin B12 status in relation to an adverse pregnancy outcome such as preterm birth (PTB), at first the frequency of this complication was investigated depending on the vitamin intake as a food supplement – no vitamin B12 intake, intake < 4.5ug/per day, intake > 4.5ug/per day (table 51).

Table 51 Vitamin B12 intake in pregnant women according to the duration of their pregnancy (normal birth on due date and PTB).

Pregnancy length	Vitamin B12, No intake	Vitamin B12, intake < 4.5ug/ day	Vitamin B12, intake > 4.5ug/ day
> 37 g.w.	34.67% (n=78)	18.22% (n=41)	47.11% (n=106)
< 37 g.w. (PTB)	37.50% (n=12)	21.86%(n=7)	40.63% (n=13)
Total	35.02% (n=90)	18.68% (n=48)	46.30% (n=119)

Of all pregnant women who delivered preterm, 37.50% reported no additional vitamin B12 intake from supplements, 21.86% were supplemented with a dose below the required minimum for the country during pregnancy, and 40.43% were taking an as-recommended dose of 4.5ug/day [344]. To determine whether there was a relationship between vitamin B12 status of pregnant women and the occurrence of preterm birth, we first described the incidence of PTB according to supplementation intake during pregnancy (vitamin B12 intake<4.5ug and >4.5ug). To detect significant differences between vitamin intake categories, we used crosstabulation and non-parametric chi-square analysis (X2= 0.519, p=0.771). Figure 31 demonstrates the frequency comparative analysis between groups of pregnant women and vitamin B12 intake from supplements. The graphical representation of the data showed that pregnant women with no or inadequate supplementation with the vitamin occurred among all three main groups (healthy, GDM, preeclampsia), but it is clear from Figure 32, depicting only intake among those who gave birth before age 37, that the percentage of pregnant women who delivered preterm and were supplemented with an optimal dose of vitamin B12 was very small.

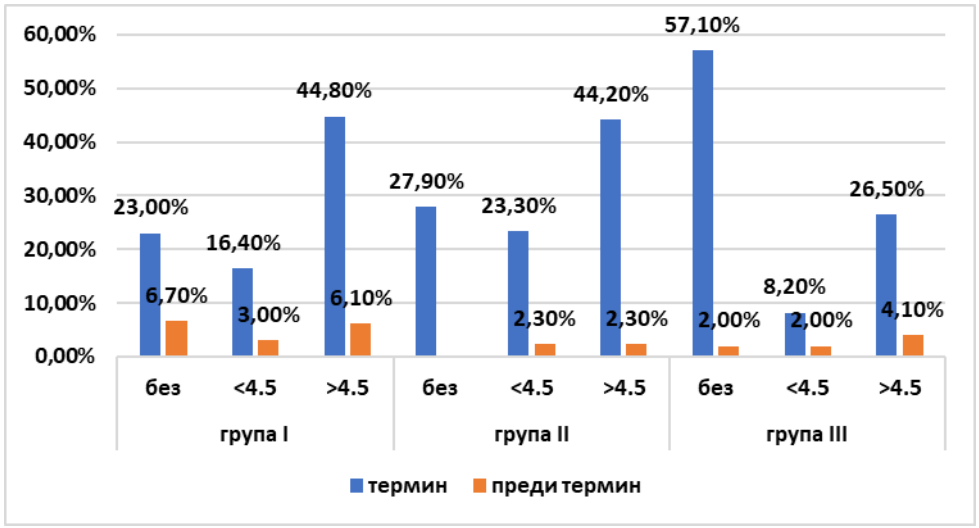


Fig. 31 Frequency distribution of pregnant women according to vitamin B12 intake and duration of pregnancy by group (group I - healthy; group II - women with GDM; group III - women with PE)

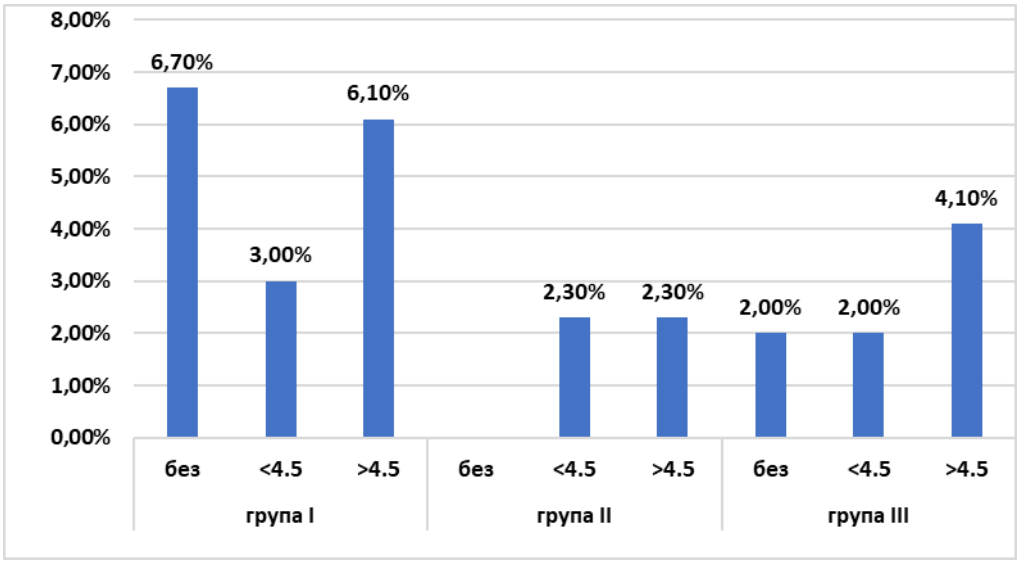


Fig. 32 Frequency distribution of pregnant women according to vitamin B12 and PTB intake in the three main study groups (group I - healthy; group II - women with GDM; group III - women with PE)

We next examined and compared the mean serum concentrations of total vitamin B12, active vitamin B12, and MMA in pregnant women of normal gestational length and with PTB (table 52).

Table 52 Results of total vitamin B12, active vitamin B12 and MMA in pregnant women with normal pregnancy duration and PTB.

Parameter	Pregnancy length	Mean ± SD	t-test/p-level	95% CI
TB12 pmol/l	Normal	179.31±93.92	t=0.899, p=0.370	-18.27; 48.98
	PTB	163.96±58.39		
AB12 pmol/l	Normal	74.06±55.16	t=0.929, p=0.354	-10.45’ 29.13
	PTB	64.72±35.94		
MMA nmol/l	Normal	247.45±157.07	t=-0.448, p=0.655	-78.52; 49.44
	PTB	261.99±141.05		

TB12 – total vitamin B12, AB12 – active vitamin B12, MMA – methylmalonic acid, Mean – average value, SD – standard deviation, 95% CI – 95% confidence interval

In spite of the fact that the differences in the measured mean serum concentrations of the parameter total vitamin B12, active vitamin B12 and MMA in pregnant women with PTB and normal pregnancy length were confirmed, credibility in statistics between the groups was not proved.

There was an attempt to confirm the role and importance of vitamin B12 for the pregnancy length by conducting a correlation analysis following Spearman’s method. The results did not detect credibility in statistics between the factors investigated ($p>0.005$), and the tested correlation dependencies are represented in table 53.

Table 53 Correlation dependencies between the serum levels of total vitamin B12, active vitamin B12, MMA and the pregnancy duration.

Healthy pregnant women						
			Normal /PTB	TB12 pmol/l	AB12 pmol/l	MMA nmol/l
Spearman's rho	Normal /PTB	rho	1.000	0.014	-0.104	0.011
		p	.	0.862	0.185	0.891
	TB12 pmol/l	rho	0.014	1.000	0.574**	-0.076
		p	0.862	.	0.000	0.354
	AB12 pmol/l	rho	-0.104	0.574**	1.000	-0.042
		p	0.185	0.000	.	0.611
	MMA nmol/l	rho	0.011	-0.076	-0.042	1.000
		p	0.891	0.354	0.611	.
Pregnant women with GDM						
			Normal /PTB	TB12 pmol/l	AB12 pmol/l	MMA nmol/l
Spearman's rho	Normal /PTB	rho	1.000	-0.089	0.018	-0.186
		p	.	0.570	0.910	0.291
	TB12 pmol/l	rho	-0.089	1.000	0.618**	0.005
		p	0.570	.	0.000	0.977
	AB12 pmol/l	rho	0.018	0.618**	1.000	0.035
		p	0.910	0.000	.	0.846
	MMA nmol/l	rho	-0.186	0.005	0.035	1.000
		p	0.291	0.977	0.846	.
Pregnant women with preeclampsia						
			Normal /PTB	TB12 pmol/l	AB12 pmol/	MMA nmol/l
Spearman's rho	Normal /PTB	rho	1.000	-0.129	0.190	0.277
		p	.	0.376	0.192	0.119
	TB12 pmol/l	rho	-0.129	1.000	0.556**	-0.079
		p	0.376	.	0.000	0.663
	AB12 pmol/l	rho	0.190	0.556**	1.000	-0.015
		p	0.192	0.000	.	0.935
	MMA nmol/l	rho	0.277	-0.079	-0.015	1.000
		p	0.119	0.663	0.935	.

TB12 – total vitamin B12, AB12 – active vitamin B12, MMA – methylmalonic acid, PTB – preterm birth

No significant statistical dependencies were found between the pregnancy duration and the parameters that reflect vitamin B12 status of pregnant women in the two groups compared (with normal pregnancy length and with PTB) ($p>0.05$).

A similar approach to the one already described was used to assess the effect of vitamin B12 status on pregnant women in relation to another adverse pregnancy outcome, namely – the birth of a newborn with low birth weight (<2500g).

Table 54 represents some information about the percentage distribution of pregnant women who gave birth to newborns with normal and low weight depending on the intake of vitamin

B12 from food supplements – no intake, with vitamin B12 intake < 4.5ug/per day and with vitamin B12 intake >4.5ug/per day.

Table 54 Frequency of vitamin B12 supplementation in pregnant women according to the weight of the newborn at birth.

Weight at birth	No vitamin B12, intake	Vitamin B12, intake < 4.5ug/per day	Vitamin B12, intake > 4.5ug/per day
Normal weight (>2500g)	76 (34.08%)	39 (17.49)	108 (48.43%)
Low weight (<2500g)	12 (35.29%)	10 (29.41%)	12 (35.29%)
Total	88 (34.24%)	49 (19.07%)	120 (46.69%)

Of all the women who gave birth to a newborn with low birth weight, 35.29% do not share that they have taken vitamin B12 supplements, and 29.41% took a dose lower than the one recommended during pregnancy. In order to establish if there exists a dependency between the vitamin B12 status of the participants and the birth of a child with low weight, at first a frequency comparative analysis between the groups of pregnant women and their supplementation with the vitamin was applied (figure 33). The graphic representation shows lack or insufficient intake of vitamin B12 among all the pregnant women. The largest amount is observed among the mothers who gave birth to children with LBW in the group of healthy women – 7.3% and a comparatively similar presentation in pregnant women who developed preeclampsia– 4.10% and GDM – 2.3% (figure 34).

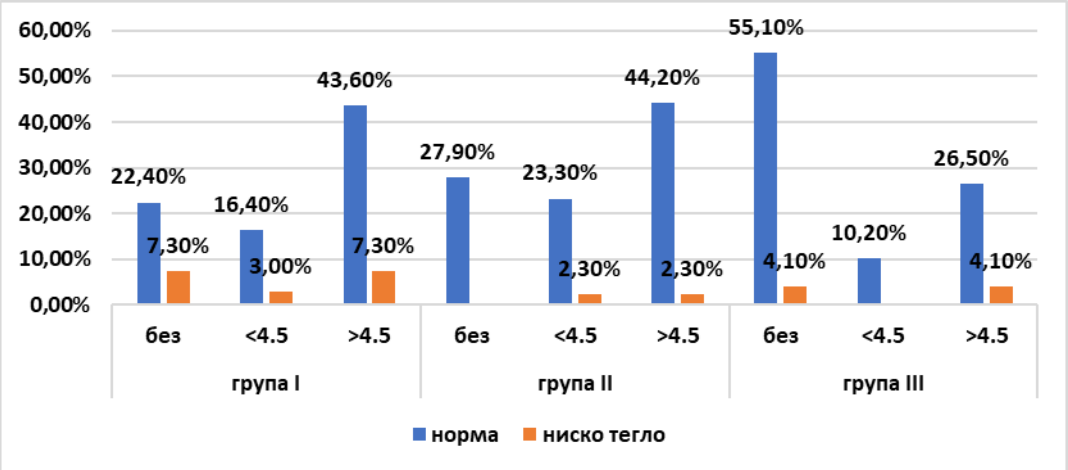


Figure 33 Frequency distribution of pregnant women according to vitamin B12 intake and newborn LBW and normal birth weight among pregnant women in the three main groups (group I - healthy; group II - women with GDM; group III - women with pre-eclampsia).

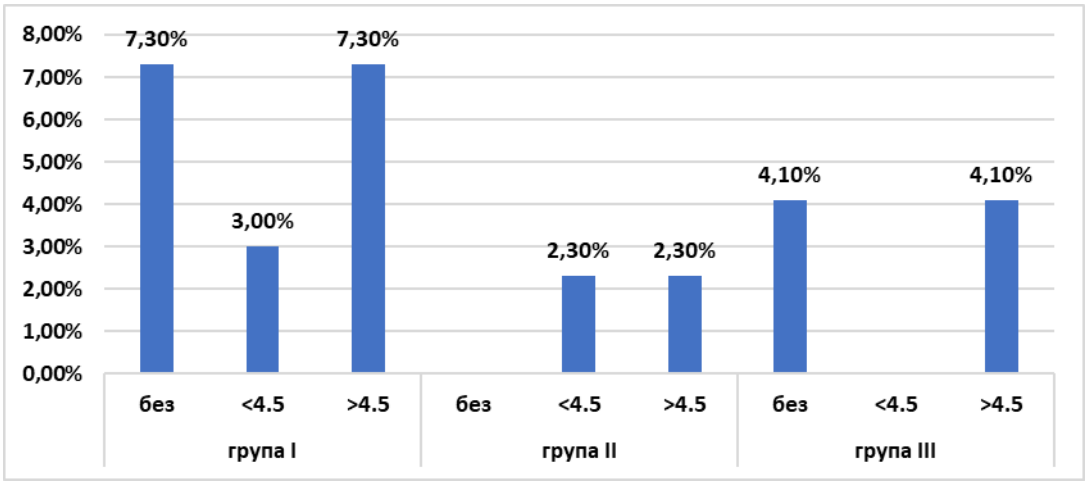


Figure 34 Frequency distribution of pregnant women according to vitamin B12 intake and low birth weight in the three main groups (group I - healthy; group II - women with GDM; group III - women with PE).

Using independent samples t-test, we compared the mean measured concentrations of total vitamin B12, active vitamin B12 and MMA. In women who gave birth to infants weighing less than 2500 g, the mean serum concentrations of the parameters total and active vitamin B12 were slightly lower, and the mean serum concentrations of matabolite MMA were slightly higher, respectively, compared with those in women who gave birth to normal-weight infants (table 55), but the results between the two groups compared did not show significant statistical significance.

Table 55 Mean measured values of total vitamin B12, active vitamin B12 and MMA in women delivering normal weight and LBW newborns.

Parameter	Weight	Mean±SD	t-test/p-level	95%CI
TB12 pmol/l	Normal	179.71±94.24	t=1.033, p=0.303	[-15.5; 49.31]
	LBW	162.74±58.58		
AB12 pmol/l	Norma	75.09±55.49	t=-1.668, p=0.091	[-2.90; 35.06]
	LBW	59.01±32.41		
MMA nmol/l	Norma	244.69±154.40	t=-1.050, p=0.295	[-90.81; 27.70]
	LBW	276.24±158.47		

TB12 – total vitamin B12, AB12 – active vitamin B12, MMA – methylmalonic acid, Mean – average value, SD – standard deviation, 95% CI – 95% confidence interval, LBW – low birth weight

The extent to which the low birth weight of the newborns is related to the particular risk group that the pregnant women belong to, was assessed by means of a correlation that

followed Spearman's method. The results are illustrated in table 56. A strong and positive dependency between the probability of giving birth to a newborn with LBW in increasing deficiency of total vitamin B12 was proved in women who developed preeclampsia (rho=0.499, p=0.009). A weak but significant connection between active vitamin B12 and the risk of giving birth to a child with low weight was identified in the control group of healthy pregnant women (rho=-0.157, p=0.004).

Table 56 Correlation and dependencies between the serum levels of total vitamin B12, active vitamin B12 and MMA and risk of LBW.

Healthy pregnant women						
			Weight	TB12 pmol/l	AB12 pmol/l	MMA nmol/l
Spearman's rho	Weight	rho	1.000	0.011	-0.157*	-0.011
		p	.	0.886	0.044	0.894
	TB12 pmol/l	rho	0.011	1.000	0.574**	-0.076
		p	0.886	.	0.000	0.354
	AB12 pmol/l	rho	-0.157*	0.574**	1.000	-0.042
		p	0.044	0.000	.	0.611
	MMA nmol/l	rho	-0.011	-0.076	-0.042	1.000
		p	0.894	0.354	0.611	.
Pregnant women with GDM						
			Weight	TB12 pmol/l	AB12 pmol/l	MMA nmol/l
Spearman's rho	Weight	rho	1.000	-0.089	0.018	-0.186
		p	.	0.570	0.910	0.291
	TB12 pmol/l	rho	-0.089	1.000	0.618**	0.005
		p	0.570	.	0.000	0.977
	AB12 pmol/l	rho	0.018	0.618**	1.000	0.035
		p	0.910	0.000	.	0.846
	MMA nmol/l	rho	-0.186	0.005	0.035	1.000
		p	0.291	0.977	0.846	.
Pregnant women with preeclampsia						
			Weight	TB12pmol/l	AB12 pmol/l	MMA nmol/l
Spearman's rho	Weight	rho	1.000	-0.192	0.000	0.449**
		p	.	0.185	1.000	0.009
	TB12 pmol/l	rho	-0.192	1.000	0.556**	-0.079
		p	0.185	.	0.000	0.663
	AB12 pmol/l	rho	0.000	0.556**	1.000	-0.015
		p	1.000	0.000	.	0.935
	MMA pmol/l	rho	0.449**	-0.079	-0.015	1.000
		p	0.009	0.663	0.935	.

TB12 – total vitamin B12, AB12 – active vitamin B12, MMA – methylmalonic acid.

V. Discussion

1. Pregnancy and risk factors for the occurrence of pregnancy complications.

Pregnancy is a physiological process during which a number of changes take place in the female body that affect almost all organs and systems. The health and style of life of the women of childbearing age are important determinants for the healthy pregnancy and birth. On a world scale, great efforts are made to develop programs for optimizing the health of mothers in order to improve the results of pregnancy and birth. [24]. The good perinatal care, the healthy way of life and wholesome diet reduce to a minimum the possible risks of developing complications and are of a substantial significance for the favorable growth and development condition of the offspring at the moment of birth and in the future as well [32].

1.1. Age of the pregnant woman.

A general tendency has been observed over the last few years for increasing the average age of the pregnant women at the moment of giving birth to the newborn. In Italy, for instance, as a country with a good socio-economic status, the average age of women giving birth has increased from 25.2 years of age in 1981 to 31.7 years of age in 2015 [185]. The average childbearing age of the women in Sweden is higher in comparison to the one of the women in China (30.9±5.6 years of age vs 28.6±4.6 years of age), while the age of the pregnant women in India at the moment of giving birth in 2020 is even lower – 27.4 years of age [349]. According to the latest data available at the National Statistical Institute, the average age of pregnant women in the Republic of Bulgaria for the year of 2021 was 29.0 years of age [350]. The average age estimated for all the pregnant women in this clinical study is 30.51±5.35 years of age (ranging from 19 to 49 years of age), which shows that the increase rate observed in the rest of the European countries manifests a similar trend in Bulgaria as well.

According to data from the scientific literature, the more advanced the age of the future mother is, the greater the frequency of complications, that arise in the course of pregnancy and birth, becomes. A statistically credible difference has been observed in this study (one-way ANOVA: $F=4.027$, $p=0.019$) between the average values of the three examined groups. The lowest average age has been established for the group of healthy pregnant women (29.84±4.79 years of age), and the highest for the group of pregnant women with preeclampsia (32.12±7.14 years of age). The difference between them is statistically significant ($p=0.023$).

It is generally accepted in the scientific literature that women are divided according to age into young (under 35 years of age) and those at an advanced age (over 35 years of age, advanced maternal age) [132]. Research among European and American populations shows that the percentage of women who give birth after the age of 35, has increased 4-8 times for the last 30-40 years [115]. 15.44% of the pregnant women who participated in this research happen to be over 35 years of age. This percentage is higher than the average for the country and is characteristic of bigger cities where women with a university degree tend to prevail. It is interesting that in the group of healthy pregnant women, the percentage of women of advanced maternal age is 12.57%, in the group with GDM it is 16.28%, and in the women with preeclampsia, it amounts to nearly one quarter (24.49%). This corresponds to the observations proved by a great number of studies, namely that the risk of emerging complications in pregnancy increases with age.

A conclusion can be drawn that the age of the pregnant woman is an independent risk factor for both emerging complications in the course of pregnancy and adverse obstetric and perinatal outcomes [185, 236].

1.2. BMI of the pregnant woman measured at the time of getting pregnant and gestational weight gain.

The increased BMI is also a risk factor for the emerging of complications in pregnant women and their newborns. According to WHO, the incidence of obesity during pregnancy varies from 1.8 to 25.3% [109]. According to the reviewed literature, the frequency of overweight and obese women, among the ones of reproductive age, tends to increase at the speed of an epidemic. At the same time, the overweight and obese pregnant women are more prone to pregnancy complications [47, 253]. Large cohort studies report a threefold increased risk of developing GDM [85], preeclampsia, cardiovascular diseases, neural tube disorders and congenital malformations [253] in mothers with high BMI. Following the recommendations of the Royal College of Obstetricians and Gynecologists (RCOG), the weight and BMI of women of a childbearing age have to be strictly monitored and their optimization, way before pregnancy is highly recommended. [85].

Depending on regional and ethnic specific features, differences are observed in the frequency of overweight and obese women. In Sweden, for example, BMI of women of a childbearing age is 24.6, while in China – 21.9 [325]. In this study the average BMI estimated for the whole group of pregnant women is 23.08±3.93 (median 30.11; ranging from 15.24 to 44.98).

This is a place in the middle between a country in Western Europe with a high standard of living and a country from Asia with a lower standard of living.

An increase of the mean value of BMI is observed in the three major groups examined (22.69±3.69, 23.06±4.91, 24.25±3.66) respectively, for the healthy pregnant women and those with GDM and preeclampsia.

The one-way ANOVA test found a tendency of statistical difference (F=2.752, p=0.065), and the difference between the healthy and the pregnant women with preeclampsia almost reached a statistical significance (p=0.051). Although there is no significant difference in the average BMI between the healthy pregnant women and those with GDM, the interesting fact remains that the highest maximum value of BMI (44.98) is observed namely in the group of women with GDM.

GWG is another important factor for the normal course of pregnancy and birth. The data from the studies are disconcerting and the world tendencies indicate that nearly half of the pregnant women manifest gestational weight gain higher than the acceptable gain in kilograms for the corresponding duration of pregnancy [296]. The data obtained from this study are similar to the ones described in the literature. For the whole examined cohort, in total, the pregnant women with GWG, which is higher than the acceptable limit for the respective gestational age, are 105 altogether, that is the percentage reached is comparatively high– 40.54%. As expected this percentage is higher for the women with pregnancy complications – 51.09% (out of all 92 participants with GDM or preeclampsia, 47 women present with GWG above the acceptable limit), and in the group of pregnant women with preeclampsia this percentage is much higher – 63.3%.

By using nonparametric chi square analysis it was checked whether the complications during pregnancy were more likely in women who were overweight or obese (BMI ≥25) before conception and with GWG above the acceptable limit for the corresponding g.w. The outcomes from the frequency distribution of data showed credible/reliable statistical dependencies for both indicators – BMI and GWG (X^2=6.046, p<0.049). Nearly half of the women with BMI ≥25 (43.3%) and a little bit more than one third of the pregnant women with GWG above the acceptable limit (35.9%) face the risk of developing complications in the course of pregnancy. The strength of the relationship between the BMI and GWG indicators was checked and the likelihood of emerging complications was assessed.

A correlation analysis following the method of Spearman was used for this purpose which proved that there exists a positive, directly proportional dependence between the high BMI (≥25) and the probability of developing GDM and preeclampsia (rho=0.124, p=0.046).

In addition, it was also established that GWG is an important and serious factor because there exists a parallel increase of BMI and GWG, as a result of which the risk of complications in the pregnant woman's condition also becomes higher (rho=0.134, p=0.031). The data obtained support the ones that have been described in the reviewed literature. It is established in them that the mother's and neonatal complications are definitely proven to be connected to the women's overweight or obese condition before the pregnancy and the abnormal GWG during pregnancy [172, 294].

By way of conclusion it may be summarized that the BMI estimated at the moment of conception and the excessive GWG are important risk factors for the occurrence of adverse consequences for the pregnant woman and offspring [296], while the efforts of the contemporary society for optimizing the weight of the women of reproductive age have to continue.

1.3. Seasonality

The season could also be a risk factor for emerging complications in the time of pregnancy. Around two thirds of all the pregnant women included in the study (64.09%), have been examined in the winter and spring half of the year and this ratio remains mostly the same in the three major groups. While the first two groups are comprised of women of gestational age mainly between 24^{th} and 28^{th} g.w., who were monitored on an outpatient basis, the group with preeclampsia was represented by women hospitalized and monitored on an inpatient basis because of a developing complication that threatened pregnancy notwithstanding their gestational age. It is an interesting fact that this serious hypertensive complication, whether it is accompanied by albuminurea or not, occurs mainly in winter. The obtained results correspond to a large part of the published data in literature where the increased frequency of the illness in the cold and damp months of the year is discussed [162]. The reasons for the availability of such seasonal variations in the occurrence of preeclampsia have not yet been sufficiently clarified and understood. Since the pathophysiology of the illness is associated with vasoconstriction, the increased frequency in the cold months of the year can be explained with the effect of temperature and humidity on the blood vessels spasm and the secretion of vasoactive substances [162, 273]. An additional factor for this

seasonality in the emerging of the disease could be vitamin D deficiency or insufficiency. The serum concentrations of the vitamin during pregnancy play an important role in the pathogenesis of the complication. By maintaining the immune homeostasis, vitamin D interferes in the blood pressure regulation through the renin-angiotensin-aldosterone system and prevents the placental vasoconstriction thus regulating the proliferation of the endothelial and the vascular smooth muscle cells and eventually the preeclampsia itself [146]. In this respect the seasonal changes in the frequency of preeclampsia occurrence can be expected in connection with the different brightness of the sun and the different and various exposure to the sun's rays. Rohr Thomsen and his co-author (2020) established significant seasonal fluctuations in the development of hypertensive disorders of pregnancy and think that the seasonal change of vitamin D status could explain the causal-consequential relationship [260].

Unlike pre-eclampsia, the incidence of GDM as a complication of pregnancy does not vary significantly at different times of the year. However, there are authors in the reviewed literature who found seasonality in the diagnosis of the disease [276]. In our study, outpatient follow-up of pregnant women included in the study, healthy and with gestational diabetes, was almost twice as high in the winter months of the year (64.67% and 62.79%, respectively) than in the summer half of the year. The lack of difference in the observed seasonal incidence in these two groups could support the idea that the higher incidence of GDM found in the cold months of the year compared to that found in the warm season (37.21%) is the result of random factors rather than of regular phenomena, also proven by the chi-square analysis performed, rejecting seasonality as a statistically significant factor determining a higher risk of GDM occurrence in winter.

Searching further for a correlation between seasonality and the likelihood of developing a pregnancy complication such as GDM and pre-eclampsia also did not prove a pattern (Spearman rho=0.024, p=0.641) in our cohort of pregnant women studied.

2. Vitamin D status of pregnant women.

In recent decades, particular attention has been paid to the widespread prevalence of vitamin D deficiency or insufficiency in the population. Altered lifestyle and diet are considered as the main reason for this observed phenomenon. It is important to stress that vitamin D deficiency or insufficiency is increasingly linked to the development of a variety of health problems and diseases. The literature reviewed shows that low serum levels, as

measured by 25(OH)D, are not only common in the general population but are also widespread among pregnant women [227].

2.1. Evaluation of serum 25(OH)D levels in the study cohort

For routine clinical and laboratory practice, measurement of 25(OH)D as the gold standard for determining vitamin D status is mainly performed by immunological methods because of their easier applicability and the possibility of producing large series of samples. In recent years, LC-MS/MS methods, accepted as definitive/reference methods, have been increasingly applied worldwide [62].

In our study of 259 pregnant women from the Northeast, serum 25(OH)D levels were examined by liquid chromatography-mass spectrometric detection (LC-MS). The mean measured 25(OH)D in all 259 pregnant women was 76.26□38.27 nmol/l (median 107.24 nmol/l with a range from 10.25 nmol/l to 204.23 nmol/l). Table 57 shows data published in recent years on the vitamin D status of Bulgarian women.

Table 57 Data on serum 25(OH)D levels for Bulgarian female populations.

Study	N; Years (Min – Max range or Mean±SD years)	Physiological status of women	Season	25(OH)D [nmol/l] Mean±SD	Method
Borissova, 2013 [44]	n=1076; *20-80	Unspecified	January-Februari, 2012	36.29±17.16	LC-MS/MS
Генова, 2022 [343]	n=29; 20-40 n=24; 24±7	Healthy non-pregnant Healthy pregnant	February-May, 2018	41±13 60±22	LC-MS/MS
Borissova, 2020 [45]	n=547; 18-47 (30±5)	Healthy pregnant	**25.09 - 06.11, 2019	***64.65±23.65	CLIA

*41.4% (n=446) от изследваните жени са във възрастовия диапазон 20-44 години
The **study design was planned to be conducted in October (an appropriate month, according to the authors, to determine vitamin D status before the onset of the winter season)
***Data in the original article are given in ng/ml (25.86±9.46)
(Min - Max range - age range encompassing minimum to maximum reported age, Mean - mean, SD - standard deviation)

The table shows that one of the surveys was carried out entirely in the winter season [44], the next was done mainly but not entirely in the same season [343], and the third at the end of the summer half [45]. In the present study, about two-thirds of the women were examined during

the winter period and their mean 25(OH)D was 71.40±36.15 nmol/l. It is rather surprising that this value is almost twice as high as the quoted value (36.29±17.16 nmol/l) from the study by Borisova et al [44], although both studies used chromatographic methods for 25(OH)D determination. In the cited study, the study cohort was subdivided into three age groups (20-44, 45-69, and ≥60 years), but no age differences in mean 25(OH)D values were found between these groups. Considering also the fact that women in the first age group constituted 41.4% of all women studied and their age was comparable to that of our study, age could not be considered as a determining factor for the observed difference between the two studies. The physiological condition of the women studied was not mentioned (presence of chronic diseases and/or pregnancy), and there was also no evidence of supplementation with vitamin D preparations. Apart from these factors, it is important to note that as a large population-based study, it included women from large and small towns and villages with different socioeconomic status, whereas our study was dominated by women from a large city (Varna) with good social status. All these facts could explain this significant difference in the mean winter 25(OH)D values between the two studies. The study by Genova et al [343] included a relatively small group of women studied (29 healthy non-pregnant and 24 healthy pregnant women), with no data on their socioeconomic status. It was conducted in the late winter period of 2018, but also covered one of the warmer months (May), and was again methodologically comparable to our study (a chromatographic method was used to determine 25(OH)D). An interesting fact from this study is that the mean 25(OH)D value for healthy non-pregnant women is statistically significantly lower compared to that of pregnant women (41±13 nmol/l vs 60±22 nmol/l respectively, $p<0.001$) and is similar to that reported in our country's first population-based study on vitamin D status by the research team of Prof. Dr. Anna-Maria Borisova [44]. Data on supplementation of healthy non-pregnant women are lacking. The mean 25(OH)D value for healthy pregnant women is similar to that for healthy pregnant women in our study for the winter period (60±22 vs. 72.95±37.95 nmol/l), but is again lower, probably due to the lower percentage of supplemented women (33% for the study by Genova et al. vs. 67% for the present study). In the third Bulgarian study [45], covering a relatively large number of pregnant women studied (n=547) from 10 cities and their adjacent villages (84 villages in total), 25(OH)D sampling and testing was performed in October of 2019 and is thought to reflect the accumulation of vitamin D in the bodies of the women studied during the summer season. Again, there was a significant difference between the mean 25(OH)D value obtained in this study compared to that for the summer half of the present study (64.65±23.65 nmol/l vs 85.30±40.63 nmol/l). This difference could be

explained by the relatively lower percentage of supplementation found in that study compared with ours (50.82% vs 67.18%, respectively), again probably reflecting the different socioeconomic status of the women included in these two studies. In addition, there is a methodological difference: serum 25(OH)D levels were determined by different types of methods, an immunochemical method in the study by Borisova et al. (2020) and a chromatographic method in the present study, respectively, which makes comparison somewhat difficult.

Comparing our results with data on average 25(OH)D levels from other countries and regions of the world is even more complex and ambiguous. The systematic review by Saraf et al [266] pooled data from 95 studies covering different and WHO-defined regions of the world (Americas, Europe, Eastern Mediterranean, Southeast Asia, Western Pacific, and Africa). In addition to being conducted in different regions of the world, they cover a very wide time span, from 1959 to 2012, with 33% conducted before 2000 and 66% after. All of this determines a large variation in the lifestyle and standard of living of the individuals studied, which to a large extent significantly influences 25(OH)D levels at the organism level (biological variation), but there is also a prerequisite for a significant analytical variation, as over the years there has been a varying possibility of using different methods for the determination of 25(OH)D, some with not very good analytical reliability. For example, only nine of the 95 studies used an LC-MS/MS method. Data on the mean 25(OH)D values for the different regions are summarised in table 58.

Table 58 Serum 25(OH)D levels in studies from different regions of the world.

Region	Number of studies	Time period for conducting the studies	25(OH)D [nmol/l]
North and South America	24	1959-2007	52-60
Europe	33	1978-2010	15-72
Eastern Mediterranean	13	1997-2009	13-60
Southeast Asia	7	1997-2007	20-52
Western Pacific	16	1980-2012	42-72
Africa	2	2000-2004	92

While the values for the Americas vary within a relatively narrow range, the wide variation for Europe is striking, with the lowest values measured for the UK in the 1980s (15±13 nmol/l) and much higher values in the following decade - 50±15 nmol/l. The highest average values for Europe are reported for Denmark for the period 2008-2010, 73.3±30.7 nmol/l measured using LC-MS/MS [290]. It is with these values that the results of our study are comparable.

Regarding the serum 25(OH)D levels in the three main groups we studied: healthy pregnant women (76.72±37.14 nmol/l), women with GDM (73.98±37.96 nmol/l) and women with PE (76.85±42.92 nmol/l) we found no statistically significant differences between them. Conflicting data were found in the literature. E.g., Burris et al. pointed out in their 2014 review that several studies from Iran, Australia, the United States, etc. found significantly lower serum 25(OH)D levels in women with GDM compared with healthy pregnant women, whereas other studies conducted in India, the United Kingdom, and North Carolina found no such difference [51]. It should be noted that a more thorough review of the studies referred to by Burris revealed both differences regarding the criteria for the diagnosis of GDM and the use of different methods for 25(OH)D determination. We had similar difficulties in comparing our data on mean 25(OH)D values in healthy pregnant women and those with pre-eclampsia with those in the literature. The comprehensive review by Karpova et al [157] reported results from multiple studies, some of which had indistinguishable mean 25(OH)D values in the two groups studied, and even slightly higher values in women with PE (mainly for the first trimester of pregnancy, but with examples available for the other trimesters). In another part of the studies, significant differences (higher values in healthy pregnant women compared to those of women with PE) were found, and this difference was particularly highlighted when healthy pregnant women were compared to women with severe PE. Such divergent results may be explained by the different design of the studies, the different number and characteristics of the participants recruited for them, the different criteria for diagnosing PE and subdividing it into severe and mild forms, etc. As an example supporting these observations, the results of Baca et al. (2016) could be cited: a mean value for 25(OH)D of 64.6 (95% CI, 64.4-64.8) nmol/L was obtained for healthy pregnant women and 57.8 (95% CI, 57.3-58.3) nmol/L for women with preeclampsia. At the same time, it was emphasized that healthy women were predominantly Caucasian, of normal weight, and with private health insurance, whereas women with PE were predominantly African-American, with higher BMI, lower education, and lower social status, all known factors affecting serum 25(OH)D levels [20]. The authors concluded that vitamin D deficiency increases the risk of preeclampsia. In

contrast to all the studies mentioned above, the women in our study were significantly more homogeneous in terms of social characteristics and ethnicity.

2.2. Determination of vitamin D status and ascertainment of the prevalence of vitamin D deficiency and/or insufficiency for the entire cohort as well as for the three study groups.

Regarding vitamin D saturation levels, in our study we applied the scale proposed by Hollis (2011), according to which levels above 80 nmol/l are considered optimal, levels between 50-80 nmol/l are suboptimal and define vitamin D insufficiency, levels between 25-50 nmol/l indicate marked insufficiency, and levels below 25 nmol/l define vitamin D deficiency. According to this scale, 54.05% of the entire study cohort had suboptimal levels of 80 nmol/l, i.e. slightly more than half of the women had varying degrees of vitamin D insufficiency and deficiency. Worryingly, a quarter of all women studied (25.09%) were severely deficient and insufficient, i.e. with levels below 50 nmol/l. Our result is not significantly different from that published in the study by Vorisova et al. (2020) [45]. For women with levels <50 nmol/l, they found a prevalence of 27.06%, but recall that their study cohort was of more diverse social status. Globally, the prevalence of vitamin D deficiency varies between countries and ranges widely, with 25(OH)D levels <50 nmol/l being documented in 24% of the US population, 37% for Canada and 40% for Europe [61, 63, 267, 270]. An interesting observation for our continent is the finding of higher serum 25(OH)D concentrations in more northern countries than in more southern countries and higher levels in western than in eastern countries [300]. The phenomenon is explained by the intake of vitamin D fortified foods, as well as the intake of vitamin D preparations and supplements in countries with better vitamin D status. Compared with these literature data, we rank among the countries with a relatively lower proportion of pregnant women with marked deficiency and insufficiency.

In determining the vitamin D status of pregnant women in the three main study groups, the proportion of women with optimal 25(OH)D levels >80 nmol/l was found to be highest in the group of healthy pregnant women (47.31%), while the proportion of women with preeclampsia was lower at 44.89% and the proportion of women with GDM was lowest at 39.53%. Levels of 25(OH)D <50 nmol/l (most commonly used in the literature when it comes to comparing healthy pregnant women with women with a pregnancy complication) occurred in 24.55% for the group of healthy pregnant women, 26.53% in women with PE and 27.91% in women with GDM. From the data analyzed in this way, it was found that the best

vitamin D status was in healthy pregnant women, with a more unfavorable status in women with PE and with the most unfavorable status in women with GDM, but the differences between groups did not reach statistical significance. In contrast to our data are the results published by Zang et al [334] for a US sample of pregnant women, which showed statistically significant differences in vitamin D status of healthy women, of whom only 14% had serum 25(OH)D levels <50 nmol/l compared with women with GDM, in whom the proportion of women with levels <50 nmol/l was about 2.5-fold higher (33%, p<0.001). The authors conclude that marked vitamin D insufficiency and deficiency may be associated with an increased risk of developing GDM. The opposite results were reached by Farrant et al [100]. Their study cohort was composed of different ethnicities with different socioeconomic status and religion (Hindus - 57%, Muslims - 34%, Christians - 9%, with median 25(OH)D levels of 40.0 nmol/l, 37.5 nmol/l and 41.0 nmol/l, respectively), among whom they found no statistically significant differences and with the characteristic feature that 66% of them had 25(OH)D levels <50.0 nmol/l. The proportion of women with GDM for the entire study group was 7%, absolutely identical to that of women with GDM with 25(OH)D levels <50.0 nmol/l (7%), thus suggesting that there is no association between vitamin D status and the occurrence of GDM. In the study by Makgoba et al [190], no statistically significant differences were found for both the mean 25(OH)D levels in healthy women and those with GDM, 47.6□26.7 and 47.2□26.7, respectively (p=0.863), and the percentage of healthy women with 25(OH)D levels <50.0 nmol/l and those with GDM (57.0% vs. 62.2%, respectively, p=0.502). As this UK population is very heterogeneous by ethnicity and BMI, further statistical processing was performed on the data to averaging the p values, but this did not result in a change in significance. It is important to stress that in all three studies cited, the criteria used to make the diagnosis of GDM were different from the currently recommended criteria of the IADPSG (International Association of Diabetes and Pregnancy Study Groups), as were the methods used to determine 25(OH)D. A more recent study of Saudi Arabian women that met IADPSG criteria concluded that there was an increased risk of developing GDM in those with 25(OH)D levels <50.0 nmol/l, indicating that the proportion of healthy women with such adverse vitamin D status was significantly lower than that of women with GDM (82.5% vs 93.1%, respectively, p=0.006) [5].

Regarding pre-eclampsia and vitamin D status, our data did not demonstrate significant differences: both mean 25(OH)D levels and the proportion of women with levels <50.0 nmol/l in healthy women and those with PE were approximately the same. In contrast, Baker et al. (2010) demonstrated significant differences for both mean levels (98 nmol/l vs. 75

nmol/l for healthy and those with PE, respectively, p<0.01) and the percentage of women below <50.0 nmol/l - healthy women were two and a half times less than those with PE (10% vs. 26%, respectively, p<0.01) [24]. In contrast to Baker et al. and similar to the data from the present study, Shand et al. (2010), studying a Canadian population, and Fernández-Alonso et al. (2012), studying a Spanish population, found no associations between vitamin D status and risk of preeclampsia [274, 104]. One possible explanation for the lack of differences in vitamin D status in our study could be that, in our study, sampling in ambulatory healthy women was mainly taken at 24 - 28 yr, whereas in women with PE, sampling was performed at a later stage, at the time of their hospitalization (mainly at 34 - 36 y), and it is possible that 25(OH)D levels increased during pregnancy. Supporting this hypothesis is the study by Bärebring et al. (2016), who found a rise in 25(OH)D levels in the last trimester, more pronounced for women of Swedish origin than for women of non-European origin [27]. Numerous studies have claimed that there are no such changes over the course of pregnancy, and Fernández-Alonso et al. (2012) even found that 25(OH)D levels decreased significantly from the first to the third trimester [104].

2.3. Evaluation of the influence of season on the vitamin D status of the study cohort.

Season is a well-known factor that influences serum 25(OH)D levels in study subjects. In the present study, the mean measured 25(OH)D value in all pregnant women studied during the winter half-year was 71.40±36.15 nmol/l, which was statistically significantly different from the mean measured 25(OH)D value in all pregnant women studied during the summer half-year (85.30±40.63 nmol/l, p=0.0109), i.e. seasonal variations were found to be present and valid for the entire study cohort of women. We have already commented on the fact that the mean values we found for the results of the whole cohort, regardless of the season, differ from those found in other Bulgarian studies. As far as seasonal differences are concerned, in our study optimal vitamin D levels were reached during the summer season, whereas in the study by Borisova et al [45], they were significantly lower (64.65±23.65 nmol/l) and were relatively far from the optimal limit of 80 nmol/l. We attributed this to the different social status of the women in the two studies, the lower percentage of vitamin D supplementation (50.82% vs. 67.18%), and the different methods used for vitamin D determination. Regarding the winter period for our country, we can only compare with the study of Genova et al [343]. The mean 25(OH)D value for healthy pregnant women is close to that of our study for the winter period (60±22 vs. 72.95±37.04 nmol/l), but again it is lower, probably because of the

too small number of pregnant women examined in that study, and because of the lower percentage of supplemented women (33% vs. 67%).

It is not surprising that in the present study and frequency distribution of pregnant women in terms of vitamin D status was dependent and more favorable in the summer half than in the winter half. Women with optimal 25(OH)D status (>80 nmol/l) were 15.55% (or 1.4-fold) more in the summer period than in the winter period, and conversely, women with critical 25(OH)D levels <25 nmol/l were almost threefold more in percentage terms in the winter half compared with the summer half (6.02% vs 2.15%).

From the evidence thus analyzed, it can be summarized that season is a significant factor determining serum 25(OH)D levels with higher mean levels in the summer half-year for the entire study cohort as well as for the three main groups. A favourable summer season also improved the frequency distribution of women according to their vitamin D status.

There are numerous literatures from all regions of the world that have also demonstrated seasonal variations in serum 25(OH)D levels in pregnant women. Higher serum levels during the summer season are naturally attributed to the prolonged exposure of pregnant women to the sun, whose rays at this time of year also fall at a more suitable angle to the earth's surface. Intriguing in this regard is the publication on Canadian pregnant women by Shand et al. (2010), who found no statistically significant differences in serum 25(OH)D levels between periods of the year [274], probably due to the fact that 96% of the women they studied were supplemented with vitamin D preparations, and 49% of them were also taking them before the onset of pregnancy.

2.4. Evaluation of the influence of body mass index on vitamin D status for the whole cohort as well as in the three groups studied.

As already noted in section 1.2 of the discussion, one of the risk factors for adverse pregnancy complications for both mother and newborn is elevated BMI. In terms of the average estimated BMI for the entire group of pregnant women studied by us (23.08□3.93 kg/m2), we occupy an intermediate position among the various countries of the world characterized by different rates of increase in BMI among pregnant women.

Vitner et al [305] summarized data on the prevalence of overweight and obesity in pregnant women for the previous two decades as reported by various obstetrician-gynecologist associations around the world (table 59):

Table 59 Prevalence of overweight and obesity in pregnant women worldwide.

RANZCOG (Royal Australian and New Zealand College of Obstetricians and Gynecologists)	**RCOG** (Royal College of Obstetrics and Gynecology, UK)	**SOGC** (Society of Obstetrics and Gynecologists of Canada)
50% (overweight and obese women included)	16-19% (only obese women included))	10.2% (only obese women included)

The overall prevalence of overweight and obese women in our study was 28.57%, and that of obese women was 4.63%. Compared to developed countries, the frequency of obesity in the cohort studied by us is two to four times lower. From the only so far population study on vitamin D status and pregnant women for our country, data on BMI of pregnant women were published at the last National Symposium of the Bulgarian Society of Endocrinology [347]. In this report, the proportion of women with BMI ≥25 kg/m2 was 30.3%, higher than that observed in our cohort of studied women – 28.57%. Interestingly, the proportion of obese pregnant women from this study was more than twice that of the current study (10.2% vs. 4.63%, respectively) and identical to that in Canada. Relationships between BMI and serum vitamin D levels have not yet been reported from the cited population study. As for our study, we found no significant differences in serum 25(OH)D levels in the normal-weight group and the overweight group (76.59±39.53 nmol/L vs 74.43±34.91 nmol/L, respectively, p= 0.907). It is interesting to note that when examining these dependencies in the three main groups, statistically significant differences were found for healthy pregnant women (p=0.035) and for those with preeclampsia (p=0.011), in contrast to the GDM group, in which does not reach a statistically significant difference. A possible explanation for the finding in the group with GDM is not only that it is the smallest, but also that the women in this group take the highest daily dose of vitamin D preparations compared to the other two groups. Another interesting fact is that intergroup differences were statistically significant in overweight women, with the lowest values in women with PE, whose mean BMI was the highest and almost reached a significant difference with that of healthy pregnant women (24.25 kg/ m2 vs. 22.69 kg/m2 respectively, p=0.051). Between-group differences in women of normal body weight were not statistically significant. It could be concluded that an increased BMI is an adverse factor regarding the vitamin D status of the studied pregnant women. The comparison of our data regarding BMI and vitamin D status with data from the world literature is again difficult for a number of reasons, some of which have already been highlighted: different methods of measuring serum 25(OH)D levels, different criteria for determining vitamin D deficiency, and even different WHO BMI criteria for different regions

of the world. For example, the WHO defines a BMI value of ≥24 kg/m2 as a criterion for overweight for women of Asian origin. Interestingly, the study by Shand et al. [274] on a Canadian population of pregnant women that found no statistically significant difference in median serum 25(OH)D levels among women of different BMIs, again recalling the high level of supplementation in this population. Conversely, Zhang et al. [335] found a statistically significant difference in BMI in Chinese women with self-perceived adequate 25(OH)D levels ≥50 nmol/l compared to those with 25(OH)D levels <50 nmol/l (20.71± 2.58 kg/m2 vs. 21.09±2.73 kg/m2 respectively, $p < 0.0001$).

2.6. Evaluation of the dependence of vitamin D status on the intake of vitamin D as a pharmaceutical product and/or nutritional supplements containing the vitamin for the entire cohort as well as in the three studied groups.

As a result of the numerous scientific studies and publications of the last two and more decades, revealing on the one hand the non-calcemic effects of vitamin D, and on the other the widespread prevalence of its deficiency or insufficiency, a change in attitude has been reached regarding the need for supplementation with vitamin D preparations of different target groups – from healthy individuals to individuals affected by chronic metabolic, infectious, autoimmune and neoplastic diseases. This applies to a large extent to pregnant women as well. In the present study, more than two-thirds of the pregnant women studied were supplemented and the mean value of their measured serum 25(OH)D levels (86.41±38.50 nmol/l) was in the zone of optimal vitamin D status. In contrast, unsupplemented women have a significantly lower mean value (56.05±29.78 nmol/l), which is close to the cutoff value of 50 nmol/l, differentiating marked vitamin D deficiency from milder vitamin D deficiency. It is natural that supplementation with vitamin D preparations also leads to differences in the frequency distribution of women according to their vitamin D status. With supplementation, the percentage gradually increases with improvement of vitamin D status - about 12% are in a state of deficiency and marked insufficiency (ie <50 nmol/l), and nearly 60% are in a state of sufficiency, while in unsupplemented women over 50% are in a state of deficiency and marked insufficiency and only 21% are in a state of sufficiency. We have already mentioned that in the study by Borisova et al. [45] the percentage of supplemented pregnant women was lower compared to ours (50.82% vs 67.18% respectively), which also led to differences in the frequency distribution of women according to their vitamin D status in the two studies. For example, the percentage of

supplemented women who reached a state of sufficiency from the cited study is almost twice (1.86 times) lower than that in the present study (31.29% vs. 58.05% respectively), as well as women with values below 50 nmol//l were 21.94% versus 12.06% of the current study, again almost a two-fold difference (1.82 times). In the said study, only the percentage of supplemented women was indicated, and no information was given about their daily intake of vitamin D preparations. We hypothesize that the lower social status of the women in this study, covering both large and small settlements, also determined the lower doses of supplements taken, which could explain the observed differences in the two studies. Regarding the daily intake of vitamin D preparations, in our study it varied quite widely from 0 IU to 3400 IU, with the average daily intake being different in the three main studied groups and if for healthy pregnant women and those with GDM it was relatively the same. that for women with PE is almost three times lower. In this group, the frequency of women not taking vitamin D preparations at all is almost twice as high as in the other two studied groups. The indicated differences are statistically significant.

The frequency distribution of women according to their daily intake of vitamin D preparations and according to their body weight was also investigated. When comparing the data from Table 31 on page 48 for the entire study cohort, it appears that women with a higher BMI have a lower attitude to supplement, but non-parametric Chi-square analysis showed no statistically significant differences ($X2=4.50$, $p=0.1054$) between the groups thus subdivided. Figure 22 on page 49 shows the frequency distribution of pregnant women in the three main groups according to BMI and their intake of vitamin D preparations, which clearly shows that overweight women in all three studied groups have a lower attitude to are supplemented with vitamin D preparations, especially manifested in the group of pregnant women with PE. Of note is the significant fact that in the group of pregnant women with PE, the ratio of women with excess BMI compared to normal BMI, as well as with gestational gain above the permissible compared to normal GWG, was twice that of the groups of healthy women and those with GDM. In addition, the average age of women in this group was the highest, significantly different from that of healthy pregnant women. All these factors and the inadequate supplementation with vitamin D preparations in this main group of studied women determined a significantly lower mean value of serum 25(OH)D levels in those with an excess BMI compared to women with a normal BMI (60.47 ± 31.85 vs. 91.19 ± 46.73 respectively, $p<0.011$). Regarding supplementation with vitamin D preparations, we could argue that we have regional differences in the country and that a better socio-

economic status of women predetermines a more conscious attitude and attitude towards supplementation with vitamin D preparations, in doses often exceeding the recommended at this time by health regulators 600 IU/day. Regarding vitamin D supplementation attitudes of pregnant women of different ethnicities in Western and Northern European countries as well as in North America, there are numerous publications. For example, in the study by Eggemoen et al. [95] reported for Norway that two-thirds of European and East Asian women took vitamin D supplements, while only 50% of women from other ethnic groups supplemented. Serum 25(OH)D levels varied widely from <12 nmol/l to 148 nmol/l with substantial differences between different ethnicities. Thus, the frequency of values <50 nmol/l in women of South Asian origin is 84%, from the Middle East - 79%, from Africa - 75%, from East Asia - 43% and from Western Europe - 20%. As the main factors leading to these significant differences between pregnant women of different ethnicities and indicated by the authors are, in addition to the season, education and supplementation with vitamin D preparations. Compared with the reported data from that study, the current study's data on vitamin D supplementation approximate the results for women of European descent in Norway, with only one-third not supplementing and two-thirds taking vitamin D preparations (85 vs. 174 women respectively).

2.6. To evaluate the influence of vitamin D status on adverse pregnancy outcome in relation to the newborn and the occurrence of preterm birth and low birth weight in healthy pregnant women and women with pregnancy complications.

Preterm birth (PTB) is the most common cause of neonatal death worldwide and the second most common cause of death in children under 5 years of age [312]. According to Agarwal et al. vitamin D deficiency during pregnancy and the associated suppressed immune function could influence the pathophysiological mechanisms of PTB [4]. The results of studies in the field are again contradictory. In a meta-analysis by Lian et al. (2021), the correlation between vitamin D deficiency in pregnant women during the first, second or third trimester of pregnancy and the development of PTB was not statistically significant. In this meta-analysis, 13 of a total of 24 observational studies clearly reported no association between low serum 25(OH)D levels in pregnancy and the occurrence of the complication [177]. On the other hand, there are also studies that find an association between deficient levels of the vitamin in the mother and PTB [21,160] or demonstrate a 60% lower risk of PTB in pregnant women with vitamin sufficiency (25(OH)D>100nmol/l) [4, 196]. In our

study, the incidence of PTB was 12.45%, or out of a total of 257 newborns, 32 were premature, with approximately two-thirds of them (n=22) being born to women with PE. From the conducted correlation analysis for the dependence of the occurrence of the PTB complication on the intake of vitamin D and on the serum concentrations of 25(OH)D, the most significant dependence was found in the group of pregnant women with PE. In this group, the lowest attitude towards supplementation with vitamin D preparations was found (57.14% of them do not supplement at all), and this is the group with the highest average value of BMI. Almost half of women with PE (42.86%) have a BMI≥25, in which the lowest values of the median and IQR were also established – 53.80 (42.23 – 67.05). Furthermore, according to Tamblyn et al. (2017) there is an accelerated metabolism of 25(OH)D in the placenta of women with PE, converting it to its inactive metabolite 24,25(OH)2D and/or epimerizing it to 3-epi-1,25(OH)2D. The latter has the ability to bind to VDR, but is not capable of triggering VDR signaling to the same extent as the active hormone 1,25(OH)2D. All these observations to some extent elucidate at least part of the vitamin D-related pathophysiological mechanisms developing in preeclampsia [295].

Another unfavorable outcome of pregnancy is the birth of a child with a low weight, i.e. <2500g. A variety of factors could account for such a pregnancy outcome, with a number of studies investigating the relationship between pregnant women's serum 25(OH)D levels and newborn birth weight. As the vitamin plays an important role in fetal growth by regulating calcium homeostasis and PTH levels [142], a low maternal 25(OH)D concentration could lead to suboptimal bone size and density and cause the birth of an infant with LBW [303]. Another possible mechanism explaining the correlation between vitamin D levels and newborn birth weight could be the anti-inflammatory effect of the vitamin and its role in the regulation of placental inflammation [74]. In their study, Chen et al. (2015) suggested that vitamin D deficiency, by reducing IGF-1 levels, could be associated with LBW delivery [75]. In several studies, maternal 25(OH)D levels were tested at different periods of pregnancy and a positive correlation was found between vitamin concentrations and newborn birth weight [209]. Other studies in the field did not prove the existence of such a relationship [11, 258]. The incidence of LBW among newborns in our study was 13.23% (n=34). The highest complication rate was found in pregnant women with preeclampsia – 33.33% (n=16). The frequency of LBW in participants with GDM was 13.95% (n=6), and in healthy subjects – 7.23% (n=12). An interesting fact is that a frequency distribution of pregnant women

according to the intake of vitamin D preparations for the entire studied cohort and the weight of their newborns showed that the intake of vitamin D was a significant factor for the birth of a child with a low weight ($\chi 2=38.02$, $p< 0.0001$). This was also confirmed when we followed the frequency distribution in the three main groups of pregnant women studied, especially clearly manifested in healthy pregnant women and those with PE. When comparing the average values of vitamin D intake in pregnant women who gave birth to children of normal weight compared to those who gave birth to children of low weight, it was found that in the first group the intake was 824.26±2167.65, and in the second it was 525.00±810.39, distinguishing over 1.5 times. Statistical significance of this difference was not reached, possibly because of the large variation in units of vitamin D supplements taken. Unfortunately, no difference was reached in the measured serum levels of 25(OH)D in the two groups thus divided (76.17±38.33 nmol/l vs 75.31±40.40 nmol/l respectively, t=0.121, p=0.904). We hypothesize that this lack of difference is due to the multiple factors that influence serum 25(OH)D levels such as seasonality, BMI, lifestyle, etc. The mothers of the relatively small group of LBW infants could not be subdivided according to all of these factors to try to determine which of them all contributed to the failure to detect our expected difference in serum 25(OH)D levels. Evidence from the large number of randomized controlled trials regarding vitamin D supplementation and the effect on birth weight is mixed, but this is most likely due to the heterogeneity of the studies. However, in most systematic reviews and meta-analyses, such as that of Maugeri et al. (2019) confirmed the positive association between vitamin D supplementation of mothers and the weight of their newborns [195]. Whether deficient serum vitamin D levels are relevant to the occurrence of pregnancy and delivery complications such as preeclampsia, GDM, PTV and LBW is a question that deserves further studies. Historically, supplementation with this important micronutrient has led to significant achievements in the past century, such as overcoming the global rickets problem. At present, the non-calcemic effects of vitamin D on the general health of individuals, including that of pregnant women and newborns, remain to be more definitively confirmed. Lifestyle changes and vitamin D supplementation could make a significant contribution to improving public health.

3. Vitamin B12 status of pregnant women.

3.1. Serum levels of total vitamin B12, active vitamin B12 and methylmalonic acid. Vitamin B12 status of pregnant women and prevalence of vitamin B12

deficiency and/or insufficiency for the entire cohort as well as for the three main study groups.

In modern society, the consumption of animal products is often limited, and this predisposes to the occurrence and wider prevalence of vitamin B12 deficiency or insufficiency [117]. In addition to dietary and supplement intake, vitamin levels depend on intestinal absorption. Malabsorption of vitamin B12 can also be a cause of insufficiency or deficiency and associated adverse clinical manifestations [297]. The widespread prevalence of subclinical vitamin B12 deficiency in the general population provides a strong enough argument that vitamin status may also be compromised in pregnancy, as well as by increased metabolic demands on the fetus. Pregnant and lactating women following a vegetarian or vegan diet, including those from developing regions of the world with poor access to animal foods, are at relatively high risk of developing vitamin B12 deficiency [252]. Because the vitamin occupies a central role in the processes of neural myelination and brain development, adequate concentrations during pregnancy are paramount for normal fetal growth and development [31, 291, 292, 328, 330].

Literature data on the prevalence of vitamin B12 deficiency and insufficiency are often conflicting and vary depending on the laboratory parameter chosen for follow-up. This is not surprising as each of the markers determining vitamin status responds in a specific manner to the stage of deficiency and the age of the patient. As an initial screening marker for cobalamin deficiency, total serum vitamin B12 is used in practice. This is also the most commonly analyzed laboratory parameter. If further refinement of vitamin status is needed, determination of additional parameters - active vitamin B12, MMA and homocysteine - is recommended. In accordance with the reviewed literature, an available vitamin B12 deficiency is assumed when serum total vitamin B12 concentrations < 148 pmol/L, and vitamin insufficiency or borderline deficiency, at concentrations from 148 pmol/L to 250 pmol/L [8, 56]. In our study, the mean measured values of the parameter total vitamin B12 for the whole cohort were 177.58±90.24 pmol/L or concentrations falling in the vitamin deficiency region. According to literature data, total vitamin B12 levels decrease during pregnancy by approximately 20% from 20 to 36 yr, but this isolated decrease in values in the second and third trimesters of pregnancy is not due to true depletion of the vitamin [126]. The decrease in parameter concentrations is explained by altered binding of cobalamin to the protein haptocorrin. Interestingly, no changes in the levels of active vitamin B12 were found in the course of pregnancy [145]. The latter represents 20 - 30% of total serum vitamin levels and is accepted as a potentially better marker for adequacy and estimation of cobalamin

metabolic functions. In practice, active B12 (holo-TC) values between 35 and 45 pmol/l define a state of present vitamin deficiency. In individuals with impaired renal function, concentrations of the active vitamin B12 parameter have been found to increase, but in the pregnant state, levels do not change and can be used as a good laboratory marker to determine the status of pregnant women [8]. In relation to the above, vitamin B12 deficiency present in our study participants was decided to be taken at serum concentrations of active vitamin B12 <35 pmol/l. The mean measured values for the holo-TC parameter in all women included in the study were 73.26±53.25 pmol/l or concentrations falling within the vitamin sufficiency range.

When holo-TC levels ranged between 35 and 50 pmol/l, the results obtained for the functional parameter, MMA, were also taken into account. The metabolite is not affected by folate or other B vitamin concentrations and represents a relatively specific and sensitive biomarker for determining vitamin B12 status. When vitamin B12 deficiency is present, serum concentrations of MMA are increased [125]. In relation to the literature reviewed, vitamin B12 deficiency was assumed for serum MMA concentrations > 300 nmol/l and vitamin B12 insufficiency status was assumed for participants with active vitamin B12 > 50 pmol/l and MMA > 300 nmol/l. In our study, mean measured MMA values for the entire cohort of pregnant women were 249.53±154.73 nmol/l, or concentrations again falling within the vitamin sufficiency region.

In relation to the new criteria for determining vitamin B12 status (holo-TC < 35 pmol/l or holo-TC 35 - 50 pmol/l and MMA > 300 nmol/l) in the total group of pregnant women we studied, vitamin B12 deficiency was found in 22.78% (n=59) and insufficiency (holo-TC > 50 pmol/l and MMA > 300 nmol/l) in 10.42% (n=27).

Worldwide, the prevalence of vitamin B12 deficiency in the course of pregnancy varies. In the southeastern region of Turkey, vitamin B12 deficiency has been reported in 72% of mothers and 41% of newborns, and severe deficiency in 48% of women and 23% of children [252]. In the United States, vitamin deficiency ranges from 6% to 25% depending on ethnicity [8], in the United Kingdom about 20% [2], and in Canada, 10% to 21% of pregnant women at 16 yr and 23% to 35% of women at 36 yr, respectively, report deficiency [317]. In a study by Finkelstein et al. (2017) conducted among pregnant women in India, the prevalence of deficiency was even higher, with 51% of mothers deficient and 42% found to have impaired vitamin B12 status [108]. Data on the prevalence of vitamin B12 deficiency among pregnant women in Bulgaria have not been published to date. To the best of our

knowledge, the study we conducted is the first for the country. The results obtained for the prevalence of vitamin B12 deficiency and insufficiency (22.78% and 10.42%, respectively) place Bulgaria among the developed countries of the world, where the lifestyle and nutrition of pregnant women are in line with the requirements and global recommendations, and the care of future offspring is of paramount importance.

The presence of vitamin B12 deficiency during pregnancy is associated with the onset of various complications in pregnant women, with the development of pre-eclampsia remaining one of the most severe [237]. Low cobalamin and folate levels causing hyperhomocysteinemia are known to contribute to increased oxidative stress and endothelial damage [271] and may be associated with the onset of the complication [192]. In the study by Serrano et al. (2018), researchers found that elevated serum homocysteine concentrations during pregnancy are a risk factor for PE, but the relationship between the vitamin B12 deficiency status of pregnant women and the complication remains unresolved [271]. A recent meta-analysis covering a total of 21 studies on 3211 pregnant women (1390 cases and 1821 controls) found that serum total vitamin B12 concentrations in women with pre-eclampsia were significantly lower compared to levels in healthy controls, but individual studies pooled in the meta-analysis also reported mixed results [192]. In our study, the mean measured total vitamin B12 parameter in participants with pre-eclampsia was 159.68 ± 68.97 pmol/l (median 246 pmol/l ; range 76 - 416 pmol/l). Slightly higher results were obtained in healthy subjects - 175.43 ± 87.72 pmol/l (median 434.5 pmol/l; range 87 - 782 pmol/l). The mean serum concentrations for the holo-TC parameter were similar in the two groups studied, and for MMA there was a tendency to differ, with the mean value for women with pre-eclampsia (271.61 nmol/l) approaching the cut-off value of 300 nmol/l. Consistent with the new criteria chosen for vitamin B12 deficiency intake, a higher percentage of the condition was found in women with pre-eclampsia, 26.53%, compared with 23.95% for the group of healthy controls. In our study of vitamin B12 status of pregnant women, there was no statistically significant difference between the mean measured serum concentrations for active vitamin B12 in the group with pre-eclampsia and healthy controls, whereas there was a trend for statistical difference for MMA. Similar results have been reported by other investigators. Most probably, the development of the complication does not depend only on the measurable vitamin deficiency but also on the totality of many other and different etiological and pathogenetic factors.

In addition to the development of hypertensive complications, vitamin B12 deficiency in pregnancy is also associated with the development of GDM. Again, in this area of study, data in the literature are controversial and heterogeneous [167]. In tracking the results of 15 clinical studies, Chen et al. (2022) found no association between the development of the complication and vitamin B12 deficiency status during the first trimester of pregnancy, but did find that low vitamin levels measured in the second or third trimester were directly associated with an increased risk of disease [73]. Similar results were obtained in another meta-analysis [307]. In the study by Li et al. (2019), among pregnant women in China, the authors found that the highest risk of GDM existed in mothers with a combination of vitamin B12 deficiency and high serum folate levels (OR=3.08), and high concentrations of cobalamin alone, resulted in a decreased likelihood of the complication (OR=0.30) [176]. In the same clinical study, a greater risk of GDM was shown to be present in participants in whom high folate and vitamin B12 deficiency status were accompanied by additional factors, such as older age of pregnant women and higher preconception BMI. In our study, the mean measured total vitamin B12 parameter in the group of pregnant women with GDM was 206.32±113.72 pmol/l (median 338.5 pmol/l; range 89 - 588 pmol/l), that of holo-TC was 88.18±61.85 pmol/l (median 187.7 pmol/l; range 16.40 - 359 pmol/l), and the metabolic parameter MMA was 245.07±146.47 nmol/l , (median 387.15 nmol/l; range 82.95 - 691.35 nmol/l). Mean measured concentrations for total and active vitamin B12 obtained in healthy controls were lower than those measured in participants with GDM, and the prevalence of vitamin B12 deficiency in the group of healthy pregnant women (23.95%) was higher than the prevalence of deficiency in pregnant women who developed the complication (18.60%). Thus, an association between vitamin B12 deficiency status of participants and the occurrence of gestational diabetes mellitus was not demonstrated in our study. Similar results were obtained by other investigators [71]. Data regarding the association of vitamin B12 status of pregnant women and the development of GDM remain controversial. It is likely that nutritional imbalances do not act alone but synergistically with other maternal risk factors leading to an increased risk of the complication [188].

3.2. Assessing the influence of season on vitamin B12 status of pregnant women studied.

There is a paucity of data in the literature on the influence of season on the levels of parameters reflecting vitamin B12 status. The vitamin enters the human body mainly through food and dietary supplements. Since the main sources of vitamin B12 are mainly food

products of animal origin, the status of the vitamin will be influenced mainly by the intake of these foods. In the study by Tong (2021) et al, researchers found higher serum vitamin B12 concentrations during the winter months of the year and correspondingly lower levels of the vitamin measured in the summer and fall ($p < 0.05$) [297]. Similarly, in our study, the measured mean values of the laboratory markers total and active vitamin B12 of pregnant women included in the study were higher in the winter half of the year compared with the summer half of the year (186.93±100.25pmol/l and 78.02±61.77pmol/l vs 160.88±64.94pmol/l and 64.78±30.55pmol/l), but measured mean serum concentrations of the metabolic parameter MMA did not differ (248.89±147.21nmol/l vs 250.78±167.69nmol/l). The data obtained showed a trend towards better vitamin B12 status of the participants included in the study during the cold months of the year compared to the summer half of the year. Since vitamin B12 cannot be synthesized in the human body and its levels depend entirely on its acquisition through exogenous sources and their absorption [10], the better vitamin B12 status of pregnant women included in the study during the winter half of the year may be explained by changes in dietary pattern towards increased intake of foods of animal origin in which the cobalamin content is high.

3.3. To assess the impact of body mass index on vitamin B12 status for the entire cohort as well as in the three groups of pregnant women studied.

Globally, across all age groups, there has been an increasing trend in overweight and obesity. Weight gain in the reproductive age population is also increasing at a relatively rapid rate. In the literature reviewed, there is a wealth of evidence supporting the claim that micronutrient deficiencies play an important role in the process of adipogenesis [2]. It is known that in the oxidation of fatty acids at the mitochondrial level, vitamin B12 acts as a coenzyme required for the conversion of methylmalonyl-CoA to succinyl-CoA. Deficiency of the vitamin inhibits the process and results in enhanced lipogenesis. Adipose tissue-derived circulating microRNAs are altered and in turn mediate an adipogenic and insulin-resistant phenotype that leads to excessive lipid accumulation and the development of obesity [3]. In relation to these mechanisms, the literature has formed the assumption that individuals with vitamin B12 deficiency have a greater propensity to obesity compared to individuals with normal vitamin status. Several observational and epidemiological studies conducted in elderly patients have demonstrated that low serum vitamin B12 levels are associated with high BMI [3], and a series of studies in pregnancy have found an intriguing association between cobalamin deficiency in pregnant women and the development of obesity [164, 291,

292]. In a recent study by Knight et al. (2015), the investigators found that low serum concentrations of the parameter total vitamin B12 (<150pmol/l) measured at 28 yr were closely associated with high BMI of pregnant women ($r = -0.25$; $p<0.001$), and normal levels of the parameter, respectively, with normal BMI. The authors assumed that for every 1% increase in BMI, a 0.6% decrease in circulating total vitamin B12 would be expected and discussed two possible interpretations of the results - vitamin B12 deficiency contributes to the development of obesity or obesity causes a decrease in serum total vitamin B12 concentrations [164]. In contrast to the correlations between BMI and vitamin B12 status found in the reviewed literature, pregnant women with high and normal BMI in our study had similar mean levels for total vitamin B12 parameters [167.74±82.86pmol/l vs 202.19±101.86pmol/l), active vitamin B12 (69.88±41.86pmol/l vs 81.36±73.46pmol/p) and MMA (266.83±162.76 nmol/l vs 201.79± 115.11nmol/l). Most likely, the number of participants included in the study as well as their unequal distribution in the two subgroups are related to the inability to demonstrate statistically significant reliability of the results. Laboratory parameters reflecting the vitamin B12 status of the two groups of pregnant women were compared by Spearman's method, but no statistically significant correlation was found ($p>0.05$).

In addition to the general group of pregnant women, the association between BMI and vitamin B12 status of the participants was also sought in the three main groups studied - healthy controls, women with GDM and with pre-eclampsia. Only for the group of participants who developed gestational diabetes mellitus was it found that overweight and obese pregnant women had a poorer vitamin B12 status compared to pregnant women with normal BMI. The values obtained were as follows: for total vitamin B12 - 144.33±54.65pmol/l vs 153.65±47.33pmol/l, for active vitamin B12 - 55.10±25.63pmol/l vs 62.08±30.08pmol/l and for MMA - 317.16±192.89nmol/l vs 307.64±159.29nmol/l. Despite the differences found in the mean measured concentrations of laboratory parameters reflecting vitamin B12 status, statistical significance between the results obtained in the participants of the two subgroups was not found ($p>0.05$). However, in view of the literature reviewed, it would be beneficial to assess vitamin B12 status in women planning pregnancy, especially overweight and obese women.

3.4. Assess the dependence of vitamin B12 status on intake of supplements containing the vitamin for the entire cohort as well as in the three study groups.

Vitamin B12 is critical for normal cell division and differentiation and is required for the development and myelination of the central nervous system. Total fetal requirements for the gestation period are estimated to be 50 ug, and maternal stores in women on a mixed diet are more than 1000 ug [252]. Fully and healthily nourished pregnant women, have sufficient body stores of cobalamin to adequately and optimally meet the increased requirements during pregnancy. Pregnant and lactating women following a vegetarian or vegan diet and those from developing regions of the world with poor access to animal foods are at high risk of vitamin deficiency [252]. Despite reports that vitamin B12 concentrations in the pregnant woman and fetus correlate, there are authors in the reviewed literature who suggest that cobalamin intake during pregnancy is a more important and determinant factor of vitamin status in the developing infant [92]. According to the current recommendations in Bulgaria, the daily intake of vitamin B12 for the period of pregnancy and lactation is higher than that for non-pregnant women and is 4.5 ug/day and 5.0 ug/day, respectively [344].

Various studies have examined the effect of daily oral vitamin B12 supplementation on serum levels of the vitamin, including during pregnancy. In the randomized, placebo-controlled clinical trial of Duggan et al. (2014), researchers found that, pregnant women taking cobalamin supplementation compared to those on placebo had significantly higher serum total vitamin B12 levels in both the second (216 pmol/l vs 111 pmol/l respectively, $p<0.001$) and third trimesters of pregnancy (184 pmol/l vs 105 pmol/l respectively, $p<0.001$) [92]. Similarly, in the study by Knight et al. (2015), measured total vitamin B12 concentrations were higher in pregnant women who took vitamin supplements during pregnancy compared with non-supplemented women (224 pmol/l vs 195 pmol/l, respectively, $p<0.001$) [164]. In our study, approximately one third of participants reported no vitamin B12 supplementation (34.75%, n=90). The remaining pregnant women confirmed vitamin supplementation, with a dose ≥4.5ug/day taken by 46.33% (n=120) of participants. In both the cited literature and our study, the mean measured value for the parameter total vitamin B12 in the group of participants with supplementation was higher compared to that measured for the group of participants not taking supplements (total vitamin B12 189.48pmol/l vs 155.25pmol/l). Similar results were obtained for the parameter active vitamin B12 (active vitamin B12 76.26pmol/l vs 67.34pmol/l), and for the functional laboratory parameter MMA, as expected, lower mean measured values were obtained in the group of supplemented participants (MMA 191.57nmol/l vs 290.29nmol/l). In the study by Bhowmik et al (2021), vitamin B12 deficiency (total vitamin B12 <148pmol/ll) from 15% in the control group, decreased to 5% in the intervention group taking cobalamin supplementation [35].

Supplementation with vitamin B12 during pregnancy has a beneficial effect on the vitamin status of the expectant mother and most likely similarly on fetal vitamin levels. The latter, as already mentioned, are of utmost importance for the neurological development and cognitive function of the future offspring [67], so the aim is to maintain them in optimal concentrations.

In addition to the general group of pregnant women, the prevalence of vitamin B12 supplementation was studied in the three main study groups. In healthy controls, 29.34% of participants (n=49) reported not taking vitamin B12 supplementation, and in the second group of women with GDM, the percentage was slightly lower at 27.90% (n=12). The highest percentage of unsupplemented pregnant women was found in the group of participants with pre-eclampsia, 59.18% (n=29). Percentage differences in vitamin use emerged between the three main groups of study participants and were statistically significant (X2= 16.80, p=0.002). Similarly, for both the total group and the three main study groups, the mean measured values of total and active vitamin B12 parameters were lower in the unsupplemented compared to the supplemented pregnant women, respectively. For the MMA parameter, an association between vitamin B12 intake from supplements and measured values in participants was found only for the group of pregnant women with pre-eclampsia.

When we examined the frequency distribution of women according to both vitamin B12 intake and BMI, we found that more than half (59.46%) of overweight and obese pregnant women had insufficient vitamin B12 intake.

3.5. To assess the impact of vitamin B12 status on adverse pregnancy outcomes in relation to neonatal outcomes and the occurrence of preterm birth and low birth weight infants in healthy pregnant women and women with pregnancy complications.

A body of evidence is found in the literature supporting the claim that vitamin B12 status of pregnant women, affects the growth and development of newborns. There is some suggestion that vitamin deficiency influences the duration of pregnancy and is associated with the occurrence of preterm birth [259], but studies conducted have had mixed results. A woman's optimal vitamin B12 status before conception was associated with a lower risk of preterm birth in one Chinese study [263], but other investigators did not confirm this relationship [259]. A meta-analysis by Rogne et al. (2017) covering 18 clinical trials examining the association between vitamin B12 status of pregnant women and gestation length also reported mixed results. For example, in one of them, higher measured concentrations of total vitamin B12 in expectant mothers were associated with normal

gestation length and reduced risk of PTB. A drawback of the study is that the sample size was small. In another study, the authors found no reliable evidence of an association between measured serum total vitamin B12 concentrations and pregnancy duration [259]. In our study, a correlation was also sought between the levels of parameters reflecting the vitamin B12 status of pregnant women and the likelihood of developing PTB. We found differences in the measured mean values of the studied parameters, with better vitamin B12 status found in women with normal duration of pregnancy - higher mean serum concentrations of the parameter total vitamin B12 (179.31±93.92pmol/l vs 163.96±58.39pmol/ l , p=0.37), similar results for the active vitamin B12 parameter (74.06±55.16pmol/l vs 64.72±35.94pmol/l, p=0.35) and lower mean values for the metabolic parameter MMA (247.45±157.07nmol/l vs 261.99±141.05nmol/l, p=0.65). Despite the differences found in measured parameter levels, statistical significance was not found between groups (p>0.05). The most likely reason for the lack of reliable differences lies in the relatively small number of participants enrolled in the study and developed the complication.

In addition to preterm birth, vitamin B12 deficiency in pregnant women is associated with low birth weight [259] . Again, the research data are conflicting. In a recent meta-analysis, a linear relationship between serum total vitamin B12 concentrations in pregnancy and newborn birth weight was not found, but the association of vitamin deficiency (total vitamin B12<148 pmol/L) with a higher risk of having a LBW newborn was confirmed [259]. In another study, investigators found that infants with birth weights <2500g and 2500-2999g had lower values of total vitamin B12 measured from umbilical cord immediately after birth compared with values measured in a control group of infants with birth weights >3000g (194.83 and 197.78 pmol/L vs 236.16 pmol/L, respectively, p=0.02) [31]. In the same study, the authors also found an existing correlation between serum vitamin B12 concentrations in pregnant women in the second and third trimesters of pregnancy and the vitamin values obtained from the umbilical cord of the newborn at birth. In relation to the literature reviewed, we searched for correlations between the levels of total vitamin B12, active vitamin B12, and MMA measured during pregnancy in pregnant women in our study and LBW neonatal delivery. Using independent samples t-test, we compared the mean measured values of the indicators reflecting the vitamin B12 status of pregnant women, but the results showed no statistical significance between the two groups compared (LBW/normal birth weight). However, we found that the mean measured values of serum concentrations of the indicators total vitamin B12 and active vitamin B12 in women delivering infants weighing

less than 2500g were lower than those obtained in women delivering normal-weight infants (total vitamin B12 162.74±58.58 pmol/l vs 179.71±94.24 pmol/l, t=1.033, p=0.303; active vitamin B12 59.01±32.41 pmol/l vs 75.09±55.49 pmol/l, t=1.668, p=0.091). For the metabolic parameter MMA, in confirmation of the results obtained above, slightly higher mean values were found in women who delivered low birth weight infants compared to those who delivered normal weight infants (276.2±158.47 nmol/l vs 244.69±154.40 nmol/l; t=1.050, p=0.295).

We also tested whether low birth weight is related to pregnant women belonging to a certain risk group by Spearman correlation. We found a strong and positive correlation between the probability of having LBW newborns with increasing vitamin B12 deficiency for the group of pregnant women who developed pre-eclampsia (rho=0.499, p=0.009). The data obtained are consistent with those described in the literature. In the large prospective cohort study conducted by Liu (2021) et al. it was found that a higher percentage of LBW was found among pregnant women who developed hypertensive complications and pre-eclampsia compared to healthy controls [183]. Pre-eclampsia as an obstetric emergency condition often requires induction of labour to preserve the life of the parturient and the newborn, and this in turn is associated with a greater risk of delivering a low birth weight infant.

According to Duggan et al. (2014), it is possible that vitamin B12 supplementation with subsequent reduction in serum homocysteine concentrations could lead to optimal birth weight and normal gestation length [92]. However, in two randomized controlled trials related to cobalamin supplementation during pregnancy, the authors reported higher serum total vitamin B12 levels in the group of women taking supplements, but did not report a reduction in measured homocysteine levels over the course of the study. The investigators also found no differences in newborn birth weight, gestation length, or incidence of low-birth-weight and preterm births in the group of pregnant women taking vitamin B12 supplements compared with the control group in either clinical trial [92, 277].

Vitamin B12 supplementation during pregnancy is known to have a beneficial effect on the neuro-cognitive development of the offspring and leads to a reduced risk of neural tube defects. Further studies with large numbers of pregnant women enrolled are also likely to demonstrate the effect of the vitamin on gestation length and birth weight of newborns. Supplementation with cobalamin during pregnancy and lactation would contribute to increased societal benefits.

VI. Conclusions

Pregnancy and risk factors for adverse complications:

1. Age of pregnant women, BMI calculated at the time of pregnancy, and gestational weight gain are important risk factors for adverse pregnancy complications such as GDM and pre-eclampsia.

2. Season can be considered as a potential risk factor for the occurrence of adverse pregnancy complications, mainly for the condition pre-eclampsia.

Vitamin D status and pregnant women:

1. The vitamin D status of pregnant women from. The vitamin D status of pregnant women in the region is much better than the vitamin D status determined for other areas of our country and is comparable to the vitamin D status of women with better social status from developed countries of the world.

2. There was a seasonal dependence of serum 25(OH)D levels in the study cohort with optimal levels in the summer half-year and suboptimal levels in the winter half-year.

3. Increased BMI was found to be an adverse factor regarding the vitamin D status of the pregnant women studied.

4. There was no difference in the mean serum 25(OH)D levels for the three main groups of pregnant women studied, nor in their frequency distribution according to vitamin D status.

5. More than two-thirds of the women studied were supplemented with vitamin D preparations, with almost 22% taking doses many times higher than the 600 IU/day recommended by health regulators, which significantly improved their vitamin D status.

6. According to their attitudes towards supplementation with vitamin D preparations, pregnant women from Varna and the region show a more conscious attitude compared to pregnant women from other regions of the country and are closer to women from developed countries of the world with better social status.

7. Women with pre-eclampsia and overweight have a lower attitude towards supplementation with vitamin D preparations, which predetermines their significantly worse vitamin D status.

8. The highest incidence and risk of preterm birth was found in women with pre-eclampsia, correlated with both serum 25(OH)D levels and vitamin D preparations intake.

9. The highest incidence and risk of low birth weight childbirth was found in women with pre-eclampsia, with vitamin D intake being a significant factor in low birth weight childbirth.

Vitamin B12 status and pregnant women:

1. The prevalence of vitamin B12 deficiency and insufficiency found in the study cohort was comparable to those seen in developed countries worldwide.

2. The winter season was found to be more favourable in terms of vitamin B12 status and the parameters that determine it.

3. BMI did not predict statistically significant differences in the parameters determining vitamin B12 status.

4. Two-thirds of the women in the study cohort were taking vitamin B12 supplementation, and in about half of them the dose taken was above the recommended daily intake.

5. In women with pre-eclampsia, there was no significant difference between serum concentrations for active vitamin B12 with those of healthy controls, whereas there was a trend for a statistical difference for MMA.

6. Risk of gestational diabetes mellitus according to vitamin B12 status was not found.

7. Mean measured values of parameters reflecting vitamin B12 status did not differ in pregnant women who delivered preterm compared with women of normal gestational length.

8. Mean measured values of parameters reflecting vitamin B12 status did not differ in pregnant women who delivered a low birth weight child compared with women of normal gestation length, but there was a statistically significant increased risk of delivering a low birth weight child with worsening B12 status in women with pre-eclampsia.

VII. Contributions

Contributions of an original nature

1. For the first time in Bulgaria, a study was conducted to investigate the vitamin D status of pregnant women - healthy and with pathological complications of pregnancy, by investigating serum 25(OH)D concentrations using high-performance liquid chromatography with mass spectrometric detection (LC-MS).

2. For the first time in our country, the possible correlations between vitamin D deficiency and/or insufficiency and the development of adverse complications in pregnant women, such as pre-eclampsia and gestational diabetes mellitus, as well as the development of adverse complications in the newborn, such as PTB and LBW, were investigated.

3. For the first time in our country, vitamin B12 status of a specific target group was determined by three interrelated laboratory parameters - total vitamin B12, active B12 and methylmalonic acid, the latter determined by high-performance liquid chromatography with mass spectrometric detection (LC-MS).

4. For the first time in Bulgaria a study was conducted to investigate the vitamin B12 status of pregnant women - healthy and with pathological complications of pregnancy.

5. For the first time in Bulgaria, the possible correlations between vitamin B12 deficiency and/or insufficiency and the development of adverse complications in pregnant women such as pre-eclampsia and gestational diabetes mellitus, as well as the development of adverse complications in the newborn such as PTB and LBW were studied.

Contributions with practical application

1. Data on vitamin D status of pregnant women in Northeastern Bulgaria were obtained, which were previously lacking.

2. Data on vitamin B12 status of pregnant women in Northeastern Bulgaria were obtained, which were previously lacking.

3. The seasonal dependence of serum 25(OH)D concentrations was confirmed, which must be considered when analysing the results.

4. The results of the study will allow to deepen the knowledge of the role of vitamin D and vitamin B12 in the normal course of pregnancy, fetal development and neonatal birth.

5. The results of the study may warrant a change in the standard of care during pregnancy by adding tests to the mandatory panel of laboratory tests to determine vitamin D and vitamin B12 levels.

6. The results of the study may warrant supplementation with vitamin D and vitamin B12 containing supplements as early as possible after confirmation of pregnancy to avoid the likelihood of adverse complications developing in pregnant women and their newborns.

VIII. Scientific publications related to the dissertation work

1. **Todorova M.**, Gerova D., Galunska B. "Vitamin D Deficiency During Pregnancy", Scripta Scientifica Medica, 2021
2. **М. Тодорова**, Д. Герова, Б. Галунска, „Ефекти на витамин B12 дефицит или недоста-тъчност върху бременността", сп. Мединфо, Август 2022
3. **М. Тодорова**, Ст. Фъртунов, „Гестационен захарен диабет и витамини", сп. Варненски медицински форум т.12, 2023, online first

Participation in scientific forums connected to the dissertation

1. Ст. Фъртунов, Я. Стефанов, П. Косев, **М. Тодорова**, Ж. Русева, С. Христова, В. Александрова, Р. Алексовска, В. Маджова. „Хипертония при бременност в общата медицинска практика", XI-та Научна среща – Обучение на СОИБОМ, 27 – 29.09.2019г., х-л Метрополитан, София
2. **М. Тодорова**, Д. Герова, Ст. Фъртунов, „Статус на витамин B12 при бременни жени", XII Национална Конференция по Клинична Лаборатория 4 – 6 Октомври 2019, Ахелой, България
3. **Todorova M**, Gerova D. „Vitamin D levels in adult outpatients for a period of 8 months", XXVIII Balkan Clinical Laboratory Federation Meeting and XIII National Conference of Clinical Laboratory, National Palace of Culture, Sofia, Bulgaria, 08 – 11 September 2021
4. **Todorova M**, Gerova D, Galunska B. "Vitamin D status during pregnancy and the role of some modifiable factors", 6th International Vitamin Conference, Copenhagen, 22 – 24 September 2021
5. **Todorova M**, Gerova D, Fartunov S. "Pregnancy and Gestational Diabetes Mellitus", IXth International Conference of Young Scientists, 14 – 15 July 2022, Plovdiv, Bulgaria
6. **М. Тодорова**, Д. Герова. „ Витамин B12 статус при бременни жени и връзката му със затлъстяване и развитие на гестационен захарен диабет", XVI Национална Конференция по Клинична Лаборатория, 14 – 16 Октомври 2022, Пловдив, България

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