Daiga Auziņa

**Analysis of Prognostic Significance
of Biochemical Markers and
Morphological Parameters
in the Development of Bone Disease
in Myeloma Patients**

Summary of the Doctoral Thesis
for obtaining a doctoral degree (*Ph.D.*)

Sector – Clinical Medicine
Sub-sector – Oncology and Hematology

Riga, 2021

RĪGA STRADIŅŠ
UNIVERSITY

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Abbreviations

Abbreviation	Full name
ALP	<i>alkaline phosphatase</i>
ASCT	<i>autologous stem-cell transplantation</i>
bALP	<i>bone-specific Alkaline phosphatase</i>
CRAB	<i>hyper<u>calc</u>emia, <u>re</u>nal failure, <u>a</u>nemia, <u>b</u>one lesions</i>
CrCl	<i>creatinine clearance</i>
CT	<i>computed tomography</i>
DEXA	<i>dual-energy X-ray absorptiometry</i>
DKK-1	<i>Dickkopf-1</i>
DSS	<i>Durie and Salmon System</i>
ECOG	<i>the Eastern Cooperative Oncology Group</i>
ESMO	<i>the European Society for Medical Oncology</i>
FISH	<i>fluorescence in situ hybridization</i>
GFR	<i>glomerular filtration rate</i>
IL-1	<i>interleukin-1</i>
IL-6	<i>interleukin- 6</i>
IMWG	<i>the International Myeloma Working Group</i>
MWG	<i>the International Myeloma Working Group</i>
ISS	<i>International Staging System</i>
KMBM	<i>bone turnover biochemical markers</i>
BMI	<i>body mass index</i>
LDH	<i>lactate dehydrogenase</i>
MGUS	<i>monoclonal gammopathy of undetermined significance</i>
MIP-1 α	<i>macrophage inflammatory protein -1 α</i>
MM	<i>multiple myeloma</i>
MRI	<i>magnetic resonance imaging</i>
MRONJ	<i>medication-related osteonecrosis of the jaw</i>

NCCN	<i>National Comprehensive Cancer Network</i>
NCI	<i>National Cancer Institute</i>
NTX	<i>amino-terminal cross-linking telopeptide</i>
OB	<i>osteoblast</i>
OC	<i>osteoclast</i>
ONJ	<i>osteonecrosis of the jaw</i>
OPG	<i>osteoprotegerin</i>
P1CP	<i>total procollagen type 1 C-terminal propeptide</i>
P1NP	<i>total procollagen type 1 N-terminal propeptide</i>
PC	<i>plasma cell</i>
PET/CT	<i>positron emission tomography/computed tomography</i>
PTHrP	<i>parathyroid hormone-related peptide</i>
RAKUS	<i>Riga East Clinical University Hospital (in Latvian: Rīgas Austrumu klīniskā universitātes slimnīca)</i>
RANK	<i>receptor activator of nuclear factor kappa B</i>
RANKL	<i>receptor activator of nuclear factor kappa B ligand</i>
R-ISS	<i>Revised International Staging System</i>
SMM	<i>smoldering myeloma</i>
SREs	<i>skeletal-related events</i>
ICD-10	<i>International Classification of Diseases, 10th Revision</i>
TNF	<i>tumor necrosis factor</i>
VEGF	<i>vascular endothelial growth factor</i>
VTE	<i>venous thromboembolism</i>
WBLDCT	<i>whole- body low dose CT</i>
WBXR	<i>whole- body X-rays</i>
Wnt signālcēļš	<i>wingless- type signaling</i>
β2-M	<i>β2-microglobulin</i>
β-CTX	<i>carboxy-terminal cross-linking telopeptide</i>

Introduction

Actuality of the study

Multiple myeloma (MM) is a primary bone marrow malignancy characterized by uncontrolled growth and proliferation of mature and immature plasma cells that secrete monoclonal immunoglobulins in the blood and/or urine (Roodman, 2009; Terpos, 2018; Rajkumar, 2014; Kyle, 2003; Kyle, Morgan, 2012; Swerdlow, 2016; *Луговская*, 2014). Since plasma cells develop from B lymphocytes, MM is sometimes referred to as B-cell malignancy.

During the course of the disease, bone lesions with abnormal bone fractures, neurological syndromes due to spinal cord injury, anemia, fatigue, weakness, kidney damage, and secondary infections develop (McKenna et al., 2008; van Rhee et al., 2010; Rajkumar et al., 2014).

Around 100 patients with MM are registered in Latvia every year (5 cases per 100,000 population). Given the current demographic trends of the world's population, the incidence of myeloma is projected to increase to 12 cases per 100,000 population in the United Kingdom by 2035 (Smittenaar et al., 2016).

MM is the only nosology of hemato-oncological diseases with secondary skeletal damage that is often found already at the time of diagnosis. Bone disease, especially lytic destruction of bone, is a very common clinical manifestation of MM, which significantly affects the patient's quality of life (Kiely et al., 2017; Mateos et al., 2020).

Osteolytic bone damage is observed in 70-80% of patients at the time of diagnosis, but up to 90% of patients develop bone lesions during the course of the disease (Terpos et al., 2013; Sezer, 2009; Melton et al., 2005).

Bone disease can cause skeletal-related events (SREs) with abnormal fractures, spinal cord compression, hypercalcemia, resulting in the need for radiological and surgical treatment (O'Donnell et al., 2017).

Unfortunately, there are no studies that identify patients at increased risk of developing bone lesions (Gupta et al., 2020). Conventional radiological examination methods are usually used to detect bone damage. However, they do not provide information on bone formation and resorption. Current guidelines for patient monitoring state that X-rays, CT scans, and MRI scans should only be performed if the patient has complaints. But changes in the bones appear already before the patient's complaints and before the bone changes have been radiologically confirmed. PET/CT can be used to detect early bone lesions, but their widespread use is limited by the expensive cost of the examination. In addition, the patient is often given a radioactive contrast agent during radiological examinations, and the many examinations result in the patient receiving an increased dose of radiation. The patient might also have trouble lying down during examinations due to pain and possible claustrophobia. Diabetics require special preparation.

Since bisphosphonate therapy reduces the formation of new bone lesions due to the inhibition of osteoclast activity, it is included in standard therapy for myeloma patients (Terpos et al., 2013; Morgan et al., 2010).

However, with the increase in complications following the long-term use of bisphosphonates (kidney damage, osteonecrosis of the jaw, hip fracture), the number of studies on methods for diagnosing and treating bone lesions is also increasing.

The pathogenesis of MM and bone metabolism should be borne in mind when considering the earliest possible detection of bone lesions or the assessment of the potential risk of bone lesions. Myeloma cells are known to produce a number of factors that stimulate osteoclast formation and activity, thereby increasing bone resorption and, conversely, inhibiting osteoblast function by inhibiting bone formation. This results in osteolytic bone lesions (Sezer, 2009; Abe, 2011; Roodman, 2009, 2011; Terpos et al., 2005; Yaccoby, 2010).

Bone markers are divided into resorption and bone formation markers. Bone resorption markers (β -CTX) are products of osteoclast or collagen degradation, while bone formation markers (e.g. bone alkaline phosphatase – bALP) are produced by osteoblasts. They can be determined to assess the risk of bone lesions/fractures and the effectiveness of treatment during the specific therapy of patients (Lund et al., 2010; Hlaing et al., 2014).

In practice, they are used to evaluate the effectiveness of osteoporosis therapy, but until now they have not been used in the case of MM.

The Wnt signaling system is known to play an important role in osteoblast growth and differentiation, and Dickkopf-1 (DKK-1) is the inhibitor of this signaling system (Ray et al., 2016; Spaan et al., 2018). DKK-1 is able to bind to Wnt signaling system receptors, block osteoblast differentiation and proliferation, leading to decreased bone formation and lytic bone changes (Terpos et al., 2018; Qiang et al., 2008; Heath et al., 2009; Menezes et al., 2012). DKK-1 can be detected by immunohistochemical methods in myeloma cells in the bone marrow, and the degree of DKK-1 expression is related to the severity of bone damage in MM patients. Several studies have confirmed increased expression of DKK-1 in MM cells, and especially in patients with severe bone lesions (Heath et al., 2009; Tian et al., 2003).

In addition, morphological examination of myeloma cells in trephine biopsy material can determine the severity of plasma cell malignancy associated with the development of bone lesions (Bartl et al., 1987).

Although MM is still an incurable disease, improvements in therapy, including new targeted therapies, monoclonal antibodies, and the increasing use of stem cell transplantation in the treatment of MM, contribute to improving patients' quality of life and reducing MM symptoms (Thorsteinsdottir et al., 2014; Turesson et al., 2018; Zhang et al., 2012; Anderson et al., 2011; Yi Q, 2009; Suzuki et al., 2020).

It is therefore important to study, collect and analyze data on the development of bone lesions in order to assess the effectiveness of early diagnosis and treatment, as well as the risk of bone lesions. The biochemical and immunohistochemical markers of bone metabolism may be an alternative to quantify the bone status in patients with MM and to reflect bone resorption and formation activity.

Aim of the study

To prove the prognostic significance of biochemical markers and morphological indicators of bone disease development in myeloma patients

Objectives of the study

1. To determine the level of β -CTX at the time of MM diagnosis and during the disease course, as well as to compare it with the level of control group individuals and to analyze its association with the severity of bone lesions.
2. To determine the level of β -CTX in patients with different therapeutic effects.
3. To determine the level of bALP at the time of diagnosis of MM and during the disease course, as well as to compare it with the level of control group individuals and to analyze its association with the severity of bone lesions.
4. To determine the level of bALP in patients with different therapeutic effects.
5. To determine the level of DKK-1 expression in myeloma cells and to evaluate its association with osteolytic bone lesions.
6. To determine the morphological features of plasma cells and analyze their association with osteolytic bone lesions.

Hypotheses of the study

1. Patients with high plasma cell malignancy and more pronounced expression of DKK-1 have a higher risk of developing bone lesions, which can be detected already at the time of diagnosis.
2. The biochemical markers of bone metabolism allow the detection of bone disease in patients at the time of diagnosis of myeloma and allow to predict the dynamics of bone disease development.

Scientific and practical novelty of the study

1. An in-depth study of morphological features of plasma cells and DKK- 1 expression in myeloma cells has been performed.
2. The study obtained data on markers of bone metabolism in patients with myeloma – both at the time of diagnosis of MM and in the dynamic of the treatment process. This allows the diagnosis of MM patients at increased risk of developing bone lesions and allows the right decision to be made on the initiation and duration of bisphosphonate treatment to reduce the risk of developing treatment complications.

The structure of the thesis and the author's contribution

The thesis is written in Latvian. Structure of the dissertation: introduction, aim of the study, objectives, hypotheses, literature review, materials and methods, results, discussion, conclusions, references. The volume of the thesis is 108 pages, including 15 tables, 49 figures, and 2 appendices. The bibliography includes 331 titles.

The author of the thesis has performed research planning, literature analysis, examination of all patients included in the study, bone marrow aspiration, and trephine biopsy to confirm the diagnosis, and knows the methodology of plasma cell morphological features and DKK-1 expression in myeloma cells. The author has got acquainted with and mastered the laboratory methods used for the analysis of biochemical markers of bone metabolism. The author of the thesis has summarized, systematized, and analyzed the results of the performed examinations. The author has written this thesis and the related publications.

Ethical aspects of work

The research work was performed based on the Declaration of Helsinki of 1964 and the permission of the Ethics Committee of Rīga Stradiņš University issued for the study on June 10, 2014. All patients were orally briefed on the progress of the study, provided with written material, answered questions, if they had any, and they voluntarily completed and signed an informed consent form. All stages of the study were conducted in accordance with ethical principles, maintaining the confidentiality and anonymity of the respondents.

1 Materials and methods

The clinically analytical study “Analysis of prognostic significance of biochemical markers and morphological parameters in the development of bone disease in myeloma patients” is a retrospective case-control study. The research was performed with the permission of the Ethics Committee of Rīga Stradiņš University; the date of the meeting of the Ethics Committee: 10.06.2014.

Place of the conduction of the study

1. Riga East Clinical University Hospital (in Latvian: *Rīgas Austrumu klīniskā universitātes slimnīca* — RAKUS), Oncology Centre of Latvia, The Clinic of Chemotherapy and Hematology
2. Rīga Stradiņš University, the Institute of Anatomy and Anthropology
3. LLC “E. Gulbja laboratory”

Technical support

Technical means were utilized for the research, using the possibilities of RAKUS, Clinic of Chemotherapy and Hematology; Rīga Stradiņš University, Institute of Anatomy and Anthropology, and LLC “E. Gulbja laboratory”, and with the support of the Rīga Stradiņš University Doctoral Study Grant, Roche Academy and the Support Fund of the Riga East Clinical University Hospital.

1.1 Characteristics of study and control groups

MM patient study group

The study patient group consisted of 123 newly diagnosed MM patients treated at RAKUS, The Clinic of Chemotherapy and Hematology from June 2014 to June 2016 with a clinically and histologically confirmed myeloma disease.

The study included newly diagnosed patients with myeloma who had not yet received chemotherapy. The diagnosis of MM and the severity of the disease were determined according to generally accepted defined diagnostic criteria. Patients underwent tests and analyses to confirm the diagnosis and determine the stage of the disease. Functional status was determined for each patient. At the time of diagnosis and during the investigation, patients with and without bone lesions were identified.

Patient grouping:

Group 1 – patients with bone lesions,

Group 2 – patients without bone lesions.

Biochemical markers of bone metabolism were determined in all patients before treatment, and then specific chemotherapy as well as the treatment of bone disease were initiated. Repeated investigations were done after 6 and 12 months.

49 of the enrolled patients, who underwent routine bone marrow trephine biopsy at the time of diagnosis, had their plasma cell morphology evaluated and DKK-1 expression in myeloma cells determined. These patients were also divided into two groups:

Group 1 – patients with bone lesions,

Group 2 – patients without bone lesions.

Control group

Exclusion criteria were developed for the selection of patients for the study control group, excluding as many co-factors and confounders as possible that could affect the results of the study. 1128 age- and sex-matched outpatients, who had no data of osteoporosis or diseases that could alter bone metabolism (e.g. rheumatic diseases, primary hyperparathyroidism, Paget’s disease) and who had not received or were not receiving medications with potential to cause osteoporosis, were used for control. All patients had submitted analyzes to the E. Gulbja laboratory.

1.2 Research scheme

The research scheme is shown in Figure 1.1.

Figure 1.1. Research scheme

* They are monitored at the time of diagnosis of the disease, as well as after 6 and 12 months.

1.3 Criteria for inclusion and exclusion of the study subjects

Inclusion and exclusion criteria were developed for the selection of patients for the study, which reduce the impact of possible systematic errors on the study results.

Inclusion criteria: patients with diagnosis code C90.0 – myeloma disease according to the ICD-10 (International Statistical Classification of Diseases and Related Health Problems, 10th edition). Patients were newly diagnosed, and they were not receiving specific therapy.

Consent to participate in the study was obtained from all patients.

Exclusion criteria:

1. Patients with a history of confirmed osteoporosis and patients who have received specific osteoporosis therapy;
2. Patients with a history of oncological diseases, excluding local non-melanoma skin tumors;
3. Patients who have received glucocorticoid therapy in the last year (for rheumatological diseases, bronchial asthma, chronic obstructive pulmonary disease, neurological diseases);
4. Patients with a $GFR < 30 \text{ ml/min/1.73 m}^2$ were excluded from the study due to the renal excretion of β -CTX as it could give erroneous results.

1.4 Analysis of radiological data

Bone lesions were identified by X-ray, CT, MRI, PET/CT and were classified into severity degrees 0–3 according to the classification of M. Alexandrakis.

Severity degrees of bone lesions 0–3 (M. Alexandrakis, 2002):

- 0) – No bone lesions,
- 1) – ≤ 3 bone lesions,

- 2) – ≥ 3 bone lesions,
- 3) – Vertebral compression fractures.

At the time of diagnosis, patients with and without bone lesions were identified.

Disease progression was defined as one or several new bone lesions.

1.5 Analysis of laboratory parameters

Clinical blood tests

A complete blood test was performed with an automatic analyzer. Blood counts were analyzed at the time of diagnosis, after 6 and 12 months.

Biochemical blood tests

Biochemical parameters determined by an automatic analyzer:

Lactate dehydrogenase (LDH) (N 200–480 U/L), creatinine (N 30–80 $\mu\text{mol/l}$), GFR (N $> 90 \text{ ml/min/1.73m}^2$), total protein (N 65–85 g/l), albumin level (N 34–52 g/l), paraprotein in the blood (N 0g/l) and urine (N 0g/l), CRP (N $< 8 \text{ mg/l}$), β_2 microglobulin (N $< 2 \text{ mg/L}$), calcium (Ca) level (N 2.2–2.6 mmol/l).

Biochemical parameters were analyzed at the time of diagnosis, after 6 and 12 months.

1.6 Analysis of biochemical markers of bone metabolism

Biochemical markers of bone metabolism were identified in all patients prior to treatment and then specific chemotherapy was initiated, including treatment of bone disease. Each patient had 3 blood samples taken from them in a fixed order (at the time of diagnosis, after 6 and 12 months) during the study.

All analyses were performed in one laboratory (E. Gulbis laboratory).

Blood sampling was performed in the morning on an empty stomach at 7.00 before diagnostic procedures and medication. The total amount of blood was 13 ml.

For long-term studies, samples were taken under the same conditions as the original sample, as serum β -CTX levels are subject to circadian rhythm.

The samples were immediately delivered to E. Gulbis laboratory, where no later than 30 minutes after blood sampling, they were centrifuged at 3000 rpm for 10 minutes.

Due to renal excretion of β -CTX, patients with $\text{GFR} < 30 \text{ ml/min/1.73 m}^2$ were excluded from determination of β -CTX levels.

Biochemical markers of bone metabolism were examined by the enzyme-linked immunosorbent assay with a high-specificity monoclonal antibody variant.

The level of **bone alkaline phosphatase** was determined by the immunochemical luminescence method in the LIAISON analyzer (DiaSorin, Inc., USA).

The LIAISON BAP OSTASE immunoassay is a direct, two-step “sandwich-type” assay that uses two affinity-purified mouse monoclonal antibodies to attract and determine bALP. A 50 μ L human serum sample is used in the analysis, which

- 1) is incubated with paramagnetic particles coated with bALP monoclonal antibody;
- 2) after incubation, a second isoluminol-conjugated monoclonal antibody is added to the reaction for a short incubation period;
- 3) after incubation, the unbound material is removed during a wash cycle;
- 4) then the initial reagents are added and thus the chemiluminescence reaction is initiated. The light signal is measured by

the photoelectron multiplier as relative light units and is proportional to the concentration of bALP in the calibrators, controls, or samples.

Determination of **β -CTX levels** was performed by the electrochemical luminescence immunoassay “ECLIA” in Cobas e 601 immunoassay analyzers (Roche Diagnostics, Germany). The principle is almost identical to bALP, however,

- 1) the luminescence detection is slightly different because during the first incubation the antigens in the sample bind to biotinylated monoclonal antibodies (beta-CrossLaps);
- 2) during the second incubation with the addition of streptavidin-coated microparticles and a monoclonal beta-CrossLaps-specific antibody, a “sandwich complex” is formed, which is bound to the solid phase by the interaction of biotin and streptavidin (a very close chemical bond that normally binds different biomolecules to other molecules or a hard surface);
- 3) the reaction mixture is sucked into a measuring chamber where the particles are magnetically detected by the electrode surface;
- 4) when a voltage is applied to the electrode, chemical luminescence is emitted, which is detected by the photomultiplier;
- 5) the results are determined by the calibration curve. Calibration is performed with a known concentration of the substance, and the concentration of the substance in the sample can be determined from the curve.

1.7 Determination of patients' functional status

The functional status of oncology patients was assessed using the performance status scales of the Eastern Cooperative Oncology Group (ECOG) and Karnofsky.

Table 1.1

ECOG performance status

Grade	ECOG performance status
0	Fully active, able to carry on all pre-disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light housework, office work
2	Ambulatory and capable of all self-care but unable to carry out any work activities; up and about more than 50% of waking hours
3	Capable of only limited self-care; confined to bed or chair more than 50% of waking hours
4	Completely disabled; cannot carry on any self-care; totally confined to bed or chair
5	Dead

* Modified according to: Oken et al., 1982.

Table 1.2

Karnofsky performance status

Index	Karnofsky performance status
100	no complaints; no evidence of disease
90	able to carry normal activity; minor signs or symptoms of the disease
80	normal activity with effort; some signs or symptoms of disease
70	cares for self; unable to carry on normal activity or to do active work
60	requires occasional assistance but is able to care for most personal needs
50	requires considerable assistance and frequent medical care
40	disabled; requires special care and assistance
30	severely disabled; hospital admission indicated although death not imminent
20	very sick; hospital admission necessary; active supportive treatment necessary
10	dying
0	dead

* Modified according to: Karnofsky, 1949.

The comparison of the ECOG Performance Status and the Karnofsky Performance Status is shown in Table 1.3.

Table 1.3

**The comparison of the ECOG and
the Karnofsky performance status scales**

ECOG performance status	Karnofsky performance status
0	100–90
1	80–70
2	60–50
3	40–30
4	20–10
5	0

* Modified according to: Karnofsky, 1949; Oken et al., 1982.

1.8 Determination of the disease stage

The Durie and Salmon and the International Staging System (ISS) classifications were used to determine the stages of MM disease.

The criteria for the Durie and Salmon Staging System of myeloma and the Revised International Staging System (R-ISS) for Multiple Myeloma are shown in Table 1.4.

Table 1.4

The criteria of the Durie and Salmon and the Revised International Staging System classifications

Stage	Durie and Salmon Staging System	R-ISS
I	Hemoglobin > 100 g/L Normal calcium levels No bone lesions Low paraprotein levels: IgA < 50 g/L or IgG < 30 g/L Bence-Jones protein in urine < 4 g/day	<ul style="list-style-type: none"> ● ISS I stage (β2 microglobulin levels < 3.5 mg/L; serum albumin > 3.5g/dl) ● No high-risk cytogenetic changes ● Normal LDH
II	Parameters between I and III	Parameters between I and III
III	Hemoglobin < 85 g/L Hypercalcaemia (> 3 mmol/L) > 3 lytic bone damage High paraprotein levels: IgA > 70 g/L or IgG > 50 g/L Bence-Jones protein in urine > 12 g/day	<ul style="list-style-type: none"> ● ISS III stage (β2 microglobulin levels > 5.5 mg/L) ● High risk cytogenetic changes [t (4;14), t (14;16) or del (17p)] or elevated LDH

* Modified according to: Palumbo, et al., *J Clin Oncol*, 2015; 33: 2863–2869.

1.9 Light microscopy and immunohistochemistry of trephine biopsies

Bone marrow trephine biopsy specimens were obtained from 49 patients who were with newly diagnosed MM. Procedures were performed with a trephine biopsy needle or with an automatic biopsy device shown in Figure 1.2.

Figure 1.2 **a) automatic device; b) trephine biopsy needle**

The procedure was preceded by skin disinfection followed by local anesthesia.

The procedure was performed in a place where the red bone marrow is technically more easily accessible (the bone is more protruding), most often, *spina iliaca posterior superior* (see Figure 1.3). The duration of the procedure was 15–45 minutes.

Figure 1.3. **Bone marrow examination**
(modified according to: myeloma.org.uk, 2017)

For routine histological staining and immunohistochemical methods, the research material was processed at the Institute of Anatomy and Anthropology of Rīga Stradiņš University.

Bone marrow trephine biopsy tissue samples were fixed in 10% formalin, dehydrated in increasing concentrations of ethyl alcohol, and embedded in paraffin for further histopathological and immunohistochemical examination. Subsequently, 4–6 μm thick histological sections were made from the hardened paraffin-embedded tissue material with a microtome and placed on SuperFrost Plus slides (MenzelGmbH, Germany). The sections were deparaffinized and rehydrated by placing them in xylene and various concentrations of alcohol

(from higher to weaker) and in distilled water. After deparaffinization and rehydration, the sections were placed in a methanolic solution with 0.3% H₂O₂ to neutralize endogenous peroxidase activity. Antigen epitope retrieval and amplification was performed in a 10 mM sodium citrate buffer, pH 6.0, at 100 °C for 5 minutes in the microwave. The immunohistochemical reaction was performed conventionally using a polyclonal rabbit anti-human DKK-1 antibody (Sigma Life Science, Inc., Sigma, Aldrich, St. Louis, USA, HPA018995, 1:20) that binds to the DKK-1 precursor recombinant protein antigen. Amplification of the primary antibody was performed using a HiDef Detection HRP Polymer system, and a diaminobenzidine-tetrahydrochloride substrate kit (Cell Marque, Rocklin, CA, USA) was used to visualize the reaction. The sections were then stained with Mayer's hematoxylin, rinsed under running water, and covered with coverslips. The prepared and stained microproducts were photographed with a Leitz DMRB bright-field microscope using a DFC 450C digital camera and a Glissando slide (micropreparation) scanner (Objective Imaging Ltd., Cambridge, UK 0.5 µm/pixel resolution with 20× magnification lens and 0.2755 µm/pixel resolution, using a 40× magnification lens).

Histopathological and immunohistochemical evaluation criteria

Plasma cell morphology (number, size, shape and structure) was analyzed in microarrays stained routinely with hematoxylin and eosin using the Bartl scoring system. Myeloma cells were divided into six types: 1) Marschalko, 2) small cell, 3) cleaved, 4) polymorphic, 5) asynchronous, 6) plasmoblastic cell type. These types were then divided into three myeloma cell prognostic groups: low malignancy (Marschalko and small cells), moderate malignancy (cleaved cells, polymorphic and asynchronous cells), and high malignancy (plasmoblastic cells) (Bartl et al., 1987).

The amount of immunoreactive cells was counted using a semi-quantitative counting method. Cells whose cytoplasm was marked brown in the cytoplasmic immunohistochemical reaction were considered immunoreactive (immunopositive). DDK-1 immunopositivity was assessed semi-quantitatively in four randomly selected fields of vision: negative – no cells were stained (0%); low expression (1–10%); moderate expression (11–50%) and pronounced expression (51–100%).

The result consisted of the findings of two independent pathologists who did not know the patients' clinical and radiological data.

1.10 Statistical analysis of data

The graphics, calculations, and statistical analyses included in the study were performed using R v.4.0.1 (R Foundation for Statistical Computing, Vienna, Austria) and Medcalc v.14.

The normal distribution of the data was checked by the Kolmogorov–Smirnov sample test. If the sample size distribution corresponded to the normal distribution, they were plotted as mean (M) and standard deviation ($\pm$ SD). Otherwise, data are presented as median (Md) and interquartile range (IQR).

Statistical analysis of normal distribution data (parametric data): mean values between two separate groups were compared using Student's t-test; mean values between three and more individual groups were compared using one-way analysis of variance (ANOVA).

Statistical analysis of data whose values did not correspond to the normal distribution (non-parametric data): the mean values between the two separate groups were compared using the Mann–Whitney U-test; means between three and more individual groups were compared using the Kruskal–Wallis H-test. Wilcoxon or Friedman statistical tests were used to analyze the dependent samples.

Qualitative data were analyzed using the Pearson chi square statistical test if the expected frequency was > 5 , otherwise the Fisher exact test was used.

The method of correlation analysis was used to study the relationships of quantitative variables. Depending on the data distribution, parametric Pearson (denoted – r) or nonparametric Spearman (denotes – r_s) correlation analysis was used.

The following parameters were used to characterize the diagnostic markers: ROC curve, area under the ROC curve, limit value, test sensitivity (Se) and specificity (Sp), positive (PPV) and negative predictive value (NPV) when reaching the respective limit values. The accuracy of the diagnostic test was assessed by the area under the ROC curve according to the following classification: 0.90–1 = excellent; 0.80–0.90 = good; 0.70–0.80 = satisfactory; 0.60–0.70 = poor; and < 0.60 = not diagnostic.

A two-tailed p-value of less than 0.05 ($p < 0.05$) was considered statistically significant for all statistical tests used.

2 Results

2.1 Characteristics of the patients and control group individuals of the study

The study involved 123 patients – 70 (56.91%) women and 53 (43.09%) men, the minimum age was 33 years, the maximum was 86 years, the age range was 53 years, the mean age of the patients was $M = 67.70$; $SD = 10.40$ years, the modal or the most common age was 71 year, the median age was 69 years, the first age quartile was 60.5 years, and the third was 75 years, and the age-related interquartile distribution was 14.50 years (Table 2.1). Patients' age histogram and box diagram are shown in Figure 2.1 a), b).

Figure 2.1. **Patient age a) histogram, b) box chart**

Statistical analysis revealed that the median age did not differ statistically significantly between the study and control groups ($p > 0.05$), nor did the proportion of men and women differ statistically significantly between the control and study groups ($p > 0.05$). The basic demographic parameters are shown in Table 2.1.

Table 2.1

Basic demographic parameters

–	Control (N=1128)	Study (N=123)
Age, years		
M (SD), min–max	65.0 (9.15); 51.0– 91.0	67.7 (10.4); 33.0– 86.0
Median [Q1– Q3]	64.0 [57.0–72.0]	69.0 [60.5–75.0]
Gender		
Female	706 (62.5%)	70 (56.9%)
Male	422 (37.5%)	53 (43.1%)

2.1.1 ssessment of patients' disease stage

According to Durie and Salmon classification: stage 1 was observed in 24 (19.51%), stage 2 – in 17 (13.82%), and stage 3 – in 82 (66.67%) patients, but according to ISS classification: stage 3 was observed in 57 (46.34%), stage 2 – in 31 (25.20%), stage 3 – in 35 (28.46%) patients (Figure 2.2).

Figure 2.2. **Patient assessment according to Durie and Salmon and ISS classification**

2.1.2 Assessment of patients' functional status

Evaluating the functional condition of patients according to the Karnofsky index, it was found that the most common Karnofsky index for patients is 70; it is found in almost half of the patients – 50 (40.65%), see Figure 2.3 a). However, dividing patients according to the cut-off value of Karnofsky index below and

above 80, it was found that index above 80 is detectable in 36 (29.30%) cases, see 2.3 b).

Figure 2.3. **Karnofsky index: a) overall quantitative estimate, b) cut-off value below and above 80**

Evaluating the classification of patients according to the ECOG index (Fig. 2.4 a)), it was found that the most common index is 2, which is found in almost half of the patients – 55 (44.72%), but the least number of patients had an index of 4, which was 3 (2.44%) patients. In contrast, the ECOG index above 2 was found in 107 (86.99%) patients, see Figure 2.4 b).

Figure 2.4. **ECOG index: a) overall quantitative estimate, (b) cut-off value below and above 2**

2.2 Analysis of radiological data

All patients underwent X-ray and/or CT, but some underwent MRI (13 patients), PET/CT (2 patients), and bone scintigraphy (78 patients). Of the patients enrolled in the study, one-third – 37 (30.08%) – had no bone lesions

at the time of diagnosis, and more than half of the patients – 69 (56.10%) – had 3rd degree bone lesions with vertebral compression fractures. 1st and 2nd bone lesions were observed in 17 (13.82%) patients, see Figure 2.5.

Figure 2.5. **Quantitative assessment of the degree of bone lesions**

Analyzing the distribution of bone lesion localization, it was found that most bone lesions are found in the vertebrae (100 localizations), followed by the ribs (20 localizations) and the pelvis (16 localizations), see Figure 2.6.

Figure 2.6. Localization of patient bone lesions

Analyzing the distribution of vertebral lesions in patients, it was found that 82% of vertebral lesions were in Th8-L5 and 50% of them were found in Th11-L1, see Figure 2.7.

Figure 2.7. **Distribution of vertebral lesions in patients**

2.3 Analysis of laboratory parameters

Laboratory parameter values of patients are shown in Table 2.2.

Table 2.2

Laboratory parameters of patients at the time of diagnosis of the disease

Parameter	–
β2 microgl. (mg/l)	
M (SD), min–max	5.79 (6.01); 1.27–30.0
Median [Q1–Q3]	3.36 [2.41–5.69]
Albumin (g/l)	
M (SD), min–max	37.0 (6.38); 21.0–50.5
Median [Q1–Q3]	37.4 [31.8–41.6]
Plasma cells in the bone marrow (%)	
M (SD), min–max	39.2 (18.3); 10.0–80.0
Median [Q1–Q3]	40.0 [25.0–50.0]
LDH (U/L)	
M (SD), min–max	372 (128); 2.88–763
Median [Q1–Q3]	363 [280–435]
Hb (g/dl)	
M (SD), min–max	106 (23.3); 12.1–157
Median [Q1–Q3]	107 [89.5–124]
Ca (mmol/l)	
M (SD), min–max	2.35 (0.299); 1.78–4.07
Median [Q1–Q3]	2.29 [2.17–2.45]
Creatinine (μmol/l)	
M (SD), min–max	134 (172); 20.0–1140
Median [Q1–Q3]	83.0 [62.0–121]
GFR (ml/min/1.73)	
M (SD), min–max	75.8 (48.2); 4.00–348
Median [Q1–Q3]	69.0 [46.5–99.0]
Paraprotein in the blood (g/l)	
M (SD), min–max	25.6 (17.8); 0–76.1
Median [Q1–Q3]	23.4 [13.5–35.0]

The most common Ig class in patients was Ig G, in total – 106, but non-secreting myeloma was observed in 7 patients (Figure 2.8).

Figure 2.8. **Distribution of paraprotein immunological subclasses**

In analysis of renal insufficiency parameters, it was revealed that $\text{GFR} < 60$ (ml/min/1.73) was present in 81 (65.85%) patients, see Figure 2.9.

Figure 2.9. **Number of patients with kidney disease**

Mild anemia ($\text{Hb} > 100$ g/l) was observed in 75 (60.98%) patients, moderate anemia in 26 (21.14%) and severe anemia ($\text{Hb} < 85$ g/l) in 22 (17.89%) patients (Figure 2.10).

Figure 2.10. **Distribution of anemia severity in patients**

Laboratorically hypercalcaemia is classified as mild (Ca 2.6–2.9 mmol/l), moderate (2.9–3.1 mmol/l) and severe (> 3.1 mmol/l). At the time of diagnosis, one-sixth or 18 patients (14.63%) were diagnosed with hypercalcaemia, and, evaluating patients according to the severity of hypercalcaemia, 1st degree (10.55%) was observed in half of the hypercalcaemia patients (Figure 2.11).

Figure 2.11. Number of patients with a) hypercalcaemia, b) distribution of hypercalcaemia severity

2.4 Analysis of biochemical markers of bone metabolism

2.4.1 β -CTX level analysis

β -CTX levels were determined in patients at the time of diagnosis (β -CTX1), after 6 months (β -CTX2), and after 1 year (β -CTX3).

In MM patients without bone lesions at the time of diagnosis, the median β -CTX was 0.62 [0.48–0.89] ng/mL, but with 1st, 2nd and 3rd degree bone lesions, the median was 0.80, 0.89 and 1.10 ng/mL. Detailed information can be found in Table 2.3.

Table 2.3

β -CTX values at different degrees of bone lesions

Degree of bone lesion	Absent (N=37)	1 (N=6)	2 (N=11)	3 (N=69)
M (SD), min–max	0.837 (0.638); 0.114–3.11	1.02 (0.464); 0.534–1.90	0.990 (0.282); 0.670–1.71	1.29 (0.563); 0.550–3.22
Median [Q1–Q3]	0.620 [0.488–0.890]	0.80 [0.76–0.985]	0.890 [0.865–0.976]	1.10 [0.920–1.42]

Analysis of the Kruskal–Wallis statistical test indicated that the median β -CTX differed statistically significantly at different degrees of bone lesions ($p = 0.001$). Secondary analysis (post-hoc) showed no statistically significant

difference ($p > 0.05$) between absent bone lesions and 1st degree bone lesions, but a statistically significant difference ($p < 0.05$) between the other degrees of bone lesions, see Figure 2.12. Assessing the relationship between the severity of bone lesions and the level of β -CTX, it was found to be moderate, positive and statistically significant ($r_s = 0.51$; 95% CI: 0.35–0.62; $p < 0.001$).

Figure 2.12. Bone lesion degree and β -CTX changes:
a) box diagram and b) average values

The analysis of the Friedman statistical test showed that β -CTX decreased statistically significantly ($p < 0.001$) and decreased by 58% ($p < 0.05$) after 6 months of treatment, but by 29.2% after 1 year ($p < 0.05$), see Figure 2.13 a), b).

Figure 2.13. Changes in β -CTX over time: a) after 6 months (β -CTX1 – β -CTX2), b) from 6 months to a year (β -CTX2 – β -CTX3)

After 6 months of treatment, β -CTX decreased in 65 (69.89%) but increased in 28 (30.11%) patients, while, in between 6 months to 1 year of treatment, β -CTX decreased in 47 (66.20%) patients and increased in 24 (33.80%) patients. Detailed box diagrams of β -CTX changes are shown in Figure 2.14 a), b).

Figure 2.14. Changes in β -CTX a) after 6 months, b) from 6 months to 1 year

In patients who responded to treatment, β -CTX was statistically significantly reduced ($p < 0.001$) from baseline ($Md = -0.77$). In contrast, in patients who did not respond to treatment, β -CTX increased from baseline after 6 months of treatment ($Md = 0.28$), see Table 2.4 and Figure 2.15.

Table 2.4

Analysis of the difference in β -CTX changes after 6 months

–	Increased (N=28)	Decreased (N=65)
β-CTX difference after 6 months		
M (SD), min–max	0.287 (0.240); 0.029–0.960	–0.772 (0.443); –2.06–0.045
Median [Q1–Q3]	0.230 [0.109–0.386]	–0.756 [–0.907– –0.501]

Figure 2.15. The difference in β -CTX change after 6 months in patients with increasing and decreasing β -CTX

The mean β -CTX level depending on the effect of treatment is shown in Table 2.5.

Table 2.5

Dynamics of β -CTX depending on the effect of therapy

Patients	β-CTX M(SD) min–max	Difference (95% CI)	p-value
Responsive to therapy			
At the time of diagnosis (N=114)	1.12 (0.64) 0.11–4.09	–0.65 (0.41–0.66)	< 0.001
After 6 months (N=84)	0.47 (0.36) 0.03–1.77		
Non-responsive to therapy			
At the time of diagnosis (N=9)	1.38 (0.74) 0.86–3.22	0.42 (0.21–0.62)	< 0.001
After 6 months (N=9)	1.80 (0.92) 1.10–3.98		

2.4.2 bALP analysis

bALP levels were determined in patients at the time of diagnosis (bALP1), after 6 months (bALP2) and after 1 year (bALP3).

Analyzing the changes in bALP over time, it was found that bALP1 and bALP2 differed statistically significantly ($p < 0.001$), however, from 6 months to a year bALP3 no longer differed statistically significantly ($p = 0.06$), see Table 2.6 and Figure 2.16.

Figure 2.16. **bALP changes over time: a) after 6 months, b) from 6 months to 1 year**

Table 2.6

Changes in bALP values at the time of diagnosis, after 6 months and after 1 year

—	Values
bALP1 $\mu\text{g/L}$	
M (SD), min–max	13.0 (6.78); 4.20–45.0
Median [Q1–Q3]	11.2 [8.73–16.0]
bALP2 $\mu\text{g/L}$	
M (SD), min–max	11.1 (8.20); 3.50–50.7
Median [Q1–Q3]	8.70 [6.70–12.2]
bALP3 $\mu\text{g/L}$	
M (SD), min–max	9.97 (5.83); 3.10–39.1
Median [Q1–Q3]	9.00 [6.38–11.5]

Statistical analysis of the Kruskal–Wallis test showed no statistically significant difference in bALP levels between different degrees of bone lesion severity ($p = 0.95$), see Table 2.7 and Figure 2.17.

Table 2.7

**bALP values at different degrees of bone lesions at the time
of disease diagnosis**

Degree of bone lesion	Absent (N = 37)	1 (N = 6)	2 (N = 11)	3 (N = 69)
bALP1 $\mu\text{g/L}$				
M (SD), Min–max	13.6 (7.70); 5.10–45.0	13.6 (4.39); 10.4–22.4	12.1 (4.50); 5.60–23.5	12.8 (6.80); 4.20–42.8
Median [Q1–Q3]	10.5 [8.10–19.0]	12.1 [11.8–12.8]	11.3 [10.1–13.1]	11.0 [8.50–15.3]

Figure 2.17. bALP changes at different degrees of bone lesions

After 6 months of treatment, the value of bALP decreased in 60 patients or 64.52%, but increased in 33 (35.48%) patients (Figure 2.18).

Figure 2.18. bALP changes after 6 months

2.4.3 Analysis of β -CTX correlations with biochemical parameters, disease stage and the functional status of patients

The study investigated the possible correlation between β -CTX and β 2 microglobulin, albumin, LDH, calcium, Karnofsky index, ECOG, plasma cell infiltration in the bone marrow, Hb and GFR levels (see Figure 2.19).

Figure 2.19. β -CTX correlation diagrams with a) albumin, b) paraprotein in blood, c) Ca, d) LDH, e) Karnofsky index, f) GFA in blood

The analysis of the Spearman correlation coefficient showed that there is a weak, negative and statistically significant correlation between β -CTX and albumin ($r_s = -0.24$; 95% CI; $p < 0.01$), between β -CTX and plasma cell infiltration – a weak, positive and statistically significant correlation ($r_s = 0.24$; $p < 0.01$), between β -CTX and Hb – a weak, negative and statistically significant correlation ($r_s = -0.25$; $p < 0.01$), there is a positive, moderate and statistically significant correlation between β -CTX and Ca ($r_s = 0.37$; $p < 0.0011$), there is

a negative, moderate and statistically significant correlation between β -CTX and Karnofsky index ($r_s = -0.36$; $p < 0.001$), there is a weak, positive and statistically significant correlation between β -CTX and ECOG ($r_s = 0.28$; $p = 0.001$), there is a weak, positive and statistically significant correlation between β -CTX and paraprotein ($r_s = 0.20$; $p = 0.01$), and there is a weak, positive and statistically significant correlation between β -CTX and $\beta 2$ microglobulin ($r_s = 0.29$; $p < 0.01$). But no statistically significant correlation was found between β -CTX and LDH levels ($p = 0.62$).

In the correlation analysis, it can be observed that with increasing ECOG, β -CTX also increases (Figure 2.20), which is also confirmed by the calculated Spearman correlation coefficient ($r_s = 0.28$; $p = 0.001$).

Figure 2.20. β -CTX changes at different ECOGs

2.4.4 Evaluation of diagnostic tests

Analyzing the suitability of bALP for the diagnosis of MM patients using the ROC curve, it was found that bALP is not suitable for diagnosis ($AUC = 0.5$; $p > 0.05$), see Figure 2.21 a). In contrast, β -CTX was found to be an excellent

diagnostic indicator of MM bone disease (AUC = 0.91; $p < 0.001$; 95% CI: 0.88–0.94), see Figure 2.21 b).

Figure 2.21. a) bALP and b) β -CTX marker ROC curve of the diagnostic test for all subjects in the study

Table 2.8

β -CTX cut-off values for control/MM patients by sex and age

Gender	Age group, years	AUC; p-value (95% CI)	Cut-off value	Se% (95% CI)	Sp% (95% CI)	PPV% (95% CI)	NPV% (95% CI)
Females	51–70	0.91; <0.001; (0.85–0.97)	0.79	80 (63–91)	92 (79–88)	58 (43–71)	97 (94–99)
	>70	0.87; <0.001; (0.79–0.96)	0.82	68 (56–83)	92 (84–96)	73 (54–87)	90 (54–87)
Males	51–70	0.92; <0.001; (0.84–0.99)	0.79	75 (53–90)	93 (82–98)	85 (63–96)	88 (76–96)
	>70	0.80; <0.001; (0.67–0.94)	0.87	62 (40–81)	85 (87–98)	88 (63–98)	57 (34–78)

The calculated cut-off value for β -CTX for all patients regardless of gender and age for control group/MM patient diagnosis was 0.79 ng/ml (Sp = 91% (95% CI: 88-94); Se = 82% (95% CI: 69- 85); PPV = 72% (95% CI: 63– 79;); NPV = 93% (95% CI: 90-95)).

Of the total number of patients, β -CTX was less than 0.79 ng/ml in 27 (22.13%) patients and greater than 0.79 ng/ml in 95 (77.87%) patients. Analysis of the β -CTX cut-off value of 0.79 ng/ml in relation to the degree of bone damage revealed a statistically significant association between the two traits ($p < 0.001$). In patients without bone lesions, 22 patients had β -CTX less than 0.79 ng/ml and 15 patients had β -CTX greater than 0.79 ng/ml (see Table 2.9).

Table 2.9

Cut-off values for β -CTX at different degrees of bone lesions

β-CTX	≤ 0.79 ng/ml (N = 27; 22.13%)	> 0.79 ng/ml (N = 95; 77.87%)	p
Degree of bone lesion			
Absent	22 (81.5%)	15 (15.8%)	–
1	1 (3.7%)	5 (5.3%)	< 0.001
2	1 (3.7%)	10 (10.5%)	–
3	3 (11.1%)	65 (68.4%)	–

2.4.5 Comparison of β -CTX and bALP with control group

Analyzing the β -CTX and bALP values of the control and study groups (see Table 2.10), it was found that the median value of β -CTX for the study group was statistically significantly higher than for the control group ($p < 0.001$), while the median bALP for the control and study groups did not differ significantly ($p = 0.55$). The bALP and β -CTX box diagrams are shown in Figure 2.22.

Table 2.10

**Characterization of MM and control group β -CTX
and bALP at the time of disease diagnosis**

–	MM (N = 123)	Controls (N = 1128)	p
β-CTX, ng/mL			
M (SD), min–max	1.11 (0.596); 0.114–3.22	0.410 (0.242); 0.03–2.50	–
Median [Q1–Q3]	0.975 [0.813–1.29]	0.371 [0.234–0.543]	< 0.001
bALP, μg/L			
M (SD), min–max	13.0 (6.78); 4.20–45.0	13.5 (12.8); 1.50–217	–
Median [Q1–Q3]	11.2 [8.73–16.0]	10.9 [8.40–15.2]	0.55

Figure 2.22. **Box diagrams of MM patients and control groups:**
a) β - CTX, b) bALP

2.5 Plasma cell structure analysis

Plasma cells were divided into 6 types: 1) Marschalko, 2) small cell, 3) cleaved, 4) polymorphic, 5) asynchronous, and 6) plasmoblastic cell type. These types were further divided into 3 myeloma cell prognostic groups using the Bartl scoring system (Bartl et al., 1987): low malignancy (Marschalko and small cell type), moderate malignancy (cleaved cell, polymorphic and asynchronous cell type) and high malignancy (plasmoblastic cell type).

Marschalko-type – the most common type. Most of the cells have an oval or round shape, an eccentrically arranged nucleus with chromatin arranged similarly to the course of the wheel spokes, the perinuclear Golgi apparatus area,

and the basophilic cytoplasm (Figure 2.23). Cell size is around 20–21 μm . In some cases, mitosis is seen. About 10% of Marschalko plasma cells show the broadest cytoplasm with organoids.

Figure 2.23. **Marschalko-type (hematoxylin / eosin staining, $\times 250$)**

The **small cell type** is dominated by small (on average 13 μm), round cells. The cell nucleus is characterized by a dense arrangement of chromatin grains, the cytoplasm surrounds the cell nucleus in a narrow layer (Figure 2.24). Similar to Marschalko-type plasma cells, the perinuclear Golgi apparatus can be seen, but the nucleolus of the cell nucleus is usually invisible. Mitotic figures are usually not seen either.

Figure 2.24. **Small cell type (hematoxylin/eosin staining, ×250)**

The **cleaved cell type** – most plasma cells are characterized by a divided, broken, sometimes curved nucleus that varies greatly in size. Cytoplasm is smaller than in a Marschalko-type plasma cell, but the area of the Golgi apparatus is often visible.

The **polymorphic cell type** is characterized by cellular polymorphism, often seen as multinucleated and giant cells (black arrow). Plasma cells with the perinuclear Golgi apparatus area can be identified (Figure 2.25).

Figure 2.25. **Polymorphic cell type (hematoxylin/eosin staining, ×250)**

Asynchronous cell-type plasma cells are characterized by large, eccentrically arranged nuclei with pronounced nucleoli and basophilic cytoplasm. The Golgi apparatus area is also pronounced. Lymphocytes and Marschalko-type plasma cells can also be detected.

Plasmoblastic type – plasmoblasts with a large nucleus, with a pronounced, centrally located nucleolus. The Golgi complex area is usually not seen. Marschalko-type cells and lymphocytes can be seen.

Analyzing the distribution of plasma cell malignancy in patients with and without bone lesions, it was found that in patients with bone lesions, moderate plasma cell malignancy grade is 1.6 times more common than in patients without bone lesions and this difference is statistically significant, see Figure 2.26. In contrast, patients with high plasma cell malignancy grade are 5 times more likely to experience bone lesions than no bone lesions, and this difference is statistically significant, see Figure 2.26.

Figure 2.26. **Distribution of plasma cell malignancy grades in patients**
1) with bone lesions, 2) without bone lesions

2.6 Analysis of DKK-1 expression in myeloma cells

Plasma cells were evaluated based on morphological examination.

DKK-1 expression levels are shown in Figures 2.27, 2.28 and 2.29.

Figure 2.27. **Low expression of DKK-1**

Figure 2.28. **Moderate expression of DKK-1**

Figure 2.29. **Pronounced expression of DKK-1**

Using Fisher's exact statistical test, a statistically significant association was found between DKK-1 expression levels and patients with and without bone lesions ($p = 0.02$). The expression level of DKK-1 is higher in patients with bone lesions and they have more cases of moderate and pronounced expression. In contrast, pronounced levels of DKK-1 expression were not observed in patients without bone lesions, see Figure 2.30.

Figure 2.30. Distribution of DKK-1 expression levels in patients with and without bone lesions

Pronounced expression of DKK-1 is more common in patients with moderate plasma cell malignancy. In patients with a high grade of plasma cell malignancy, DKK-1 is expressed in all cases (Figure 2.31).

Figure 2.31. Distribution of DKK-1 expression levels at plasma cell malignancy levels

Analyzing the correlation between the plasma cell malignancy grade and β -CTX, no statistically significant correlation was found ($p > 0.05$), however, as shown in Figure 2.32, there is a tendency for β -CTX to increase at a higher grade of malignancy.

Figure 2.32. **Box diagrams of β -CTXs at different grades of plasma cell malignancy**

A similar trend, although not statistically significant ($p > 0.05$) – an increase in β -CTX at a more pronounced level of DKK-1 expression – was also observed, see Figure 2.33.

Figure 2.33. **Box diagrams of β -CTX at different levels of DKK1 expression**

Evaluation of β -CTX at different levels of DKK-1 expression in patients with bone lesions and without bone lesions showed that patients with bone lesions tend to have an increase in β -CTX with a higher degree of DKK-1 expression, whereas patients without bone lesions do not show such a visually observable trend, see Figure 2.34.

Figure 2.34. **Box diagrams of β -CTX at different levels of DKK-1 expression 1) with bone lesions, 2) without bone lesions**

Similarly, there was an observable trend of increase in β -CTX with a higher grade of plasma cell malignancy in patients with bone damage, whereas no such correlation was observed in patients without bone damage, see Figure 2.35.

Figure 2.35. Box diagrams of β -CTX at different grades of plasma cell malignancy in patients a) with bone lesions, b) without bone lesions

Statistical analysis using Fisher's exact test revealed that no statistically significant association was observed between DKK-1 and β -CTX above or below the cut-off value (0.79 ng/mL) ($p = 0.25$), however, it was observed (Fig. 2.36) that there are more patients with moderate to severe DKK-1 expression above the value of 0.79 ng/mL.

Figure 2.36. **DKK1 expression distribution at β -CTX a) below the cut-off value (0.79 ng/mL), b) above the cut-off value (0.79 ng/mL)**

Statistical analysis using Fisher's exact test showed that there was no statistically significant association ($p = 0.88$) between plasma cell malignancy levels and β -CTX below or above the cut-off value (0.79 ng/mL), however, (Figure 2.37) it was observed that there were more patients with moderate to high grade plasma cell malignancy above the cut-off value of 0.79 ng/mL.

Figure 2.37. **Plasma cell malignancy distribution at β -CTX cut-off value of 0.79 ng/mL**

Correlation analysis between plasma cell malignancy and calcium (Ca) did not show a statistically significant correlation ($p = 0.16$), nor was a statistically significant difference between plasma cell malignancy and Ca ($p = 0.16$) observed in the group comparison using the Wilcoxon test ($p > 0.05$), see Figure 2.38.

Figure 2.38. **Box diagram of Ca levels at different grades of plasma cell malignancy**

No statistically significant correlation was found in the correlation analysis between DKK-1 and calcium (Ca) ($p = 0.06$), nor was there a statistically significant difference between DKK-1 and Ca ($p > 0.05$) in the group comparison using the Wilcoxon test, see Figure 2.39.

Figure 2.39. **Box diagrams of Ca levels at different levels of DKK-1 expression**

3 Discussion

The doctoral thesis is devoted to MM, which is the only nosology of oncological haematological diseases with secondary skeletal damage, which is often found already at the time of diagnosis in up to 80% of patients, but during the disease course it develops in up to 90% of patients. Bone disease, especially pathological fractures, significantly affects the patient's quality of life. Although imaging plays an important role in the diagnosis and treatment of MM patients, in most cases it does not contribute to early detection of bone disease and does not provide information on bone formation and resorption; the study aimed to identify optimal laboratory and molecular biological methods for early detection and monitoring of MM bone disease. Osteolysis, osteopenia, pathological fractures, or a combination thereof are usually detected radiologically. 15–20% of patients have abnormal fractures and 5% experience spinal cord compression.

Current guidelines state that X-rays, CT scans, MRIs should be performed at the time of diagnosis of MM or only if the patient has complaints. However, changes in bone occur before the patient complaints and before radiologically confirmed bone changes. PET/CT can be used to detect early bone damage, but their widespread use is limited by the high cost of the examination. It is also important to note that osteoporosis is not included in the criteria for symptomatic MM, and Muchtar (2017) in his study confirms the ineffectiveness of densitometry in the diagnosis of MM patients.

The doctoral thesis consists of two parts of the research, which are united by the study of prognostic factors of multiple myeloma bone disease. Two biochemical markers of bone metabolism and the grade of myeloma cell malignancy, and DKK1 expression, determined by bone marrow trephine biopsy, were studied.

MM is typical for middle-aged or elderly patients and similar results were obtained in this study, where it was proved that the average age of patients in Latvia is 67.65 years (from 33 to 86 years). Of the patients enrolled in the study, 70 (56.3%) were women and this differs from that described in the literature, which states that myeloma is more common in men (Landgren et al., 2009; Kristinsson, 2007; Kyle, 2003; Thorsteinsdottir, 2020). According to the data available in the database of the Central Statistical Bureau (CSB), there are 269,564 women and 128,364 men in the age group over 65 among the Latvian population (i.e., a ratio of 2.1:1). This may explain the higher prevalence of myeloma among women in Latvia.

Knowledge of the pathogenesis of MM has grown rapidly over the past decade and therapeutic options have evolved (Pawlyn, 2017). Therefore, research into bone disease is very important. Bone metabolism is a dynamic process that can be detected by a variety of biochemical markers in the blood or urine and by immunohistochemistry, obtaining tissue samples during an invasive procedure. The pathogenesis of bone disease is associated with generalized osteoclast (OC) activation. Samples of bone marrow biopsies from MM patients show a strong association between tumor, OC number, and resorption surface. Although altered function of OC is a major factor in the development of bone disease, reduced trabecular thickness and low calcification in bone marrow biopsy samples with osteolysis and reduced osteoblast (OB) counts indicate that OB activity is also impaired. This confirms that bone metabolism, in which OC and OB activity are closely related, is highly altered in MM patients.

There are currently no high-sensitivity and specificity methods that can solve the clinical problem associated with early diagnosis and monitoring of bone disease in patients with MM. In recent years, there has been a significant increase in interest in non-invasive and inexpensive diagnostic and monitoring methods for MM bone lesions based on biochemical parameters. In 2010, the IMWG recommended markers of bone metabolism in clinical practice for the treatment

of MM patients (Terpos et al., 2010). These laboratory tests can assess the intensity of bone formation and bone resorption and serve to identify individuals with negative bone formation out of the total number of MM patients before the onset of clinical complaints or diagnostic signs of bone disease.

"Markers of bone metabolism" is a broad-spectrum term for factors secreted by bone cells or which are products of bone degradation. They are not disease-specific and are used in both diagnosis and disease monitoring to improve the treatment of patients with various bone diseases such as Paget's disease, primary osteoporosis, tumor-induced osteomalacia, rheumatoid arthritis, type 2 diabetes and primary hyperparathyroidism. Based on the work of many authors, the determination of the dynamics of these markers allows the detection of a group of patients at increased risk of developing bone disease (Lipton et al., 2007; Penq et al., 2015; Ting, 2016).

Blood-derived CTX, NTX and urinary deoxypyridinoline (DPD), CTX, NTX, and others are known as markers of resorption. When considering the design of the study, the possibility of detecting DPD in urine was initially considered. However, based on the recommendations of the International Osteoporosis Foundation for osteoporosis and data from many authors (Park et al., 2018; Gurban et al., 2019) on the most accurate and best marker of resorption, it was decided to identify and analyze CTX in blood. A large proportion of MM patients have significant renal impairment and CTX is known to have a lower interaction with renal function compared to DPD. Therefore, in my work I chose to study CTX, which is a marker of bone resorption, and bALP, which is a marker of bone formation.

Since the detection of biochemical markers is not invasive, monthly monitoring of the patient is therefore possible. Our study has conclusively demonstrated that β -CTX levels are higher in MM patients compared to control group individuals. In addition, β -CTX levels in MM patients at the time of diagnosis were lower in patients without bone damage and increased with

the degree of bone damage. In our study, 114 patients who received treatment showed positive dynamics. The study showed that in the context of effective chemotherapy and bisphosphonates, 84 of 114 patients had a significant reduction in median β -CTX compared to the pre-treatment level after 6 months of treatment. This confirms the sensitivity and specificity of this marker as a bone resorption marker, showing a reduction in osteolysis due to its inhibition by chemotherapy and bisphosphonates. At the same time, a reliable median increase in β -CTX was observed in 9 patients with advanced bone disease. The study also showed that patients with progressing bone disease had higher levels of β -CTX at the time of diagnosis compared with patients who later had a good response to treatment. High level of osteolysis marker correlated with clinical complaints and laboratory parameters. Elevated level of β -CTX in patients with disease progression indicates intense bone resorption against ineffective MM therapy.

Ting (2016) and Pecoraro (2015) have also confirmed in their studies that the level of resorption marker (β -CTX) is higher in primary MM patients compared to control and remission patients and it decreases during treatment. This has also been confirmed in the works of other authors (Vallet et al., 2018; Liu Y et al., 2016). Ting in his study showed that β -CTX levels increased 3–6 months before a relapse was confirmed, affirming that β -CTX can detect preclinical signs of bone disease before it can be detected by laboratory and radiological methods. This, in turn, would help avoid skeletal-related events with bone compression fractures. The authors also confirmed that β -CTX is elevated in MM patients during relapse, independent of bisphosphonate therapy.

The cut-off value of β -CTX calculated in the doctoral thesis is 0.79 ng/ml and it differs depending on gender and age. Our study showed that of the total number of patients, β -CTX was less than 0.79 ng/ml in 22.13% of patients and greater than the cut-off value in 77.87% of patients. Analysis of the β -CTX cut-off value for bone damage revealed a statistically significant association between the two traits. In patients without bone lesions, β -CTX was below the cut-off

value in 81% of patients, and in 68% of patients with 3rd degree bone lesions, β -CTX was below the cut-off. However, in a proportion of patients without bone damage, β -CTX was above the cut-off value. Probably one of the reasons for the increase in β -CTX in patients without bone disease is the systemic effect of MM disease on bone tissue, which is accompanied by an intensive increase in osteolysis. Therefore, in patients without confirmed bone lesions, increased bone metabolism cannot be ruled out due to early diagnostic difficulties. It has been shown in practice that patients with elevated markers usually develop bone disease.

In our study, the diagnostic significance of β -CTX was determined based on the sensitivity and specificity of this parameter. β -CTX has marked sensitivity and specificity, which is confirmed by the data of our study, where the diagnostic sensitivity was 82% and the specificity was 91%, respectively. This confirms that β -CTX is an excellent diagnostic indicator of MM bone disease.

Monoclonal gammopathy of undetermined significance (MGUS) is known to progress to myeloma disease in many patients. Vallet et al. in their 2018 study of 241 patients found that markers of bone metabolism could be identified to predict the development of bone lesions in patients with MGUS at high risk of progression to myeloma. These results demonstrate that the bone resorption marker β -CTX is significantly higher in MM patients compared to MGUS patients. In addition, the lack of correlation with radiological evidence of bone loss may confirm early changes in β -CTX level. β -CTX level correlates with bone lesions detected by MRI and is a sensitive marker for predicting bone disease before severe damage develops. The authors' 10-year follow-up data confirm that asymptomatic MGUS patients without an increase in β -CTX can be considered stable. As we also have data on many MGUS patients, it would be useful for this group of patients to determine the dynamics of β -CTX level in order to diagnose patients at high risk of progression to myeloma with bone lesions.

The main treatment for MM patients is chemotherapy and bisphosphonates. Bisphosphonates are known to be inhibitors of osteoclast-induced bone resorption, which significantly reduces bone pain, the incidence of abnormal fractures and other complications in patients with MM, and significantly reduces the level of resorption markers. In vitro data confirm that bisphosphonates also have a direct cytotoxic effect on tumor cells and inhibit tumor-stromal cell interactions. However, despite positive results in vitro and in vivo, neither clinical studies confirm either a reduction in the development of symptomatic MM or a prolongation of survival in patients receiving bisphosphonates.

Sometimes it is difficult to find the best time to start these medications. Several studies (Corso, 2001) have shown that even in asymptomatic patients without any clinically or radiologically confirmed bone damage, bone metabolism may be altered. In addition, there has been a recent trend to start chemotherapy in such patients as early as possible. It is still unclear whether bisphosphonates should be prescribed to patients with dormant myeloma or MGUS, or stage 1 MM without lytic bone lesions who are not receiving concomitant chemotherapy. Markers of bone metabolism may help in this regard (Diamond et al., 2001). On the other hand, the population of asymptomatic patients is not homogeneous and the time to progression is from several months to several years. For example, 50% of patients with dormant MM do not progress to myeloma disease within 5 years, but 30% do not progress after 10 years.

Numerous studies have analyzed results that suggest the clinical significance of markers for evaluating the efficacy of bisphosphonate therapy (Kyle, 2007; Coleman et al., 2012; Pozzi et al., 2011; Soe et al., 2014; Lee et al., 2012).

Studies (Terpos, 2005) have confirmed that there is a large group of patients who do not respond and are refractory to bisphosphonate therapy and this is confirmed by an increase in β -CTX. These patients have a higher risk of

skeletal-related events and disease progression compared to patients who have responded to bisphosphonate therapy and whose bone resorption has returned to normal.

The median survival of MM patients has increased in recent years due to new and effective specific chemotherapy and bisphosphonate therapies.

Because long-term use of bisphosphonates can cause osteonecrosis of the jaw, stress fractures, and decreased renal function, there is a need to find a method to determine patients who can safely discontinue bisphosphonate therapy (Patel et al., 2014; Oh et al., 2007; Weide et al., 2010; Soe et al., 2014). Bone resorption markers could be used to identify patients with increased bone metabolism and an increased risk of developing osteonecrosis of the jaw. Unfortunately, currently there are insufficient data on the routine use of these markers to predict the development of osteonecrosis of the jaw.

Analyzing the association of β -CTX with laboratory parameters, our study had a positive correlation between β -CTX and β 2 microglobulin, calcium and PP levels, plasma cell infiltration in the bone marrow and ECOG, but a negative correlation between β -CTX and albumin, Hb levels, Karnofsky index and calcium levels. This differs from the data of the study of Terpos (2014), where only a weak correlation was found between β -CTX and β 2 microglobulin levels, and albumin levels. In contrast, no correlation was found between β -CTX and LDH levels in our study.

The obtained clinical data of β -CTX, a specific indicator for monitoring of bone resorption, demonstrate the efficacy of treatment. It is therefore appropriate to include it in the monitoring plan for patients with and without bone disease in order to increase the awareness and economic benefits of monitoring, as well as to improve treatment outcomes and patients' quality of life. It should be noted that our data are consistent with the results of other authors, which show the clinical significance of the β -CTX marker. In case of progression of bone

damage, regular determination of β -CTX is necessary to evaluate the effectiveness of treatment and to optimize treatment tactics.

The NCC guidelines recommend the detection of β -CTX at 6-12 month intervals in MM patients with plateau phase of the disease who have favorable prognostic factors (low disease stage, ECOG up to grade 2, effective treatment). However, in patients with adverse prognostic factors (high disease stage, ECOG grade 3 and higher), it is recommended that β -CTX be measured every 3 months for early detection of disease progression.

The biggest controversy in the literature is about the role of the bone formation (bALP) marker in the early diagnosis and monitoring of bone damage in MM. In our study, no difference in bALP levels was observed between MM patients and control group individuals. Similarly, this was not observed in the studies of Ting (2016), Annibali (2015), and Kowalska (2010). In our study, when analyzing the median bALP, there were no statistically significant differences between the different degrees of bone lesion severity either at the time of diagnosis or when evaluating their level during the disease course. This could be explained by a decrease in bone formation due to imbalance in resorption and formation. Such results were also found in the Ting's (2016) study. Two independent studies (Dizdar, 2005, Politou, 2004) confirmed that patients with MM and bone lesions had lower bALP levels compared to controls and patients with MGUS. This was explained with a decrease in bone formation in MM patients. In some studies (Lund et al., 2010), bALP levels increased in some cases and decreased in others during treatment. Zangari (2011) confirmed in their study that bALP levels increased in patients after a positive response to chemotherapy and that this increase correlated with a decrease in serum DKK-1 levels. Terpos (2014) in his study demonstrated that chemotherapy reduces bone resorption but does not affect bone formation.

Therefore, in our study, β -CTX has shown a greater prognostic role in the development of bone lesions and in the evaluation of patients' treatment efficacy compared to bALP.

Various requirements for the use of bone metabolism markers in clinical practice differ from scientific studies. Bone resorption markers are associated with circadian rhythms and decrease after eating. Szulc (2017) recommends taking the patients' analyses from 7.30 to 10.00 on an empty stomach. This must be done in a tube with EDTA. In contrast, bone formation markers do not have a strict circadian rhythm and analyses can be taken at any time of the day. Since these markers have large diurnal variations and they are not utilised often enough to be a good standard for both interpretation of results and performing analyses, they are not currently widely used in clinical practice. To standardize our study, analyses were performed on all patients at 7 in the morning on an empty stomach. It was recommended that outpatient patients be tested by 9 a.m. on an empty stomach.

Clinicians should consider the need for fasting, recent fractures, age, menopausal period in women, the renal and hepatic status when prescribing and interpreting markers of bone metabolism as these factors could provide erroneous results. Patients with severe renal impairment have significantly higher levels of β -CTX compared to patients with normal renal function. Ting (2016) recommends that β -CTX should be determined with caution in patients with $\text{GFR} < 30 \text{ ml/min/1.73 m}^2$. In contrast, patients with moderate renal impairment ($\text{GFR } 30\text{--}60 \text{ ml/min/1.73}$) do not differ in β -CTX levels from patients with normal renal function.

Markers of bone metabolism can be detected in both blood and urine. As all of these markers have a high individual variability, estimated at about 10% in the blood and 15–25% in the urine, this limits the use of the markers in daily practice.

Evidence-based literature also demonstrates an important role for vitamin D in bone metabolism, although the exact mechanism is unknown. Some studies (Khadka et al., 2018) have found that patients with vitamin D deficiency have higher levels of bone metabolism markers (CTX, bALP). In many of our patients we were unable to obtain data on vitamin D, therefore there are no data on vitamin D and its correlation with bone metabolism markers.

The combination of markers of bone resorption and formation and other laboratory parameters as well as imaging techniques could improve the examination and treatment of MM patients.

Because all MM patients undergo bone marrow histology for diagnostic purposes, simultaneously osteoclast and osteoblast activity can be estimated quantitatively at the time of diagnosis, but it cannot be used for regular bone disease monitoring (Bataille et al., 1992; Abildgaard et al., 2004; Eastell et al., 2018).

Bone marrow examination provides the necessary information about the degree of plasma cell infiltration in the bone marrow and their morphological characteristics. It, together with immunohistochemistry, is included in the guidelines for diagnostic tests of both the International Myeloma Group and the NCCN.

DKK1 is a protein that inhibits the Wnt signaling pathway, which in turn plays an important role in embryogenesis, organogenesis and homeostasis. This cascade of the signaling system is the basis of normal physiological processes, such as cell proliferation, tissue regeneration, embryonic development, and it is regulated in various ways. Therefore, changes in the signaling pathway cause complications and may contribute to the development of various diseases such as tumors, bone diseases, autoimmune diseases, diabetes, and Alzheimer's disease (Huang Yu et al., 2018). DKK1 is expressed in bone cells – osteoblasts and osteocytes, mesenchymal stem cells, as well as in the skin, prostate, placenta and endothelium (Ke et al., 2012).

Immunohistochemical examination of plasma cells is very important in the diagnosis of MM. When working on the protocol and design of the study, the idea of the importance of double staining was considered. Based on the results of previous studies (Tian et al., 2003; Kristensen et al., 2014), the DKK1 protein, which is expressed in myeloma cells in patients with bone disease, was selected. The surface marker CD138 (syndecan-1) is often used in plasma cell studies. However, analysis of the literature suggests that myeloma cells often poorly express CD138 (Kawano et al., 2012). The role of another plasma membrane molecule, namely the CD56 molecule, in the detection of plasma cells was also considered in this study. Membrane glycoprotein CD56 is expressed by NK cells and osteoblasts as well as myeloma cells (Harrington et al., 2009). Considering the technical difficulties in identifying and quantifying the products of the double staining reaction, determining the cytoplasmic and membranous reaction, as well as the significance of this reaction, it was decided to perform the determination of DKK-1 immune expression.

Several studies have shown that DKK-1 plays a major role in the development of lytic bone lesions (Liu, 2018; Hameed, 2014). In addition, a persistently high level of DKK-1 in the bone marrow in MM patients has been shown to reduce the activity of osteoblast progenitors.

Our study determined the level of DKK 1 expression at different stages of MM disease, suggesting the role of DKK 1 in the development of bone lesions using bone marrow trephine biopsy material obtained from 49 MM patients. These patients had both bone lesions and no bone lesions.

The study found that moderate to severe DKK-1 expression was significantly more common in bone marrow samples of the patients with bone lesions compared to samples from patients without osteolytic bone lesions. These results are consistent with the results of Ida B. Kristensen in 2014 and Heath in 2009, which showed that DKK1 expression in plasma cells correlated with bone

damage. As in the study published by Heath (2009), no pronounced DKK1 expression was observed in this study in patients without bone lesions.

The Wnt signaling pathway is involved in several aspects of myeloma disease. By secreting Wnt antagonists, including DKK-1, myeloma cells upset the balance between bone formation and bone resorption. Subsequent disorders of bone homeostasis result in osteolytic bone lesions. This not only causes the direct and indirect growth of MM cells, which in turn leads to tumor progression, but is also the cause of bone pain and pathological fractures in MM patients. There are reports (Spaan et al., 2018) that the intrinsic canonical Wnt/ β -catenin signaling pathway affects myeloma cell survival and thus promotes disease progression. In addition, the Wnt signaling pathway is associated with acquired drug resistance. This is most likely due to the interaction between MM cells and bone marrow stromal cells. Resistance to conventional therapies is a clinical problem and adversely affects patients' treatment outcomes. Therefore, Wnt signaling medication may be attractive as the choice of a new therapy. This could increase MM cell apoptosis and reduce DKK-1 secretion in the bone marrow microenvironment, thus contributing to a more successful and sustained response to therapy.

In recent years, personalized therapy for MM patients has become relevant. This includes identifying the risk of bone disease development so that the most appropriate treatment can be found for each patient.

Terpos (2018) has confirmed in his article that by reducing DKK-1 levels, it is possible to improve the efficacy of therapy and normalize bone metabolism. Other authors (Spaan, 2018, and Heath, 2009) have shown that anti-DKK-1 therapy increases the rate of bone formation by 25% and the mineralizing surface by 28%, and have suggested that restoring osteoblast differentiation may prevent the development of osteolytic bone lesions, providing an effective therapeutic approach to the treatment of this disease.

Based on plasma cell morphology, myeloma is divided into mature, immature plasma cell- and plasmoblastic variants. In our study, the structure of malignant plasma cells was investigated and the morphological variants of plasma cells were confirmed. Based on Bartl's (1987) study, 6 histological variants of plasma cells were confirmed, which were divided into 3 plasma cell malignancy grades. The study confirmed the correlation of plasma cell morphological variants with the severity of bone damage in MM patients.

Because there were more patients with bone lesions in the study, more plasma cell malignancy grades were determined in patients with bone damage than in patients without bone damage. Patients with bone lesions have more high grade malignancy plasma cells compared to patients without bone lesions.

The results of studies by Bartl (1987) and Fujino (2018) confirm reduced survival and poorer prognosis in patients with higher plasma cell infiltration in the bone marrow and with immature and plasmoblastic plasma cell morphology. Because our study has shown an increased risk of bone disease development in patients with plasmoblastic cells, it can be concluded that these patients have a reduced average survival compared to patients with mature plasma cells in the bone marrow. A study by Subramanian (2009) has shown that bone marrow examination is necessary not only for diagnostic, but also for prognostic purposes. Plasma cell morphology and the degree of infiltration are significantly correlated with the stage of the disease.

At the same time, our results show that DKK-1 expression levels differ significantly compared to low, medium, and high plasma cell malignancy levels. Expression of DKK-1 is more common in patients with moderate plasma cell malignancy. In patients with high grade plasma cell malignancy, DKK-1 is expressed in all cases.

In our study, when analyzing the correlation between plasma cell malignancy and β -CTX, no statistically significant correlation was found ($p > 0.05$), however, a tendency for β -CTX to increase at higher malignancy

grade was observed. There is also a similar tendency for β -CTX to increase at a more pronounced level of DKK-1 expression.

Evaluation of β -CTX at different levels of DKK-1 expression in individual patients with and without bone lesions revealed a tendency for an increase in β -CTX in patients with bone lesions with more pronounced DKK-1 expression, but no such visual trend was observed in patients without bone lesions.

Similarly, there was a trend for an increase in β -CTX with a higher grade of plasma cell malignancy in patients with bone lesions, whereas no such correlation was observed in patients without bone lesions.

Although no statistically significant association was found ($p = 0.25$) between DKK-1 and β -CTX above or below the cut-off value (0.79), more patients with moderate to severe DKK1 expression were observed above the cut-off value. There was also no statistically significant ($p = 0.88$) association between plasma cell malignancy and β -CTX above or below the cut-off value (0.79), but more patients with moderate to high plasma cell malignancy were observed above the cut-off value.

In conclusion, evaluation of bone marrow biopsy results remains an essential component of the diagnosis of MM. As bone disease is the most common complication of MM, the detection of DKK-1 in biopsies may provide additional information for the interpretation of this clinically important aspect of the disease.

Thus, routine trephine biopsy in all primary MM patients can be used to determine the grade of myeloma cell malignancy and the level of DKK-1 expression, which correlates with bone damage in MM patients.

In contrast, the use of biochemical markers of bone metabolism can determine the risk of bone lesion development both at the time of diagnosis and in the dynamics of the disease and evaluate the effectiveness of therapy.

Based on the fact that plasma cells are formed from B lymphocytes, MM belongs to the group of B cell malignancies, which also includes non-Hodgkin's lymphoma. Today, non-Hodgkin's lymphomas are histologically classified according to the WHO's 2016 classification of lymphoid tumors (Swerdlow et al., 2016), which has a clinical and prognostic significance. Therefore, the use of plasma cell malignancy and the degree of DKK-1 expression in the histological classification of MM would be recommended.

As a result, combining blood biochemical markers and other laboratory parameters, bone marrow DKK-1 expression in plasma cells and plasma cell morphological variants with radiological studies, it is possible to assess the risk of bone lesion development in patients, determine treatment efficacy and help decide when to start and stop bisphosphonate therapy in patients with MM disease.

In recent years, the prognosis of MM patients has improved significantly due to the widespread use of autologous stem cell transplants and new drugs that affect both myeloma cells and the microenvironment. However, bone disease control remains a therapeutic challenge for these patients, since a multidisciplinary team of hematologists, radiologists, pathologists, neurologists and neurosurgeons is involved in their treatment.

Conclusions

1. The levels of β -CTX, the marker of bone metabolism resorption, are higher in MM patients compared to control patients. β -CTX levels are statistically significantly correlated with the severity of bone lesions in multiple myeloma.
2. Patients who responded to treatment had a reduction in β -CTX levels compared to baseline. In contrast, there was an increase in β -CTX levels in patients who did not respond to treatment.
3. The level of bALP, the marker of bone metabolism formation, does not differ between MM patients and controls. Changes in bALP levels do not correlate with the development of bone disease or the severity of bone lesions.
4. The expression level of DKK-1 is higher in MM patients with bone lesions than in MM patients without them.
5. Patients with bone lesions have more high-grade malignancy plasma cells compared to patients without bone lesions.

Practical recommendations

To assess the risk of bone disease and its development in the case of myeloma, it is recommended:

1. To determine the level of β -CTX in the blood, as well as the degree of DKK-1 expression and the grade of plasma cell malignancy in the histological examination of the bone marrow for all patients at the time of myeloma diagnosis.
2. To measure β -CTX levels during the course of the disease (every 6 months) in order to assess the effectiveness of therapy.

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