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„PROF. DR. PARASKEV STOYANOV“ – VARNA

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Vladimir Todorov Gerov, MD

BIOMARKERS FOR THE ASSESSMENT OF BONE DISEASE IN MULTIPLE MYELOMA

ABSTRACT

of a dissertation paper
for the award of educational and scientific degree "Doctor"
Programme „Haematology and blood transfusion“

Scientific Director:

Prof. Ilina Dimitrova Micheva, MD, PhD.

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The dissertation contains a total of 184 pages, illustrated by 52 figures and 50 tables. The bibliography contains 424 reference sources in Latin alphabet, 1 in Cyrillic and 2 web sites.

The dissertation was discussed, by the Departmental Council of the Second Department of Internal Medicine at Medical University "Prof. Dr. Paraskev Stoyanov" - Varna (№ 3/12.03.2024). It has been accepted and directed for defense before the Scientific panel composed of:

External members:

Prof. Vesselina Goranova, M.D., PhD, MU Plovdiv

Prof. Krasimira Ikonomova, M.D., PhD, Sofia

Assoc. Prof. Vanya Slavcheva, M.D., PhD, MU Pleven

Internal members:

Prof. Valeria Kaleva, M.D., PhD, MU Varna

Assoc. Prof. Eleonora Dimitrova-Gospodinova, M.D., PhD, MU Varna

Back-up members:

Assoc Prof. Antonio Antonov, M.D., PhD, MU Pleven (external member)

Assoc. Prof. Trifon Chervenkov, M.D., PhD, MU Varna (internal member)

The defense

The materials on the defense are available in the library of the Medical University “Prof. Dr. P. Stoyanov” – Varna and in electronic format on the website of MU – Varna.

Notes of the members of the scientific panel

CONTENTS:

ABBREVIATIONS USED

ASCT	autologous stem cell transplantation
bALP	bone alkaline phosphatase
β2MG	beta-2-microglobulin
BMI	bone marrow infiltration
BMP	bone morphogenetic protein
BMSCs	bone marrow mesenchymal stromal cells
CR	complete response
Dkk-1	Dickkopf-1
ECM	extracellular matrix
FDG-PET/CT	f
ISS	International staging system for multiple myeloma
LRP	low-density lipoprotein receptor-related protein
MBD	myeloma-induced bone disease
MGUS	monoclonal gammopathy of undetermined significance
MM	multiple myeloma
MMP	matrix metalloproteases
MPCs	myeloma plasma cells
MRI	magnetic resonance imaging
NDMM	newly diagnosed MM patients
NF-kB	nuclear factor kB
NTX	N-terminal cross-linking telopeptide of type 1 collagen
OBs	osteoblasts
OCs	osteoclasts
OPG	osteoprotegerin
OPN	osteopontin
PON	periostin
	partial response
PTH	parathyroid hormone
RANK	receptor activator of NF-kB
RANKL	receptor activator of NF-kB ligand
mRANKL	membrane RANKL
sRANKL	soluble RANKL
Scl	sclerostin
SCID	severe combined immunodeficiency
	stable disease
SMM	smoldering multiple myeloma
TNF	tumor necrosis factor
	very good partial response
WBLDCT	whole-body low-dose computed tomography

I. INTRODUCTION

Multiple myeloma (MM) is one of the most common haematological malignancies, whose frequency both worldwide and in Bulgaria. The reasons for the observed increase in MM incidence are not yet fully understood. Probably they are related, on the one hand, to the improved diagnostics of the

All this requires the search for suitable biomarkers to effectively monitor MBD. In the long term, this is a prerequisite for the development of new biological agents for targeted therapy and adequate treatment of bone disease, which would improve the quality of life and prolong the survival of patients with MBD.

II. AIMS AND OBJECTIVES

Aim:

To evaluate the role of the bone biomarkers sclerostin, Dkk-1, sRANKL, osteopontin and periostin in the development of bone disease in newly diagnosed multiple myeloma patients and to follow their dynamics in the course of treatment.

Objectives:

1. To determine the serum levels of sclerostin, Dkk-1, sRANKL, osteopontin and periostin in newly diagnosed patients with multiple myeloma prior to treatment initiation.
2. To analyse the serum levels of sclerostin, Dkk-1, sRANKL, osteopontin and periostin in newly diagnosed patients with multiple myeloma depending on the stage of the disease defined according to the ISS.
3. To analyse the serum levels of sclerostin, Dkk-1, sRANKL, osteopontin and periostin in newly diagnosed patients with multiple myeloma depending on the severity of the myeloma bone disease.
4. To analyse the serum levels of sclerostin, Dkk-1, sRANKL, osteopontin and periostin in newly diagnosed patients with multiple myeloma depending on bone marrow infiltration by MPCs.
5. To study the relationships between the serum levels of sclerostin, Dkk-1, sRANKL, osteopontin and periostin with routine hematological, morphological and biochemical indicators.
6. To establish the serum levels of sclerostin, Dkk-1, sRANKL, osteopontin and periostin in newly diagnosed patients with multiple myeloma in the course of their treatment – after four courses of chemotherapy, after eight courses of chemotherapy and three months after ASCT.
7. To analyse the serum levels of sclerostin, Dkk-1, sRANKL, osteopontin and periostin depending on the therapeutic response and to assess their predictive role as independent markers of response and outcome.
8. To elucidate the diagnostic reliability of sclerostin, Dkk-1, sRANKL, osteopontin and periostin as markers for assessment of myeloma bone disease and to assess their individual effect on myeloma bone disease progression.

III. MATERIALS AND METHODS

1. Participants in the study

The research performed is prospective in its character and is carried out in the Clinic of Haematology, at St. Marina University Hospital, Varna from 01.06.2021 to 31.12.2022. A total of 74 subjects are examined, 41 of them newly diagnosed patients with multiple myeloma and 33 healthy subjects who comprise the control group. After the clarification of the aims and objectives of the study all the participants received and signed an informed consent document according to the requirements of the Commission for Ethics of Scientific Research at Varna Medical University. The personal data of the research participants as well as the results of their investigations were obtained, processed and saved in accordance with the Law on personal data Protection and the Code of ethics for healthcare professionals and researchers.

1.1. Patient selection

The patients are selected according to the inclusion and exclusion criteria of the research.

1.1.1. Inclusion criteria:

- Age above 18
- Newly diagnosed patients with a confirmed diagnosis of MM /Multiple Myeloma according to the revised criteria of the International Myeloma Working Group (IMWG)
- Presence of the CRAB (Hyper**C**alcemia, **R**enal insufficiency, **A**nemia, **B**one lesions) criteria for the beginning of a treatment
- Signed informed consent

1.1.2. Exclusion criteria:

- Age above 80
- Patients with other malignant diseases
- Patients with autoimmune diseases
- Patients with acute systemic and local infections
- Patients with neurological and mental disorders that are difficult to deal with
- Intake of steroid medications for the past 6 months
- Intake of bisphosphonates and denosumab for the past 6 months

1.2. Selection of controls

The control group comprises 33 clinically healthy individuals similar in demographic characteristics to the patients and selected according to study inclusion and exclusion criteria.

1.2.1. Inclusion criteria:

- Age above 18
- Signed informed consent

1.2.2. Exclusion criteria:

- Age above 80
- Patients with established malignant diseases
- Patients with autoimmune diseases
- Patients with acute systemic and local infections
- Patients with neurological and mental disorders that are difficult to deal with
- Intake of steroid medications for the past 6 months
- Intake of bisphosphonates and denosumab for the past 6 months

The recruitment and selection of control individuals starts at the start of the study. A sample of venous blood was drawn from them for determination of serum levels of sclerostin, Dkk-1, sRANKL, osteopontin and periostin.

2. Scientific research design

The research design includes the following stages:

- **Stage T0**

At diagnosis and prior to the onset of the treatment, venous blood and urine samples are taken from all patients to investigate routine laboratory parameters (CBC complete blood count, differential blood count, biochemical parameters, serum paraprotein, light chains of immunoglobulin in serum and urine) as well as to determine the serum levels of sclerostin, Dkk-1, sRANKL, osteopontin and periostin (T0). Bone marrow aspirate is performed for a morphological assessment and establishing the percentage % of plasma cell infiltration as well as for flow cytometric estimation of clonality. On the basis of the results of the basic investigation, the patients are distributed according to the international staging system (ISS) and divided into three groups – patients in ISS-1, ISS-2 and ISS-3. The assessment of bone disease is done with whole-body low-dose computed tomography (WBLDCT) scan and the patients are distributed into two groups: group G1 with absence of osteolytic lesions or up to 3 osteolytic lesions and G 2 – patients with more than 3 osteolytic lesions and/or the presence of a pathological fracture / pathological fractures. After the diagnosis is made, all patients begin therapy following the VCD protocol: Bortezomib (Velcade) – 1.3 mg/m^2 D1, D4, D8, D11; Cyclophosphamide – 400 mg/m^2 D1, D8, D15; Dexamethasone – 40 mg D1-D4, D8-D11 (in patients above 75 years of age – 20 mg D1, D8, D15, D22). This cycle is repeated after 28 days. All patients take bisphosphonate (Zoledronic acid) once a month related to the serum creatinine. To patients with serum creatinine values >265 μmol/L, bisphosphonate is not applied.

- **Stage T1**

After finishing the first four cycles of chemotherapy, the effect of the treatment is assessed according to the unified criteria of IMWG /international myeloma working group/ for response assessment. Venous blood and urine are taken for investigating routine laboratory parameters (CBC complete blood count, differential blood count, biochemical parameters, serum paraprotein, light chains of immunoglobulin in serum and urine) as well as for determining the serum levels of sclerostin, Dkk-1, sRANKL, osteopontin and periostin (T1). Bone marrow aspirate is performed for a morphological assessment and establishment of the plasma cell infiltration. The therapeutic response is assessed as follows – complete response (CR), very good partial response (VGPR), partial response (PR) and stable disease (SD). The patients who present with evident progression of the disease, drop out of the research. At this stage the patients are divided into two groups: those below 65 years of age that have achieved CR and VGPR are referred to undergoing an autologous stem-cell transplant (ASCT), while all the rest continue their treatment with additional 4 courses of chemotherapy following the VCD protocol.

- **Stage T2**

The endpoint of monitoring the patients who are not eligible for ASCT is after completing four additional cycles of chemotherapy. Venous blood and urine are taken for investigating routine laboratory parameters (CBC complete blood count, differential blood count, biochemical parameters, serum paraprotein, light chains of immunoglobulin in serum and urine) as well as for determining the serum levels of sclerostin, Dkk-1, sRANKL, osteopontin and periostin (T2). Bone marrow aspirate is

performed for a morphological assessment and establishment of plasma cell infiltration. The patients are stratified again according to their treatment response.

- **Stage TA**

The endpoint of monitoring the patients who are supposed to undergo a transplant is three months after a high-dose chemotherapy and ASCT. Venous blood and urine are taken for investigating routine laboratory parameters (CBC complete blood count, differential blood count, biochemical parameters, serum paraprotein, light chains of immunoglobulin in serum and urine) as well as for determining the serum levels of sclerostin, Dkk-1, sRANKL, osteopontin and periostin (TA). Bone marrow puncture is performed. The bone marrow aspirate is used for a morphological assessment of the bone marrow and for establishing the percentage % of plasma cell infiltration. The patients are stratified again according to the achieved treatment response.

The study design is presented schematically in Figure 5.

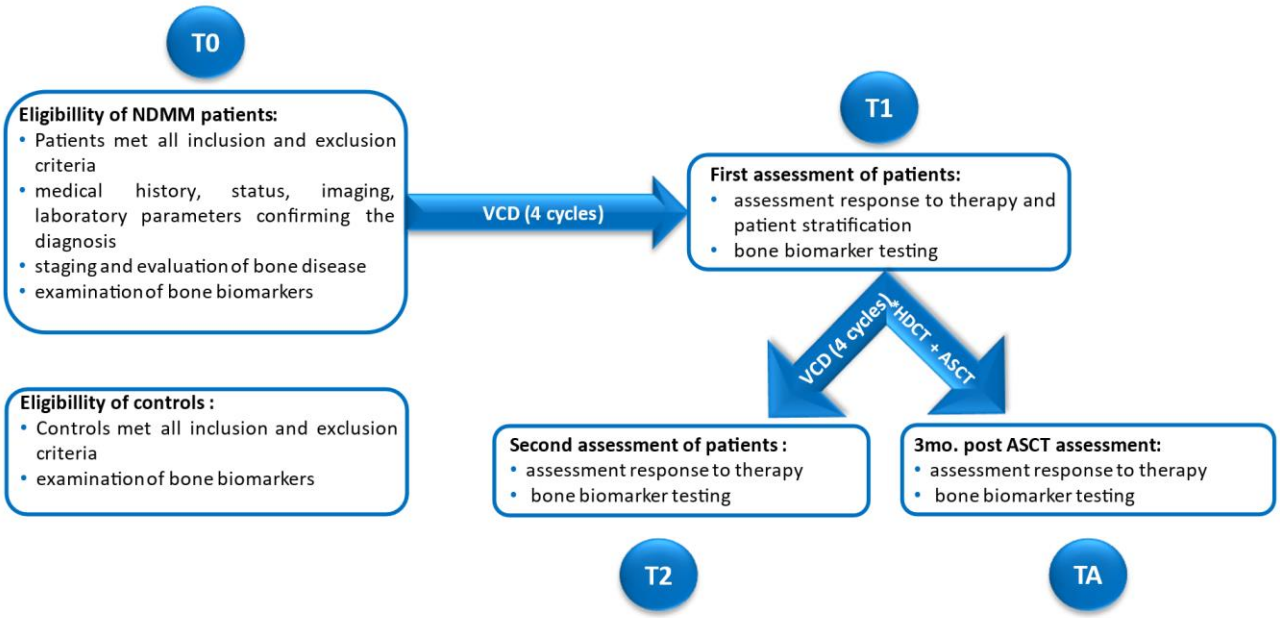

Figure 5. Study design. (*HDCT + ASCT).

3. Clinical examination

All research participants have undergone a thorough primary clinical examination, including medical history and status, in order to establish the presence/absence of inclusion or exclusion research criteria.

4. Laboratory methods

4.1. Biological material, obtaining and storing the samples

Blood is taken in the present study according to standardized conditions-in the morning, on an empty stomach, after 12 hours fasting. The patients are instructed to collect urine for 24 hours. Within half an hour, the biological material thus taken is sent to the Clinical laboratory at "St Marina" University hospital in Varna, for determining the routine laboratory indicators. Two serum vacutainers of 5 ml with gel separator are additionally taken to determine the bone biomarkers. The blood samples are processed on the Hematology clinic premises. After a 20-minute pause and centrifuging at 3000 revolutions per minute (rpm), the serum is separated and aliquoted in equal quantities in five polypropylene test tubes of the "Eppendorf" type which are transferred immediately to the Clinical immunology laboratory to be stored at -80°C until the analysis is carried out.

The bone marrow aspiration is performed in a typical place (the intercostal space between the second and third ribs of the sternum) after preliminary administration of lidocaine local anesthetic. Part of the bone marrow material placed in a vacutainer with anticoagulant K_2EDTA is sent to the Clinical immunology laboratory of "St Marina" University hospital in Varna for flow cytometric investigation. Another part of the material is used to perform a swab test for bone marrow for morphological investigation. The swabs are colored by using the Giemsa staining method in the Clinical laboratory at "St Marina" University hospital in Varna while the morphological assessment is done at the Haematology clinic.

4.2. Routine parametres

- Complete blood picture test with differential blood count is performed from a vacutainer with anticoagulant K_2EDTA of automatic hematology analyzer Sysmex XN 1000, Sysmex Corporation, Kobe, Japan.
- The biochemical indicators general protein, albumin, glucose, urea, creatinine, uric acid, LDH, electrolytes, common calcium, beta2-microglobulin (B2M) are processed from a serum vacutainer with gel separator of automatic biochemical analyzer ADVIA 1800 Chemistry System, Siemens Healthineers, Germany.
- Capillary electrophoresis for determining the M-protein in serum is performed by Sebia Capillarys 2 Flex Piercing, Sebia, France to separate M-protein from other serum proteins and determine its type by immunofixation with reagents from Sebia, France.
- A latex-enhanced immunoturbidimetric method (Diazyme's Human Kappa (κ) and Lambda (λ) Free Light Chain assays, Diazyme Laboratories, Inc., USA) was used to determine the amount and type of light chains in serum and 24-hour urine on a Roche Cobas C 501 automated biochemical analyzer, Roche Diagnostics GmbH, Germany.

4.3. Specific biomarkers

The bone biomarkers are investigated in serum with the help of ELISA (enzyme-linked immunosorbent assay) methods with readymade trade kits of Shanghai Sunred Biological Technology Co., Ltd, China. They are based on a noncompetitive "sandwich" principle / immunoassay. The measurements are taken at the Department of Biochemistry, Molecular Medicine and Nutrigenomics of the Faculty of Pharmacy, Medical University, Varna by strictly following the producer instructions. The results are reported with the help of Biotek Synergy 2 Multi-Mode Plate Reader, USA. The trade kits and participants' samples are tempered on the day of the measurement, prior to the beginning of

the experiment. All standards and the samples of the research participants are added drop by drop twice into two adjacent wells of ELISA plate and the arithmetic mean is calculated.

A short description of the procedure (identical for each one of the investigated proteins)**:**

To the wells of the ELISA plate, factory coated with adhesive monoclonal antibody against human sclerostin / Dkk-1 / sRANKL / osteopontin / periostin 40 µl of the samples are inserted drop by drop which contain the antigen looked for (sclerostin / Dkk-1 / sRANKL / osteopontin/periostin). 10 µl solution of the detection antibody is introduced subsequently drop by drop (the corresponding antisclerostin / anti-Dkk-1 / anti-sRANKL / anti-osteopontin / anti-periostin antibody), bound to biotin and 50 µl solution of anti-immunoglobulin antibody, marked by enzyme (Streptavidin-Horseradish peroxidase), which binds with the detection antibody. The plate is covered with a self-adhesive foil then it is placed on a laboratory incubator shaker and is incubated for 60 minutes at 37°C for the immune complexes to form. After the incubation period is over the extra fluid is removed and the plates are washed fivefold with 350 µl diluted washing solution. 50 µl of the homogenous solution A is added to each well and the homogenous solution B and after careful shaking, the plate is incubated in a dark place for 10 more minutes at 37°C. The color of the content in the wells turns blue as its intensity depends on the antigen concentration. Right at the end of the incubation period, 50 µl of Stop Solution is inserted drop by drop to stop the enzymatic reaction, at which the color changes from blue to yellow. Within 15 minutes, the extinction of each well is detected by an automatic ELISA reader with a wavelength of 450 nm. The concentration of the antigen sought (sclerostin / Dkk-1/ sRANKL / osteopontin / periostin) for each sample is estimated with the help of 5-parameter, logistic, nonlinear regression that resulted from the standard curve, created on the basis of the detected extinctions of the standard solutions, parallelly inserted drop by drop with the samples of the research participants in separate wells of the ELISA plate.

The parameter reliability of the ELISA methods used, as guaranteed by the producer for each one of the following bone biomarkers are presented in table 3.

Table 3. Analytical reliability of the ELISA methods used**.**

Biomarker	**LOD***	**Cross reactivity**	**Linearity**	**Reproducibility (CV%)**
Sclerostin	37.5 ng/mL	not indicated	62.5 – 4000 ng/mL	not indicated
Dkk-1	0.979 ng/mL	not indicated	12.51 – 400 ng/mL	(intra-assay): CV<9% (inter-assay): CV<11%
sRANKL	1.56 pg/mL	not indicated	0 – 60 pg/mL	(intra-assay): CV<9% (inter-assay): CV<11%
Osteopontin	46.9 ng/mL	Clinically significant cross reactivity with osteopontin analogs is not established	78 – 5000 ng/mL	(intra-assay): CV<9% (inter-assay): CV<11%

Periostin	31.0 pg/mL	not indicated	78 – 5000 pg/mL	(intra-assay): CV<10% (inter-assay): CV<12%

*****LOD (limit of detection) – the least quantity of the determinable sample component which can be detected*

5. Imaging investigation

The research was conducted on a Siemens Somatom Force, dual source 384 slice (2x192) CT machine. The protocol includes 1800mm long topogram, FOV scanning 500 mm and a slice thickness of 3 mm. A bone reconstruction algorithm is used structures with Kernel Bg 59 and a second soft-tissue one with Kernel Br 32. The reconstructions of bone window are performed in all three planes, for convenience and improved visibility of the findings are divided into several segments - head and neck; thoracic cage and upper limbs; abdomen and small pelvis; and lower limbs. Reconstructions of soft tissue window are in the axial plane only.

6. Statistical methods

The statistical program GraphPad Prism, version 8.0.2 for Windows, GraphPad Software, La Jolla California USA is used for the statistical processing of the data and the following methods are applied:

- Descriptive analysis for presenting the frequency distribution of quality variables as absolute values (n) and relative frequencies (%) are included.
- Shapiro–Wilk's test for assessment of the normalcy of the continuous variables' distribution. The results are normally distributed when an absence of statistical significance is detected according to the Shapiro–Wilk's ($p>0.05$) test and the values of asymmetricity (skewness) are within the acceptable range (-1/+1). The excess (kurtosis) is believed not to influence the results of the statistical tests and that is why a rule for its interpretation does not exist.
- A variational analysis of the quantity variables for assessing the characteristics of the central tendency and dispersion of the results. The data reflecting the continuous variables with normal distribution are presented as an average value (mean) ± standard deviation (SD). The continuous variables with non-Gaussian distribution are presented with the help of the average position value (median) and interquartile range that varies from the 25^{th} to the 75^{th} percentile.
- Comparative analysis – the parametric t-test of Student-Fisher is used to compare two independent groups, whose results are with normal distribution and the nonparametric Mann–Whitney test and Kruskal–Wallis test for comparing two or more independent groups respectively, whose results are characterized by Non-Gaussian distribution.
- Chi-Square (χ^2) test for goodness of fit (Chi-Square χ^2 test for coherence) – nonparametric statistical test for comparing the proportion of observations of a sample of the hypothetical (theoretical) values.
- Correlation analysis for detecting the dependence between two variables – in normal distribution of the results Pearson correlation coefficient analysis is used, while in the non-Gaussian distribution – Spearman's rank correlation analysis is applied. The degree of correlation dependence is measured according to the value of the correlation coefficient in relation to a 5-tier rating scale:
  - $0 < R < 0.3$ – weak correlation
  - $0.3 < R < 0.5$ – moderate correlation
  - $0.5 < R < 0.7$ – significant correlation
  - $0.7 < R < 0.\ 9$ – strong/high correlation
  - $0.9 < R < 1$ – very strong/high correlation

- Linear regression analysis to determine the degree of dependence and predict the values of the dependent variable.
- Odds ratio (OR) to assess the relationship between odds and probability (risk assessment) of the factors included in the study.
- Receiver-Operating Characteristic (ROC) analysis for assessing the diagnostic reliability of the investigated research indicators/biomarkers and for determining their threshold (cut-off) values.
- Graphic analysis for visual representation of the obtained results. In the statistical analysis made, $\alpha=0.05$ is considered a critical error. The zero hypothesis is rejected and statistical significance is established when $p<0.05$.

This dissertation work is written as a result of the activities in scientific project №19009 of the Science Fund at Medical university "Paraskev Stoyanov" Varna, on the topic "New molecular biomarkers for assessing the bone disease in multiple myeloma". The study protocol was approved by the Research Ethics Committee at Medical University, Varna (protocol № 94/25.06.2020).

IV. RESULTS

1. Demographic and clinical laboratory characteristics of the individuals investigated

1.1 Demographic characteristics

Thirty-three healthy volunteers are included in the control group. They all meet the criteria for inclusion in the research. 18 of them are men and 15 – women. The age and sex characteristics are displayed in table 4

Table 4. Demographic characteristics of the control group.

Control group	Age range (years)	Mean + SD (years)	Median (IQR) (years)
Men (n=18)	49.00 – 79.00	61.94 ± 8.633	61.50 (56.00 – 67.00)
Women (n=15)	51.00 – 74.00	59.07 ± 6.065	60.00 (54.00 – 61.00)
Total (n=33)	49.00 – 79.00	60.64 ± 7.56	60.00 (53.00 – 64.50)

Forty-one newly diagnosed patients with multiple myeloma are investigated in the present research. Out of them all, 20 are men and 21 – women. The age and sex characteristics are demonstrated in table 5.

Table 5. Demographic characteristics of the patients' group.

Patients' group	Age range (years)	Mean + SD (years)	Median (IQR) (years)
Men (n=20)	40 – 80	64.08 ± 12.40	63.50 (56.50 – 74.75)
Women (n=21)	36 – 80	63.93 ± 11.30	62.00 (57.50 – 73.50)
Total (n=41)	36 – 80	64.02 ± 12.14	63.00 (57.50 – 74.50)

A Chi square analysis is performed in order to establish whether there is a different distribution by sex in both groups investigated (patients' and control). The analysis detected no difference in the frequency distribution by sex ($\chi^2 = 0.2432$, df = 1, p = 0.6219). A significant difference in the age between the healthy controls and the patients investigated (p = 0.1040) is not detected with the help of a Student-Fisher t-test.

1.2 Clinical laboratory characteristics of the individuals investigated that constitute the patient group

The patients are divided according to the stage of the disease, following the ISS criteria (International staging system for multiple myeloma). Sixteen of the patients (39.02%) are in the first clinical stage, six (14.63%) are in the second clinical stage and nineteen (46.34%) are in the third clinical stage.

The assessment of bone damage associated with MM is done with the help of a whole-body low-dose computed tomography (WBLDCT). In order to stratify the patients on the basis of the bone

lesions the Durie-Salmon staging system is used. At diagnosis, in six of the patients are established from one to three osteolytic lesions and only in three patients no osteolytic lesions are found. These nine patients (21.95%) form the group with absent to mild bone tissue involvement (G1). All the remaining 32 patients (78.05%) are included in the G2 group (severe bone involvement). Twenty-one of these patients (65.7%) are with more than three osteolytic lesions, while eleven patients (34.3%) had pathologic fractures in addition to the existing osteolytic lesions. Only one patient from the G2 group has also a soft tissue lesion at L1 vertebral level.

In twenty-three of the patients (56.10%), the type of the M-protein secreted is IgG (κ, n=14 and λ, n=9), in six of them (14.63%) it is IgA (κ, n=5 и λ, n=1). Thirteen of the patients (29.27%) are with a light chain myeloma (FLCκ, n=8 и FLCλ, n=4).

The morphological assessment of the bone marrow aspirate, plasma cells infiltration below 60% is detected in 17 of the patients (41.46%), and in the remaining 24 patients (58.54%) the plasma cell infiltration is more than 60%.

At diagnosis 12 of the patients are with creatinine above the upper reference limit. Four of them are with creatinine up to 176 μmol/L, in two of which creatinine values tend to get back to normal in the course of treatment. Eight of the patients have creatinine more than 176 μmol/L, and two of which have to go on dialysis (creatinine more than 400 μmol/L). In the course of treatment, an improvement of their kidney function is not detected and they remain on dialysis till the end of the monitoring.

Twenty-two patients reached the T1 stage. The reasons for dropping out the remaining patients from the study were:

- Withdrawal from the study – 4 patients
- Death – 4 patients as a result of COVID 19 infection and 4 patients as a result of progressive renal failure
- Disease progression – 5 patients
- Change in the therapeutic protocol – 2 patients

At stages T1, T2 and TA, depending on the achieved response the patients were subdivided into two groups: (1) – those who reached CR and VGPR and (2) – those who reached PR or were in SD. Patients in whom a progression of the illness was detected were excluded from the research. Table 6 demonstrates the patients' distribution according to their treatment response.

Table 6. Frequency distribution of the patients according to their treatment response.

Stages	CR + VGPR	PR + SD	PD
T1 – n/N (%)	10/22 (45.5)	11/22 (50.0)	1/22 (4.5)
T2 – n/N (%)	3/11 (27.3)	8/11 (72.7)	0
TA – n/N (%)	10/10 (100.0)	0	0

n – number of patients, who have achieved the respective response; N – the total number of patients, who have achieved the corresponding stage of treatment

A total number of 22 patients were investigated at stage T1. Six of them (27.3%) were with CR and four of them (18.2%) with VGPR. Eight patients (36.4%) were with PR, and three (13.6%) remained in SD. A progression of the disease (PD) was observed in one of the patients (4.5%). A total of 21 patients reached stages T2 and TA (11 were at stage T2 and 10 – at stage TA). Three patients (27.3%) reached VGPR at stage T2, five (45.5%) were with PR and three (27.2%) were in SD. Nine patients (90.0%) reached CR and only one patient (10.0%) was with VGPR at stage TA.

The clinical laboratory characteristics of the patients at the moment of their inclusion in the research are presented in a summary format in table 7.

Table 7. Clinical laboratory characteristics of the patients at their diagnosis.

Indicator/biomarker	**N (%) / Mean ± SD (Range)**
ISS stage: I II III	 16 (39.02%) 6 (14.63%) 19 (46.34%)
Bone disease: G1 G2	 9 (21.95%) 32 (78.05%)
Type of M-protein: IgG κ/λ IgA κ/λ FLC κ/λ	 23 (56.10%) 6 (14.63%) 12 (29.27%)
Bone marrow infiltration (BMI): <60% >60%	 17 (41.46%) 24 (58.54%)
Hb (g/l)	100.75 ± 25.36 (55 – 151)
WBC (x10^9/L)	6.39 ± 2.95 (2.03 – 14.58)
PLT (x10^9/L)	208.41 ± 103.31 (32 – 461)
Creatinine (µmol/L)	126.05 ± 91.53 (53 – 449)
LDH (U/L)	425.75 ±370.34 (200 – 2571)
Total protein (g/L)	92.70 ± 20.99 (54.5 – 133.1)
Albumin (g/L)	36.89 ± 7.58 (18.8 – 51.0)
B2MG (mg/L)	5.14 ± 3.13 (1.8 – 14.0)
Calcium (mmol/L)	2.42 ± 0.47 (1.8 – 4.01)

2. DICKOPFT-1 (Dkk-1)

2.1 Basic serum levels of Dkk-1 in newly diagnosed patients with multiple myeloma (NDMM)

The serum levels of Dkk-1 in NDMM patients are investigated immediately after the diagnosis and before the onset of the therapy. The use of the Shapiro-Wilk's test establishes a non-Gaussian distribution of the results which justifies the implementation of nonparametric methods in the statistical analysis of the parameter investigated.

The median and the respective interquartile range of the serum levels of Dkk-1 for the patients' group are 61.75 ng/mL (IQR: 55.11 – 71.45 ng/mL), while for the control group are 46.62 ng/mL (IQR: 42.43 – 53.32 ng/mL). Thus, the serum levels in the patients' group exceed those observed in the control group by more than 30%. The Mann-Whitney comparative analysis establishes a significant difference between the two groups investigated ($p<0.0001$, figure 6).

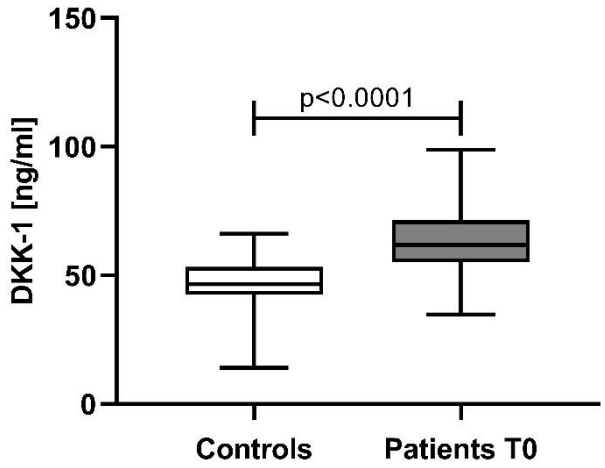

Figure 6. Comparison of serum levels of Dkk-1 in controls and patients' groups.
(The Mann-Whitney test is applied)

The influence of sex on the serum levels of Dkk-1 is investigated. In both groups (patients' and control group) no statistically significant differences are detected depending on the sex (table 8).

Table 8. Influence of the sex on the serum levels of Dkk-1 in the patients' and control groups.

Group investigated	Sex	N	DKK-1 [ng/ml]		P value
			Median	IQR	
NDMM patients	Men	20	62.05	54.84 – 71.98	0.7916
	Women	21	61.53	54.29 – 69.59	
Control individuals	Men	18	44.64	41.96 –51.51	0.2354
	Women	15	49.41	43.49 –61.21	

The Mann-Whitney test is applied in the comparative analysis.

The sex distribution does not change the significant difference between patients found for the total group and healthy individuals. Both male and female patients had significantly higher levels of Dkk-1 compared to healthy males and females respectively (figure 7).

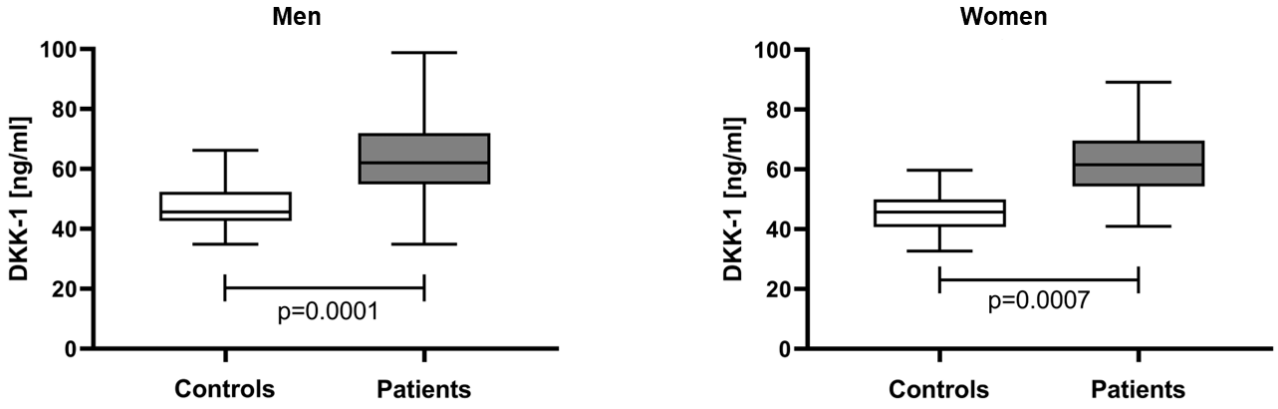

Figure 7. Comparison of Dkk-1 serum levels in the two groups depending on the sex.
(The Mann-Whitney test)

Yet it is interesting to point out that while in the NDMM patients' group the men present with negligibly small higher values of Dkk-1 in relation to the women, in the control group on the contrary the women are with higher values than the men and although they do not reach a statistical significance, the difference between the values is approximately 11%.

The influence of the age on the levels of Dkk-1 is also investigated. The patients' and control groups are subdivided into two additional subgroups according to age. Group A includes patients under 65 years of age and group B includes patients over 65 years of age. No statistically significant differences depending on the age are established in both groups investigated (table 9).

Table 9. Influence of age on the serum levels of Dkk-1 in the patients' and control group.

Group investigated	**Age**	**N**	**DKK-1 [ng/ml]**		**P value**
			Median	**IQR**	
NDMM patients	Group A (<65 years)	24	62.05	56.47 – 71.63	0.5600
	Group B (>65 years)	17	56.36	51.41 – 71.45	
Control individuals	Group A (<65 years)	25	45.66	41.96 – 54.58	0.7359
	Group B (>65 years)	8	48.29	41.43 - 55.72	

(The Mann-Whitney test is applied)

The influence of renal function on serum Dkk-1 levels is also evaluated. The patients are divided into two groups – with creatinine below and above 177μmol/L. The median and corresponding IQR for the first group are 61.04 ng/ml (54.84 – 67.90) and for the second group 72.06 ng/ml (50.27 – 81.18). The Mann-Whitney comparative test reveals no significant difference between the two groups (p=0.2275).

2.2 Serum levels of Dkk-1 in NDMM patients depending on the stage of the disease

The results of the serum levels of Dkk-1 in patients stratified into three subgroups according to the stage of the disease determined by the IMWG criteria are presented in table 10.

Table 10. Serum levels of Dkk-1 in patients stratified according to the stage of the disease.

Stage of the disease	N	DKK-1 [ng/ml]	
		Median	IQR
ISS-I	16	57.7	53.29-61.9
ISS-II	6	62.32	53.82-69.24
ISS-III	19	68.94	55.72-75.34

Gradual increase in the values of Dkk-1 is observed with the advancement of the disease stage. Thus, a statistically significant difference is established between the first and the third stage of the multiple myeloma (p=0.025). The values of Dkk-1 at stage ISS-II are higher than those at the first stage and lower than the ones at stage three but a statistical significance is not reached. The results of the comparative analysis are shown in figure 8

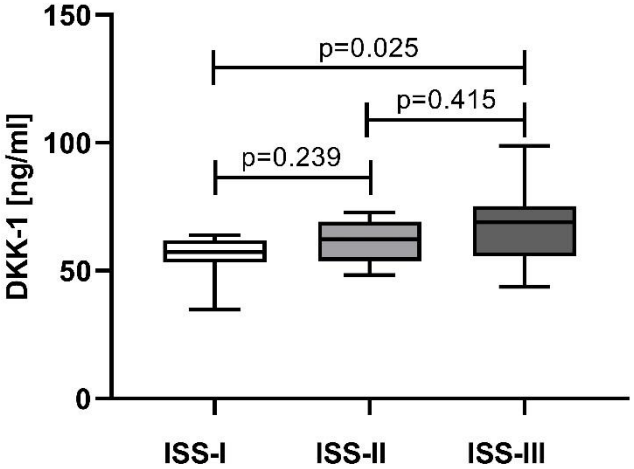

Figure 8. Comparison of the serum levels of Dkk-1 of patients stratified into three groups according to their disease stage. (The Mann-Whitney test is applied)

2.3 Serum levels of Dkk-1 in NDMM patients depending on the bone disease

Depending on the presence of bone lesions, the patients are divided into two subgroups (G1 and G2). Table 11 displays the results of serum levels of Dkk-1 found in these two subgroups.

Table 11. Dkk-1 serum levels in patients, stratified into subgroups depending on the bone disease

Bone disease	N	DKK-1 [ng/ml]	
		Median	IQR
G1	9	55.68	43.71–61.75
G2	32	62.86	56.36–71.45

(G1 – group of patients with zero up to three osteolytic lesions, G2 – group of patients with more than three osteolytic lesions and/or pathological fracture/s available)

With the aid of Mann-Whitney test, a statistically significant difference (p=0.048) is detected between the patients' subgroups distributed according to the severity of their bone disease (figure 9).

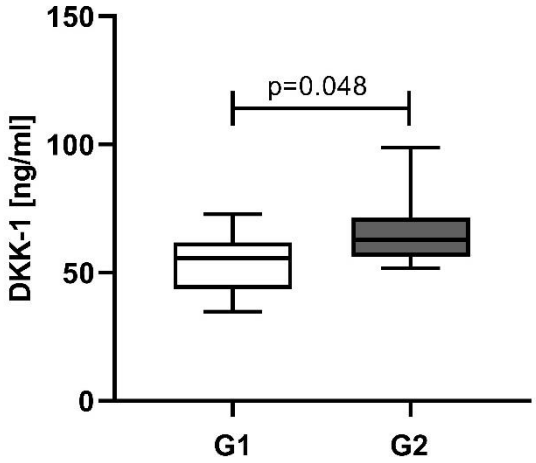

Figure 9. Comparison of serum levels of Dkk-1 in both groups investigated.

Patients from subgroup G1 had higher Dkk-1 values compared to the control group, but no statistically significant difference was reached (55.68 ng/ml vs 46.62 ng/ml respectively, p=0.3479).

2.4 Serum levels of Dkk-1 in NDMM patients depending on the bone marrow infiltration with plasma cells

In order to evaluate the influence of the tumor load on the serum levels of Dkk-1, the patients are divided into two subgroups depending on the bone marrow infiltration with plasma cells. A cut-off at 60% is used to differentiate between the subgroups. The results are displayed in table 12.

Table 12. Serum levels of Dkk-1 in patients stratified depending on the bone marrow infiltration with plasma cells.

BMI	N	DKK-1 [ng/ml]	
		Median	IQR
PCs <60%	17	61.75	48.76 – 72.30
PCs ≥60%	24	62.08	55.67–69.7

(BMI – bone marrow infiltration, PCs – plasma cells)

With Mann-Whitney test, no statistically significant difference (p=0.9843) is detected between the two subgroups investigated (figure 10).

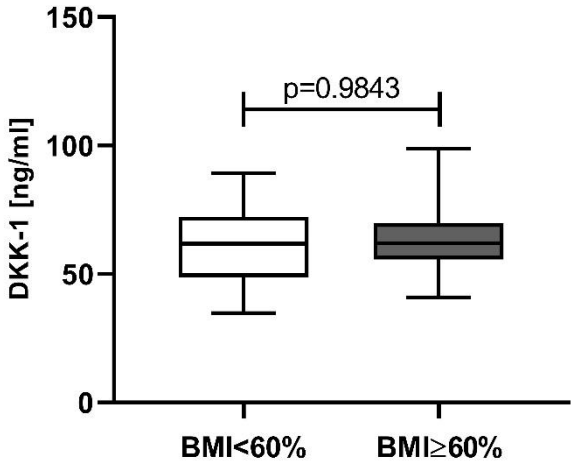

Figure 10. Comparison of the serum levels of Dkk-1 in the two groups investigated.
(The Mann-Whitney test is applied)

2.5 Investigation of the correlation interrelationships between the serum levels of Dkk-1 with routine hematological, morphological and biochemical indicators

Table 13 shows the results of the Spearman correlation analysis carried out to determine the interrelations between the serum levels of DKK-1 with routine hematological, morphological and biochemical indicators.

Table 13. Interrelations between serum levels of Dkk-1 with routine hematological, morphological and biochemical indicators.

Parameter investigated	Spearman rho	P value
Hb	**–0.3166**	**0.0496**
WBC	– 0.0439	0.7906
PLT	–0.0139	0.9327
BMI	0.05109	0.7574
β2MG	**0.3902**	**0.0155**
Creatinine	0.2228	0.1728
LDH	0.0046	0.9778
TP	0.2010	0.2198
Alb	**-0.3868**	**0.0150**
Ca^{2+}	0.3117	0.0824

Hb – hemoglobin, WBC – white blood cells, PLT – platelets / thrombocytes, Alb – albumin, TP – total protein, LDH – lactate dehydrogenase, β2MG – β2-microglobulin, BMI – bone marrow infiltration

Negative and moderate in strength statistically significant correlation is established between Dkk-1 and hemoglobin and albumin and a positive and moderate in strength statistically significant correlation is detected only with β2-microglobulin. Interdependence is not observed with the remaining parameters investigated. The significant correlations are illustrated in figure 11.

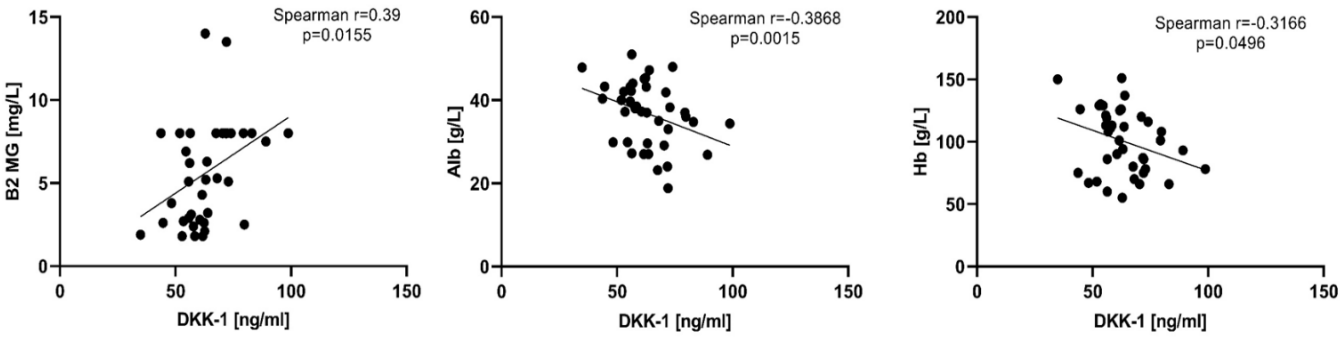

Figure 11. Correlations between Dkk-1 and β2-microglobulin, hemoglobin and albumin.
(Nonparametric Spearman correlation analysis is performed)

2.6 Serum levels of Dkk-1 in NDMM patients in the course of their treatment

According to the research design, the serum levels of Dkk-1 are investigated three times in the course of the treatment (T0, T1, T2 or TA). The results are displayed in table 14.

Table 14. Changes in the serum levels of Dkk-1 in the course of the therapy.

Stages	N	DKK-1 [ng/ml]	
		Median	IQR
T0	41	61.75	55.11 – 71.45
T1	22	54.57	50.32 – 61.04
T2	11	50.55	41.09 – 58.13
TA	10	43.90	37.20 – 47.74

The Kruskal–Wallis test is carried out, in order to compare the non-Gaussian distribution of the results of more than two independent groups. A statistically significant difference between the levels of Dkk-1 investigated at the different treatment points is established (γ^2=24.34, p<0.0001). Additionally, the Mann-Whitney comparative analysis helps to estimate more precisely between which of the treatment points there exists a statistically significant difference. Figure12 displays the results of this analysis.

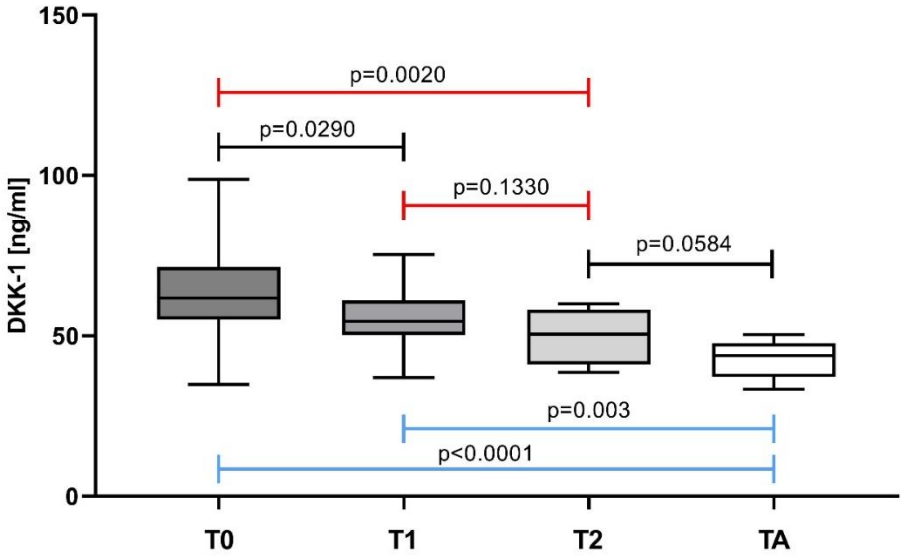

Figure 12. Differences in serum levels of Dkk-1 in the course of the treatment of NDMM patients.
(The Mann-Whitney test is applied)

The basic serum levels of Dkk-1 (T0) differ substantially in relation to those detected at all the remaining treatment points. The degree of significance gradually increases. Straight after the first four cycles of chemotherapy the levels drop significantly (p<0.0290). In the patients who have not undergone transplantation, after additional four cycles of chemotherapy, an even more significant

decrease is detected ($p<0.0020$), while in the patients who have undergone transplantation the most significant decrease of serum levels of Dkk-1 ($p<0.0001$) is observed. The levels at the T1 are not significantly different from those of the patients who have not undergone transplantation (T1 vs T2: $p=0.1330$), while a significant decrease is achieved in the patients who have undergone transplantation (T1 vs TA, $p=0.003$). Towards the end of the period investigated (treatment points T2 and TA) the levels between the two groups of patients (non-transplanted / transplanted) show a tendency for a statistically significant difference (T2 vs TA: $p=0.0584$) with higher levels in non-transplanted patients. Notwithstanding this difference between them, their levels do not differ statistically from the control group (TA vs Controls: $p=0.4561$; T2 vs Controls: $p=0.2104$). Similar convergence to control group values was not found at T1 stage (T1 vs Controls: $p=0.0043$).

The patients who have reached treatment point T1 are subdivided into two subgroups: T1(A) subgroup is formed by patients who are eligible for transplantation and T1(nonA) subgroup includes all others. The median and corresponding IQR for T1(nonA) were 59.36 ng/ml (57.50 - 62.85) and for T1(A) were 50.86 ng/ml (47.76 - 53.09). With the Kruskal–Wallis test a statistically significant difference is establishes between the Dkk-1 levels, investigated at different treatment points ($\gamma^2=30.76$, $p<0.0001$). With the Mann-Whitney test the difference in the Dkk-1 levels between T1(A) and T1(nonA) is studied. Their comparison with the levels measured at the remaining treatment points is also investigated (figure 13).

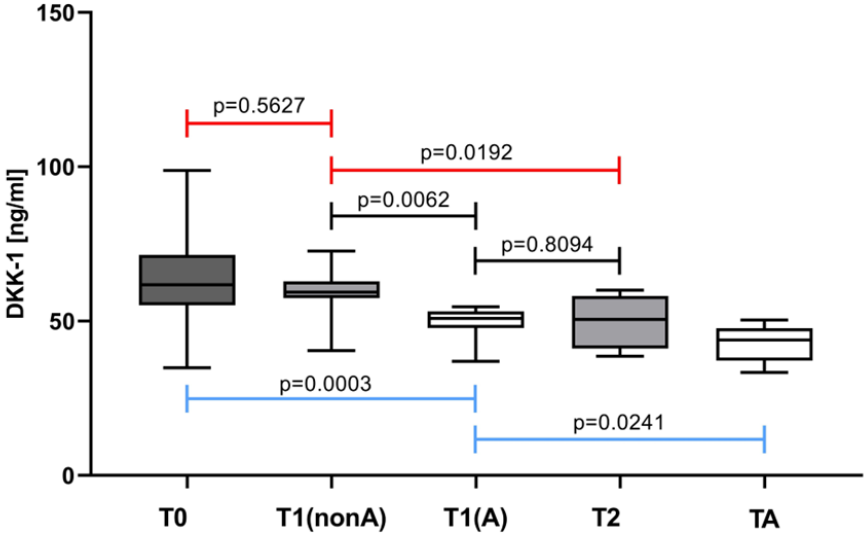

Figure 13. Comparison of Dkk-1 serum levels in T1(A) and T1(nonA) subgroups with those measured in all other treatment. (The Mann-Whitney test is applied)

Since the T1(A) patients have reached CR and VGPR at this treatment point already (T1), their levels differ significantly from the basic levels before the beginning of the (T1(A) vs T0, $p=0.0003$), but they are still significantly higher than the levels which have been achieved three months after the transplantation (T1(A) vs TA, $p=0.0241$). At this treatment point, T1(nonA) patients continue to present high serum Dkk-1 levels, which are not different from the basic levels ($p=0.5627$). At the T2 treatment point the non-transplanted patients reach a significant decrease of their Dkk-1 levels already as compared to T1(nonA), $p=0.0192$ (figure 13).

Comparing T1(A) and T1(nonA) subgroups, it is established that even at this early stage of treatment (T1) they differ significantly in their Dkk-1 levels ($p=0.0062$). The levels in subgroup T1(A) drop so significantly that they reach and do not differ from the levels of the non-transplanted patients observed at treatment point T2, $p=0.8094$ (figure 13).

2.7 Serum levels of Dkk-1 depending on the treatment response.

In order to assess the variations in Dkk-1 levels according to the therapy response, the patients are categorized into two groups: (1) patients with CR and VGPR and (2) patients who with PR and SD, notwithstanding the treatment point, in which they have achieved it (T1 + T2 + TA). Significantly lower values (p=0.0475) for the serum Dkk-1 levels are observed in the group with CR and VGPR (median 50.39 ng/mL and IQR 41.48 – 57.57) as compared to those in the group with PR and SD (median 57.72 ng/mL and IQR 50.37 – 61.04). Besides that, the patients with CR and VGPR have significantly lower levels of the parameter tested in relation to its basic levels (p<0.0001) and which do not differ from those of the control group (p=0.6295). The observations of the group with worse response to the therapy are just the opposite. The Dkk-1 levels in patients with PR and SD are not different from the basic level at stage T0 (p=0.0903) and are significantly higher than those in the control group (p=0.0051) (figure 14).

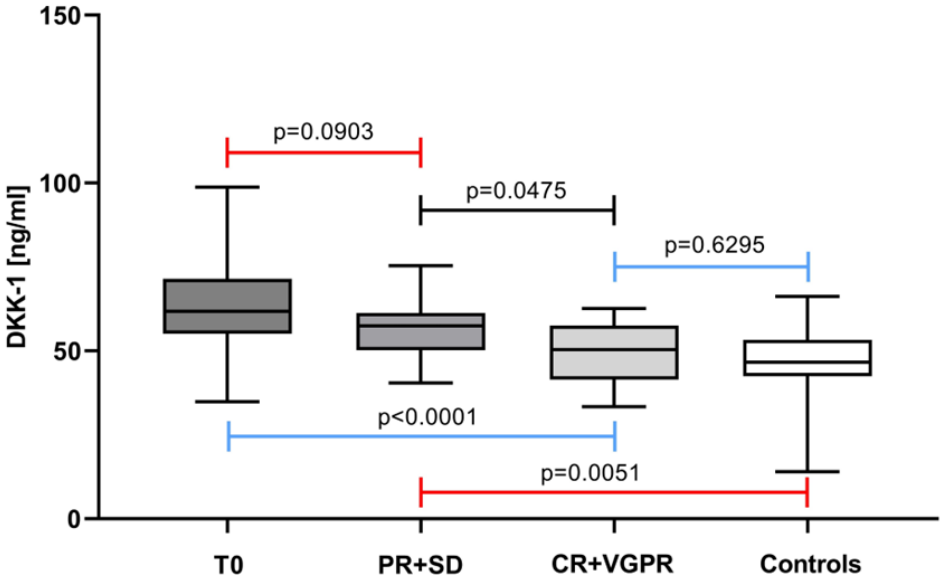

Figure 14. Differences in serum levels of Dkk-1 according to the treatment response.
(The Mann-Whitney test is applied)

For both patient groups the baseline levels (T0 stage) were monitored. For patients with achieved CR and VGPR, the median and corresponding IQR were 59.46 ng/ml (50.97 – 62.96), and for those with PR and SD, the median and corresponding IQR were 65.83 ng/ml (54.24 – 77.78). The Mann-Whitney test showed no significant difference between the two groups (p=0.1655).

3. SCLEROSTIN

3.1 Basic serum levels of Sclerostin in newly diagnosed patients with multiple myeloma (NDMM)

The serum levels of sclerostin (Scl) in NDMM patients are investigated immediately after the diagnosis and before the onset of the therapy. The use of the Shapiro-Wilk's test establishes a non-Gaussian distribution of the results which justifies the implementation of nonparametric methods in the statistical analysis of the parameter investigated.

The median and the respective interquartile range of the serum levels of Scl for the patients' group are 576.8 ng/mL (IQR: 502.1–697.8ng/mL), while for the control group are 74.76 ng/mL (IQR: 41.83–106.2 ng/mL). Thus, the serum levels in the patients' group exceed those observed in the control group by more than 7.7 times The Mann-Whitney comparative analysis establishes a significant difference between the two groups investigated ($p<0.0001$, figure 15).

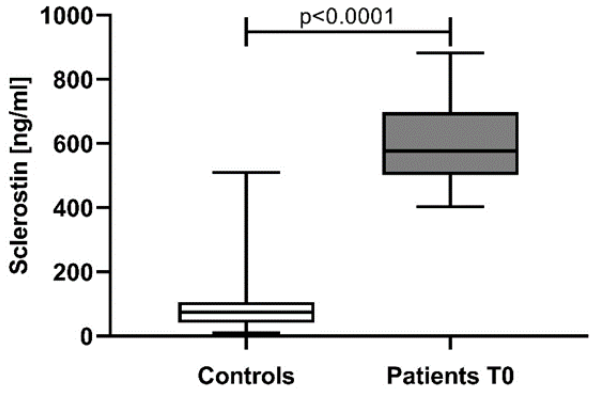

Figure 15. Comparison of serum levels of Scl in controls and patients' groups.
(The Mann-Whitney test is applied)

The influence of sex on the serum levels of Scl is investigated. In the control group, statistically significantly higher values of the tested parameter were found in men compared to women, while in the patient group no such difference was found (table 15).

Table 15. Influence of the sex on the serum levels of Scl in the patients' and control groups.

Group investigated	Sex	N	SCL [ng/ml]		P value
			Median	IQR	
NDMM patients	Men	20	580.1	546.4 – 779.1	0.2083
	Women	21	544.9	493.1 – 647.8	
Control individuals	Men	18	103.3	86.06 – 176.9	0.0148
	Women	15	52.65	41.83 – 80.41	

The Mann-Whitney test is applied in the comparative analysis.

The sex distribution does not change the significant difference between sick and healthy individuals found for the total group. Both sick men and sick women have significantly higher Scl levels compared with healthy men and women, respectively (figure 16).

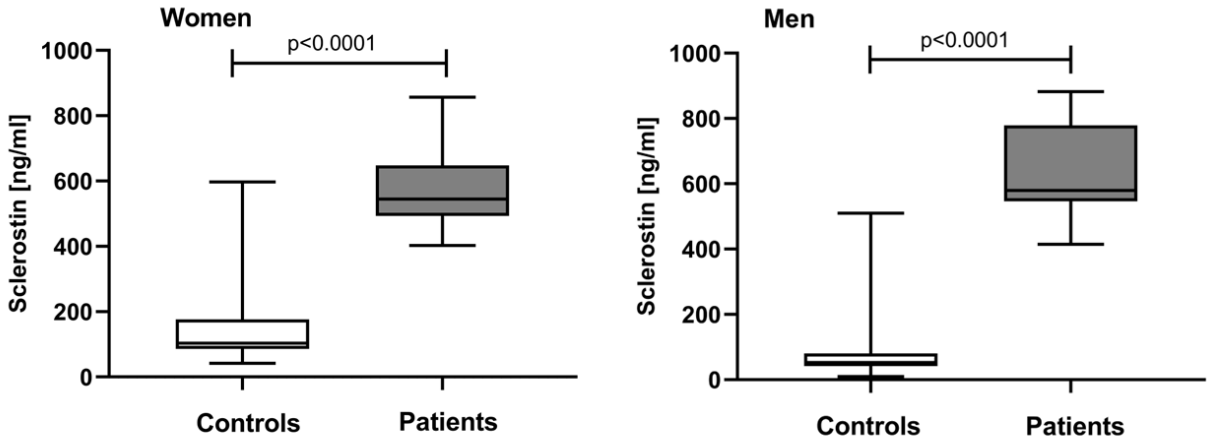

Figure 16. Comparison of serum levels of Scl in the two groups investigated depending on the sex.
(The Mann-Whitney test is applied)

The influence of the age on the levels of Scl is also investigated. The patients' and control groups are subdivided into two additional subgroups according to age. Group A includes patients under 65 years of age and group B includes patients over 65 years of age. No statistically significant differences depending on the age are established in both groups investigated (table 16).

Table 16. Influence of age on the serum levels of Scl in the patients' and control groups.

Group investigated	**Age**	**N**	**SCL [ng/ml]**		**P value**
			Median	**IQR**	
NDMM patients	Group A (<65 years)	24	565.7	490.5 – 639.2	0.3939
	Group B (>65 years)	17	579.6	544.6 – 754.0	
Control individuals	Group A (<65 years)	25	86.06	49.95 – 112.1	0.1068
	Group B (>65 years)	8	47.24	41.83 – 77.59	

(The Mann-Whitney test is applied)

The influence of renal function on serum Scl levels is also evaluated. The patients are divided into two groups – with creatinine below and above 177μmol/L. The median and corresponding IQR for the first group are 576.0 ng/ml (490.5 – 649.9) and for the second group 585.1 ng/ml (548.7 – 754.0). The Mann-Whitney comparative test reveals no significant difference between the two groups (p=0.3118).

3.2 Serum levels of Scl in NDMM patients depending on the stage of the disease

The results of the serum levels of Scl in patients stratified into three subgroups according to the stage of the disease determined by the IMWG criteria are presented in table 17.

Table 17. Serum levels of Scl in patients stratified according to the stage of the disease.

Stage of the disease	N	SCL [ng/ml]	
		Median	IQR
ISS-I	16	513.7	451.5 – 579.6
ISS-II	6	550.1	488.0 – 798.4
ISS-III	19	634.7	554.4 – 765.9

Gradual increase in the values of Scl is observed with the advancement of the disease stage. Thus, a statistically significant difference is established between the first and the third stage of the multiple myeloma (p=0.0012). The values of Scl at stage ISS-II are higher than those at the first stage and lower than the ones at stage three but a statistical significance is not reached. The results of the comparative analysis are shown in figure 17.

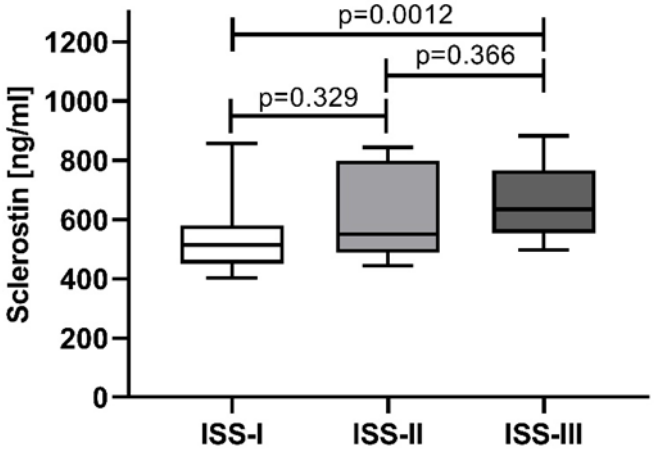

Figure 17. Comparison of the serum levels of Scl of patients stratified into three groups according to their disease stage. (The Mann-Whitney test is applied)

3.3 **Serum levels of Scl in NDMM patients depending on the bone disease**

Depending on the presence of bone lesions, the patients are divided into two subgroups (G1 and G2). Table 18 displays the results of serum levels of Scl found in these two subgroups.

Table 18. Serum levels of Scl in patients, stratified into subgroups depending on the bone disease.

Bone disease	N	SCL [ng/ml]	
		Median	IQR
G1	9	443.6	417.7– 499.8
G2	32	625.50	547.0 – 760.0

(G1 – group of patients with zero up to three osteolytic lesions, G2 – group of patients with more than three osteolytic lesions and/or pathological fracture/s available)

With the aid of Mann-Whitney test, a statistically significant difference ($p<0.0001$) is detected between the patients' subgroups distributed according to the severity of their bone disease (figure 18).

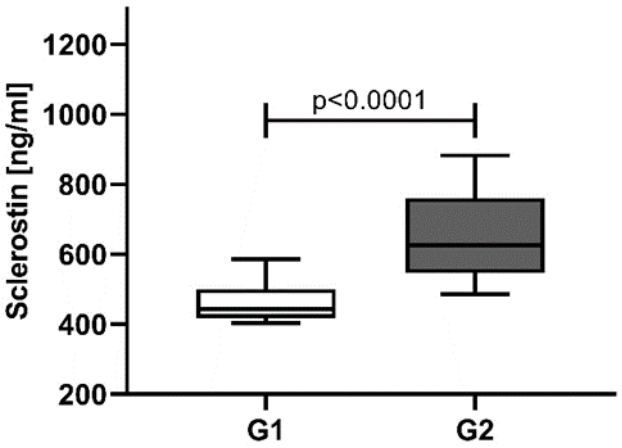

Figure 18. Comparison of serum levels of Scl in both groups investigated.
(The Mann-Whitney test is applied)

Patients in subgroup G1 had significantly higher Scl values and compared to control group (443.6 ng/ml vs 74.76 ng/ml respectively, $p<0.0001$).

3.4 Serum levels of Scl in NDMM patients depending on the bone marrow infiltration with plasma cells

In order to evaluate the influence of the tumor load on the serum levels of Scl, the patients are divided into two subgroups depending on the bone marrow infiltration with plasma cells. A cut-off at 60% is used to differentiate between the subgroups. The results are displayed in table 19.

Table 19. Serum levels of Scl in patients stratified depending on the bone marrow infiltration with plasma cells.

BMI	N	SCL [ng/ml]	
		Median	IQR
PCs <60%	17	501.4	462.9 – 569.0
PCs ≥60%	24	630.5	559.6 – 781.2

(BMI – bone marrow infiltration, PCs – plasma cells)

With Mann-Whitney test, statistically significant difference (p=0.0003) is detected between the two subgroups investigated (figure 19).

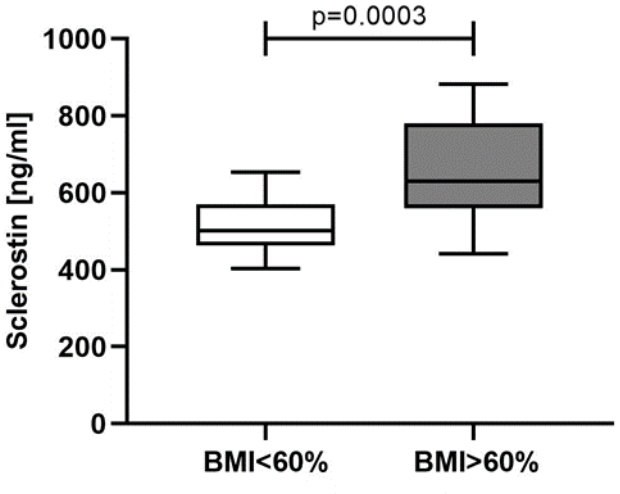

Figure 19. Comparison of the serum levels of Scl in the two groups investigated.
(The Mann-Whitney test is applied)

3.5 Investigation of the correlation interrelationships between the serum levels of Scl with routine haematological, morphological and biochemical indicators.

Table 20 shows the results of the Spearman correlation analysis carried out to determine the interrelations between the serum levels of Scl with routine haematological, morphological and biochemical indicators.

Table 20. Interrelations between serum levels of Scl with routine haematological, morphological and biochemical indicators.

Parameter investigated	Spearman rho	P value
Hb	**–0.3607**	**0.0205**
WBC	–0.06986	0.6643
PLT	**–0.4024**	**0.0091**
BMI	**0.4495**	**0.0032**
β2MG	**0.5224**	**0.0005**
Creatinine	**0.4799**	**0.0015**
LDH	-0.1755	0.2723
TP	**0.3323**	**0.0338**
Alb	**-0.3980**	**0.0100**
Ca^{2+}	-0.08124	0.6531

Hb – hemoglobin, WBC – white blood cells, PLT – platelets / thrombocytes, Alb – albumin, TP – total protein, LDH – lactate dehydrogenase, β2MG – β2-microglobulin, BMI – bone marrow infiltration

Negative and moderate in strength statistically significant correlation is established between Scl and hemoglobin, platelets and albumin and a positive and moderate in strength statistically significant correlation is detected only with β2-microglobulin, BMI, creatinine and total protein. Interdependence is not observed with the remaining parameters investigated. The significant correlations are illustrated in figure 20.

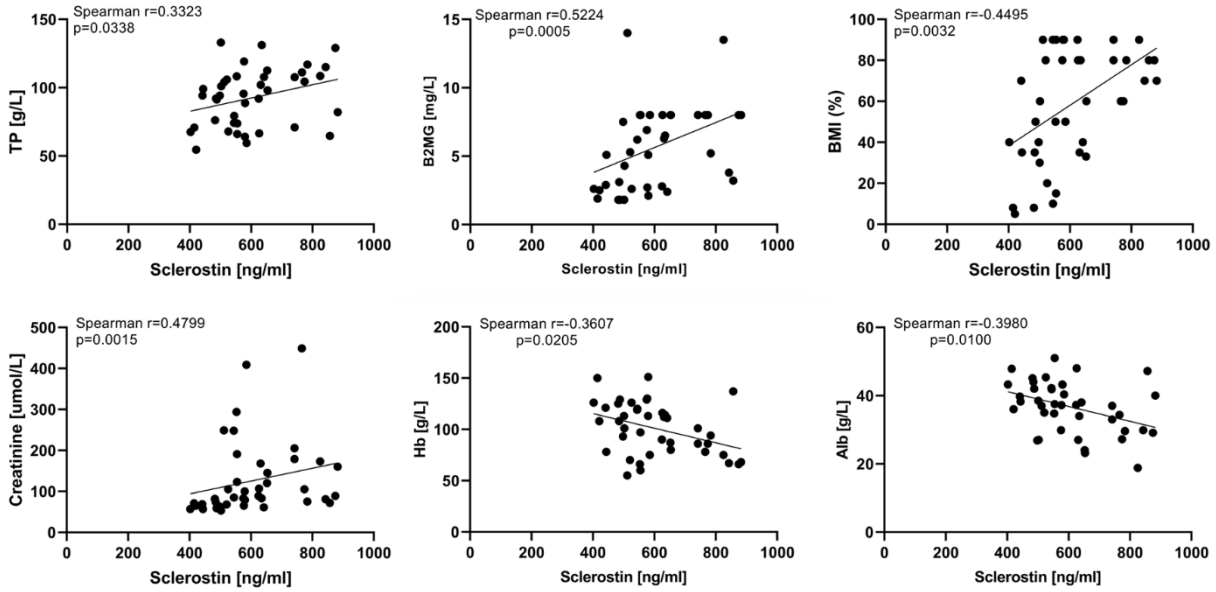

Figure 20. Correlations between Scl and β2-microglobulin, total protein, BMI, creatinine, hemoglobin and albumin. (Nonparametric Spearman correlation analysis is performed)

3.6 Serum levels of Scl in NDMM patients in the course of their treatment

According to the research design, the serum levels of Scl are investigated three times in the course of the treatment (T0, T1, T2 or TA). The results are displayed in table 21.

Table 21. Changes in the serum levels of Scl in the course of the therapy.

Stages	N	SCL [ng/ml]	
		Median	IQR
T0	41	576.8	502.1 – 697.8
T1	22	519.5	469.1 – 580.6
T2	11	481.6	311.3 – 561.5
TA	10	309.8	273.0 – 350.1

The Kruskal–Wallis test is carried out, in order to compare the non-Gaussian distribution of the results of more than two independent groups. A statistically significant difference between the levels of Scl investigated at the different treatment points is established (γ^2=31.40, p<0.0001). Additionally, the Mann-Whitney comparative analysis helps to estimate more precisely between which of the treatment points there exists a statistically significant difference. Figure 21 displays the results of this analysis.

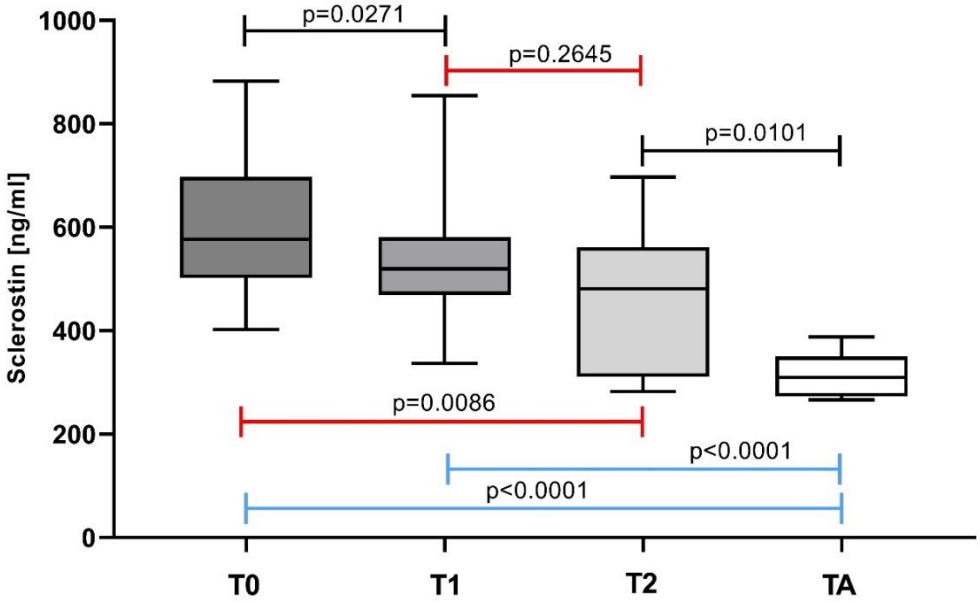

Figure 21. Differences in serum levels of Scl in the course of the treatment of NDMM patients. (The Mann-Whitney test is applied)

The basic serum levels of Scl (T0) differ substantially in relation to those detected at all the remaining treatment points. The degree of significance gradually increases. Straight after the first four cycles of chemotherapy the levels drop significantly ($p<0.03$). In the patients who have not undergone transplantation, after additional four cycles of chemotherapy, an even more significant decrease is detected ($p<0.0086$), while in the patients who have undergone transplantation the most significant decrease of serum levels of Scl ($p<0.0001$) is observed. The levels at the T1 are not significantly different from those of the patients who have not undergone transplantation (T1 vs T2: $p=0.26$), while a significant decrease is achieved in the patients who have undergone transplantation (T1 vs TA, $p<0.0001$). At T1 stage, serum Scl levels were significantly higher than those in the control group ($p<0.0001$). Towards the end of the period investigated (treatment points T2 and TA) the levels between the two groups of patients (non-transplanted / transplanted) show a statistically significant difference (T2 vs TA: $p=0.01$) with higher levels in non-transplanted patients. Their levels differ statistically from the control group (TA vs Controls: $p<0.0001$; T2 vs Controls: $p<0.0001$).

The patients who have reached treatment point T1 are subdivided into two subgroups: T1(A) subgroup is formed by patients who are eligible for transplantation and T1(nonA) subgroup includes all others. The median and corresponding IQR for T1(nonA) were 539.1 ng/ml (470.0 - 562.6) and for T1(A) were 493.2 ng/ml (408.3 - 565.6). With the Kruskal–Wallis test a statistically significant difference is establishes between the Scl levels, investigated at different treatment points ($\gamma^2=18.27$, $p<0.0004$). With the Mann-Whitney test the difference in the Scl levels between T1(A) and T1(nonA) is studied. Their comparison with the levels measured at the remaining treatment points is also investigated (figure 22).

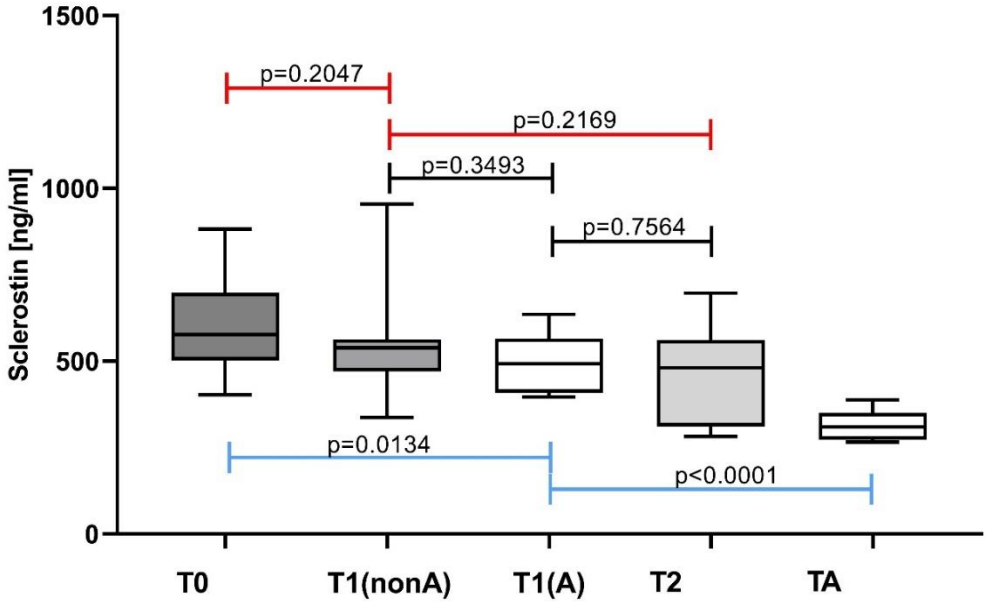

Figure 22. Comparison of Scl serum levels in T1(A) and T1(nonA) subgroups with those measured in all other treatment. (The Mann-Whitney test is applied)

Since the T1(A) patients have reached CR and VGPR at this treatment point already (T1), their levels differ significantly from the basic levels before the beginning of the (T1(A) vs T0, p=0.0134), but they are still significantly higher than the levels which have been achieved three months after the transplantation (T1(A) vs TA, p<0.0001). At this treatment point, T1(nonA) patients continue to present high serum Scl levels, which are not different from the basic levels (p=0.2047). At the T2 treatment point the non-transplanted patients do not reach a significant decrease of their Scl levels already as compared to T1(nonA), p=0.2169 (figure 22).

Comparing T1(A) and T1(nonA) subgroups, it is established that at this early stage of treatment (T1) they do not differ significantly in their Scl levels (p=0.3494). The levels in subgroup T1(A) drop so significantly that they reach and do not differ from the levels of the non-transplanted patients observed at treatment point T2, p=0.7564, but differ significantly from those in T0, p=0.0134 (figure 22).

3.7 Serum levels of Scl depending on the treatment response

In order to assess the variations in Scl levels according to the therapy response, the patients are categorized into two groups: (1) patients with CR and VGPR and (2) patients who with PR and SD, notwithstanding the treatment point, in which they have achieved it (T1 + T2 + TA). Significantly lower values (p=0.0018) for the serum Scl levels are observed in the group with CR and VGPR (median 372.2 ng/mL и IQR 310.1 – 503.2) as compared to those in the group with PR and SD (median 536.4 ng/mL и IQR 469.1 – 582.7). Besides that, the patients with CR and VGPR have significantly lower levels of the parameter tested in relation to its basic levels (p<0.0001) and differ from those of the control group (p<0.0001). The observations of the group with worse response to the therapy are just the opposite. The Scl levels in patients with PR and SD are not different from the basic level at stage T0 (p=0.0950) and are significantly higher than those in the control group (p<0.0001) (figure 23).

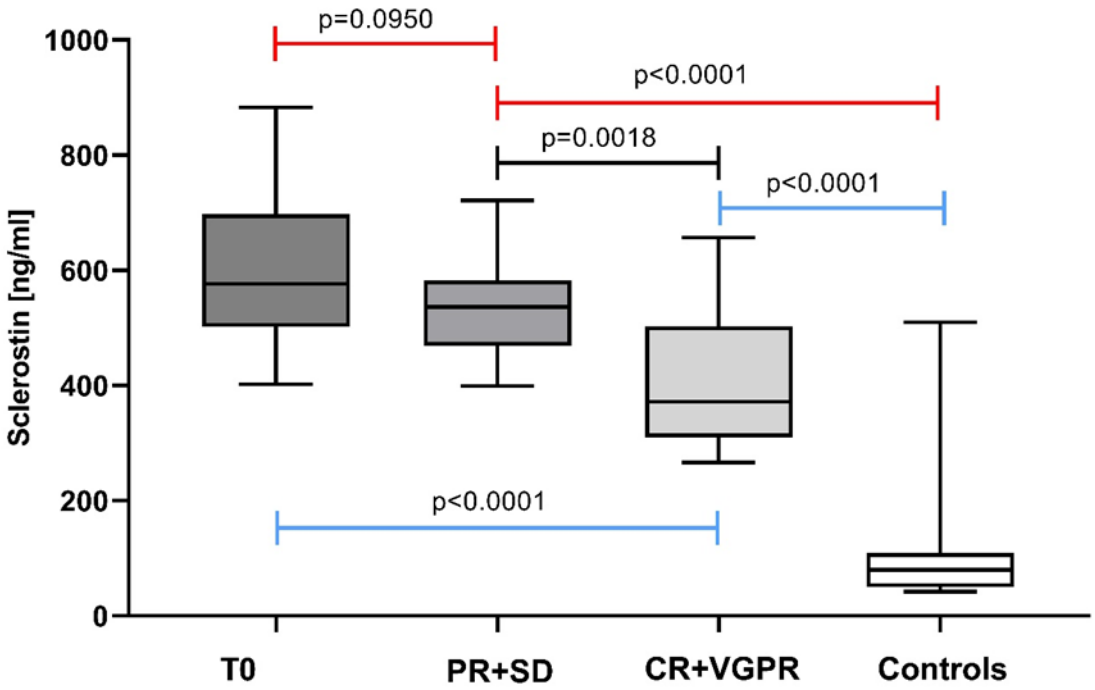

Figure 23. Differences in serum levels of Scl according to the treatment response.
(The Mann-Whitney test is applied)

For both patient groups the baseline levels (T0 stage) were monitored. For patients with achieved CR and VGPR, the median and corresponding IQR were 562.7 ng/ml (498.0 – 846.6), and for those with PR and SD, the median and corresponding IQR were 605.7 ng/ml (501.6 – 636.5). The Mann-Whitney test revealed no significant difference between them ($p>0.9999$).

4. sRANKL

4.1. Basic serum levels of sRANKL in newly diagnosed patients with multiple myeloma (NDMM)

The serum levels of sRANKL in NDMM patients are investigated immediately after the diagnosis and before the onset of the therapy. The use of the Shapiro-Wilk's test establishes a non-Gaussian distribution of the results which justifies the implementation of nonparametric methods in the statistical analysis of the parameter investigated.

The median and the respective interquartile range of the serum levels of sRANKL for the patients' group are 9.592 pg/mL (IQR: 8.033 – 10.92 pg/mL), while for the control group are 5.665 pg/mL (IQR: 5.153 – 6.441 pg/mL). Thus, the serum levels in the patients' group exceed those observed in the control group by more than 70%. The Mann-Whitney comparative analysis establishes a significant difference between the two groups investigated ($p<0.0001$, figure 24).

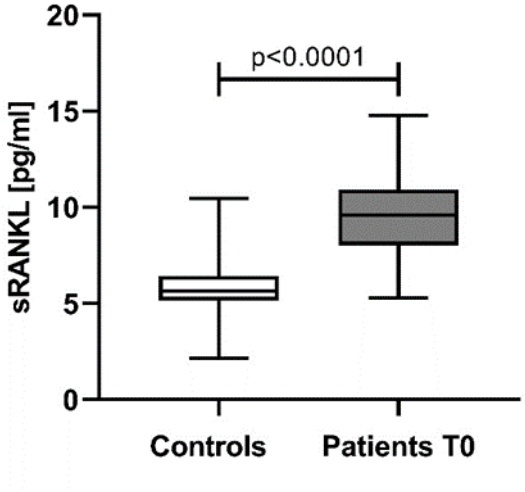

Figure 24. Comparison of serum levels of sRANKL in controls and patients' groups.
(The Mann-Whitney test is applied)

The influence of sex on the serum levels of sRANKL is investigated. In both groups (patients' and control group) no statistically significant differences are detected depending on the sex (table 23).

Table 23. Influence of the sex on the serum levels of SRANKL in the patients' and control groups.

Group investigated	Sex	N	sRANKL [pg/ml]		P value
			Median	IQR	
NDMM patients	Men	20	9.456	8.050 – 10.98	0.7914
	Women	21	9.650	8.022 – 10.92	
Control individuals	Men	18	5.409	4.519 – 6.181	0.1007
	Women	15	5.923	5.281 – 9.099	

The Mann-Whitney test is applied in the comparative analysis.

Yet it is interesting to point out that in the NDMM patients' group and in the control group the men present with negligibly small lower values of sRANKL in relation to the women.

However, the significant difference between male patients and healthy men remains, as well as between female patients and healthy women persists (figure 25).

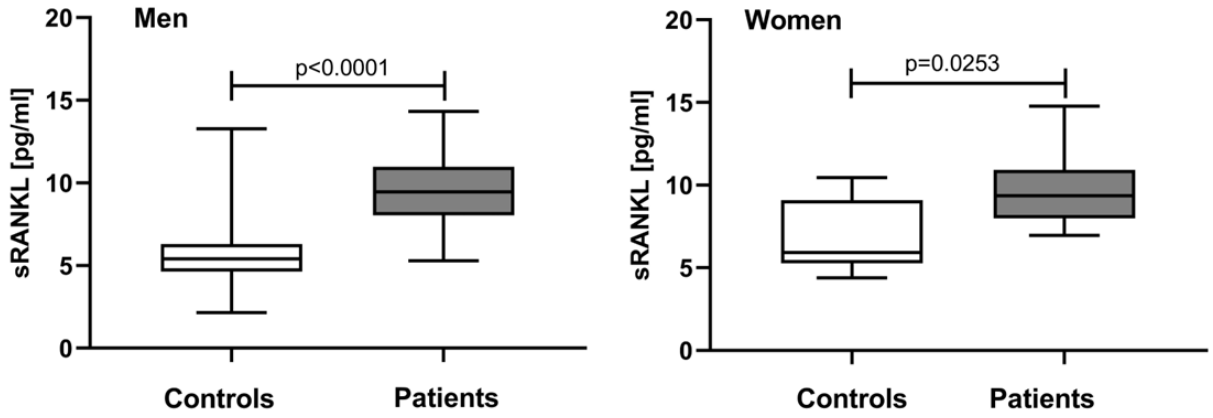

Figure 25. Comparison of serum levels of sRANKL in the two groups investigated depending on the sex. (The Mann-Whitney test is applied)

The influence of the age on the levels of sRANKL is also investigated. The patients' and control groups are subdivided into two additional subgroups according to age. Group A includes patients under 65 years of age and group B includes patients over 65 years of age. No statistically significant differences depending on the age are established in both groups investigated (table 24).

Table 24. Influence of age on the serum levels of SRANKL in the patients' and control groups.

Group investigated	Age	N	sRANKL [pg/ml]		P value
			Median	IQR	
NDMM patients	Group A (<65 years)	24	9.226	7.189 – 10.55	0.1603
	Group B (>65 years)	17	9.650	8.676 – 11.54	
Control individuals	Group A (<65 years)	25	5.665	4.772 – 7.625	0.7323
	Group B (>65 years)	8	5.923	5.345 – 6.246	

(The Mann-Whitney test is applied)

The influence of renal function on serum sRANKL levels is also evaluated. The patients are divided into two groups – with creatinine below and above 177μmol/L. The median and corresponding IQR for the first group are 9.341 pg/ml (8.016 – 10.73) and for the second group 10.55 pg/ml (9.362 – 12.67). The Mann-Whitney comparative test reveals no significant difference between the two groups (p=0.1037).

4.2 Serum levels of sRANKL in NDMM patients depending on the stage of the disease

The results of the serum levels of sRANKL in patients stratified into three subgroups according to the stage of the disease determined by the IMWG criteria are presented in table 25.

Table 25. Serum levels of sRANKL in patients stratified according to the stage of the disease.

Stage of the disease	N	sRANKL [pg/ml]	
		Median	IQR
ISS-I	16	8.033	6.967 – 9.935
ISS-II	6	9.093	8.017 – 10.92
ISS-III	19	10.28	9.320 – 12.89

Gradual increase in the values of sRANKL is observed with the advancement of the disease stage. Thus, a statistically significant difference is established between the first and the third stage of the multiple myeloma (p=0.0013). The values of sRANKL at stage ISS-II are higher than those at the first stage and lower than the ones at stage three but a statistical significance is not reached. The results of the comparative analysis are shown in figure 26.

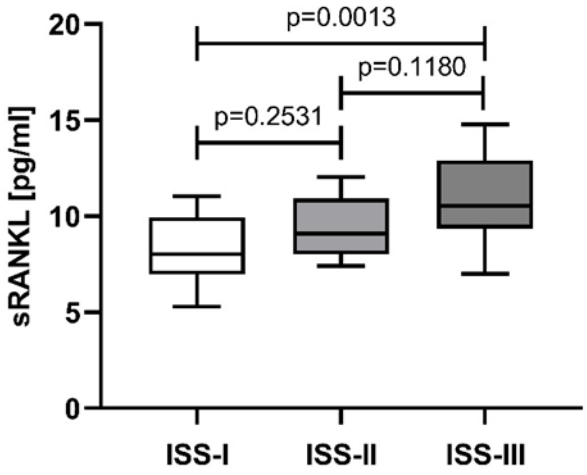

Figure 26. Comparison of the serum levels of sRANKL of patients stratified into three groups according to their disease stage. (The Mann-Whitney test is applied)

4.3 Serum levels of sRANKL in NDMM patients depending on the bone disease

Depending on the presence of bone lesions, the patients are divided into two subgroups (G1 and G2). Table 26 displays the results of serum levels of SRANKL found in these two subgroups.

Table 26. Serum levels of SRANKL in patients, stratified into subgroups depending on the bone disease.

Bone disease	N	sRANKL [pg/ml]	
		Median	IQR
G1	9	7.754	6.442 – 9.290
G2	32	9.625	8.720 – 10.98

(G1 – group of patients with zero up to three osteolytic lesions, G2 – group of patients with more than three osteolytic lesions and/or pathological fracture/s available)

With the aid of Mann-Whitney test, a statistically significant difference (p=0.0092) is detected between the patients' subgroups distributed according to the severity of their bone disease (figure 27).

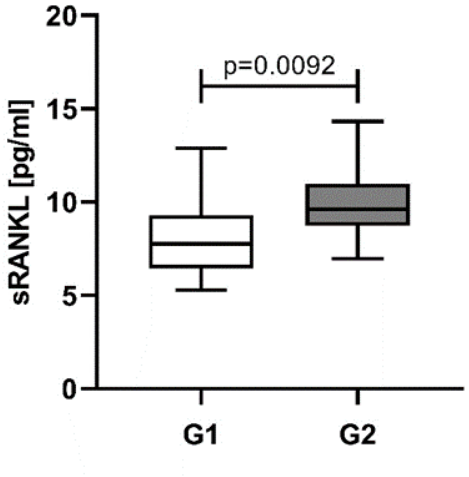

Figure 27. Comparison of serum levels of sRANKL in both groups investigated.
(The Mann-Whitney test is applied)

Patients in subgroup G1 also had significantly higher sRANKL values compared to the control group (7.754pg/ml vs 5.665 pg/ml respectively, p=0.0102).

4.4 Serum levels of sRANKL in NDMM patients depending on the bone marrow infiltration with plasma cells

In order to evaluate the influence of the tumor load on the serum levels of sRANKL, the patients are divided into two subgroups depending on the bone marrow infiltration with plasma cells. A cut-off at 60% is used to differentiate between the subgroups. The results are displayed in table 27.

Table 27. Serum levels of SRANKL in patients stratified depending on the bone marrow infiltration with plasma cells.

BMI	N	sRANKL [pg/ml]	
		Median	IQR
PCs <60%	17	8.033	6.963 – 10.55
PCs ≥60%	24	9.625	8.900 – 11.73

(BMI – bone marrow infiltration, PCs – plasma cells)

With Mann-Whitney test a statistically significant difference (p=0.0169) is detected between the two subgroups investigated (figure 28).

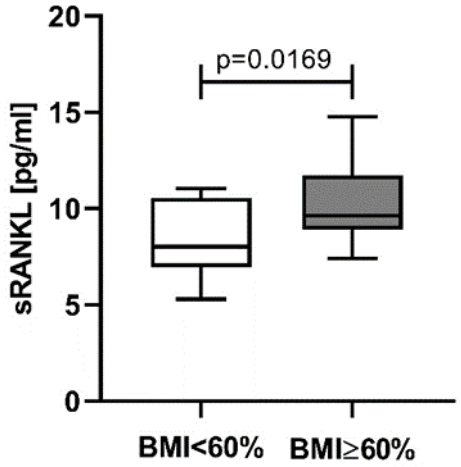

Figure 28. Comparison of the serum levels of sRANKL in the two groups investigated.
(The Mann-Whitney test is applied)

4.5 Investigation of the correlation interrelationships between the serum levels of sRANKL with routine hematological, morphological and biochemical indicators

Table 28 shows the results of the Spearman correlation analysis carried out to determine the interrelations between the serum levels of SRANKL with routine hematological, morphological and biochemical indicators.

Table 28. Interrelations between serum levels of sRANKL with routine hematological, morphological and biochemical indicators.

Parameter investigated	Spearman rho	P value
Hb	**-0.3736**	**0.0161**
WBC	-0.2237	0.1597
PLT	**-0.3946**	**0.0107**
BMI	**0.3304**	**0.0349**
β2MG	**0.5756**	**<0.0001**
Creatinine	**0.3528**	**0.0237**
LDH	0.05015	0.7555
TP	0.2089	0.1899
Alb	**-0.3179**	**0.0428**
Ca^{2+}	0.1890	0.2921

Hb – hemoglobin, WBC – white blood cells, PLT – platelets / thrombocytes, Alb – albumin, TP – total protein, LDH – lactate dehydrogenase, β2MG – β2-microglobulin, BMI – bone marrow infiltration

Negative and moderate in strength statistically significant correlation is established between sRANKL and hemoglobin, platelets and albumin and positive and significant in strength correlation is found with β2-microglobulin, while with creatinine and bone marrow infiltration the correlation is positive and moderate in strength. Interdependence is not observed with the remaining parameters investigated. The significant correlations are illustrated in figure 29.

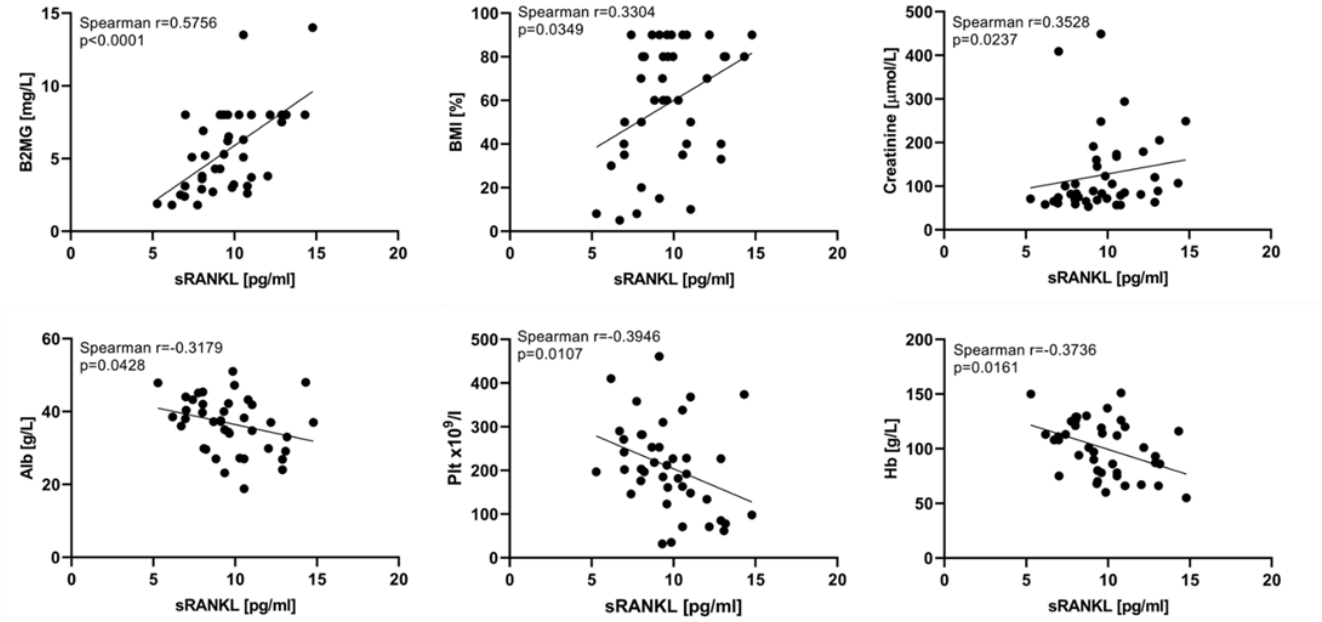

Figure 29. Correlations between sRANKL and β2- microglobulin, BMI, creatinine, albumin, hemoglobin and platelets. (Nonparametric Spearman correlation analysis is performed)

4.6 Serum levels of sRANKL in NDMM patients in the course of their treatment

According to the research design, the serum levels of sRANKL are investigated three times in the course of the treatment (T0, T1, T2 or TA). The results are displayed in table 29.

Table 29. Changes in the serum levels of sRANKL in the course of the therapy.

Stages	N	sRANKL [pg/ml]	
		Median	IQR
T0	41	9.592	8.033 – 10.92
T1	22	8.575	7.201 – 10.18
T2	11	9.210	5.925 – 10.09
TA	10	5.733	5.441 – 6.479

The Kruskal–Wallis test is carried out, in order to compare the non-Gaussian distribution of the results of more than two independent groups. A statistically significant difference between the levels of SRANKL investigated at the different treatment points is established (γ^2=20.45, p<0.0001). Additionally, the Mann-Whitney comparative analysis helps to estimate more precisely between which of the treatment points there exists a statistically significant difference. Figure30 displays the results of this analysis.

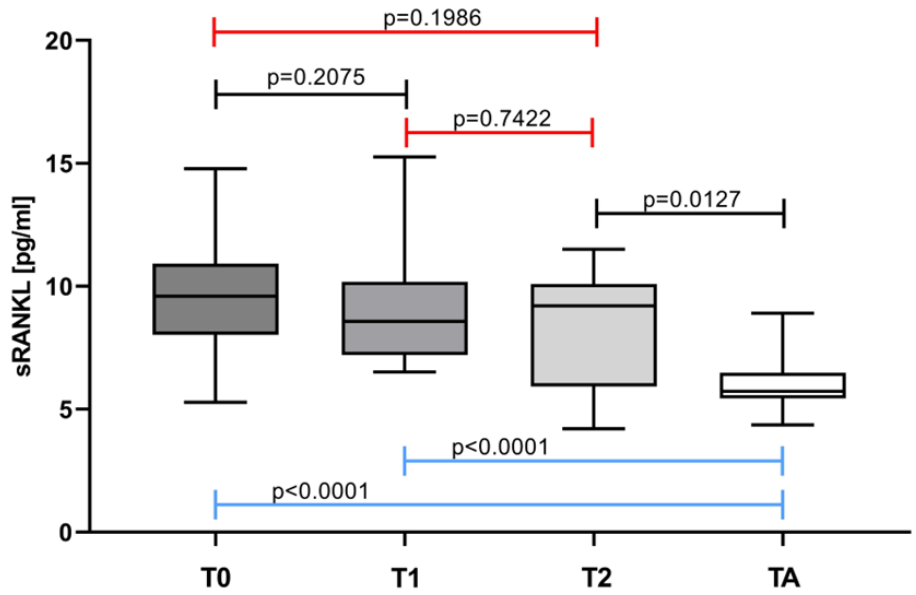

Figure 30. Differences in serum levels of sRANKL in the course of the treatment of NDMM patients. (The Mann-Whitney test is applied)

The basic serum levels of sRANKL (T0) do not differ substantially in relation to those detected after the first four cycles of chemotherapy (T1 vs T0, p=0.2075), as well as after four additional cycles (T2 vs T0, p=0.1986). Significant decrease is detected (p<0.0001) in the patients who have undergone transplantation. The levels at the T1 are not significantly different from those of the patients who have not undergone transplantation (T1 vs T2: p=0.7422), while a significant decrease is achieved in the patients who have undergone transplantation (T1 vs TA, p<0.0001). Also at T1 stage, serum sRANKL levels were significantly higher than those in the control group (p<0.0001). Towards the end of the period investigated (treatment points T2 and TA) the levels between the two groups of patients (non-transplanted / transplanted) show a statistically significant difference (T2 vs TA: p=0.0127) with higher levels in non-transplanted patients. sRANKL levels in TA approached those of the control group (TA vs Controls: p=0.5614), and those in T2 remained significantly higher than the control group (T2 vs Controls: p=0.0060 и T1 vs Controls: p<0.0001).

The patients who have reached treatment point T1 are subdivided into two subgroups: T1(A) subgroup is formed by patients who are eligible for transplantation and T1(nonA) subgroup includes all others. The median and corresponding IQR for T1(nonA) were 9.560 pg/ml (8.248 - 10.550) and for T1(A) were 7.692 pg/ml (6.580 - 8.854). With the Kruskal–Wallis test a statistically significant difference is establishes between the sRANKL levels, investigated at different treatment points (γ^2=2593, p<0.0001). With the Mann-Whitney test the difference in the sRANKL levels between

T1(A) and T1(nonA) is studied. Their comparison with the levels measured at the remaining treatment points is also investigated (figure 31).

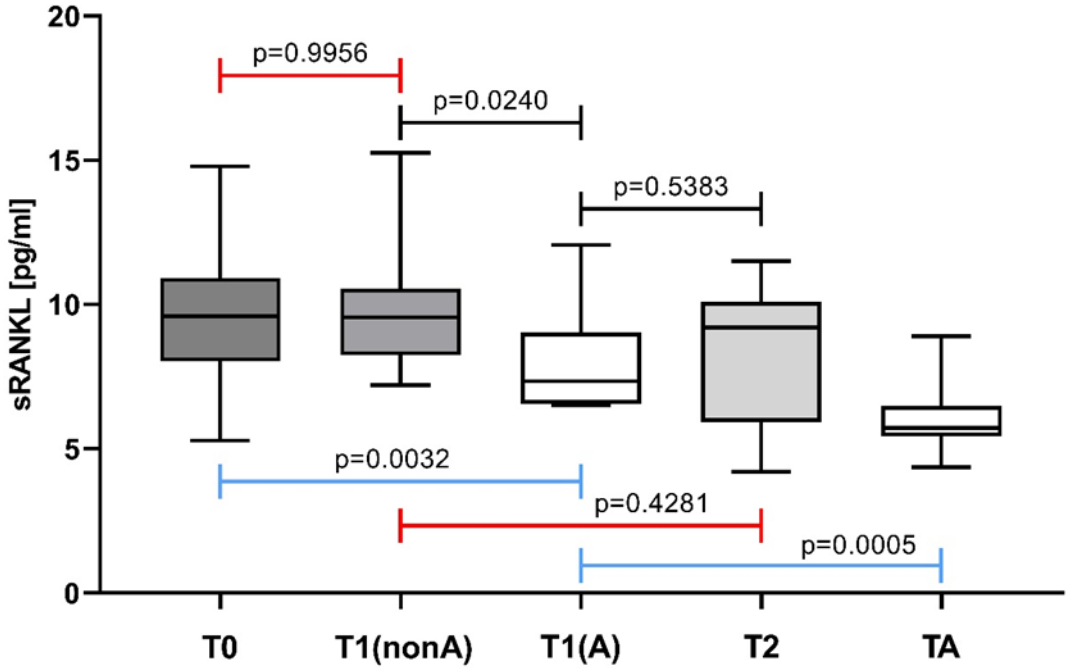

Figure 31. Comparison of sRANKL serum levels in T1(A) and T1(nonA) subgroups with those measured in all other treatment. (The Mann-Whitney test is applied)

Since the T1(A) patients have reached CR and VGPR at this treatment point already (T1), their levels differ significantly from the basic levels before the beginning of the (T1(A) vs T0, p=0.0032), but they are still significantly higher than the levels which have been achieved three months after the transplantation (T1(A) vs TA, p=0.0005). At this treatment point, T1(nonA) patients continue to present high serum sRANKL levels, which are not different from the basic levels (p=0.9956). At the T2 treatment point the non-transplanted patients do not reach a significant decrease of their SRANKL levels already as compared to T1(nonA), p=0.4281 (figure 31).

Comparing T1(A) and T1(nonA) subgroups, it is established that even at this early stage of treatment (T1) they differ significantly in their sRANKL levels (p=0.0240). The levels in subgroup T1(A) drop so significantly that they reach and do not differ from the levels of the non-transplanted patients observed at treatment point T2, p=0.5383 (figure 31).

4.7 Serum levels of sRANKL depending on the treatment response

In order to assess the variations in sRANKL levels according to the therapy response, the patients are categorized into two groups: (1) patients with CR and VGPR and (2) patients who with PR and SD, notwithstanding the treatment point, in which they have achieved it (T1 + T2 + TA). Significantly lower values (p=0.0167) for the serum sRANKL levels are observed in the group with CR and VGPR (median 6.880 pg/mL и IQR 5.923 – 9.210) as compared to those in the group with PR and SD (median 9.361 pg/mL и IQR 7.201 – 10.55). Besides that, the patients with CR and VGPR have significantly lower levels of the parameter tested in relation to its basic levels (p<0.0003) and differ from those of the control group (p=0.0036). The observations of the group with worse response to the therapy are just the opposite. The sRANKL levels in patients with PR and SD are not different

from the basic level at stage T0 (p=0.4608) and are significantly higher than those in the control group (p<0.0001) (figure 32).

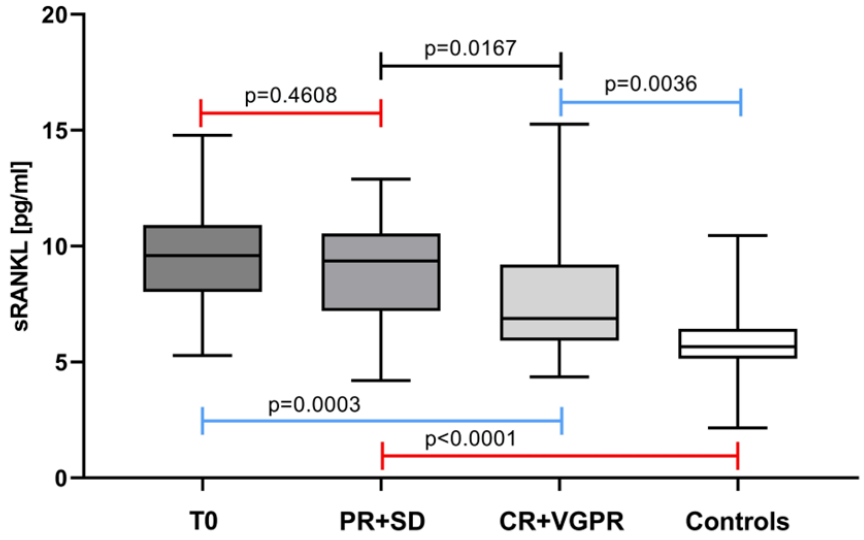

Figure 32. Differences in serum levels of sRANKL according to the treatment response.

For both patient groups the baseline levels (T0 stage) were monitored. For patients with achieved CR and VGPR, the median and corresponding IQR were 9.226 pg/ml (7.570 – 10.860), and for those with PR and SD, the median and corresponding IQR were 9.621 pg/ml (8.367 – 11.130). The Mann-Whitney test revealed no significant difference between them (p=0.4693).

5. OSTEOPONTIN

5.1 Basic serum levels of Osteopontin in newly diagnosed patients with multiple myeloma (NDMM)

The serum levels of osteopontin (OPN) in NDMM patients are investigated immediately after the diagnosis and before the onset of the therapy. The use of the Shapiro-Wilk's test establishes a non-Gaussian distribution of the results which justifies the implementation of nonparametric methods in the statistical analysis of the parameter investigated.

The median and the respective interquartile range of the serum levels of OPN for the patients' group are 596.0 ng/mL (IQR: 479.7 – 793.8 ng/mL), while for the control group are 387.0 ng/mL (IQR: 335.9 - 441.9ng/mL). Thus, the serum levels in the patients' group exceed those observed in the control group by more than 54%. The Mann-Whitney comparative analysis establishes a significant difference between the two groups investigated ($p<0.0001$, figure 33).

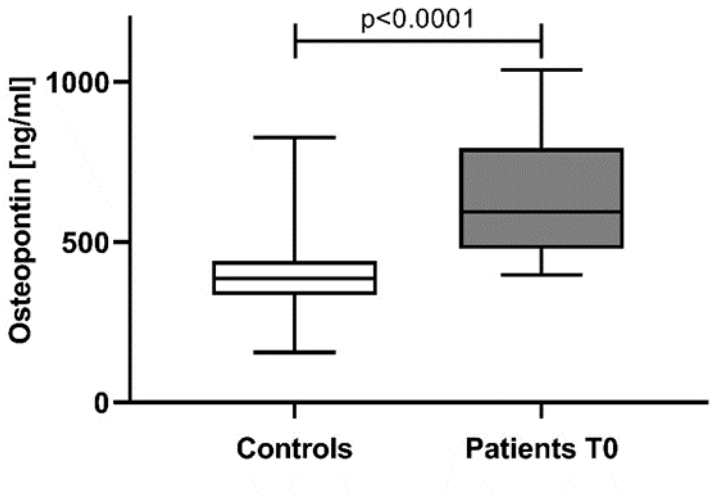

Figure 33. Comparison of serum levels of OPN in controls and patients' groups.
(The Mann-Whitney test is applied)

The influence of sex on the serum levels of OPN is investigated. In both groups (patients' and control group) no statistically significant differences are detected depending on the sex (table 30).

Table 30. Influence of the sex on the serum levels of OPN in the patients' and control groups.

Group investigated	Sex	N	OPN [ng/ml]		P value
			Median	IQR	
NDMM patients	Men	20	539.1	469.3 – 754.9	0.5439
	Women	21	552.0	490.6 – 724.0	
Control individuals	Men	18	397.5	377.1 – 438.3	0.4296
	Women	15	355.8	276.3 - 622.2	

The Mann-Whitney test is applied in the comparative analysis.

The sex distribution does not change the significant difference between patients found for the total group and healthy individuals. Both male and female patients have significantly higher levels of OPN compared to healthy males and females respectively (figure 34).

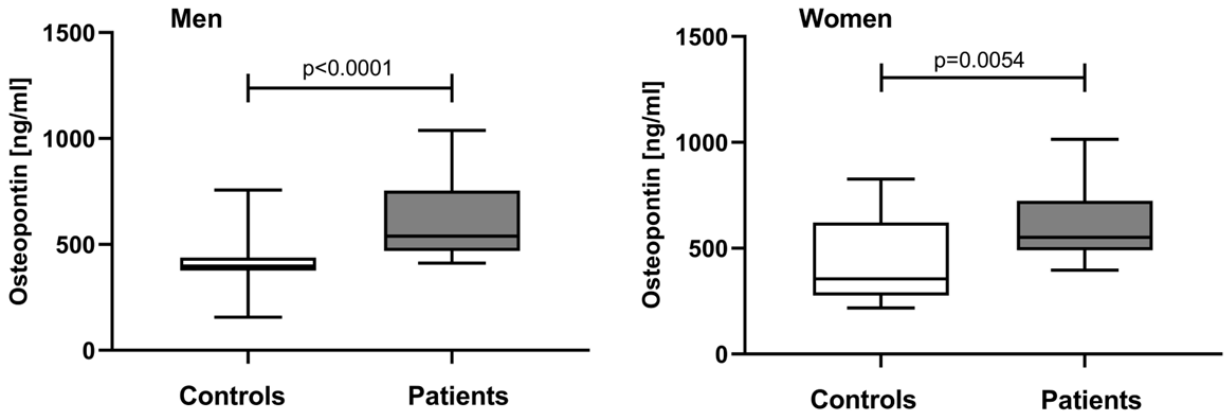

Figure 34. Comparison of serum levels of OPN in the two groups investigated depending on the sex. (The Mann-Whitney test)

Yet it is interesting to point out that while in the NDMM patients' group the men present with negligibly small lower values of OPN in relation to the women, in the control group on the contrary the women are with lower values than the men and although they do not reach a statistical significance, the difference between the values is approximately 11%.

The influence of the age on the levels of OPN is also investigated. The patients' and control groups are subdivided into two additional subgroups according to age. Group A includes patients under 65 years of age and group B includes patients over 65 years of age. No statistically significant differences depending on the age are established in both groups investigated (table 31).

Table 31. Influence of age on the serum levels of OPN in the patients' and control groups.

Group investigated	Age	N	OPN [ng/ml]		P value
			Median	IQR	
NDMM patients	Group A (<65 years)	24	555.5	479.4 – 754.5	0.7635
	Group B (>65 years)	17	551.2	468.1 – 712.0	
Control individuals	Group A (<65 years)	25	396.9	355.8 – 460.0	0.3162
	Group B (>65 years)	8	333.6	271.7 – 442.2	

(The Mann-Whitney test is applied)

The influence of renal function on serum OPN levels is also evaluated. The patients are divided into two groups – with creatinine below and above 177μmol/L. The median and corresponding IQR for the first group are 551.5 ng/ml (472.0 – 701.0) and for the second group 715.0 ng/ml (577.7 – 920.1). The Mann-Whitney comparative test reveals significant difference between the two groups (p=0.0407).

5.2 Serum levels of OPN in NDMM patients depending on the stage of the disease

The results of the serum levels of OPN in patients stratified into three subgroups according to the stage of the disease determined by the IMWG criteria are presented in table 32.

Table 32 Serum levels of OPN in patients stratified according to the stage of the disease.

Stage of the disease	N	OPN [ng/ml]	
		Median	IQR
ISS-I	16	480.0	442.8 – 562.0
ISS-II	6	526.2	477.7 – 715.0
ISS-III	19	715.0	596.0 – 920.1

Gradual increase in the values of OPN is observed with the advancement of the disease stage. Thus, a statistically significant difference is established between the first and the third stage of the multiple myeloma (p=0.0003). The values of OPN at stage ISS-II are higher than those at the first

stage without reaching significant difference (p=0.2938), but they are lower than the ones at stage III almost reaching statistical significance (p=0.05). The results of the comparative analysis are shown in figure 35.

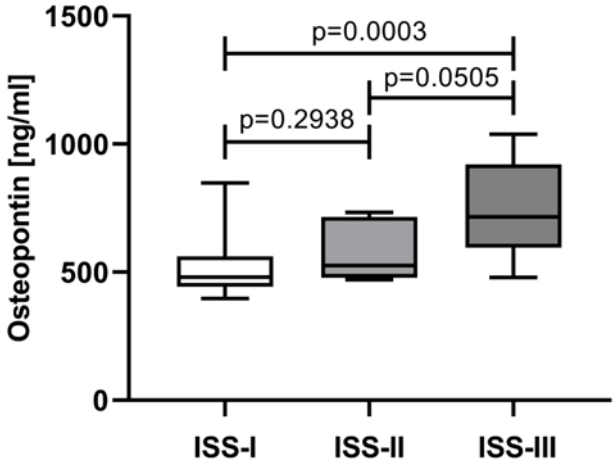

Figure 35. Comparison of the serum levels of OPN of patients stratified into three groups according to their disease stage. (The Mann-Whitney test is applied)

5.3 Serum levels of OPN in NDMM patients depending on the bone disease

Depending on the presence of bone lesions, the patients are divided into two subgroups (G1 and G2). Table 33 displays the results of serum levels of OPN found in these two subgroups.

Table 33. Serum levels of OPN in patients, stratified into subgroups depending on the bone disease.

Bone disease	N	OPN [ng/ml]	
		Median	IQR
G1	9	460.0	419.1 – 494.8
G2	32	669.8	535.5 – 843.1

(G1 – group of patients with zero up to three osteolytic lesions, G2 – group of patients with more than three osteolytic lesions and/or pathological fracture/s available)

With the aid of Mann-Whitney test, a statistically significant difference (p=0.0002) is detected between the patients' subgroups distributed according to the severity of their bone disease (figure 36).

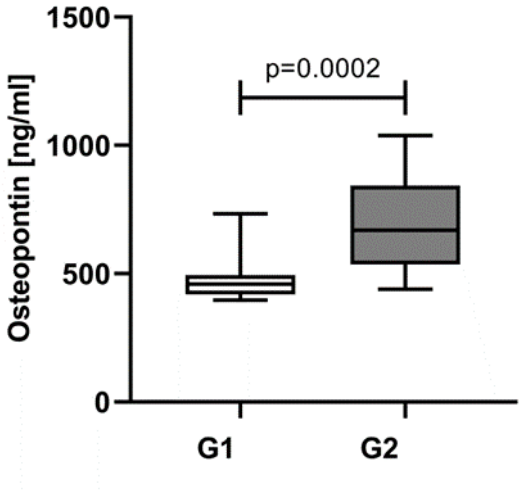

Figure 36. Comparison of serum levels of OPN in both groups investigated.
(The Mann-Whitney test is applied)

Patients in subgroup G1 also had significantly higher OPN values compared to the control group (460.0 ng/ml vs 387.0 ng/ml, respectively, p=0.0146).

5.4 Serum levels of OPN in NDMM patients depending on the bone marrow infiltration with plasma cells

In order to evaluate the influence of the tumor load on the serum levels of OPN, the patients are divided into two subgroups depending on the bone marrow infiltration with plasma cells. A cut-off at 60% is used to differentiate between the subgroups. The results are displayed in table 34.

Table 34. Serum levels of OPN in patients stratified depending on the bone marrow infiltration with plasma cells.

BMI	N	OPN [ng/ml]	
		Median	IQR
PCs <60%	17	521.2	453.9– 646.5
PCs ≥60%	24	676.7	517.7 – 834.3

(BMI – bone marrow infiltration, PCs – plasma cells)

With Mann-Whitney test, statistically significant difference (p=0.0158) is detected between the two subgroups investigated (figure 37).

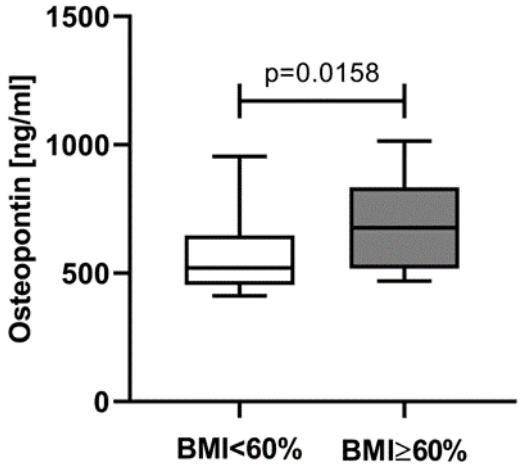

Figure 37. Comparison of the serum levels of OPN in the two groups investigated.
(The Mann-Whitney test is applied)

5.5 Investigating the correlation interrelationship between the serum levels of OPN with routine hematological, morphological and biochemical indicators

Table 35 shows the results of the Spearman correlation analysis carried out to determine the interrelations between the serum levels of OPN with routine hematological, morphological and biochemical indicators.

Table 35. Interrelations between serum levels of OPN with routine hematological, morphological and biochemical indicators.

Parameter investigated	Spearman rho	P value
Hb	**-0.3315**	**0.0342**
WBC	-0.1774	0.2673
PLT	**-0.3099**	**0.0486**
BMI	0.2607	0.0997
β2MG	**0.6968**	**<0.0001**
Creatinine	0.6047	<0.0001
LDH	-0.1802	0.2597
TP	0.1461	0.3621
Alb	-0.2791	0.0773
Ca^{2+}	0.08910	0.6219

Hb – hemoglobin, WBC – white blood cells, PLT – platelets / thrombocytes, Alb – albumin, TP – total protein, LDH – lactate dehydrogenase, β2MG – β2-microglobulin, BMI – bone marrow infiltration

Negative and moderate in strength statistically significant correlation is established between OPN and hemoglobin and platelets and a positive and moderate in strength correlation is detected only with β2-microglobulin. Interdependence is not observed with the remaining parameters investigated. The significant correlations are illustrated in figure 38.

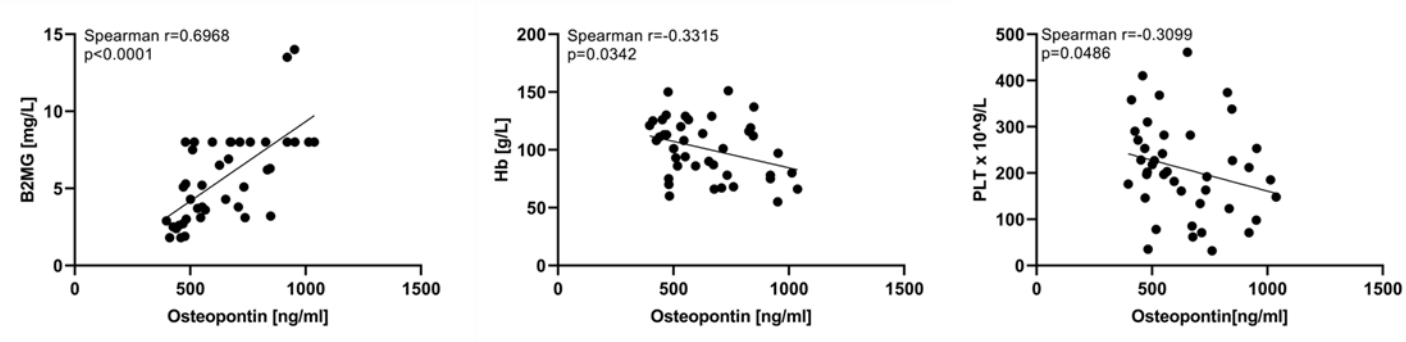

Figure 38. Correlations between OPN and β2-microglobulin, hemoglobin and platelets.
(Nonparametric Spearman correlation analysis is performed)

5.6 Serum levels of OPN in NDMM patients in the course of their treatment

According to the research design, the serum levels of OPN are investigated three times in the course of the treatment (T0, T1, T2 or TA). The results are displayed in table 36.

Table 36. Changes in the serum levels of OPN in the course of the therapy.

Stages	N	OPN [ng/ml]	
		Median	IQR
T0	41	596.0	479.7 – 793.8
T1	22	584.9	487.9– 621.1
T2	11	551.0	383.2 – 714.1
TA	10	346.6	264.9 – 387.9

The Kruskal–Wallis test is carried out, in order to compare the non-Gaussian distribution of the results of more than two independent groups. A statistically significant difference between the levels of OPN investigated at the different treatment points is established (γ^2=24.7, p<0.0001). Additionally, the Mann-Whitney comparative analysis helps to estimate more precisely between which of the treatment points there exists a statistically significant difference. Figure39 displays the results of this analysis.

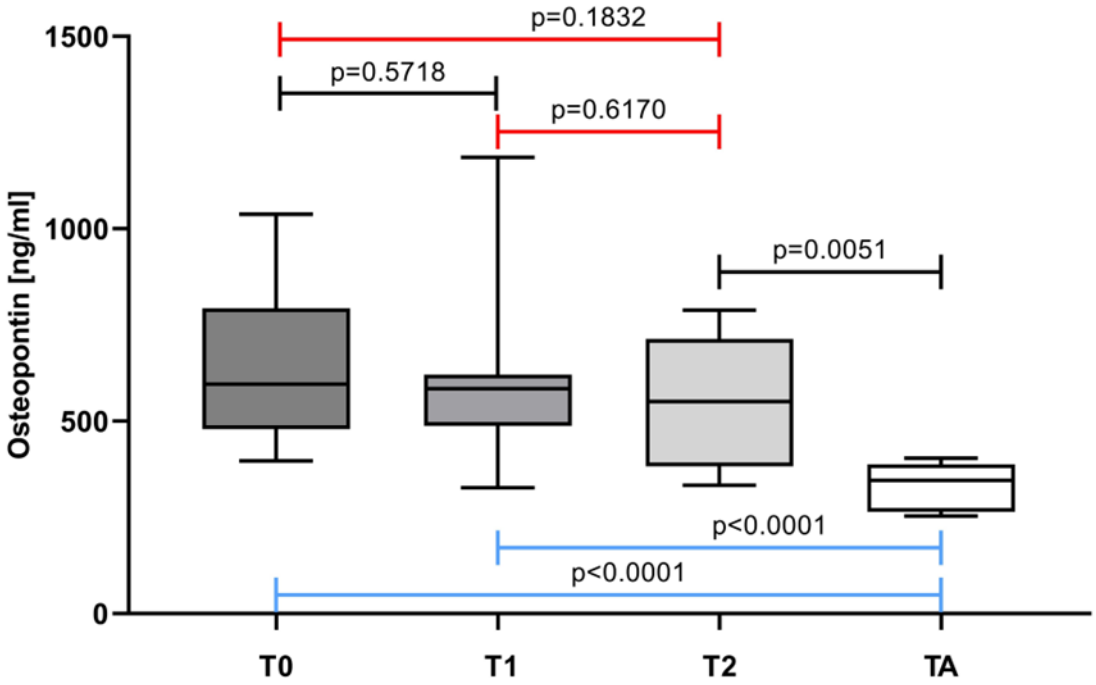

Figure 39. Differences in serum levels of OPN in the course of the treatment of NDMM patients. (The Mann-Whitney test is applied)

The basic serum levels of OPN (T0) differ substantially in relation to those detected at all the remaining treatment points. The serum levels of OPN did not reach statistical significance both after the first 4 cycles of chemotherapy ($p<0.5718$) and after the additional 4 cycles of chemotherapy ($p=0.1832$). The most significant decrease in OPN serum levels is observed in the transplanted patients ($p<0.0001$). Levels in stage T1 did not differ significantly from those of non-transplanted subjects at the end of the study period (T1 vs T2: $p=0.6170$), while a significant lower reduction is achieved in transplanted patients (T1 vs TA, $p<0.0001$). At the end of the study period (stages T2 and TA), the levels between the two groups of patients (non-transplanted / transplanted) showed a statistically significant difference between them (T2 vs TA: $p=0.0051$), with the levels of the non-transplanted being higher than those in transplanted patients. When compared to the control group, transplanted patients achieved values indistinguishable from those of control subjects (TA vs Controls: $p=0.0996$), while T2 levels showed rather significant difference (T2 vs Controls: $p=0.0506$). A similar convergence to control group values was not found at the T1 stage (T1 vs Controls: $p=0.0001$).

The patients who have reached treatment point T1 are subdivided into two subgroups: T1(A) subgroup is formed by patients who are eligible for transplantation and T1(nonA) subgroup includes all others. The median and corresponding IQR for T1(nonA) were 589.5 ng/ml (533.0 - 662.3) and for T1(A) were 522.1 ng/ml (480.3 - 585.7). With the Kruskal–Wallis test a statistically significant difference is establishes between the OPN levels, investigated at different treatment points ($\gamma^2=26.57$, $p<0.0001$). With the Mann-Whitney test the difference in the OPN levels between T1(A) and T1(nonA) is studied. Their comparison with the levels measured at the remaining treatment points is also investigated (figure 40).

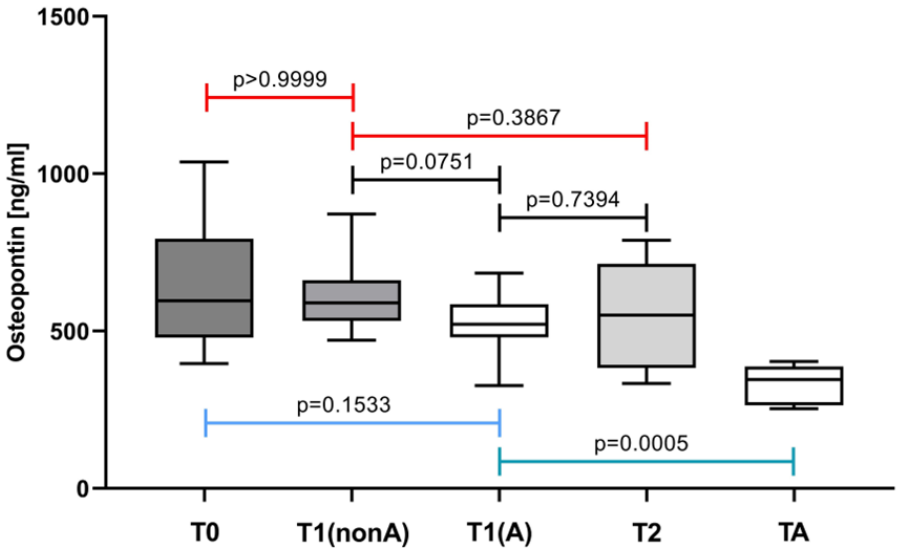

Figure 40. Comparison of OPN serum levels in T1(A) and T1(nonA) subgroups with those measured in all other treatment. (The Mann-Whitney test is applied)

Since the T1(A) patients have reached CR and VGPR at this treatment point already (T1), their levels did not differ significantly from the basic levels before the beginning of the (T1(A) vs T0, p=0.1533), but they are still significantly higher than the levels which have been achieved three months after the transplantation (T1(A) vs TA, p=0.0005). At this treatment point, T1(nonA) patients continue to present high serum OPN levels, which are not different from the basic levels (p>0.9999). At the T2 treatment point the non-transplanted patients did not reach a significant decrease of their OPN levels already as compared to T1(nonA), p=0.3867 (figure 40).

Comparing T1(A) and T1(nonA) subgroups, it is established that even at this early stage of treatment (T1) they did not differ significantly in their OPN levels (p=0.0751). The levels in subgroup T1(A) drop so significantly that they reach and do not differ from the levels of the non-transplanted patients observed at treatment point T2, p=0.7394 (figure 40).

5.7 Serum levels of OPN depending on the treatment response

In order to assess the variations in OPN levels according to the therapy response, the patients are categorized into two groups: (1) patients with CR and VGPR and (2) patients who with PR and SD, notwithstanding the treatment point, in which they have achieved it (T1 + T2 + TA). Significantly lower values (p=0.0134) for the serum OPN levels are observed in the group with CR and VGPR (median 403.8ng/mL и IQR 350.2 – 583.4) as compared to those in the group with PR and SD (median 586.9ng/mL и IQR 471.4 – 724.1). Besides that, the patients with CR and VGPR have significantly lower levels of the parameter tested in relation to its basic levels (p<0.0002) and which do not differ from those of the control group (p=0.2224). The observations of the group with worse response to the therapy are just the opposite. The OPN levels in patients with PR and SD are not different from the basic level at stage T0 (p=0.6591) and are significantly higher than those in the control group (p=0.0004) (figure 41).

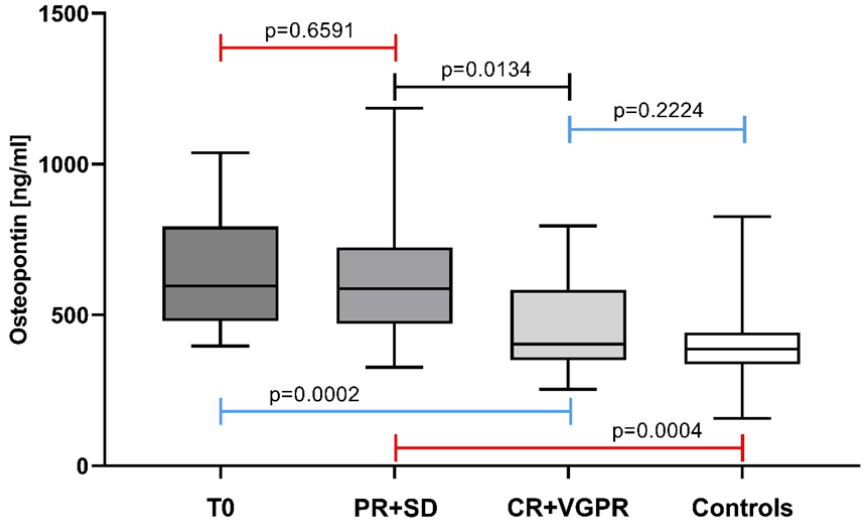

Figure 41. Differences in serum levels of OPN according to the treatment response.
(The Mann-Whitney test is applied)

For both patient groups the baseline levels (T0 stage) were monitored. For patients with achieved CR and VGPR, the median and corresponding IQR were 609.6 ng/ml (518.4 – 743.8), and for those with PR and SD, the median and corresponding IQR were 568.8 ng/ml (479.9 – 831.9). The Mann-Whitney test revealed no significant difference between them (p=0.9118).

6. PERIOSTIN

6.1 Basic serum levels of Periostin in newly diagnosed patients with multiple myeloma (NDMM)

The serum levels of periostin (PON) in NDMM patients are investigated immediately after the diagnosis and before the onset of the therapy. The use of the Shapiro-Wilk's test establishes a non-Gaussian distribution of the results which justifies the implementation of nonparametric methods in the statistical analysis of the parameter investigated.

The median and the respective interquartile range of the serum levels of PON for the patients' group are 648.4 pg/mL (IQR: 594.4 – 809.9 pg/mL), while for the control group are 396.9 pg/mL (IQR: 308.6 – 471.9 pg/mL). Thus, the serum levels in the patients' group exceed those observed in the control group by more than 63%. The Mann-Whitney comparative analysis establishes a significant difference between the two groups investigated ($p<0.0001$, figure 42).

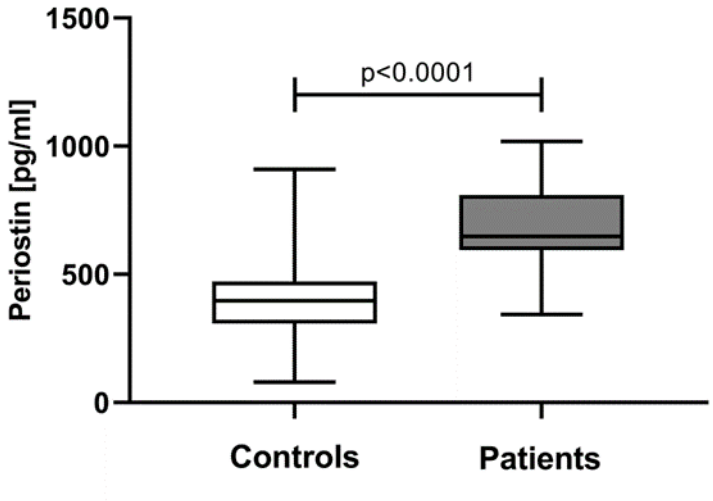

Figure 42. Comparison of serum levels of PON in controls and patients' groups.
(The Mann-Whitney test is applied)

The influence of sex on the serum levels of PON is investigated. While in the patient group no gender difference was found ($p=0.2680$), in the control group women had statistically .significantly higher values compared to men ($p=0.0176$) (table 37).

Table 37 Influence of the sex on the serum levels of PON in the patients' and control groups.

Group investigated	Sex	N	PON [pg/ml]		P value
			Median	IQR	
NDMM patients	Men	20	641.8	538.7 – 832.5	0.2680
	Women	21	554.7	492.0 – 700.2	
Control individuals	Men	18	330.6	305.5 – 456.1	0.0176
	Women	15	453.6	386.6 – 534.0	

The Mann-Whitney test is applied in the comparative analysis.

Yet it is interesting to point out that while in the NDMM patients' group the men present with negligibly small higher values of PON in relation to the women, in the control group on the contrary the women are with higher values than the men and they reach a statistical significance (p=0.0176).

The sex distribution does not change the significant difference between patients found for the total group and healthy individuals. Both male and female patients have significantly higher PON levels compared to healthy males and females respectively (figure 43).

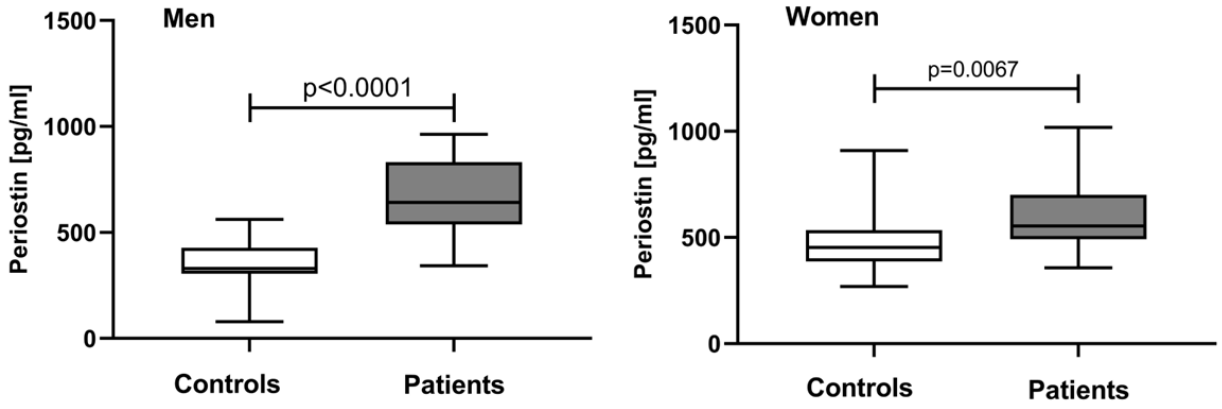

Figure 43. Comparison of serum levels of PON in the two groups investigated depending on the sex. (The Mann-Whitney test)

The influence of the age on the levels of PON is also investigated. The patients' and control groups are subdivided into two additional subgroups according to age. Group A includes patients under 65 years of age and group B includes patients over 65 years of age. No statistically significant differences depending on the age are established in both groups investigated (table 38).

Table 38. Influence of age on the serum levels of PON in the patients' and control groups.

Group investigated	Age	N	PON [pg/ml]		P value
			Median	IQR	
NDMM patients	Group A (<65 years)	24	619.7	492.4 – 720.6	0.8291
	Group B (>65 years)	17	638.1	511.4 – 820.3	
Control individuals	Group A (<65 years)	25	383.2	305.5 – 446.4	0.1668
	Group B (>65 years)	8	468.4	396.9- 498.6	

(The Mann-Whitney test is applied)

The influence of renal function on serum PON levels is also evaluated. The patients are divided into two groups – with creatinine below and above 177μmol/L. The median and corresponding IQR for the first group are 616.1 pg/ml (523.2 – 716.8) and for the second group 645.5 pg/ml (492.0 – 833.4). The Mann-Whitney comparative test reveals no significant difference between the two groups (p=0.7745).

6.2 Serum levels of PON in NDMM patients depending on the stage of the disease

The results of the serum levels of PON in patients stratified into three subgroups according to the stage of the disease determined by the IMWG criteria are presented in table 39.

Table 39. Serum levels of PON in patients stratified according to the stage of the disease.

Stage of the disease	N	PON [pg/ml]	
		Median	IQR
ISS-I	16	563.2	432.8 – 620.6
ISS-II	6	669.0	632.3 – 761.4
ISS-III	19	807.6	719.6 – 860.5

Gradual increase in the values of PON is observed with the advancement of the disease stage. Thus, a statistically significant difference is established between the first and the second stage of the multiple myeloma (p=0.0012), as well between the first and the third stages (p<0.0001). The values

of PON at stage ISS-II are higher than those at the first stage and lower than the ones at stage three but a statistical significance is reached only with stage one. The results of the comparative analysis are shown in figure 44.

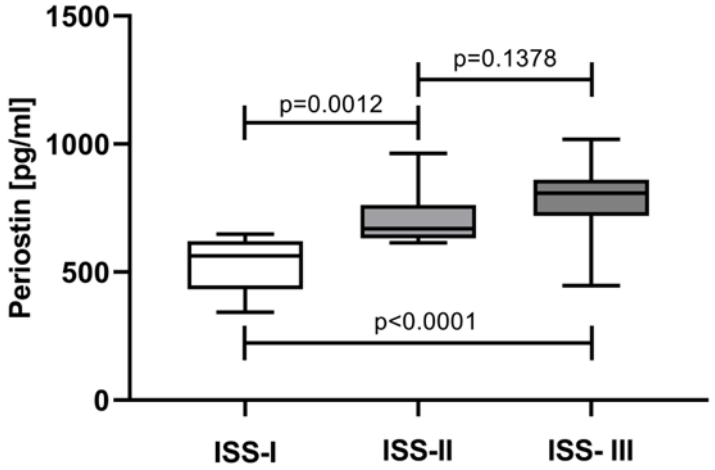

Figure 44. Comparison of the serum levels of PON of patients stratified into three groups according to their disease stage. (The Mann-Whitney test is applied)

6.3 Serum levels of PON in NDMM patients depending on the bone disease

Depending on the presence of bone lesions, the patients are divided into two subgroups (G1 and G2). Table 40 displays the results of serum levels of PON found in these two subgroups.

Table 40. Serum levels of PON in patients, stratified into subgroups depending on the bone disease.

Bone disease	N	PON [pg/ml]	
		Median	IQR
G1	9	463.8	431.0 – 596.4
G2	32	706.8	624.0 – 833.6

(G1 – group of patients with zero up to three osteolytic lesions, G2 – group of patients with more than three osteolytic lesions and/or pathological fracture/s available)

With the aid of Mann-Whitney test, a statistically significant difference (p=0.0002) is detected between the patients' subgroups distributed according to the severity of their bone disease (figure 45).

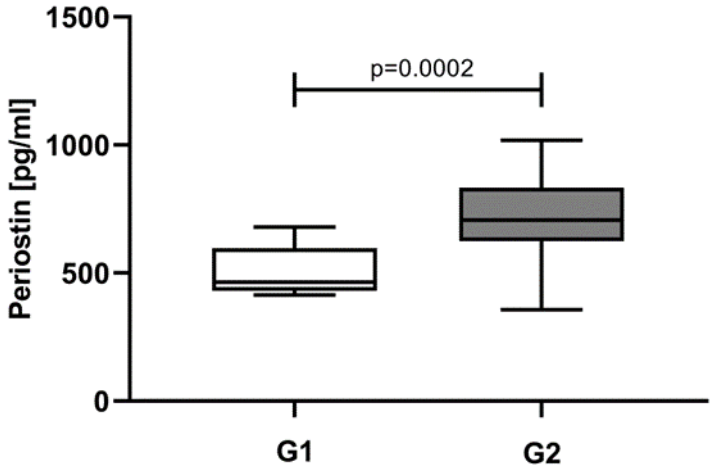

Figure 45. Comparison of serum levels of PON in both groups investigated.
(The Mann-Whitney test is applied)

Patients in subgroup G1 also had significantly higher PON values compared to the control group (463.8 pg/ml vs 396.9 pg/ml, respectively, p=0.0231).

6.4 Serum levels of PON in NDMM patients depending on the bone marrow infiltration with plasma cells

In order to evaluate the influence of the tumor load on the serum levels of PON, the patients are divided into two subgroups depending on the bone marrow infiltration with plasma cells. A cut-off at 60% is used to differentiate between the subgroups. The results are displayed in table 41.

Table 41. Serum levels of PON in patients stratified depending on the bone marrow infiltration with plasma cells.

BMI	N	PON [pg/ml]	
		Median	IQR
PCs <60%	17	585.6	436.3 – 660.1
PCs ≥60%	24	726.9	639.9 – 855.1

(BMI – bone marrow infiltration, PCs – plasma cells)

With Mann-Whitney test, statistically significant difference (p=0.9843) is detected between the two subgroups investigated (p=0.0003) (figure 46).

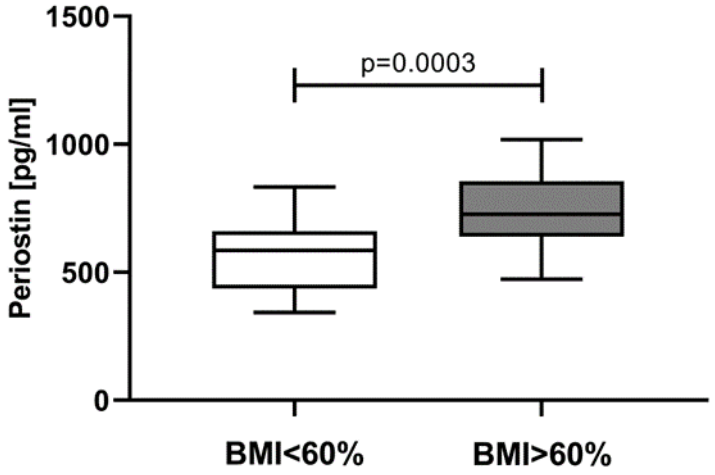

Figure 46. Comparison of the serum levels of PON in the two groups investigated.
(The Mann-Whitney test is applied)

6.5 Investigating the correlation interrelationship between the serum levels of PON with routine hematological, morphological and biochemical indicators

Table 42 shows the results of the Spearman correlation analysis carried out to determine the interrelations between the serum levels of PON with routine hematological, morphological and biochemical indicators.

Table 42. Interrelations between serum levels of PON with routine hematological, morphological and biochemical indicators.

Parameter investigated	Spearman rho	P value
Hb	**-0.4858**	**0.0013**
WBC	-0.1474	0.3578
PLT	**-0.4457**	**0.0035**
BMI	**0.4172**	**0.0067**
β2MG	**0.7791**	**<0.0001**
Creatinine	**0.5311**	**0.0004**
LDH	-0.08372	0.6028
TP	**0.4577**	**0.0026**
Alb	**-0.6540**	**<0.0001**
Ca^{2+}	-0.1451	0.4204

Hb – hemoglobin, WBC – white blood cells, PLT – platelets / thrombocytes, Alb – albumin, TP – total protein, LDH – lactate dehydrogenase, β2MG – β2-microglobulin, BMI – bone marrow infiltration

Negative and moderate in strength statistically significant correlation is established between PON and hemoglobin and platelets and with albumin – a negative and significant correlation. Positive and moderate in strength correlation is detected with BMI, whereas with β2-microglobulin and creatinine the correlation is positive and strong in strength. Interdependence is not observed with the remaining parameters investigated. The significant correlations are illustrated in figure 47.

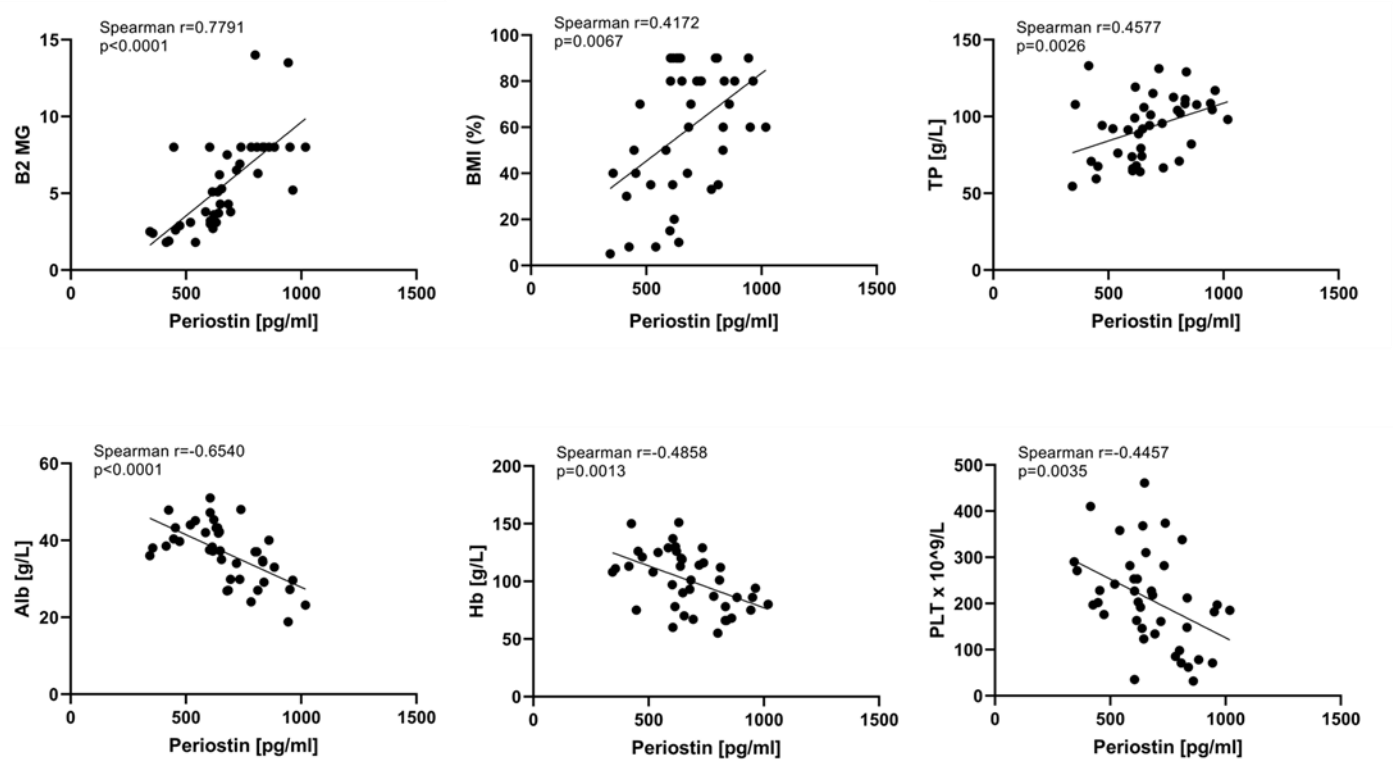

Figure 47. Correlations between PON and β2-microglobulin, hemoglobin, albumin, platelets, BMI, total protein.

6.6 Serum levels of PON in NDMM patients in the course of their treatment

According to the research design, the serum levels of PON are investigated three times in the course of the treatment (T0, T1, T2 or TA). The results are displayed in table 43.

Table 43. Changes in the serum levels of PON in the course of the therapy.

Stages	N	PON [pg/ml]	
		Median	IQR
T0	41	648.4	594.4 – 809.9
T1	22	565.4	426.9 – 633.2
T2	11	466.2	305.5 – 573.2
TA	10	407.9	322.4– 447.6

The Kruskal–Wallis test is carried out, in order to compare the non-Gaussian distribution of the results of more than two independent groups. A statistically significant difference between the levels of PON investigated at the different treatment points is established (γ^2=25.49, p<0.0001). Additionally, the Mann-Whitney comparative analysis helps to estimate more precisely between which of the treatment points there exists a statistically significant difference. Figure 48 displays the results of this analysis.

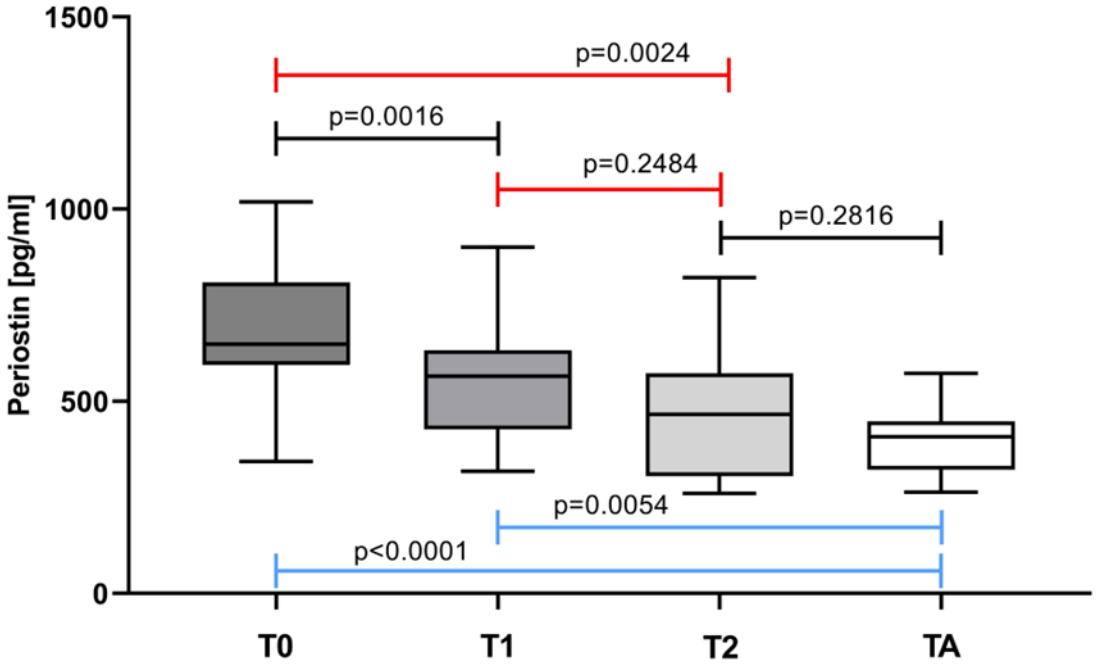

Figure 48. Differences in serum levels of PON in the course of the treatment of NDMM patients. (The Mann-Whitney test is applied)

The basic serum levels of PON (T0) differ substantially in relation to those detected at all the remaining treatment points. The degree of significance gradually increases. Straight after the first four cycles of chemotherapy the levels drop significantly (p<0.0016). In the patients who have not undergone transplantation, after additional four cycles of chemotherapy, the significant difference is preserved (p<0.0024), while in the patients who have undergone transplantation the most significant decrease of serum levels of PON (p<0.0001) is observed. The levels at the T1 are not significantly different from those of the patients who have not undergone transplantation (T1 vs T2: p=0.2484), while a significant decrease is achieved in the patients who have undergone transplantation (T1 vs TA, p=0.0054). At the end of the study period, PON levels in non-transplanted patients were higher than those of transplanted patients, but no statistically significant difference was reached (T2 vs TA: p=0.2816). Their levels do not differ statistically from the control group (TA vs Controls: p>0.9999; T2 vs Controls: p=0.1899). Similar convergence to control group values was not found at T1 stage (T1 vs Controls: p=0.0005) (figure 48).

The patients who have reached treatment point T1 are subdivided into two subgroups: T1(A) subgroup is formed by patients who are eligible for transplantation and T1(nonA) subgroup includes all others. The median and corresponding IQR for T1(nonA) were 584.8 pg/ml (409.4 – 638.1) and

for T1(A) were 500.4 pg/ml (432.5 - 559.6). With the Kruskal–Wallis test a statistically significant difference is establishes between the PON levels, investigated at different treatment points (γ^2=27.15, p<0.0001). With the Mann-Whitney test the difference in the PON levels between T1(A) and T1(nonA) is studied. Their comparison with the levels measured at the remaining treatment points is also investigated (figure 49).

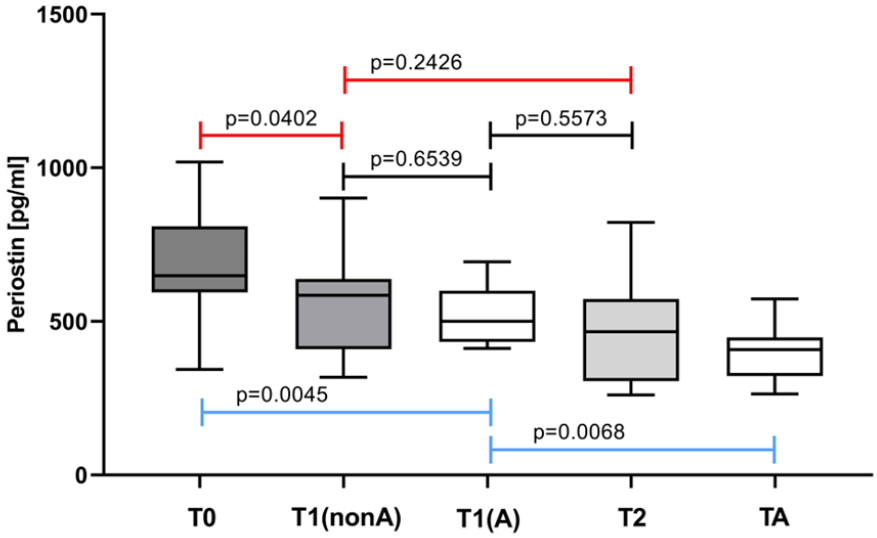

Figure 49. Comparison of PON serum levels in T1(A) and T1(nonA) subgroups with those measured in all other treatment. (The Mann-Whitney test is applied)

Since the T1(A) patients have reached CR and VGPR at this treatment point already (T1), their levels differ significantly from the baseline levels (T1(A) vs T0, p=0.0045), but they are still significantly higher than the levels which have been achieved three months after the transplantation (T1(A) vs TA, p=0.0068). Although serum PON levels in T1(nonA) patients were higher than those in T1(A), no statistical difference was reached between them (p=0.6539). It is important to note that even at this stage of treatment T1(nonA) levels are also different from baseline levels (p=0.0402). At T2 stage, non-transplanted patients did not achieve a significant decrease in their levels compared to T1(nonA), p=0.2426. The levels in subgroup T1(A) drop so significantly that they reach and do not differ from the levels of the non-transplanted patients observed at treatment point T2, p=0.5573 (figure 49).

6.7 Serum levels of PON depending on the treatment response

In order to assess the variations in PON levels according to the therapy response, the patients are categorized into two groups: (1) patients with CR and VGPR and (2) patients who with PR and SD, notwithstanding the treatment point, in which they have achieved it (T1 + T2 + TA). Significantly lower values (p=0.0510) for the serum PON levels are observed in the group with CR and VGPR (median 432.0 pg/mL and IQR 327.8 – 572.5) as compared to those in the group with PR and SD (median 511.9 pg/mL and IQR 432.7 – 638.1). Moreover, patients with CR and VGPR have significantly lower levels for the parameter tested in relation to its baseline levels (p<0.0001), and at the same time, indistinguishable from those of the control group (p=0.2408). Regarding the PON levels in the group of patients with PR and SD, they also different from the baseline levels (T0 stage: p=0.0068), but remained significantly higher than those in the control group (p=0.0009) (figure 50).

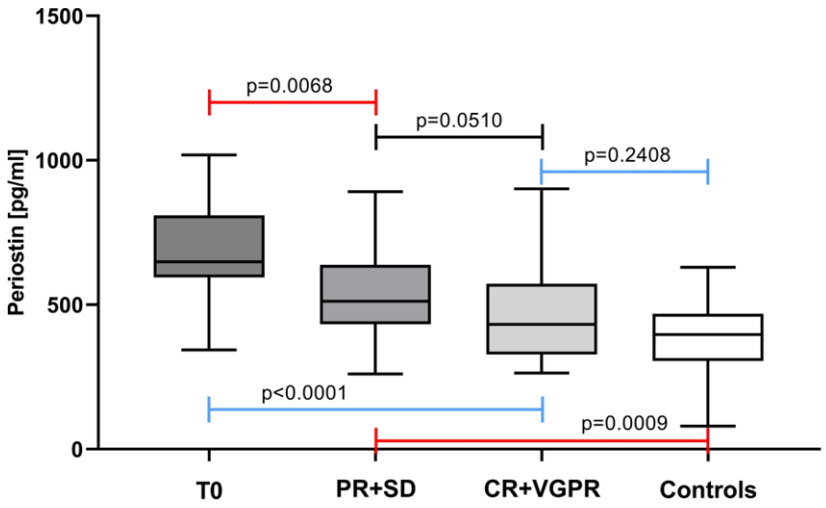

Figure 50. Differences in serum levels of PON according to the treatment response.

For both patient groups the baseline levels (T0 stage) were monitored. For patients with achieved CR and VGPR, the median and corresponding IQR were 685.4 pg/ml (585.7 – 819.0), and for those with PR and SD, the median and corresponding IQR were 546.6 pg/ml (474.3 – 739.2). The Mann-Whitney test showed no significant difference between them (p=0.1655).

7. DIAGNOSTIC RELIABILITY AND PROGNOSTIC VALUE OF DKK-1, SCLEROSTIN, SRANKL, OSTEOPONTIN AND PERIOSTIN AS MARKERS FOR ASSESSING THE MYELOMA BONE DISEASE

7.1 Diagnostic reliability of the bone biomarkers investigated

A ROC analysis is done (receiver operating characteristic – operating characteristic curves of the observer) to assess the diagnostic reliability of the biomarkers investigated, i.e. to what extent they could differentiate in a reliable manner the healthy individuals (control group) from the NDMM patients. Figure 51represents the results of the analysis performed.

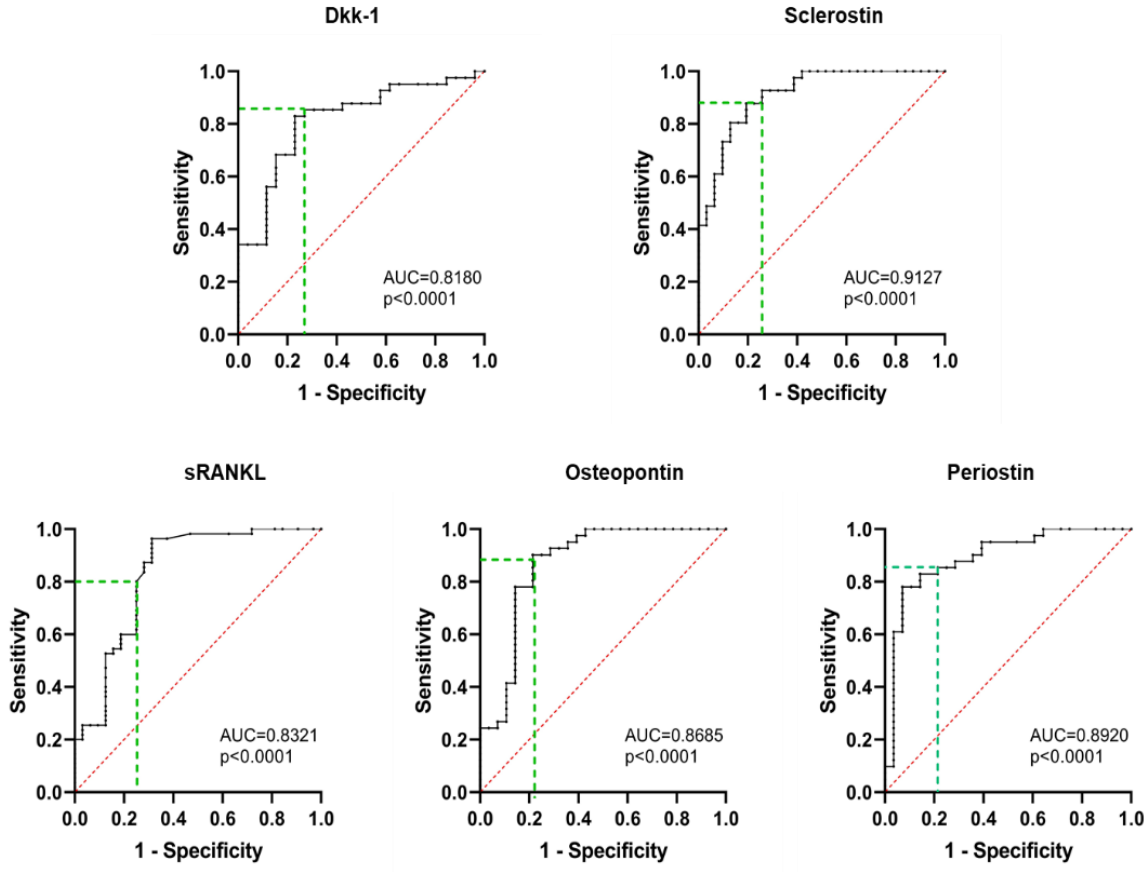

Figure 51. Diagnostic reliability of Dkk-1, sclerostin, sRANKL, osteopontin and periostin.

The area under the curve (AUC) has the value of more than 0.8 at p<0.0001 for all parameters investigated. Two of the parameters (sclerostin and periostin) reach a value close to 0.9 (for sclerostin AUC=0.9127, and for periostin AUC=0.8920). These values close to one, obviously demonstrate that the parameters investigated possess a high diagnostic reliability.

According to the results of all pairs of diagnostic sensitivity and diagnostic specificity of each of the bone biomarkers investigated, the cut-off value is also determined. It reliably distinguishes between the healthy subjects and the patients with their corresponding sensitivity and specificity (table 44).

Table 44. Cut-off values of bone biomarkers assessed through ROC analysis.

Parameter [unit]	Cut-off value	Sensitivity	95% CI	Specificity	95% CI	Likelihood ratio (LR)
Dkk-1 [ng/mL]	52.64	0.8293	0.6874 to 0.9147	0.7692	0.5795 to 0.8897	3.593
Sclerostin [ng/mL]	472.1	0.8780	0.7446 to 0.9468	0.8065	0.6372 to 0.9081	4.537
sRANKL [pg/mL]	8.067	0.8000	0.6764 to 0.8845	0.7500	0.5789 to 0.8675	3.200
Osteopontin [ng/mL]	447.1	0.9024	0.7745 to 0.9614	0.7857	0.6046 to 0.8979	4.211
Periostin [pg/mL]	473.1	0.8537	0.7156 to 0.9312	0.7857	0.6046 to 0.8979	3.984

The positive coefficient of probability ($LR = \frac{\text{sensitivity}}{1-\text{specificity}}$) for all biomarkers investigated is more than 1 and shows how many times the chance is bigger that the examined person is with bone disease if his result is more than the definite cut-off value.

7.2 Dkk-1, sclerostin, sRANKL, osteopontin and periostin as predictors for the development of myeloma bone disease

A statistically significant difference of the values is found for all the investigated parameters depending on the degree of the bone disease. This allowed us to investigate the prognostic value of each one of the parameters and their possibility to correctly distinguish the patients whose skeletal system is slightly damaged (G1) from those whose skeletal system is severely damaged (G2) by implementing ROC analysis (figure 52).

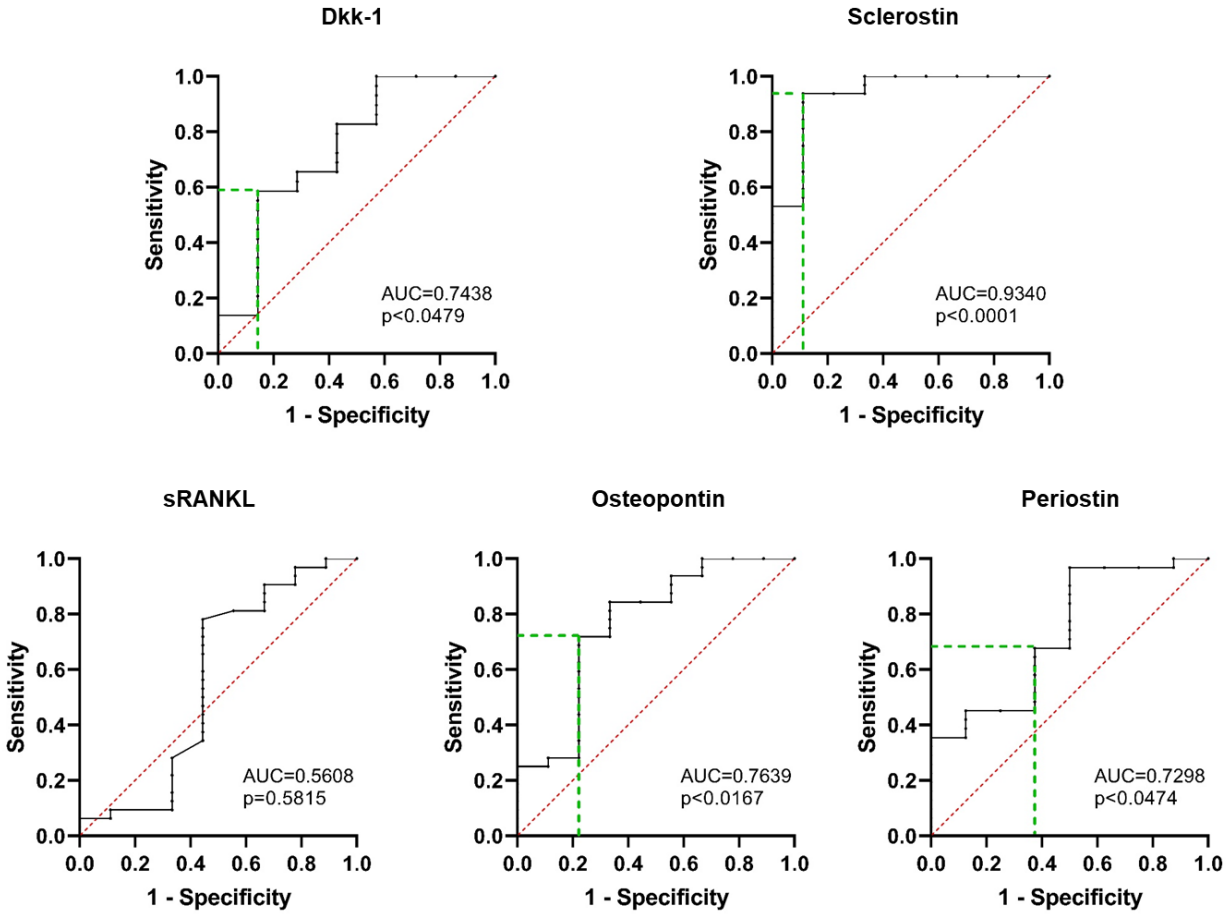

Figure 52. Dkk-1, sclerostin, sRANKL, osteopontin and periostin as predictors for development of bone disease in MM.

The ROC analysis shows the highest AUC value for sclerostin (0.9340, p<0.0001), followed by those for osteopontin (AUC=0.7639, p=0.0167), Dkk-1 (AUC=0.7438, p=0.0479 and periostin (AUC=0.7298, p=0.0474). The value for sRANKL AUC is close to 0.5 at p>0.5, which makes it unsuitable for a reliable distinguishing between patients with a different degree of bone disease. For all the remaining parameters, a cut-off value is estimated with its corresponding diagnostic specificity and diagnostic sensitivity (table 45).

Table 45. Cut-off values of Dkk-1, sclerostin, osteopontin and periostin for prediction of bone disease in MM.

Parameter [unit]	Cut-off value	Sensitivity	95% CI	Specificity	95% CI	Likelihood ratio
Dkk-1 [ng/mL]	62.05	0.5862	0.4074 to 0.7449	0.8571	0.4869 to 0.9927	4.103
Sclerostin [ng/mL]	502.1	0.9375	0.7985 to 0.9889	0.8889	0.5650 to 0.9943	8.438
Osteopontin [ng/ml]	514.0	0.7188	0.5463 to 0.8444	0.7778	0.4526 to 0.9605	3.234
Periostin [pg/ml]	545.9	0.6774	0.5014 to 0.8143	0.6250	0.3057 to 0.8632	1.806

A logistic regression analysis is applied additionally in order to assess the individual effect of the biomarkers investigated on the myeloma bone disease. Based on its non-satisfactory result from the ROC analysis, sRANKL is not included in the logistic regression. Initially, the influence of each separate parameter is analyzed as an independent predictor for successful progression of the bone disease. The results of the analyses performed are summarized in table 46.

Table 46. Summary of results from the logistic regression analysis performed separately for each one of the parameters.

Parameter [unit]	χ^2	df	P value	% correct distribution of the patients according to MBD (Overall Percentage)
Dkk-1 [ng/mL]	10.020	1	0.002	87.8
Sclerostin [ng/mL]	23.239	1	<0.001	87.8
Osteopontin [ng/ml]	13.077	1	<0.001	87.8
Periostin [pg/ml]	23.677	1	<0.001	87.8

For each independent indicator the regression model is statistically significant in relation to the prognosis. Regression models are constructed as well which combine predictors two by two. They

reflect the inhibition of the osteoblast activity or the activation of the osteoclast activity. The results of these analyses are displayed in table 47.

Table 47. Summary of results from the regression analyses when combining the predictors.

Parameter	χ^2	df	P value	% correct distribution of the patients according to MBD (Overall Percentage)
Dkk-1 + Sclerostin	25.924	2	0.002	87.8
Osteopontin + Periostin	24.007	2	<0.001	92.7

When two independent variables are reported simultaneously, they predict the correct distribution of the patients according to the degree of the bone damage with statistical significance. On examining the Wald criterion in the models thus constructed, one of the two independent variables is not statistically significant – osteopontin for the osteoclast markers and Dkk-1 for the osteoblast markers (table 48 and table 49).

Table 48. Osteoclast independent variables in the regression equation

	B	S.E.	Wald	df	Sig.	Exp(B)	95% CI for EXP(B)	
							Lower	Upper
OPN	-.003	.005	.290	1	.590	.997	.988	1.007
PON	-.020	.009	5.548	1	**.019**	.980	.964	.997
Constant	12.299	4.523	7.393	1	**.007**	219483.286		

a. Variable(s) entered: OPN, PON.

Table 49. Osteoblast independent variables in the regression equation

	B	S.E.	Wald	df	Sig.	Exp(B)	95% CI for EXP(B)	
							Lower	Upper
Scl	-.041	.018	5.073	1	**.024**	.960	.927	.995
Dkk-1	-.152	.109	1.957	1	.162	.859	.694	1.063
Constant	29.081	13.891	4.383	1	**.036**	426389167 2629.099		

a. Variable(s) entered: Scl, Dkk-1.

A new, two factor regression model is constructed that includes statistically significant independent variables from previous analyses (sclerostin and periostin). The new regression model is statistically significant: $\chi^2=29.465$, $df=2$ u $p<0.001$. The model explains 51.3% (Cox & Snell R2) and 78.7% (Nadelkerkes R2) of the dispersion and classifies correctly 90.2% of the observations of bone disease (93.8% for G2 and 77.7% for G1). On examining the Wald criterion in the model thus constructed both independent variables are statistically significant and influence substantially the prognosis regarding the degree of the bone disease. The values of their regression coefficients are: periostin – -0.019, sclerostin – -0.028, while the value of the regression constant is 24.675 (table 50).

Table 50. Osteoclast and osteoblast variables in the regression equation

	B	S.E.	Wald	df	Sig.	Exp(B)	95% C.I.for EXP(B)	
							Lower	Upper
PON	-.019	.009	4.056	1	**.044**	.982	.964	1.000
Scl	-.028	.015	3.574	1	**.050**	.972	.944	1.001
Constant	24.675	10.028	6.054	1	**.014**	520105313 48.956		

Variable(s) entered: PON, Scl.

From the step-by-step regression analyses performed, the last one is the most significant. The coefficients calculated with this regression analysis help us to generate a regression equation, which can give a correct answer with a very high probability for specific values of the biomarkers periostin and sclerostin for a given patient, what degree of bone disease he would have, i.e. whether he would be classified into group G1 or G2. The equation looks as follows:

$$p = \frac{e^{24.675\ -0.019[PON]-0.028[Scl]}}{1+\ e^{24.675\ -0.019[PON]-0.028[Scl]}}$$

The ROC analyses performed confirm the high diagnostic reliability of the bone biomarkers investigated while the regression analysis outlines two of them which could predict the bone illness with the greatest degree of probability.

V. DISCUSSION

As a metabolically active and dynamic structure, the bone tissue continuously renews itself during the individual human life by the processes of physiological bone remodeling. These processes are strictly regulated and are of a substantial importance for the preservation of the bone integrity and the maintenance of the mineral homeostasis. The imbalance in the processes of bone remodeling with a prevalence of bone resorption over bone formation is a key factor in the pathogenesis of MBD. Among the many pathogenetic factors that determine the dysregulated balance of bone remodeling processes, we have investigated several to characterize the main aspects of MBD, namely Dkk-1 and sclerostin as inhibitors of osteoblast activity, sRANKL as a major osteoclast activator, and osteopontin and periostin as important ECM proteins providing a favorable environment in the bone marrow microenvironment for the colonization, proliferation and expansion of clonal myeloma plasma cells.

1. Markers for impaired osteoblastic activity – DKK-1 and Sclerostin

Wnt signaling pathway is crucial for the regulation of the osteoblastic bone formation. The soluble antagonists of the Wnt pathway, Dkk-1 and Scl, are critical components of this system and play an important role for the suppressed osteoblast function, observed in MBD [20]. Dkk-1, secreted by OBs, BMSCs and MPCs, through the concurrent coupling with Wnt receptors suppresses the differentiation of BMSCs in mature OBs, and it also blocks the further maturation of OBs [113,363]. The other soluble Wnt inhibitor, Scl, which also regulates bone formation, is interesting is interesting in that it is not synthesized by the tumor MPCs, but solely by osteocytes and their precursors [226]. Colucci et al. in *in vitro* experimental model detect a significant decrease of bALP, osteocalcin and sialoprotein II, as well as the formation of colony forming units of OBs, which proves the important suppressive function of Scl on the OBs differentiation [64]. The inhibited osteoblastogenesis predetermines the suppressed formation of the bone organic matrix and its mineralization [109,417].

1.1 Baseline serum levels of Dkk-1 and Scl in NDMM patients

Since in the scientific literature there exist data on the influence of sex and age on the serum level values of Dkk-1 and Scl, we subdivided the two major groups (patients and controls) into subgroups depending on these biological factors. For Dkk-1 statistically significant differences are not identified depending on the sex both for the patients' group and the control group. It is obvious though that the values of Dkk-1 in the women of the control group are approximately 11% higher than those in men although they do not reach a statistically significant difference. There exist data in the literature on the estrogens as negative regulators of the gene expression of Dkk-1. For instance increased expression of Dkk-1 is detected in ovariectomized rats [304]. In research with human subjects, higher levels of Dkk-1 in women in comparison to men are also found [92] . The observed trend for slightly higher values in the women we studied could be explained by the fact that most of them are in menopause, in which the possibility of estrogens to suppress the Dkk-1 expression disappears. Regarding Scl, no significant sex differences were found in the patient group, whereas in the control group significantly higher values were observed in men than in women. A similar difference with higher serum levels of Scl in men are described by other authors as well [9,92]. It should be pointed out that the division according to sex does not change the established significant difference between sick and healthy individuals in the whole group. Both sick men and sick women present with a significantly higher values for both Scl and Dkk-1 in comparison to the healthy men and women respectively.

According to most of the literature data, the values of both studied parameters increase with increasing age [9,13,25,92,284]. Such correlation both in the patients' and control group is not detected in this investigation. This is most probably due to the fact that both age subgroups encompass only the age range above 50 years of age while the studies mentioned includes individuals that cover a much wider age range which is a prerequisite for the presence of age differences.

It can be concluded that the pathological process in the investigated cohort of NDMM patients is stronger than the influence of biological factors such as age and sex and has a decisive importance for the significantly higher levels of Dkk-1 and Scl observed at the diagnosis. This allows us to not take into consideration sex and age in the further discussions of the changes in the serum levels of the tested proteins.

At the diagnosis for both tested parameters, significantly higher values are identified in the patients' group as compared to the control group. Thus Dkk-1values in the patients' group exceed those of the control group ($p<0.0001$) by more than 30%, while the Scl values are multiple times higher (about 8 times) than the ones of the individuals in the control group ($p<0.0001$). In 2010, Heiland et al., while investigating the pathogenesis of bone loss in inflammatory conditions proved in an experimental model the role of the proinflammatory cytokine TNF-α in the suppression of bone tissue formation. Since TNF-α is an important inducer of the expression of both Wnt inhibitors Dkk-1 и Scl, the authors analyse interactions among these three cytokines – TNF-α, Dkk-1 and Scl. When influencing primary osteoblasts with TNF-α the expression of sclerostin mRNA significantly increases as well as the expression of the protein itself. Besides, the blocking of Dkk-1 through the application of anti-Dkk-1 antibody entirely neutralizes the Scl synthesis induced by TNF-α. This suggests that Dkk-1 probably takes part in the induction of the expression of the SOST gene (the gene for Scl). On the other hand the addition of recombinant Dkk-1 to OBs drastically increases the levels of Scl and this effect is prevented by adding anti- Dkk-1 antibody [131]. Later these observations are confirmed by Eda et al. already in an experimental mouse model of MM. Immunodeficient xenograft mice are injected intravenously with human MPCs, which provokes the development of MM. A significant increase of mice but not human sclerostin is detected in the blood plasma of the mice which suggests that the mice bone marrow microenvironment is the source of sclerostin and not the human MPCs established in it. It is known that Scl is synthesized by osteocytes nevertheless the viable osteocytes and mature OBs significantly diminish in number in MM. Consequently, it is unlikely that the osteocytes are the only probable source of the elevated Scl. By investigating the source of sclerostin secretion the authors prove that OBs precursors are the main source of protein. The effect of MPCs on OBs differentiation is investigated in the above-mentioned research. It is established that it is inhibited as a result of the stimulated expression of the sclerostin mRNA in OBs, and this is not due to cell-to-cell interaction with MPCs. This suggests the presence of a cytokine-mediated mechanism. Since Dkk-1 stimulates the sclerostin mRNA in mature OBs and bearing in mind that Dkk-1 is secreted by MPCs, the following hypothesis is made: Dkk-1 is the cytokine responsible for the regulation of Scl synthesis by OBs. An anti-Dkk-1 neutralizing antibody is used which inhibits the effect of MPCs on the expression of sclerostin mRNA in OBs. Thus a mechanism is established for stimulating Scl secretion by the immature OBs with the participation of Dkk-1, produced by MPCs [95]. The outcomes of these experimental works support the multiple times higher serum values of Scl established by us compared to the Dkk-1 values in NDMM patients. It is highly likely that the initially slightly elevated Dkk-1 will be an important stimulator of the Scl biosynthesis both by the mature, but as a consequence of the illness decreasing osteocytes and by the immature

OBs. With disease progression and increased Dkk-1 biosynthesis by MPCs, an exponential increase in Scl would be expected. Thus, a self-perpetuating vicious cycle is created that ensures favorable conditions for the growth and proliferation of MPCs in the bone marrow niches leading to the disease progression.

The significantly high serum levels of Dkk-1 in the patients' group, identified by us, in comparison to the ones that belong to the individuals from the control group, confirm the data for pathologically elevated serum levels of Dkk-1 in NDMM patients mentioned in earlier scientific investigations [104,164,265,343]. For example, Politou et al. identify that the Dkk-1 serum levels in 32 NDMM patients are considerably higher not only than the ones of the control group but also higher than those in MGUS patients. On the basis of these findings, the authors suggest that the elevated OCs activity in MGUS patients is compensated by the still normal function of the OBs and therefore no osteolytic lesions develop at this early stage of the disease. The authors also suggest that it is possible that local bone marrow levels of Dkk-1, which have not been investigated in this research, may begin to rise at this early stage but their slight changes may not lead to changes in serum levels [265]. Thus Keiser et al. use MGUS patients as a control group and detect also significantly higher serum levels of Dkk-1 in NDMM patients than those in MGUS controls [164]. Later Terpos et al. demonstrate, in a clinical research, higher levels of Dkk-1 in symptomatic MM compared to MGUS patients but also compared to a control group of healthy patients. In the last two groups a substantial difference in Dkk-1 levels has not been established [344].

In regard to Scl, the data from the scientific literature are scarce. Nonetheless the established significant difference in the serum levels of Scl between patients and controls is in accordance with most investigations published up to now. Terpos et al. first initiate clinical research about the role of Scl in MM. The authors find out that the levels of the circulating Scl are significantly higher in NDMM patients than in the controls and MGUS patients without the presence of considerable differences for Scl between MGUS patients and the control group [344]. In a later investigation the observation that Scl increases only when MM is active, is confirmed but it does not occur in the earlier stages of the disease [95]. Contrary to them, Brunetti et al. do not prove any difference in the serum levels of Scl among MGUS patients, admitted as controls, and those with symptomatic MM. Thus they presuppose that probably the small number of investigated patients /controls has predetermined the absence of statistical difference [33].

In the present research we found out a continuous step by step increase of the values of both Wnt inhibitors with the disease progression, i.e. the greater the stage of the MM, the higher values of both investigated parameters are observed. The values in the third clinical stage according to ISS reach statistically significant difference in relation to those in the first clinical stage. Thus, for Dkk-1 they have increased by about 19.5% ($p=0.025$), while for Scl – by 23.6% ($p=0.0012$). The present observation corresponds to the results obtained by other research studies investigating the dependence of Wnt inhibitors on MM stage. For instance, in the research [164,265,343] significantly higher levels of Dkk-1 at stages ISS-3 and ISS-2 are detected compared to stage ISS-1.

A small number of clinical research works have investigated the connection between the clinical stage of MM and sclerostin. An investigation carried out by Terpos et al. shows findings corresponding to our data. This investigation proves that the serum Scl levels in patients at ISS-3 stage are significantly higher than those at ISS-1 and ISS-2 stages [344]. The investigation done by Wang et al. has found a significant difference in the serum levels of Scl between the patients at ISS-3 and ISS-2 stages, as well as between them and the control individuals [387].

One of the common and severe complications of MM is renal involvement (the so-called myeloma kidney), and it is estimated that at diagnosis of MM, about 50% of patients already have renal involvement [115,376]. In our research, 22% (9 patients) of all NDMM patients have creatinine above 176 μmol/L. Comparing the two groups of patients (with creatinine below and above 176 μmol/L respectively) we did not observe a significant difference both in the serum values of Dkk-1 ($p=0.2275$), and in those of Scl ($p=0.3118$). An investigation that focuses on the circulating serum levels of Scl and Dkk-1 in patients with chronic kidney disease (CKD) finds out that only Scl increases, but not Dkk-1, as the kidney disease progresses. Thus the authors conclude that Scl could be used as a biomarker for monitoring CKD, while Dkk-1 is not appropriate to for this goal [23]. Bearing in mind this research, we think that it is very likely for the higher levels of Dkk-1 at stage ISS-3 to reflect solely the MM progression and be independent of the kidney function. As for Scl, the investigation of Terpos et al. detects a significant difference in the serum levels of Scl between the patients at III-B stage according to Durie-Salmon (with creatinine >2 mg/dl or 176 μmol/L) and those at III-A stage (with creatinine <2 mg/dl) [340]. Our investigation differs from the one done by Terpos, it does not find a difference between the values of Scl between the two groups which probably is due to the small number of patients with creatinine above 176 μmol/L (9 patients). Notwithstanding this difference, it is important to highlight the fact that in both investigations, the one carried by us and the investigation done by Terpos, there exists a significant difference in the values of Scl depending on the stage of MM, that is, it is highly likely that the serum levels of Scl reflect to a greater degree more adequately the stage of MM, than the impaired kidney function.

Both parameters, Dkk-1 and Scl, show significantly higher serum levels in group G2 (>3 osteolytic lesions) in comparison with group G1 (≤ 3 osteolytic lesions). It is interesting that while Dkk-1 values of patients from subgroup G1 are higher than the ones from the control individuals despite of the fact that they do not reach a statistically significant difference ($p=0.3479$), for Scl, the individuals from G1 subgroup are significantly different from the controls ($p<0.0001$). In one of the first clinical investigations that explore Dkk-1 in MM, Politou et al., do not find correlation between the serum levels of DKK-1 and MBD. According to the authors this result could be explained by the relatively small number of patients, as well as by the limitations of the imaging techniques (plain X-rays) that is used in detecting osteolytic lesions [265]. In subsequent studies, however, a clear correlation was found between Dkk-1 and the degree of bone involvement. For example, in the research carried out by Kaiser et al. it is established that the patients without bone lesions have significantly lower levels than the ones with existing lesions ($p=0.003$). Besides, when patients with ≤3 and those with >3 osteolytic lesions are compared, again significantly higher levels of Dkk-1 are detected in patients with a heavily damaged bone system ($p=0.002$) [164]. Terpos et al. also state in their investigation the same correlation with the bone disease – patients with ≤3 lesions have significantly lower levels of Dkk-1 as compared to those who have more than >3 osteolytic lesions. [357]. Thus we can conclude that our results for Dkk-1 are not different from most of the results published in the scientific literature. In the few existing publications on Scl and MBD we come across results similar to ours. The outcomes of the investigation of Terpos et al. are not that definitive because their patients with advanced bone disease (>3 osteolytic lesions and/or pathological fractures) the Scl levels display a tendency to increase but do not reach a statistically significant difference in relation to the rest of the patients ($p=0.078$). In an additional subanalysis though it is proved that the patients with bone fractures while being diagnosed have significantly higher levels of the circulating Scl compared to all the rest ($p<0.01$) [344]. More convincing data are collected in the investigation carried out by Wang et al. As for MBD, their patients are also subdivided into patients with ≤3 и >3 osteolytic

lesions, and the two groups have considerable differences ($p<0.001$) – higher Scl levels are registered in the second subgroup. It is interesting that the Scl levels in the first subgroup are considerably higher than the ones observed in the control group and this is the same as the findings in our investigation [388].

The changes observed in the levels of the Wnt inhibitors both in the above-mentioned investigations and our own research provide evidence for their pathogenetic role in relation to the MBD onset and progression. As inhibitors of OB differentiation, they prevent normal processes of bone remodeling and create conditions for prevalence of the osteoclastogenesis over osteoblastogenesis. Elevated levels of the routine laboratory biomarkers for bone resorption (e.g. CTX) and lower levels of biomarkers for bone formation (e.g. bALP and osteocalcin) are detected frequently in the clinical investigations, together with elevated levels of the Wnt inhibitors. This additionally proves their inhibitor role in relation to the OB differentiation [343,357,388]. Thus, in conclusion it can be stated that the Wnt inhibitors Dkk-1 and Scl examined, precisely reflect the degree of bone damage and could be introduced in the routine laboratory practice for MBD monitoring.

The present research also investigates the interrelationship of Dkk-1 and Scl with the important hematological, morphological and biochemical indicators used to diagnose the MM as well as to monitor the disease progression. A negative and moderate in strength statistically significant correlation is detected for Dkk-1 with haemoglobin and albumin, while a positive and moderate in strength correlation is detected solely with β2-microglobulin. A negative and moderate in strength statistically significant correlation is observed for Scl with haemoglobin and albumin, while a positive and moderate in strength statistically significant correlation is detected with β2-microglobulin, bone marrow infiltration, creatinine and the total protein. We did not find research in the scientific literature that examines the relationship between the bone marrow infiltration (BMI) in diagnosis of MM and the inhibitors of the Wnt signaling pathway. The prognostic meaning of BMI has not been well defined until recently. In 2020 Al Saleh demonstrated that MPC>60% at the diagnosis is a good prognostic marker for PFS and OS in low and high risk patients [5]. We found significantly lower levels of Scl of BMI<60% and higher levels of BMI>60%, as for the Dkk-1 there was no difference in the levels ($p=0.9843$). Besides, in contrast to Scl in which we found a moderate positive correlation with BMI, for Dkk-1 correlation dependency is not detected. The relationships between the bone marrow micrienvironment and the MPCs are mediated by cell-to-cell interactions and paracrine / autocrine mediators. In their research, Eda et al. show that the MPCs are able to stimulate the expression of Scl in OBs by secretion of DKK-1 [95]. It can be assumed that the minimal local increase of DKK-1 in bone marrow could accelerate the biosynthesis of sclerostin in an exponential way which in turn could lead to its more pronounced increase in blood and in serum respectively. This could explain, at least partially, the proven correlation relationship in one of the proteins and the absence of such in the other. The results thus achieved doubtlessly prove that the changes in serum levels of Dkk-1 and Scl, properly reflect the substantial characteristics of the myeloma disease, namely anemia, the paraprotein production, bone marrow infiltration by MPCs and kidney damage.

1.2 Serum levels of Dkk-1 and Scl, detected in the course of the medical treatment

According to the research design the patients investigated are monitored within the period of eight months. In order to investigate the dynamic of the circulating levels of the examined bone biomarkers, Dkk-1 and Scl, serum was taken three times – before the beginning of the therapy (stage T0); at the end of the first 4 courses of therapy following the VCD protocol (stage T1); after finishing

the additional 4 courses following the VCD protocol for patients not eligible for transplantation (stage T2), while for the transplant patients– three months after the ASCT.

One of the medicaments of the induction protocol used in the treatment of the patients from the present study is bortezomib, which possesses a proven anti-tumor efficacy [330]. The interesting thing about this proteasome inhibitor is that it not only effectively destroys MPCs, but it also improves the bone remodeling through the induction of OBs differentiation. Thus it inhibits the osteolytic progression in patients with MM [274,367].

The Kruskal-Wallis test found that there exists a statistically significant difference between the levels of the two parameters investigated in the course of the applied therapy (Dkk-1: $\gamma 2=24.34$, $p<0.0001$; Scl: $\gamma 2=31.40$, $p<0.0001$). The additional *post hoc* analysis with Mann-Whitney discerned the differences of the separate stages of the study. At each stage of the monitoring a significant decrease of the serum levels of Dkk-1 and Scl is detected in relation to their baseline levels and the degree of significance increases (T1 vs T0: $p=0.0290$; T2 vs T0: $p=0.0020$ and TA vs T0: $p<0.0001$ for Dkk-1 and T1 vs T0: $p=0.0271$; T2 vs T0: $p=0.0086$ and TA vs T0: $p<0.0001$ for Scl). When levels at the end of the study period in both arms are compared to those measured at T1, it can be seen that in the transplant patients their decrease is bigger and reaches the values of a statistical significance (TA vs T1: $p=0.003$for Dkk-1 and $p<0.0001$ for Scl), while in non-transplant patients after the second course of treatment the levels continue to decrease but not to the extent that they would reach a statistically significant difference (T2 vs T1: $p=0.1330$ for Dkk-1 and $p=0.2645$ for Scl).

The values of Dkk-1 at stage TA do not differ statistically from the values in the control group ($p=0.4561$), which is probably due to the significant decrease in the tumor mass achieved by high dose chemotherapy. Despite the fact that the decrease of Dkk-1 at stage T2 is not significant in relation to the whole group investigated at stage T1, it is sufficient to reach values close to the ones of the control group ($p=0.2104$). The dynamic of decrease of Scl is similar to the one of the Dkk-1 but at the end of the study period its levels both at stages TA and T2 continue to be significantly higher than those of the control individuals. It is necessary to emphasize that the initial levels of Scl at T0 are nearly eight times higher than those of the control group.

Bearing in mind this fact as well as the fact that it has already been definitively proven that not the MPCs but the osteocytes and OBs precursors are responsible for the synthesis of Scl [226] we make the following hypothesis – since the therapy targets primarily tumor cells, the decrease of Scl is probably due to not so much the direct effect of the medicaments on the MPCs, as to the regulatory signals that come from the bone marrow microenvironment which are most likely induced by the treatment in an indirect manner and so these initially multiple times increased values of Scl in relation to the control group levels could decrease more gradually and with greater difficulty.

Since at the stage of T1 the patients eligible for transplantation are selected, an additional subanalysis is also done by dividing the patients into two subgroups T1(A) and T1(nonA). A significant difference is detected in the values between the two subgroups for Dkk-1 ($p=0.0062$). Straight after the first cycle of treatment is over, part of the patients, the ones from subgroup T1(A), reach CR and VGPR and their Dkk-1 levels significantly differ from their baseline levels before the beginning of the therapy (T1(A) vs T0, $p=0.0003$) and they get close to the ones achieved by the non-transplant patients at the later stage T2 (T1(A) vs T2: $p=0.8094$). Despite that, they are still significantly higher than the levels which are reached three months after the transplantation (T1(A) vs TA, $p=0.0241$). In contrast to them the T1(nonA) patients who do not present with a good response

at the end of the first cycle of treatment, maintain high serum levels of Dkk-1, statistically indistinguishable from their baseline levels (T1(nonA) vs T0: p=0.5627). The additional cycle of 4 courses of VCD though are beneficial for the non-transplant patients at stage T2 to reach a significant decrease in Dkk-1 levels in relation to those detected at stage T1 (T2 vs T1(nonA): p=0.0192). The dynamic of Scl levels follows a slightly different course of action. Thus, in spite of the fact that the T1 (A) patients have reached CR and VGPR, their Scl levels do not differ significantly from those at stage T1(nonA) (p=0.3493), but they are significantly different from their baseline levels (p=0.0134). In contrast to them, the Scl levels at T1 (nonA) patients do not attain a significant difference from the baseline levels (p=0.2047). The additional cycle of 4 courses of VCD lower the levels of Scl, but they still do not differ from the ones achieved at stage T1 (T2 vs T1(nonA): p=0.2169). Again, it can be observed that the decrease of Scl levels follows a more gradual and slowed down course.

As a summary for both proteins Dkk-1 and Scl, it could be concluded that both factors: the number of therapeutic courses and the individual characteristics of the patients that presuppose the achievement of better or worse response to the therapy, contribute to the significant decrease in their levels in the course of the treatment.

The results in relation to the effect of the treatment of MM on the serum levels of Dkk-1 available in the scientific literature are not unambiguous. For example, an investigation carried out by Terpos et al. establishes that patients treated with lenalidomide and dexamethasone (RD) and achieved more than a partial response to the treatment even demonstrate a slight increase of Dkk-1 levels with 9%, while in the patients who did not respond to the therapy, the increase amounts to 91% [343]. The results that do not correspond to ours could be explained by the different mechanism of activity of the immunomodulating medications (IMiDs). In experimental models with chicken embryos and with human embryonic fibroblasts, it is found that thalidomide induces Dkk-1 expression [186]. Another research also proves that thalidomide and lenalidomide enhance the expression of Dkk-1 by activating the JNK signaling pathway in cell cultures with primary human myeloma plasma cells [62]. In 2014 Terpos et al. again confirm the results for Dkk-1 in patients with refractive MM or relapse of the disease and patients treated with RD. After three and six cycles of patients' treatment, no significant differences are found in the Dkk-1 serum levels, either at the retrospective or prospective part of their research. At the same time when the proteasome inhibitor bortezomib (VRD) is also added to the therapy protocol, a statistically significant reduction of Dkk-1 is achieved. After the third cycle the decrease is almost double (44% decrease in the values). After the sixth cycle the Dkk-1 levels continue to drop and are significantly lower in relation to the baseline levels (59% decrease), while compared to the levels of the 3rd cycle, they are 26% lower. These changes in the Dkk-1 values are accompanied also by a significant increase of the levels of the bone formation biomarkers the (osteocalcin – the increase is statistically significant after the 3rd month already, while for bALP – after the 6th month) [357]. The outcomes of this research fully correspond to our results which detect a continuous decrease of Dkk-1 levels in the course of treatment.

As for the Scl, the results of the several investigations that carry out research on the influence of the applied treatment on its levels are also not unambiguous. Terpos et al. investigate the changes in the Scl levels in MM patients after treatment with regimens that include bortezomib or thalidomide at the plateau phase of the disease that is defined as a state with stable M-protein for at least 6 months without criteria for progression. The Scl values at this phase are significantly higher not only than the ones in the control group but also than those detected before the beginning of the treatment (p=0.02). In contrast, the Dkk-1 levels at this phase are considerably lower (p<0.001) than the values at the time

of the diagnosis and do not differ from the ones in the control group. The authors conclude that the elevated Scl levels at this phase are the reason for the continuous OBs dysfunction which is observed for a long time after the applied treatment is over [352]. It is impossible to compare these results with ours which are achieved by investigating a serum obtained as soon as the therapy comes to an end. Another research by Terpos et al. demonstrates that a treatment with 4 courses of bortezomib as a monotherapy in patients with relapse of MM leads to a significant decrease of the Scl levels both for the patients who responded to this treatment and those who did not respond to it to almost the same extent and no difference is observed between them ($p=0.67$). According to the authors, the decrease in Scl levels proves the presence of yet another mechanism of activity of bortezomib which is important for overcoming the osteoblast dysfunction in MM. Most likely this effect of bortezomib is due to its inhibiting effect on the Dkk-1 expression on one hand, as it is proved to increase the Scl expression and on the other hand this could be an independent effect of bortezomib on the osteocytes [346].

In previous research by Heider et al. the Dkk-1 serum levels were investigated in patients with MM, who followed different treatment regimens: with adriamycin / idarubicin and dexamethasone (AD/ID); with bortezomib; with lenalidomide; with thalidomide; as well as with high dose chemotherapy followed by ASCT. Three months after the treatment applied all therapeutic regimens lead to a significant decrease of the Dkk-1 serum levels with the exception of the patients treated with thalidomide whose decrease is insignificant [130]. The authors point out that the treatment with thalidomide in their investigation does not lead to the expected increase in the Dkk-1 levels which is exactly opposite to the increased protein expression stimulated by thalidomide and proven in other investigations. But they do not give a possible explanation about this fact. What is more, the essential thing is that this investigation proves the importance of the high dose chemotherapy, followed by ASCT to achieve the best possible therapeutic effects for the patient including the bone disease. This type of therapeutic approach brings about the most significant decrease in the Dkk-1 values which is a presupposition for the recovery of the OBs proliferation, differentiation and the normal mechanisms of regulation of the bone remodeling. These specific results fully correspond to our results which also demonstrate the greatest decrease of Dkk-1 levels after an autologous bone marrow transplant is performed.

Besides Heider's research, a number of other investigations establish the role of the high dose chemotherapy for the significant decrease of Dkk-1. For instance, in the investigation carried out by Politou et al. the values of Dkk-1 three months after ASCT reach the ones of the control group. What is more the authors find out that the routine bone formation biomarkers osteocalcin and bALP begin to get back to normal not earlier than the 8^{th} month after the transplantation, while the bone resorption markers NTX and TRACP5 remain elevated till the third month [265]. Thus, it could be concluded that the changes in the serum levels of Dkk-1 are more dynamic and reflect the changes in the bone structure earlier than the routine biomarkers. In an interesting study tracking the bone biomarkers for the period of 12 months after ASCT, after which a consolidation treatment is carried out following the VTD protocol, an additional decrease of the Dkk-1 serum levels is not registered. In this study the levels of Dkk-1 reached after the transplantation differ from the ones observed in their control group [359].

As far as we know, in the world database so far, there exist published results of only one investigation that analyses the effect of ASCT followed by a subsequent consolidation treatment with bortezomib and lenalidomide (VR) without dexamethasone and bisphosphonates on the serum levels

of Scl. The Scl levels were investigated immediately before the transplantation, three months after the transplantation and after the consolidation treatment is over. A significant decrease of Scl levels is detected at each stage of the investigation ($p<0.001$). At the same time a decrease in the serum levels of the bone resorption markers was not observed and changes in the levels of Dkk-1, bALP and osteocalcin were not detected either for the period investigated. It is important to emphasize that during the period of monitoring the patients within an average of 62- month- period after the consolidation therapy performed, no pathologic bone events/fractures were observed despite the absence of bisphosphonates application [341].

1.3 Serum levels of Dkk-1 and Scl depending on the therapeutic response

Besides the fact that Dkk-1 and Scl levels change in the course of the treatment, our investigation proved that they are directly dependent on the response achieved in relation to the therapy. For instance, Dkk-1 values in the group of patients with achieved CR and VGPR they are considerably lower as compared to the ones belonging to the patients with PR and SD ($p=0.0475$). They are not different from the values of the control group ($p=0.6295$) and are considerably lower than their levels at diagnosis ($p<0.0001$). Patients with bad response to the therapy have opposite results – the Dkk-1 levels in patients with PR and SD do not differ from the baseline levels ($p=0.0903$) and are considerably higher than the ones of the control group ($p=0.0051$). The results for Scl are similar. The patients with achieved CR and VGPR have significantly lower levels of Scl as compared to the patients with PR and SD ($p=0.0018$), but because of the reasons already discussed they remain higher and are different from those of the control group ($p<0.0001$). At the same time, they are considerably lower than their baseline levels at T0 ($p<0.0001$). The patients with a bad response to the therapy (PR and SD) are with Scl levels not different from their baseline levels ($p=0.0950$) and of course they are significantly higher than the ones that belong to the control group ($p=0.0051$).

Our results correspond to the results of Heider's research in which independently of the therapeutic regimen applied, even in the treatment with thalidomide, only the responders (i.e. those with CR and VGPR) have significantly lower levels of Dkk-1 three months after the beginning of the treatment as compared to the baseline levels, while the non-responders (with PR and disease progression) do not reach a statistically significant decrease [130]. The results of the investigation performed by Lemaire et al. are also interesting. These outcomes do not detect significant decrease of Dkk-1 in the 6^{th} month after the ASCT in the patients who have less than 75% decrease of the M-protein established immediately after the induction therapy is over and are pointed out by the authors as bad responders. A considerable decrease in the 6^{th} month is attained only in these patients who are with more than 75% decrease of M-protein after the induction therapy (good responders). Since the patients are monitored within a period of three years after the transplantation, the authors define the observed relapses of the disease as *early*, those that occur up to the 18^{th} month and *late* – that occur after the 18^{th} month. The Dkk-1 serum levels are identified in the 12^{th} month as well, and in the patients with late relapse of the disease an insignificant decrease of the values related to the exit levels, while in the patients with early relapse of the disease a multiple increase of Dkk-1 levels ($p<0.02$) is observed. At this stage of the monitoring, insignificantly lower levels of the M-protein are found in relation to the baseline values for both groups. There is no significant difference between the patients with early and late relapse of the disease. The authors come to the conclusion that the assessment of Dkk-1 in the 12^{th} month after the ASCT can serve as a good predictor of a future early or late relapse of MM [200]. In another survey patients with achieved CR are monitored until the disease recurrence. Dkk-1 and Scl levels are investigated up to the moment of reaching CR, four

months before the relapse of the disease and as soon as this relapse is identified. The Dkk-1and Scl serum levels increase considerably while monitoring the patients and the authors emphasize that four months before the disease recurrence, the Dkk-1and Scl levels are increased in all patients, while the M-protein is still undetected in more than half of the patients [219]. This fact is yet another confirmation of the conclusion that the Dkk-1 and Scl are dynamic and early indicators which could be used for predicting of a possible relapse of the disease. The transplant patients in our research are monitored at the third month after the ASCT, when they are still in CR and their Dkk-1 and Scl values are still low.

As has already been mentioned the data in relation to Scl are quite scarce. In an investigation that includes a heterogenous group of MM patients (newly diagnosed, with MGUS and with a relapse of the disease) there exist data for Scl levels depending on the achieved response only for those with a relapse of the disease and treated with bortezomib monotherapy. Contrary to our investigation, the decrease of Scl levels is to the same degree (about 50%) both for the responders and for the non-responders to this treatment (p=0.67) [346]. A later investigation that deals with the effect of post-ASCT consolidation therapy on Scl levels, the authors registered a more pronounced decrease of the biomarker investigated in patients with and more than VGPR [341].

Besides the fact that the Dkk-1 and Scl levels tend to change in the course of the treatment and are dependent on the achieved response, we were curious to determine whether the primary levels of the proteins could predict the response to the treatment. For this purpose, the baseline levels at T0 stage were monitored for patients who later achieved CR and VGPR, as well as those who achieved PR or were in SD. Since a significant difference was not detected in the Dkk-1 and Scl levels between the two groups (p=0.1655 and p>0.9999, respectively), we think that the initial values could not predict the treatment response. Corresponding to our findings are also the data from the research of Heider et al. who state that the Dkk-1 serum levels before treatment are not predictors for the treatment response (a statistical difference in the Dkk-1 levels measured before treatment both in patients who responded to the treatment and in those who did not, has not been detected, p=0.696) [130].

2. Markers of impaired osteoclast activity and altered bone marrow microenvironment – sRANKL, OPN, PON

A characteristic feature of MBD is pathologically activated osteoclastogenesis and suppressed osteoblastogenesis, causing active foci with prominent osteolytic processes in bone tissue. By providing a favorable environment for MPCs to interact with cellular and non-cellular elements through direct and indirect mechanisms, the bone marrow microenvironment plays a key role in the progression of MM and the development of MBD.

2.1 Basal serum levels of sRANKL, OPN, PON in NDMM patients

We did not detect any significant differences according to sex and age for sRANKL either in the patients' group or in the control group. It is interesting that the sRANKL values in women in the control group are higher than those of men by 9.5%, although they do not reach a statistically significant difference. It is difficult to compare our data with the ones from the scientific literature since controversial results are found there. For example, some authors do not encounter age-related differences [162,370], whereas others prove that sRANKL decreases with age [175,210]. Most authors prove higher serum levels of sRANKL in men [162,175,187]. It is commonly understood that estrogens suppress the expression of RANKL [239], which leads to the expectation of higher serum

levels of sRANKL in postmenopausal women. Nevertheless, Liu et al. in their own study, find out that Chinese postmenopausal women are characterized by lower serum levels of this cytokine in relation to the women in fertile age [210]. It is very likely that this could be due to racial peculiarities that presuppose a more different regulation of sRANKL expression.

During the analysis performed, no significant sex or age differences for OPN were identified. The literature sources which elaborate on the influence of the biological factors on the serum concentrations of OPN are rather limited. In a large-scale Danish study, 300 healthy individuals are examined in order to investigate different factors that could lead to variations in defining and determining OPN. In that study, as well as in our own research, no significant difference depending on the sex is detected. The age groups distributed in decades do not differ from each other according to the serum values of OPN, either. What is more, the influence of the menstrual cycle is investigated as well as the physical activity, neither of which lead to significant differences between the groups [308].

For the third protein investigated in our study, PON, only a difference between men and women in the control group ($p=0.0176$) is found, and the values in women are higher than those in men. Besides, in our control group, higher values of the protein by approximately 19% are detected in the group of people over 65 years of age. Since a statistical significance is not reached, we consider that the data correspond to the ones in the literature because most of the authors do not find influence of age on the serum concentrations of PON in healthy individuals [4,335]. Walsh et al. compare three age groups: 16-18, 30-32 and >70 years of age. The authors detect significantly higher values for PON in people in the 16-18 age range when there is an active increase of bone mass, in relation to the 30-32 age group, when the bone mass peak is reached, and the levels are at their lowest. In the age group >70 years of age the levels show a tendency to increase, compared to the group in the age range of 30-32, but a statistical difference is not reached ($p=0.08$). For this reason, the reference range determined by these authors is wide [383]. As regards sex, our data coincide with the ones of Tan et al. who detect higher values of PON in women, while other researchers do not identify any sex differences [42,335,383].

In the three proteins, investigated by us, the division according to sex does not change the significant difference, between patients and healthy individuals that is detected for the general group. The NDMM men and women display significantly higher values than the respective healthy group for each one of the parameters investigated. The subdivision of the groups according to age also did not lead to detecting significant differences for the three parameters. This could be explained with the comparatively narrow age range that characterizes the groups investigated. It could be concluded that the pathological process in the cohort of NDMM patients investigated by us, exceeds in strength the effect of the biological factors age and sex and has a decisive importance for the significantly higher values of sRANKL, OPN, PON observed at the time the diagnosis is made. This allows us not to take into consideration sex and age in the further discussions of the changes in the serum levels of these proteins.

As has already been mentioned for the three parameters investigated, significantly higher serum values in the patients' group were detected as compared to the ones in the control group. Thus, the levels of sRANKL exceed by 69% ($p<0.0001$) those of the control individuals. The role of sRANKL as a basic activating factor for OCs in MM is well researched in different animal models and cell cultures [34,80,263,324]. These primary investigations prove the pathogenic mechanisms with the

help of which MPCs turn out to be the major factors leading to upregulation of sRANKL synthesis. First, they enhance the expression of sRANKL from the bone marrow stromal cells with which they closely interact, and thus they stimulate the osteoclastogenesis in a direct way. Second, the MPCs themselves can secrete sRANKL and directly, independently of the stromal cells they can initiate the formation of OCs. Last but not least, the MPCs suppress the synthesis of the soluble receptor activator for sRANKL, OPG, in the bone marrow microenvironment and thus it does not allow it to effectively control the processes of bone remodeling. An original work written by Schmiedel et al., proves that MPCs as well as CLL malignant cells express mRANKL (membrane localized), but soluble sRANKL is secreted only by MPCs, which proves the unique role of this protein in the pathogenesis of MM and bone disease which is not observed in CLL [300]. A correlation has been proved between the bone marrow concentration of sRANKL with its serum concentration in MM [190], as well as in some bone metastasizing cancers [366]. All this proves the increase of this protein in the serum of patients with MM is not accidental. In accordance with our data for increased levels of sRANKL in NDMM are also those from a number of studies, in some of which the increase is to more than twice [118,264,310,354]. What is more, Politou et al. detect increased levels of sRANKL also in MGUS, in comparison with the controls, which presupposes that the activated osteoclastogenesis is an early event in the monoclonal gammopathies [264]. In contrast to all these studies, Kraj et al. not only do not prove statistically significant increased levels of sRANKL, but just the opposite, they detect a significant increase of the serum OPG. Despite the lack of statistical significance declared by them, when the data are analyzed in detail, it could be noticed that their patients have higher values of sRANKL as compared to the controls (mean ±SD: 19.75±53.42 and 4.47±8.03, respectively) [190]. Probably a statistical significance is not reached because of the great variation of the results in the patients' group. Another team of scientists, while investigating serum levels of sRANKL in MM, prove increased values of the parameter over the upper reference limit for the kit which they used in 43% of the patients investigated. Since there is no control group, the authors are not able to point out precisely whether this increase is significant or not [303]. Thus, it could be concluded that it is possible that methodological issues might underpin the differences in the last investigations cited.

In the present study, besides sRANKL, the serum OPN in NDMM patients is also higher than the one measured in the control group, by 54% ($p<0.0001$). In physiological conditions the OPN in bones is expressed mainly in OBs and BMSCs. In a fundamental study by Saeki et al., which investigates cultures with different B-cell malignant lines, an OPN expression and production is proved solely by the MM cell lines. The conclusion is that MPCs produce *in vivo* OPN, which additionally increases the bone resorption, as a result of the intense activity of OCs [291]. On the other hand MPCs induce the stromal cells to produce additional OPN, with which probably a favorable environment is created for retention and growth of the myeloma cells and the formation of a self-sustaining vicious cycle [326]. The outcome thus obtained by us about OPN, corresponds to those experimental studies, as well as to most of the clinical investigations in which the baseline levels of OPN in the symptomatic patients are significantly higher than those of the controls [88,232,291,326,378]. It is curious that some authors detect increased levels of OPN even in the early stages of the disease as early as the stage of MGUS [326], while others do not prove such a difference [232,291]. Contrary to these studies, Kang et al. do not find a significant difference in the levels of OPN between the patients' and control group, but this is a conclusion based on the investigation of just 13 NDMM patients and 14 healthy individuals [168]. What is more, Maaroufi et al. in their recent study of MM patients in first clinical stage, detect even significantly lower levels of OPN compared to the controls. An interesting issue in this study is that BSP (bone sialoprotein) is also investigated,

which is significantly higher in the patients' group. The authors do not discuss these puzzling outcomes [217]. It is well known that BSP and OPN belong to the SIBLING family. As matricellular proteins they show selective affinity to the cancer cells, metastasizing in the bones and they lead to a different type of bone lesions – OPN is responsible for osteolytic type of lesions, whereas BSP predetermines the osteosclerotic lesions [41].

The role of PON in the pathogenesis of MM is not well clarified. An experimental study presupposes that the fibroblast-like cells of the bone marrow microenvironment take part in the remodeling of ECM and lead to increased synthesis of different matrix proteins, including PON as well. Thus, a favorable environment is created for the growth of MPCs. Through proteome analysis of a supernatant of fibroblast-like cells, isolated from the bone marrow of patients with MGUS and such with active MM, a higher concentration of PON is detected in the supernatant that originates from the patients with active MM [320]. From this point of view our data detecting significantly elevated values of PON by 63% ($p<0.0001$) in the patients as compared to the control individuals, correspond to the experimental results of the above-mentioned study. In relation to comparing our results to those from other clinical investigations, we do not have a great choice really, because as far as we know, only one scientific team has worked on this problem and has published some data. Notwithstanding the different number of NDMM patients mentioned in their publications, always significantly higher values of PON with approximately 70% ($p<0.001$) are obtained in the patients compared to the control group. The authors have also investigated a group of patients with a relapse of the disease with approximately 75% higher levels of PON as compared to the controls ($p=0.016$) [340,347-349].

Besides the comparison with the control group, we also investigated how the tested parameters change, depending on the characteristics of the myeloma disease. At the beginning we studied the dependency of their serum concentrations on the stage of MM. With the progression of the disease, further increase of the serum levels of sRANKL is observed. The values of sRANKL at stage ISS-II take an intermediate position between the detected values at stage ISS-I and the ones at stage ISS-III without a statistically significant difference between them. The gradual increase by 13% at every transition from one stage to another leads to an overall increase of the values by 28% between ISS-I and ISS-III and reaching a significant difference between them ($p=0.0013$). The RANK/RANKL axis interferes with the MM pathogenesis since the early stages of the disease. For instance, Roux et al. investigate immunohistochemically bone marrow from healthy individuals, MGUS patients and from patients with active MM. An increasing protein expression from the stromal cells is detected, which is almost lacking in the control biopsies, higher than the ones from MGUS patients and significantly higher in the bone marrow of MM patients [287]. In addition, Heider et al. prove by flowcytometry that the MPCs in the bone marrow also express RANKL [129]. Taking into consideration the fact that the bone marrow infiltration with MPCs increases with the progression of the disease, it is not surprising that we detect gradually increasing values of sRANKL from stage ISS-I to stage ISS-III. Moreover, our data correspond to most of the data found in the scientific literature. For example, Politou et al. detect significantly higher values of sRANKL in MGUS patients in comparison to the control individuals ($p<0.0001$), who in turn are significantly lower than those in MM patients at stage ISS-I/ISS-II ($p=0.042$) [264]. Furthermore, Goranova-Marinova et al. detect a significant difference in sRANKL levels between ISS-I/ISS-II and ISS-III ($p<0.05$) [118]. Although Jakob et al. assess tRANKL (parameter that measures free and bound with OPG RANKL), the authors report results

almost identical to our results (ISS-I vs ISS-II: p=0.091; ISS-II vs ISS-III: p=0.029 and ISS-I vs ISS-III: p=0.004) [155].

As for the OPN, with the progression of the disease, a progressive increase of its serum levels is observed as well. OPN increases by approximately 10% between stages ISS-I and ISS-II without significant difference between them (p=0.293). Between stages ISS-II and ISS-III, though, the slope of values is steeper (36%), as it almost reaches the statistically significant difference (p=0.0505). This also predetermines the significant difference between stages ISS-I and ISS-III (p=0.0003), whose values differ from one another by 49%. Probably this is due to the fact that OPN creates a favorable environment for adhesion and proliferation of the myeloma cells, which in turns also produce OPN and determine higher levels of protein in the later stages of the disease. Minarik et al., while investigating serum levels of the hepatocyte growth factor (HGF), syndecan-1/CD138 (SYN) and OPN in MM patients find a tendency of increase of the OPN values depending on the stage of the disease progression [232]. The results published by Saeki et al. are more straightforward. Their patients have been staged following the Durie-Salmon staging system since 1975 and the following results are observed in their work for OPN (ng/ml): SMM vs Stage III (MM, inactive) – 383±43 vs 816±134, p<0.05; Stage III (MM, inactive) vs Stage III (MM, active) – 816±134 vs 1991±406, p<0.01 [291].

The dependency of serum concentrations of PON on the MM stage follows the ones already described for sRANKL and OPN in our study. The increase of PON between stages ISS-I and ISS-II is by 19% (p=0.0012), while between stages ISS-II and ISS-III it is by 21% (p=0.1378). Again a significant difference is observed between stages ISS-I and ISS-III (p<0.0001), the values of which differ from one another by 44%. The only scientific team that investigated PON in MM, through ANOVA analysis proves a statistically significant difference (p<0.01) in the concentrations of PON (ng/ml) with continuous growing of values with the progression of the disease – ISS-I vs ISS-II vs ISS-III: 542±196 vs 775± 500 vs 1036 ± 801 [347]. With the help of PON interaction with the integrin receptors of MPCs, a favorable environment is provided for their proliferation and survival. The interactions of MPCs with the bone marrow microenvironment lead to increased secretion of PON from both the myeloma and the stromal cells. This self-sustaining vicious cycle leads to continuous growth of protein with the disease progression.

The elevated bone resorption is a distinctive feature of MM. The osteolytic lesions are detected near the MPCs which allows us to assume that they take part in the production of cytokines which stimulate the differentiation and proliferation of OCs. Heider et al. detect for the first time a significantly higher expression of RANKL from MPCs isolated from bone marrow of MM patients with osteolytic lesions compared to those without osteolytic lesions (p<0.0005) [125]. Thus, it is not surprising that in the present study the serum values of sRANKL in group G1 (≤3 osteolytic lesions) are considerably lower in comparison to the ones in group G2 (>3 osteolytic lesions), p=0.0092. These results correspond to the data published by other authors. Terpos et al. are the first to investigate the levels of sRANKL, OPG and their ratio in MM patients with a different degree of expression of MBD and they detect a significant difference (p<0.0001) between the serum levels both of sRANKL and OPG and of their ratio in the three defined groups (without osteolytic lesions, with 1-3 lesions and >3 lesions) [354]. Significantly higher values (p<0.05) for sRANKL are later detected by Goranova-Marinova et al. in the group of patients with MBD extent „0+1“ as related to MBD extent „2+3“, as well as Sfiridaki et al., who following the same method of assessment of MBD, detect nearly 6 times higher values in patients with severe skeletal system injuries (p<0.0001) [118,310]. Although Jakob

et al. investigated tRANKL, they proved almost three times higher serum values in MM patients with osteolytic lesions in comparison to those without any lesions ($p<0.001$) [155]. All these data emphasize the importance of sRANKL as an informative biomarker that correlates with the extent of bone system damage especially when also taking into consideration the strong correlation proven by Terpos et al., between sRANKL and the confirmed markers for bone decomposition NTX and TRACP-5b ($r=0.69$, $p<0.0001$ and $r=0.87$; $p<0.0001$ respectively) [354].

Experimental research works with mice provide the basis for the understanding of the significant role of OPN in the bone homeostasis. It has been proved that mice with an OPN deficiency are protected against bone mass loss [152,410,413]. These preliminary data for OPN provoke the interest in the protein and its role in the MM bone disease. Most authors detect significantly higher levels of OPN in MM patients with advanced bone disease, on the basis of which they identify it as a potential biomarker for monitoring of MBD. The data from our research demonstrating significantly higher levels of OPN in group G2 as compared to group G1 ($p=0.0002$) support the results reported by Saeki et al. [291], who separating the patients based on: those with and without osteolytic lesions, find a significant difference between them ($p<0.01$). Similarly Sfiridaki et al. observe a significant difference in OPN levels ($p=0.006$) depending on the extent of bone disease (higher in patients with MBD stages 2 and 3 in comparison to those with 0 – 1) [309]. Comparing patients with and without osteolytic lesions, Valković et al. also detect significantly higher values of OPN for the first group ($p=0.03$) [378]. The high OPN levels do not always correlate with MBD. For instance, Standal et al., although they detect elevated levels of OPN ever since the MGUS stage, which moreover tend to increase in the more advanced stages, do not identify any connection between the extent of skeletal system damage and OPN. Regardless of this fact, the authors bearing in mind the well-known OPN function to activate OCs, confirm that OPN is important for the pathogenesis and progression of MM [326]. According to Robbiani et al. the potential value of this marker should be assessed also in combination with the molecular type of the illness. Investigating the OPN expression in several different human myeloma cell lines and in primary MPCs, isolated from the bone marrow of NDMM patients, they detect high OPN expression only in the cells that express simultaneously the transcription factors c-maf or mafB as well. It is an interesting fact that in patients whose bone marrow MPCs express high levels of OPN, there are no osteolytic lesions. The authors assume that in the cases of maf translocation, OPN counteracts the effect of osteolytic factors generated as a result of the interaction between MPCs and BMSCs. MPCs acquire an ability to overproduce OPN by an autocrine mechanism. It seems the bone marrow microenvironment rich in OPN, paradoxically suppresses the activation of OCs, which leads to a more weakly expressed MBD in these cases [383].

In relation to PON and its connection to MBD, we found out significantly higher values of the protein in the G2 group ($p=0.0002$) compared to G1. Terpos et al. prove that the serum levels of PON reflect adequately an elevated bone resorption in MM, through the established significant correlation relationship between PON and CTX ($r=0.369$, $p=0.005$). What is more, the patients with >3 osteolytic lesions and/or pathological fractures have significantly higher values of PON in comparison with all the rest ($p=0.01$) [340]. Later the same scientific team detect almost twice as high levels of PON in the bone marrow plasma ($p<0.001$) and serum ($p=0.032$) in NDMM patients with pathological fractures, as compared to all the rest [347].

In the present study, we investigated also the interrelationships of sRANKL, OPN and PON with the important hematological, morphological and biochemical indicators used for diagnosing and monitoring of MM. For all the three tested parameters the values are significantly higher in patients

with BMI>60% of MPCs in comparison to those with <60% infiltration (p=0.0169, p=0.0158 and p=0.0003 respectively for sRANKL, OPN and PON). We prove also a moderate in strength and statistically significant relationship between BMI and sRANKL (r=-0.3304 and p=0.0349), as well as between BMI and PON (r=-0.4172 and p=0.0067). Unlike them we did not detect a statistically significant relationship between OPN and BMI (r=-0.2607 and p=0.0997), despite the 30% increase of OPN in the group of patients with BMI>60%. It is considered for proven that OPN creates a favorable environment for adhesion and growth of the tumor cells [336] and from this point of view the lack of correlation and dependency between BMI and OPN is surprising. A similar lack of correlation is detected in the research done by Standal et al. [326]. β2-MG is an important serum marker that reflects the tumor load in different neoplastic diseases including MM as well. It is not by chance that β2-MG and not BMI, is included as one of the factors in staging the disease according to ISS [76]. In relation to β2-MG we detect a considerable positive correlation with each one of the three indicators investigated (sRANKL: r=0.5756, p<0.0001; OPN: r=0.6968, p<0.0001 and PON: r=0.7791, p<0.0001). This proves that the proteins investigated reflect sufficiently well basic characteristics of MM, especially when also taking into consideration the rest of the correlation relationships investigated: negative with Hb, thrombocytes and albumin, and positive with normal protein and creatinine.

It is well known that kidney damage as a result of various pathological processes, induces also secondary bone impairments. The elevated PTH levels in the secondary hyperparathyroidism induce OBs activation with increased RANKL expression and reduced OPG expression. In an *in vitro* study, performed by Huang, it is reported that the PTH considerably increases the expression of mRNA for RANKL from the stromal OBs and inhibits the gene expression of OPG at all stages of OBs differentiation [145]. There exist in the scientific literature controversial results for the RANKL levels in the chronic kidney disease (CKD), and normal, high and low levels of the protein are found in relation to the control individuals [6,14,91,311]. In a study performed by Goranova-Marinova et al. with MM patients, it was reported that the serum levels of sRANKL are not different in the groups of patients with creatinine under or over 168 μmol/l [118].We also do not detect a significant difference in the sRANKL serum levels in the patients with creatinine over 177 μmol/l and under 177 μmol/l (p=0.1037).

The renal tissue is one of the most OPN abundant [49]. There is a sufficient accumulation of experimental data about the role of OPN in the pathogenesis of a number of kidney diseases [403], but no data can be found in the scientific literature for the participation of osteopontin in the myeloma kidney disease. OPN is normally expressed in the ascending loop of Henle and in the distal convoluted tubules from the nephrons of the kidney in physiological conditions. OPN expression increases considerably after kidney damage and its expression increases in all tubular segments and in the glomeruli up to 18 times [403]. The OPN expression is increased in the kidneys, blood and urine of patients with CKD, especially in those with a diabetic kidney disease and glomerulonephritis [319]. The higher serum levels of OPN detected by us in patients with creatinine more than 177 μmol/l in comparison to those with creatinine less than 177 μmol/l (p=0.0407) are probably due to the impaired kidney function as a result of MM as well.

PON is not found in the renal tissue under normal physiological conditions [153]. Satirapoj et al. establish that the periostin is strongly expressed in the tubulointerstitial areas in the case of kidney damage. The authors think that PON in the urine is a measure for the loss of renal tubular cells, and it could serve as an early marker for kidney impairment in the case of diabetic neuropathy [294]. As

far as we know, the connection between the tubulointerstitial injuries in MM and the serum and urine levels of periostin, has not been investigated in the scientific literature. In our study, we do not detect a significant difference in the serum levels of PON in NDMM patients with creatinine over 177 μmol/l and under 177 μmol/l (p=0.7745). On the other hand, we found a significant positive correlation between the three studied proteins with creatinine, moderate in strength for sRANKL (Spearman r=0.3528, p=0.0237) and significant in strength for OPN (Spearman r=0.6047, p<0.0001) and PON (Spearman r=0.5311, p=0.0004). Goranov et al. investigated the pathogenetic relationships between bone and kidney damage in MM patients. The authors concluded that they are based on the vicious circle principle and modulate each other's clinical expression [1].

2.2 Serum levels of sRANKL, OPN и PON, detected in the course of treatment

Besides at the diagnosis (stage T0), the serum levels of sRANKL, OPN and PON are also studied in their dynamics at stages T1, T2 and TA while carrying out the treatment of NDMM patients. A gradual decrease of the values is observed in the transition from one stage to the next for all proteins investigated. Using the Kruskal-Wallis test, it was found that there was a statistically significant difference for all three tested proteins measured at different stages of the study (sRANKL: $\gamma 2=20.45$, $p<0.0001$; OPN: $\gamma 2=24.7$, $p<0.0001$ and PON: $\gamma 2=25.49$, $p<0.0001$). Several additional *post hoc* Mann-Whitney tests were conducted to determine exactly which stages differed. When comparing the serum concentrations at stages T1 and T2 with their baseline values, a statistically significant difference is detected only for PON (T1 vs T0: p=0.0016; T2 vs T0: p=0.0024), while for sRANKL and OPN, the decrease of values is insignificant. The decrease of levels is substantial also for the three proteins investigated at stage TA both in relation to the baseline levels and the ones at stage T1 (sRANKL, OPN and PON – TA vs T0: p<0.0001, p<0.0001 and p<0.0001, respectively; TA vs T1: p<0.0001, p<0.0001 and p=0.0054, respectively). The three proteins differ according to the extent of decrease of their levels in the course of the treatment applied and PON is the one that changes most dynamically. The serum levels of sRANKL, PON and OPN, three months after ASCT are so drastically reduced that they do not differ from those observed in the control group. The levels reached in the non-transplant patients at stage T2 are close to the ones of the control group only for PON (p=0.1899), while for OPN, a tendency of significant difference from the control values is observed (p=0.0506), and for sRANKL a significant difference (p=0.006) is detected. While carrying out a subgroup analysis of the data from stage T1 by additional division of the patients (eligible or not for ASCT), it is ascertained that the patients in group T1(A) have significantly lower levels in relation to T0 for sRANKL (p=0.0032) and for PON (p=0.0042), but not for OPN (p=0.1533). The patients from group T1(nonA) do not show difference in the values in relation to T0 but only for PON (p=0.0402). These data emphasize again that the therapy applied most significantly affected PON values already at T1 stage for both subgroups. In order to reach a significant decrease of sRANKL at stage T1, it is necessary for the patients to have achieved CR or VGPR, while for OPN the type of response is not defining. OPN is influenced most weakly by the conventional therapy and a significant decrease of its values is observed only after ASCT.

Besides with the proven antitumor activity [334] and induction of OBs differentiation [367], bortezomib could have an inhibiting effect on the expression of RANKL. Lin et al., in an experimental investigation, detect a reduced expression of RANKL in human myeloma cell line RPMI 8226 when treating patients with bortezomib, which depends on both time and dose [205]. In another experimental investigation with cells from a periodontal ligament, it is found out that bortezomib increases the expression of mRNA of a number of bone morphogenetic proteins, including also OPN

by increasing the cytosol accumulation and nuclear translocation of β-catenin [184]. As far as we know, no data is available in the scientific literature about the effect of bortezomib on the periostin expression. The results of the first experimental study explain, at least partially, the gradual decrease of serum levels of sRANKL observed in our investigation. This effect is most pronounced in patients who achieved CR and VGPR as well as after ASCT. The increased rather than suppressed OPN expression demonstrated in the second experimental study could explain the more non-dynamic changes in serum OPN levels we observed during the course of treatment.

Terpos et al. in two consecutive investigations detect in patients with MM relapse a significant decrease of sRANKL in the course of monotherapy with bortezomib in the first study or application of the VMDT protocol (bortezomib, melphalan, dexamethasone, thalidomide) in the second. In both studies the authors prove a significant decrease of sRANKL already in the 4^{th} course ($p=0.01$), as well as after the 8^{th} course ($p<0.001$) [339,355]. It is curious that in the second investigation the authors relate the decrease of sRANKL also to the therapy response. In contrast to them, we observe an insignificant decrease of sRANKL at stages T1 and T2, but in the additional subgroup analysis of the data from T1, we detect, same as they do, a significant decrease of sRANKL in patients who have achieved CR or VGPR. Sfiridaki et al., while applying the PAD protocol (bortezomib, adriamycin and dexamethasone), also detect a significant decrease of sRANKL only in the patients who have achieved CR and VGPR and have undergone an ASCT ($p<0.005$) [31](Sfiridaki 2011). Contrary to us, in a later investigation, Terpos et al. detect values of sRANKL in transplant patients still different from the values in their control group ($p=0.037$)[62].

Monitoring the effect of therapy on the OPN values is found in a few investigations. A study carried out by Sfiridaki et al. reports a significant decrease of OPN after a chemotherapy is given ($p<0.05$). The therapeutic regimen (medications and number of courses, as well as response achieved) is not pointed out in the article [309]. Lower values of OPN again are detected in the study done by Minarik et al. after a chemotherapy is given and a remission of the disease is achieved ($p=0.0003$) in comparison to those at the diagnosis. Various protocols are used, and 40% of the patients follow established treatment protocols that contain thalidomide, again the number of courses is not stated precisely [232]. Terpos et al. monitor patients with MM relapse in dynamics (after the 4^{th} and 8^{th} course of treatment, following the VMDT protocol). Contrary to us, the authors detect a significant decrease of OPN values, both after the 4^{th} and after the 8^{th} cycle ($p<0.01$ and $p<0.0001$, respectively) [355]. In our opinion, this rapid and considerable decrease in the OPN serum levels in the above-mentioned studies could be due to the thalidomide included in the protocols. It is established that thalidomide in a mouse model of a hepatocellular carcinoma suppresses the OPN synthesis and thus it reduces the tumor infiltration and metastatic activity [204]. We suppose that thalidomide could have a similar effect in MM, too. As far as we know, only Minarik et al. monitor the effect of ASCT on the OPN levels and detect a significant decrease in relation to the baseline levels, independently of the response achieved [232].

In the only study concerning the periostin levels in MM, there is scarce data on the effect of the therapy applied. Serum PON levels in patients in the plateau phase (6 months with stable M-protein) have been reported. The authors detect a “borderline” decrease of PON concentration, but they point out to a significant value for p ($p=0.05$). At the same time PON values continue to be elevated in comparison to the healthy controls ($p=0.013$) [347]. In other words, a positive effect of the therapy is observed on the serum PON levels. In this respect, our results are similar to those of the above-mentioned study. However, data on PON levels immediately after completion of one cycle or another

during the course of therapy are lacking, and the therapeutic regimen with which patients were treated is not mentioned. It is difficult under these circumstances to assess the dynamics of the change of concentration of the circulating protein in their research as it has been established in our investigation.

2.3 Serum levels of sRANKL, OPN и PON depending on the treatment response

The dependency of the serum levels of sRANKL, OPN and PON on the response achieved in relation to the therapy has also been monitored in the present study. For all proteins investigated, the patients who achieve CR and VGPR, have values significantly lower than those in patients with PR or in SD ($p=0.0167$, $p=0.0134$ and $p=0.0510$ respectively for sRANKL, OPN and PON). In the first group (CR and VGPR) values of OPN and PON are so significantly decreased that they are not different from the ones of the control group. Contrary to them, sRANKL levels in this patients' group are still significantly more different than the controls ($p=0.0036$). We take into consideration the fact that the patients' group who achieved CR and VGPR, is not homogenous. It includes all the patients with such a response notwithstanding the stage of treatment at which they have achieved it. The sRANKL levels of T1(A) patients, although they have already achieved CR and VGPR at stage T1, are significantly higher than the ones of TA patients ($p=0.0005$), as well as compared to the controls ($p=0.0012$). Only the TA patients are with sRANKL values, no different from those of the control individuals. Thus, it appears that high-dose chemotherapy followed by ASCT is able to normalize sRANKL levels, an important element for improving imbalances in bone remodeling processes. In contrast to us, research performed by Terpos et al. detects sRANKL values in transplant patients still different from the values in their control group. While carrying out a consolidating therapy following the VTD protocol (6 courses). The authors report a significant change only in CTX levels reaching those of controls, but not for the other bone biomarkers studied, including sRANKL [359].

As far as we know there is no data available in the scientific literature in relation to the dependency of the serum levels of OPN and PON on the response achieved in the treatment of MM.

3.3 Diagnostic reliability of the bone biomarkers investigated

Biomarkers in the modern healthcare practice are important instruments used in the diagnostic process in order to make a prognosis about the outcome of the disease and/or to monitor the treatment. The biomarker is defined as a specific feature, which can be measured objectively and serves as an indicator of normal and pathological biological processes as well as an assessment of the pharmacological response to a definite therapeutic intervention [35]. The bone is a metabolically active and dynamic structure. Physiological bone remodeling is deeply dysregulated in MM. The pathological processes taking place in the bone marrow after MPCs settlement and proliferation, lead to overexpression of various cytokines and ECM proteins, part of which enter the blood circulatory system and could be easily measured.

As an essential clinical feature of MM, the bone involvement is objectified mainly by imaging. The conventional radiology, considered in the past as a gold standard for detecting bone injuries, has been superseded by new methods of visualization which include WBLDCT, FDG-PET/CT and MRI. At present WBLDCT is a methodology widely accessible, comparatively cheap and easy to implement. It takes a short time for screening and is characterized by high sensitivity [267]. The European Myeloma Network, EMN and the European Society for Medical Oncology, ESMO recommend WBLDCT as a method of choice for a primary assessment of lytic bone lesions related to MM [414]. Nevertheless, the IMWG advises that care should be taken with overinterpretation of ambiguous or small (<5 mm) transparencies [277]. According to Baffour et al., WBLDCT has a

comparatively limited role in the evaluation of the treatment response [16]. This also explains the increased interest of the scientific community in identifying new biomarkers which could be used in the diagnostic process and monitoring of MBD.

The evaluation of the diagnostic tests is an important question in contemporary medicine not only for confirming the presence of a disease but also for excluding its presence in healthy people. The diagnostic reliability of a given laboratory method is measured with the help of probability values which characterize its ability to reliably distinguish the disease state from the healthy one. During the past four decades, the ROC analysis has become a popular method for assessing the accuracy of the diagnostic tests. An important feature of ROC analysis is that its accuracy indices do not get distorted by randomly chosen criteria, worked out by research groups. The derived generalized accuracy measure "area under the curve" (AUC), an effective and combined measure of sensitivity and specificity, defines the inherent ability of the test to distinguish the diseased from the healthy population [122].

In this research, we carried out a ROC analysis for assessing the diagnostic reliability of Dkk-1, Scl, sRANKL, OPN and PON. For all the parameters investigated AUC has a value more than 0.8 at $p<0.0001$, while for two of them (sclerostin and periostin) the AUC even reached a value close to 0.9 (for sclerostin AUC=0.9127, and for periostin AUC=0.8920). In ROC analysis, it is commonly accepted that if AUC=1, this means that the diagnostic test is perfect in distinguishing between the sick and the healthy individuals. A value that equals 1 is related only to cases when the patients' results do not coincide and overlap with those of healthy people. Thus, our results for AUC, close to 1, eloquently suggest that the indicators investigated are characterized by high diagnostic reliability. On the basis of the ROC analysis, thus performed, a cut-off value is also determined for each one of the indicators that are characterized by high sensitivity and specificity and a positive likelihood ratio (Likelihood ratio, LR) higher than 1. This shows how many times bigger the chance is that the individual examined is sick if their result is over the cut-off value determined (table 44).

Examining the dependency of serum levels of Dkk-1, Scl, sRANKL, OPN and PON on the extent of manifestation of the bone disease, a statistically significant change in the five parameters is established. This motivated us to investigate the predictive value of each of them by additional ROC analysis and their ability to correctly differentiate patients with mild skeletal involvement (G1) from those with severe involvement (G2). We determined the highest AUC value for sclerostin (0.9340, $p<0.0001$), followed by the ones for osteopontin (AUC=0.7639, $p=0.0167$), Dkk-1 (AUC=0.7438, $p=0.0479$) and periostin (AUC=0.7298, $p=0.0474$). The value of AUC for sRANKL is close to 0.5 at $p>0.5$. AUC=0.5 and means random discrimination, at which the curve lies on the diagonal line in the ROC space. This result of sRANKL makes it unsuitable and inapplicable for reliable differentiation between the patients at a different stage of bone disease. For all the remaining indicators, for which we have obtained high values of AUC, a cut-off value is also estimated with its corresponding diagnostic specificity and diagnostic sensitivity which could be used for determining the MBD weight (table 45). As far as we know, no research data is available in the scientific literature for conducting a ROC analysis of the indicators, which are the subject matter of this dissertation. This does not allow us to make a comparative analysis of our results with similar ones.

On the basis of the results of the ROC analysis, a logistic regression analysis was also used to assess and evaluate the individual effect of the biomarkers examined on the progression of the myeloma bone disease. The gradual regression analyses carried out show that the combination of the Scl and PON parameters is most significant, regarding prediction of the bone disease progression. A

regression equation was constructed, which could give a correct answer with a very high probability of specific values of the biomarkers periostin and sclerostin for a given patient, what could be the severity of their bone injury, that is whether he would be in group G1 or G2. The equation looks as follows:

$$p = \frac{e^{24.675\ -0.019[PON]-0.028[Scl]}}{1 +\ e^{24.675\ -0.019[PON]-0.028[Scl]}}$$

The ROC analyses, thus conducted, confirm the high diagnostic significance of the bone biomarkers examined while the regression analysis especially highlights two of them which could predict the bone disease with maximum likelihood.

VI. CONCLUSIONS

1. The serum levels of Dkk-1, sclerostin, sRANKL, osteopontin and periostin are significantly higher in NDMM patients compared with control subjects.
2. The serum levels of Dkk-1 and sclerostin in NDMM patients increase with advancing clinical stage of MM, reflecting suppressed osteoblastic activity in the bone marrow.
3. The serum levels of sRANKL, osteopontin and periostin in NDMM patients increase with advancing clinical stage of MM, reflecting an altered bone marrow microenvironment predisposing to increased osteoclast activity.
4. The serum levels of Dkk-1, sclerostin, sRANKL, osteopontin and periostin, in NDMM correlate with the severity of the myeloma bone disease.
5. The serum levels of Dkk-1, sclerostin, osteopontin and periostin reliably discriminate patients with milder bone involvement from those with more severe bone involvement and could be used as an additional tool to assess bone disease.
6. The positive correlation of the biomarkers with bone marrow infiltration and B2MG and the negative correlation with hemoglobin probably reflect tumor growth and disease progression.
7. The biomarkers investigated are characterized by a very good diagnostic potential and they reliably discriminate patients from healthy individuals, as demonstrated by ROC analysis.
8. The biomarkers investigated are dynamic indicators and change in the course of the treatment, with their serum levels decreasing as a function of the number of treatment courses carried out.
9. Bortezomib-based therapy not only suppresses the tumour process, but also has the potential to interfere with bone remodeling processes and improve the imbalance between osteoclastogenesis and osteoblastogenesis.
10. The lowest serum levels of Dkk-1, sclerostin, sRANKL, osteopontin and periostin during the course of the treatment are found after ASCT, demonstrating the role of high-dose chemotherapy not only in reducing tumor burden but also its beneficial effect on bone metabolism.
11. The levels of the biomarkers tested are dependent on the response achieved to therapy and are significantly lower in patients with CR and VGPR compared to other patients who responded to treatment. They could serve as an additional tool to assess the response to therapy.
12. Of all the biomarkers studied, sclerostin and periostin are the most sensitive and significant in predicting the progression of myeloma-induced bone disease.

VII. CONTRIBUTION

Original scientific contributions:

- For the first time, the dynamics of serum levels of new bone biomarkers during the course of therapy was investigated and their dependence on response to therapy was analysed.

Contributions of applied scientific value:

VIII. SCIENTIFIC PUBLICATIONS RELATED TO THE DISSERTATION

1. **Геров В.**, Герова Д., Мичева И., Галунска Б. Костни биомаркери при множествен миелом. "Мединфо" 2022, 04;80-86.
2. **В. Геров**, Д. Герова, И. Мичева, Б. Галунска. Дисрегулация на костното ремоделиране – основен патогенетичен механизъм на костната болест при множествения миелом. Хематология, vol. LIX, 2022, № 3-4:12-21.
3. **Gerov V,** Gerova D, Micheva I, Nikolova M, Mihaylova G, Galunska B. Dynamics of Bone Disease Biomarkers Dickkopf-1 and Sclerostin in Patients with Multiple Myeloma. J Clin Med. 2023 Jul 1;12(13):4440 (**5-Year Impact Factor: 4.1**).
4. **Gerov V,** Gerova D, Micheva I, Nikolova M, Pasheva M, Nazifova N, Galunska B. Circulating sRANKL, Periostin, and Osteopontin as Biomarkers for the Assessment of Activated Osteoclastogenesis in Myeloma Related Bone Disease. Cancers 2023 Nov 24;15(23):5562 (**5-Year Impact Factor: 5.6**).

IX. PARTICIPATION IN SCIENTIFIC FORUMS RELATED TO THE DISSERTATION

еров В., Мичева И., Галунска Б., Герова Д. Роля на периостин за костната болест при мултиплен миелом. Дванадесети национален конгрес по хематология, 05-08.10 2023 г., Казанлък (доклад).

еров В., Мичева И., Герова Д. Dickkopf-1 и склеростин – инхибитори на остеобластната функция при миеломната костна болест. Дванадесети национален конгрес по хематология, 05-08.10 2023 г., Казанлък (постер).

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