MEDICAL UNIVERSITY - PLEVEN

FACULTY OF PHARMACY

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APPLICATION OF ANALYTICAL METHODS FOR ASSESSING THE RELATIONSHIP BETWEEN BONE DENSITY, BIOGENIC ELEMENT LEVELS, AND OXIDATIVE STRESS IN OSTEOPOROSIS

ABSTRACT

of the dissertation

for awarding **the educational and scientific degree „Doctor“**

Professional direction 4.2. Chemical Sciences
Doctoral program „Analytical Chemistry”

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Pleven, 2024

The dissertation is presented in 209 pages. It contains 39 figures, 44 tables, and 5 graphs. The bibliography comprises 174 sources, including 7 appendices.

The doctorate candidate is a lecturer at the sub-department of Chemistry at the Medical University of Pleven

Serum samples were supplied by the Rheumatological centre „Sveta Irina" – Sofia.
The samples were taken in accordance with the requirements for ensuring the quality of clinical laboratory analyses.
The atomic absorption analysis for determining calcium, copper, zinc, magnesium, and iron was conducted at the Clinical Laboratory of „Alexandrovska" University Hospital – Sofia.
The spectrophotometric analysis for determining oxidative stress was conducted in the research laboratory of the Medical University – Sofia, Department of „Medical Physics and Biophysics".

The public defence of the dissertation will take place on 19.09.2024 at 13:00 at 113 hall, faculty „Pharmacy", Medical University of Pleven.

LIST OF ABBREVIATIONS USED

CA - Cluster Analysis
PCA - Principal Component Analysis
AAS - Atomic Absorption Spectroscopy
AOA - Antioxidant Activity
DEXA - (Dual-energy X-ray absorptiometry)
BMI – Body Mass Index
BMD – Bone Mineral Density
T-Score - the number of standard deviations from the mean bone density of healthy individuals of the same gender at age 30
TE - Trolox Equivalent
RSA - Radical Scavenging Activity
ROS - Reactive Oxygen Species

CONTENTS

INTRODUCTION

Osteoporosis is a socially and economically significant disease. It disables a large number of people. Enormous resources are allocated to surgeries for fractures and deformities, which may shorten the lives of some elderly patients.

The condition of patients with osteoporosis is described by many clinical factors simultaneously, which determine the severity of the disease. Examining these factors one by one leads to low information levels. It is necessary to classify and model the large volume of data obtained. Interpreting all factors simultaneously and finding objective relationships between them forms the basis for both successful diagnosis and individualized treatment approach for each patient. For this purpose, statistical approaches such as correlation analysis, regression analysis, and multivariate statistical methods such as CA and PCA are applied to data from various clinical and laboratory analyses.

All of the above methods were used to interpret data sets from patients with osteoporosis and healthy volunteers, including: bone density; selected routine clinical indicators; own laboratory results for trace element composition (calcium, copper, zinc, magnesium, and iron content); biomarkers for oxidative stress levels in serum.

There are numerous factors influencing the calcium-phosphorus metabolism, especially in combination with negative lifestyle factors, dietary habits, and accompanying diseases. The role of trace elements such as copper, zinc, iron, and magnesium is not yet fully understood. However, increasing evidence suggests they play a significant role in calcium-phosphorus metabolism and oxidative stress levels. The results for biogenic element levels were obtained using Flame AAS, while oxidative stress levels were assessed using photometric ABTS analysis.

Results published by other authors on serum levels of trace elements in relation to decreased bone density are not conclusive, primarily due to inconsistent participant selection criteria in studies. Additionally, there is a lack of research on the relationship between oxidative stress levels in the body, concentrations of these biogenic elements, and bone density.

This motivated us to conduct a study involving women from the Bulgarian population, carefully selected using exclusion criteria, in order to determine the influence of RSA and concentrations of biogenic elements on BMD.

AIMS AND OBJECTIVES OF THIS DISSERTATION

The aim of the current dissertation work is: investigation of the relationships between bone density, levels of the biogenic elements calcium, magnesium, iron, copper, and zinc, and oxidative stress in menopausal women using analytical and chemometric approaches.

The objectives of the current dissertation work are:

1. Analytical determination of serum concentrations of calcium, magnesium, iron, copper, and zinc, as well as the level of oxidative stress in patients with osteoporosis;
2. The discovery of mutual relationships between the studied biogenic elements, oxidative stress, and bone mineral density, through correlation, regression, and multivariate statistical analysis;
3. Statistical identification of patterns of similarity (phenotypes) among the studied patient groups and defining discriminative parameters responsible for forming these similarity groups;
4. To analyse the results of a survey regarding family history and detrimental household habits with negative effects on bone density in menopausal women patients.

MATERIALS AND METHODS

The current prospective study, conducted from June 2018 to 2023, includes a total of 118 women with an mean age of 62.75 ± 8.64 years from the Sofia region. Reduced bone density was diagnosed in 95 of them. The remaining 23 women were found to have normal bone density and form the control group.

The groups of women studied were selected according to the following criteria: women in menopause or post menopause without treatment or therapy for osteoporosis; without diseases of the parathyroid gland, kidney diseases, and diabetes; without systemic intake of Ca, Cu, Zn, Mg, Fe, P, vitamin D, and collagen supplements. The individuals in the study groups do not have blood relations with each other.

We conducted an experimental-statistical study with the following stages:

- Conducting a questionnaire to assess the risk of osteoporosis;
- Determination of BMD using the DEXA method. Based on the T-Score result, women were divided into three groups: osteoporosis (below -2.5 SD), osteopenia (between -1.0 and -2.5 SD), and a control group with normal density (above -1.0 SD);
- Analytical determination of the levels of total calcium, copper, zinc, magnesium, iron, and AOA in serum.

Each participant in the study signed informed consent.

Analytical methods

Serum concentrations of biogenic elements **calcium**, **magnesium**, **copper**, **zinc**, and **iron** were determined using Flame AAS with a Perkin-Elmer AAnalyst 300 instrument. Each element was analysed under appropriate conditions: serum dilution, suitable additives, lamp current strength for the monochromatic light source, concentration of aqueous calibration standards, and instrumental parameters.

The **AOA** of the serum was determined using the spectrophotometric ABTS assay. This method relies on spectrophotometric measurement of the change in absorption of the chromophore cation-radical $ABTS^{\bullet+}$ in response to free radical processes with substances exhibiting radical-scavenging activity. As a result, the concentration of the radical in the

sample decreases, affecting the colour intensity of the solution and the measured absorbance. The decrease in absorbance corresponds to the RSA of the sample.

To construct a calibration curve, the RSA effect of a water-soluble analogy of vitamin E, Trolox, was used. Various amounts of a standard Trolox solution at a concentration of 1 mmol/L were added. The data obtained were used to calculate the antioxidant capacity of the serum in TE.

Statistical methods

Statistical analysis was performed using the STATISTICA 7,0 software program.

Correlation analysis: calculation of the correlation coefficient; testing the significance of the correlation dependency.

Regression analysis**:** defining a regression model

Methods of multivariate statistics

Cluster analysis: z-transformation of the input data; calculation of the Euclidean distance for similarity between subjects; clustering of subjects into similarity clusters using the Ward method; graphical representation of the obtained clusters (dendrograms); determination of the statistical significance of the clusters using the Sneath criterion; interpretation of clusters by subjects and variables.

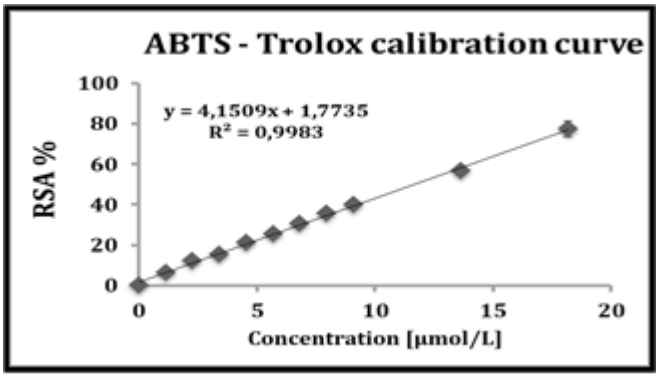
PCA: z-transformation of the input data; determination of the optimal number of principal components (through a scree plot); varimax rotation method.

The following techniques were also used for data analysis: descriptive analysis, analysis of variance (ANOVA), and one-way ANOVA. The significance level at which the null hypothesis was rejected was set at $P < 0.05$.

RESULTS AND DISCUSSION

1. Study of the levels of oxidative stress

The first step of the study on AOA in blood serum involves assessing the effectiveness of the standard reference Trolox in reducing the concentration of the utilized radical 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) when different concentrations of the antioxidant are added. Based on the obtained results of sample absorbance, the RSA was determined for each concentration and a calibration curve was constructed.



Graph 1. Concentration dependence of radical-scavenging activity of the reference Trolox in a model system containing ABTS radical

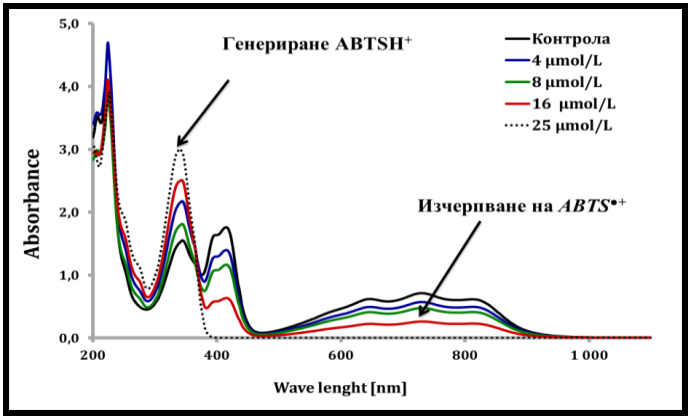


Figure 1. Absorption spectra of ABTS radical before and after interaction with Trolox

The obtained data clearly demonstrate a pronounced increase in RSA with increasing concentration of the reference Trolox in the samples. When recording the absorption spectrum at a wavelength of 734 nm at the maximum tested concentration of 25 μmol/L, an extinction of 0.0024 was recorded, corresponding to complete decolorization and depletion of the radical in the sample. At a concentration of 18 μmol/L, the RSA determined by the formula was approximately 80%.

Additional analytical information provided on the figure includes the functional form of the equation describing the relationship between concentration and RSA, as well as R^2. Based on the equation **$y = 4.1509x + 1.7735$** and **$R^2 = 0.9983$**, the linearity is observed to be very good.

The full results obtained from the study on blood serum of patients and healthy volunteers using the ABTS method to determine RSA, as well as the results from atomic absorption analysis for biogenic elements and anthropometric indicators, are detailed extensively in the dissertation.

2. Study of the dependencies between the mean values of biogenic elements, total AOA, and BMD

In the study, we included 83 participants who were closely matched in age and BMI. This sample selection from the study population reduces the influence of age and BMI when investigating biogenic elements and total antioxidant activity, and facilitates comparisons between the studied groups.

To investigate the correlation between copper and zinc, we introduce a new indicator, K - the ratio of Cu, μmol/L to Zn, μmol/L.

Table 1. Mean values (±SD) of serum levels of Cu, Zn, Mg and AOA

Parameter	Total	Number of patients with reduced BMD	Patients with osteopenia	Patients with osteoporosis	Controls
Number of patients	83	71	18	53	12
Age	63.57 ± 8.07	64.18 ± 7.96	64.44 ± 9.33	64.09 ±7.54	59.92 ± 8.07

BMI	24.58 ± 3.69	24.30 ± 3.73	24.39 ± 4.33	24.27 ± 3.54	26.24 ± 3.10
BMD, g/cm^2	0.761 ± 0.152	0.709 ± 0.07	0.726 ± 0.071	0.703 ± 0.069	1.072 ± 0.135
Cu, µmol/L	21.18 ± 5.24	21.71 ± 5.43	23.21 ± 5.83	21.2 ± 5.25	18.02 ± 2
Zn, µmol/L	13.84 ± 2.46	13.95 ± 2.55	14.39 ± 3.10	13.80 ± 2.35	13.14 ± 1.80
$Zn, \frac{\mu mol}{L}$ / BMI	0.5751 ± 0.1307	0.5866 ± 0.1329	0.6103 ± 0.1728	0.5785 ± 0.1173	0.5073 ± 0.0954
K = *Cu/Zn*	1.58 ± 0.51	1.62 ± 0.54	1.68 ± 0.58	1.59 ± 0.54	1.39 ± 0.2
Mg, mmol/L	0.90 ± 0.17	0.92 ± 0.18	0.97 ± 0.12	0.90 ± 0.19	0.79 ± 0.09
AOA,% (TE)	68.80 ± 11.13 (6.78 ± 0.95)	71.12 ± 10.35 (7.02 ± 0.87)	67.29 ± 7.28 (6.63 ± 0.56)	72.42 ± 10.95 (7.15 ± 0.93)	55.10 ± 1.5 (5.40 ± 0.03)

In regards to BMI and age, the groups have no statistically significant differences, despite the clear tendency for lower BMI in patients with osteoporosis.

The serum concentrations of *copper* and *magnesium* in all patients are elevated compared to the controls, and the differences are statistically significant. However, there is no statistical difference between the osteoporosis and osteopenia groups for these indicators.

We observed elevated serum *zinc* levels in the groups with reduced bone density compared to the control group, but these differences are not statistically significant. When including the indicator Zn µmol/L per unit of BMI, we found a statistical difference ($p = 0.05$) between the group with reduced bone density and the controls.

In patients with normal bone density, the copper to zinc ratio is 1.4, whereas in patients with osteoporosis, it is 1.6. This value changes consistently with the severity of the disease.

Patients with osteoporosis have higher antioxidant activity in response to increased free radical processes in the body.

With decreasing bone density, the level of RSA increases, resulting from the elevated production of ROS. The higher level of radicals likely enhances the production of liver enzymes, such as Cu,Zn-SOD. On the other hand, the kinetics of radical reactions give us reason to believe that the overproduction of radicals damages the protein structure of oxidoreductases, especially in patients with slower biochemical mechanisms.

The serum concentration of copper is elevated in patients, which increases RSA. During the destruction of metalloproteins, redox-active metal ions are released. The higher level of radicals also damages cell membranes, facilitating the release of free copper into the blood. The increased concentration of copper ions in the serum initiates secondary radical processes and further increases the RSA of patients.

3. Correlation and regression analysis

An assessment of the correlation coefficients was made for a group of 100 patients with osteoporosis and osteopenia, described by the following 9 variables: age, T-score, BMI, BMD, serum levels of Ca, Mg, Cu, Zn, and AOA. Table 2 presents the values of the correlation coefficients, with statistically significant ones marked as follows: bold font for coefficients showing significant correlation and italics for coefficients showing moderate correlation. Unmarked coefficients are statistically insignificant.

Table 2. Correlation Coefficients for 9 Variables

	Ca	Mg	Cu	Zn	AOA	Age	T - score	BMI	BMD
Ca	***1.00***								
Mg	0.05	***1.00***							
Cu	*0.20*	**0.35**	***1.00***						
Zn	- 0.11	**0.30**	*-0.16*	***1.00***					
AOA	0.09	0.10	**-0.31**	0.10	***1.00***				
Age	0.07	-0.05	-0.01	-0.07	**0.30**	***1.00***			
T-score	0.02	**0.29**	-0.01	0.12	**0.62**	**0.29**	***1.00***		
BMI	0.03	*-0.18*	-0.003	0.02	**-0.32**	**-0.38**	**-0.38**	***1.00***	
BMD	-0.01	**-0.28**	-0.05	-0.09	**-0.54**	**-0.30**	**-0.83**	**0.39**	***1.00***

The following conclusions can be made:

- Ca does not have significant correlations with any of the other variables, making it practically impossible to seek causal relationships involving Ca;
- Mg is significantly correlated with Cu, Zn, T-score, and BMD, suggesting that this mineral component may participate in a multiple regression model for BMD and mineral components;
- Cu is significantly correlated with AOA;
- Zn demonstrates significant correlation solely with Mg;
- AOA is significantly correlated with a few other variables – Cu, age, T-score, BMI, BMD; therefore, a multiple regression model is feasible for examining the relationship between AOA, BMD, and other parameters;
- The Age parameter is significantly correlated with T-score, BMI, BMD; hence, this parameter is also a potential dependent variable for a regression model of bone density;

- T-score and BMI are other potential independent variables for a regression model concerning bone density (due to significant correlations with BMD).

From the correlation analysis data, it is clear that a multiple regression model could be proposed to examine the relationship between bone density and parameters such as magnesium, AOA, T-score, BMI, and age.

In the current study, it was valuable to ascertain the feasibility of constructing a suitable model to explore the relationship between bone mineral density BMD (a dependent variable) and one or more independent variables from the dataset. The goal is to obtain a convenient method for predicting bone density in patients based on readily accessible independent variables such as mineral components, AOA and BMI.

The preliminary correlation analysis indicated potential stochastic relationships as a basis for conducting dispersion analysis. Multiple regression relationships were tested, and most of the models obtained, both single and multiple regressions, were inadequate.

However, the best model obtained in the study demonstrated good adequacy:

BMD** = 1.141 – 0.729 **AOA** + 0.136 **BMI

This shows the relationship between bone density, AOA и BMI.

The following table shows the statistical indicators for the conducted multiple regression where BMD = f(all parameters):

Table 3. Summary and statistical analysis of the multiple regression model BMD = f (all parameters)

	Beta	**Std.Err. of Beta**	**B**	**t(91)**	**p-level**
Intercept			**1,141**	6.21	0.000
Ca	-0.014	0.058	-0.012	-0.23	0.816
Mg	-0.018	0.068	-0.015	-0.26	0.795

Cu	-0.044	0.064	-0.001	-0.69	0.493
Zn	-0.012	0.062	-0.001	-0.19	0.846
AOA	**-0.729**	**0.080**	**-0.001**	**-1.42**	**0.076**
Age	-0.101	0060	-0.002	-1.67	00.98
BMI	**0.136**	0.062	0.005	**2.17**	**0.032**

The table reveals that the statistically significant regression coefficients are for the variables AOA and BMI. The T-score variable was excluded from the regression analysis as it is correlated with the dependent variable BMD and is calculated based on experimental data for BMD.

The regression model includes an intercept term. The regression coefficient for AOA has a negative sign (indicating BMD decreases with an increase in the parameter), while that for BMI has a positive sign.

The measured BMD using the DEXA method was compared with the BMD values predicted by the model.

The correlation is impressive for such a complex system: $R = 0.843$; $R^2 = 0.710$.

In principle, such a model can be used to predict bone density based on AOA and BMI data. Both independent variables are logically linked to bone density: AOA serves as a clinical indicator for patients with osteopenia or osteoporosis, while BMI, as an easily calculated body mass index, can serve as an important indicator of disease severity, with underweight indicating a risk of bone fractures.

4. Multivariate statistical analysis for determining the relationship between BMD , concentrations of Ca, Cu, Mg, Zn, Fe, and AOA in patients

The input data were standardized to avoid the influence of different scales of the included parameters on the classification procedures. Therefore, the input data with specific dimensions were transformed into dimensionless data with a specific normal distribution, having a mean value of zero and a standard deviation of ±1. Euclidean distances between the data points were used as a measure of similarity, and the Ward method was employed to cluster the parameters and subjects. In the resulting dendrogram, the significance of the clusters was determined using the Sneath's test, where clusters were considered significant if their height in the dendrogram was 1/3 or 2/3 of the maximum height, Dmax.

The dataset for this specific study consists of 59 patients (referred to as "subjects") described by 11 clinical parameters (referred to as "variables"), resulting in an initial matrix of dimensions [59x11].
The measured variables (descriptors) were: concentrations of Ca, Cu, Mg, Zn, and Fe; AOA (assuming this refers to antioxidant capacity); T-score; BMI; BMD (bone mineral density); age; and a status code (1 for osteopenia and 2 for osteoporosis).
Hierarchical grouping of variables
Figure 2 depicts the hierarchical dendrogram for clustering 11 variables.
Three primary and statistically significant clusters are formed: C1 (BMD, BMI, AOA, Age, Fe, Zn); C2 (T-score, Pen_Por); C3 (Ca, Mg, Cu).
C1 can be considered to correspond to anthropometric factors such as BMI, BMD, Age, AOA, and levels of two essential blood components (Fe, Zn). Therefore, this cluster can be termed as conditionally representing the "general health status effect." C2 is clearly associated with parameters responsible for "osteoporosis or osteopenia diagnosis," while C3 relates to levels of essential elements associated with "reduced bone density condition."

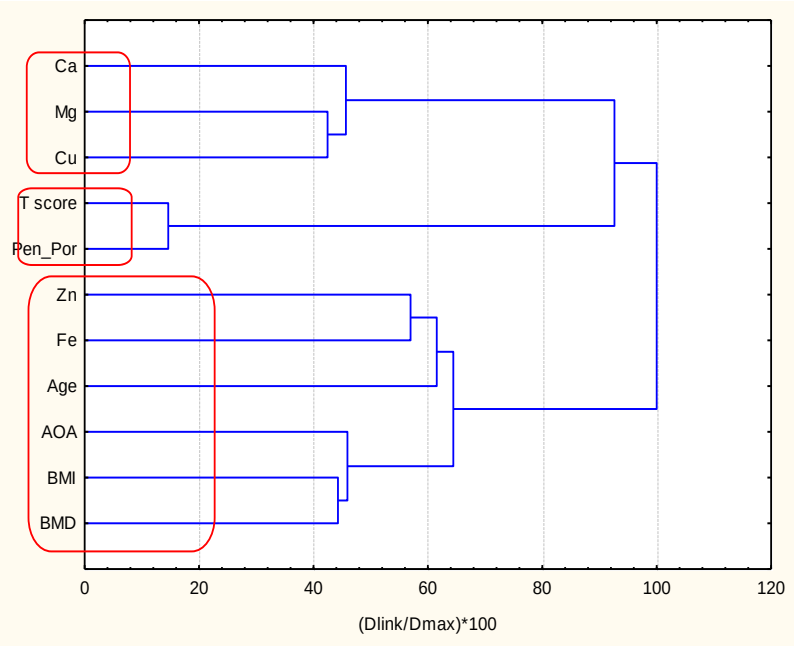


Figure 2. Hierarchical dendrogram for 11 variables

These results were confirmed by applying K-means clustering, for which a preliminary hypothesis of the existence of 3 clusters was required.

Hierarchical object grouping

In the following tables, the members of each identified cluster from the analysis are presented.

Table 4. Members of the clusters. Distance from the cluster centroid. Variables in the cluster.

Cluster 1 members and distance from the cluster centroid. The cluster contains 2 variables.	
Variables	Distance
T-score	0.355091
Pen_Por	0.355091
Cluster 2 members and distance from the cluster centroid. The cluster contains 3 variables.	
Variables	Distance

Ca	0.724779
Mg	0.716695
Cu	0.694088

Cluster 3 members and distance from the cluster centroid.
The cluster contains 6 variables.

Variables	Distance
Zn	0.948792
Fe	0.897872.
AOA	0.836456
Age	0.945784
BMI	0.788613
BMD	0.889304

The only difference with K-means clustering is the numbering of the clusters - the contents of the clusters are the same.

Therefore, it can be assumed that three factors are related to the structure of the dataset: descriptors responsible for "osteoporosis or osteopenia diagnosis"; descriptors related to "reduced bone density condition"; descriptors associated with the "general health status effect".

Figure 3 depicts the hierarchical dendrogram for clustering 59 patients.

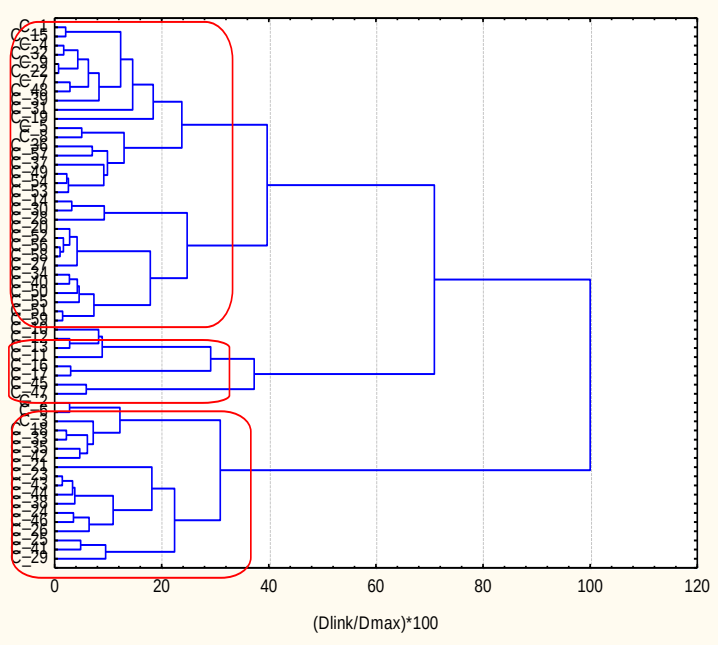


Figure 3. Hierarchical dendrogram for clustering 59 patients

Three clusters have been formed (confirmed by K-means clustering) as follows:

✓ Cluster 1 contains 12 subjects (conditional number 1, 10, 11, 12, 13, 15, 16, 17, 19, 30, 45, 47);

✓ Cluster 2 contains 29 subjects (conditional number 4, 5, 7, 8, 9, 14, 20, 22, 27, 28, 31, 32, 34, 36, 37, 39, 40, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59);

✓ Cluster 3 contains 18 subjects (conditional number 2, 3, 6, 18, 31, 23, 24, 25, 26, 29, 33, 35, 38, 41, 42, 43, 44, 46).

It is crucial to define specific descriptors for each identified cluster of patients to understand the reasons for forming these groups of similarity among different patients.

Figure 4 presents a graph of the mean values of each variable for each identified cluster of patients.

Figure 4. A graph of the mean values of each variable for each identified cluster of patients (normalised values).

The number of each cluster on the graph corresponds to the number presented in the tables detailing the distribution of patients: Cluster 1 has 12 members, Cluster 2 has 29, and Cluster 3 has 18. The sequence of variables on the graph is as follows: *Ca, Mg, Cu, Zn, Fe, AOA, Age, T-score, Pen_Por, BMI, BMD.*

Cluster 1 is characterized by the highest levels for Ca, Mg, Cu, and the lowest levels for Zn, Fe, AOA, BMI, BMD, with mean values for the parameter Age. The T-score values are close among the subjects in Cluster 2, while Pen-Por (2 for osteoporosis) is accordingly equal. It can be assumed that this phenotype represents patients with the most compromised bone metabolism. Cluster 1 is the smallest group and serves as a sample of patients with the most negative impact from osteoporosis, indicating the most significant metabolic disturbances.

Cluster 2 serves as a model for early-stage osteoporosis effects—showcasing the lowest levels of Ca, Mg, Cu, intermediate values for Zn, and the highest for Fe and AOA. The values of these indicators (Ca, Mg,

Cu, Zn, Fe, and AOA) are closer to those of Cluster 3 than to Cluster 1. Patients in Cluster 2 exhibit high levels of BMI, which are nearly identical to those in Cluster 3. T-scores are similar among subjects in Cluster 1, while Pen-Por (2 for osteoporosis) is correspondingly equal. This group is characterized by the youngest mean age (Age) and the highest levels of BMD. It represents the largest group of patients and warrants further investigation to clarify similarities and differences with Clusters 1 and 3.
Patients in Cluster 3 have Pen-Por values of 1, indicating osteopenia. They exhibit intermediate levels of Ca, Mg, Cu, Fe, and still maintain high levels of BMI and BMD. This group is characterized by the highest mean age (Age) and the highest levels of Zn. We can infer that these patients are deserving of increased attention and corresponding medical interventions.
The higher BMD values observed in Cluster 2 compared to Cluster 3 can be explained by the significant difference in bone density localisation across different disease sites. In Cluster 2, patients with disease localisation in the femur constitute 13.8%, whereas in Cluster 3, those with reduced femoral bone density make up 72%, and in Cluster 1, they are 33.34%. This defines the division of patients based on disease localisation for our future studies.

Factor analysis and principal component analysis

The table below presents the factor loadings of the variables for 3 latent factors.

Table 5. Factor loadings

Factor loadings. Extraction: Principal Components (significant loadings are marked)			
Variables	Factor - 1	Factor - 2	Factor - 3
Ca	**-0.488**	0.160	0.077
Mg	**-0.674**	-0.070	-0.132
Cu	**-0.581**	0.029	0.579
Zn	-0.010	0.046	**-0.656**
Fe	0.363	0.159	**-0.545**
AOA	**0.746**	-0.033	0.015

Age	0.064	0.057	**-0.438**
T-score	0.095	**-0.929**	-0.135
Pen_Por	0.136	**-0.838**	0.290
BMI	0.373	0.451	**0.665**
BMD	0.249	0.270	**0.736**
Explained variation %	28.5	21.8	18.4

Three latent factors (principal components) explain nearly 70% of the total variation in the system. Factor 1 (explaining over 28% of the total variation) is associated with levels of key components responsible for the condition of osteoporosis (similar to Cluster 3 in hierarchical clustering or Cluster 2 in K-means clustering). Factor 2 (explaining over 20% of the total variation) can be termed as a conditional "osteoporosis diagnostic factor" (fully consistent with the results from both clustering procedures). Factor 3 is a conditional "general health factor" (including anthropometric indicators) and corresponds to clusters from both clustering procedures.
It can be concluded that the structure of the data is defined by three latent factors, each associated with a specific clinical meaning.

Figures 5 and 6 illustrate the results of the factor analysis, with variable grouping shown in 2D and 3D graphics.

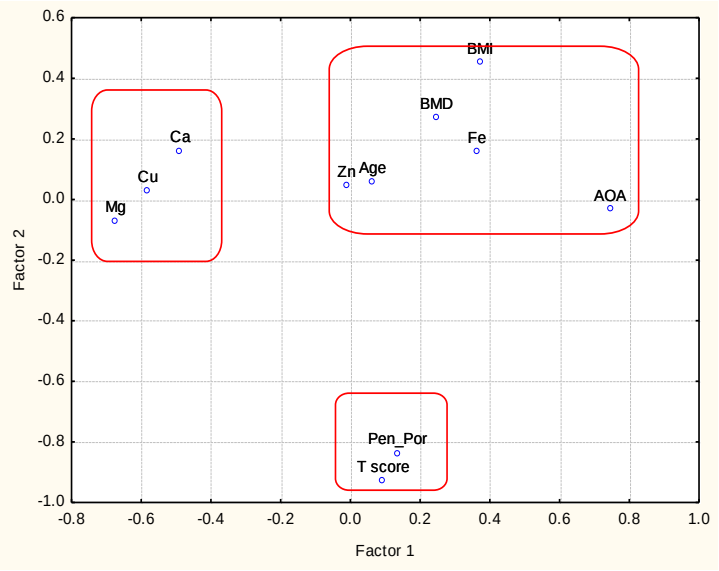


Figure 5. Diagram of factor loadings for the first 2 principal components

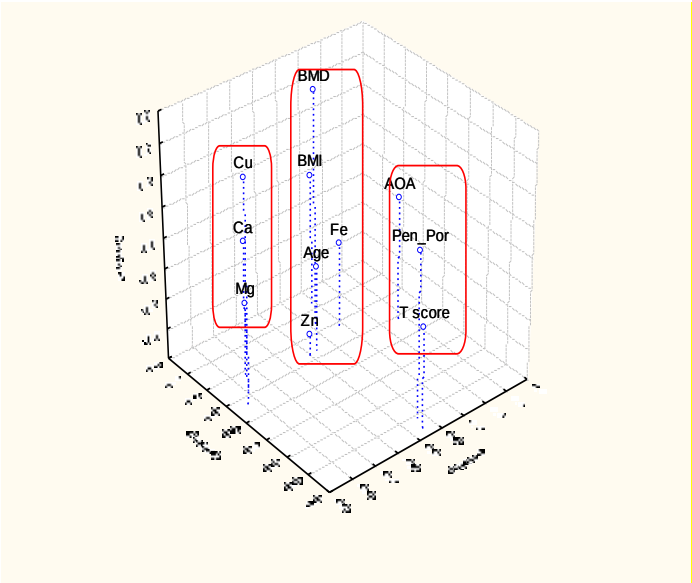


Figure 6. 3D diagram of factor loadings (first 3 principal components from factor analysis)

Principal component analysis yields results that confirm the role of 3 latent factors in explaining the structure of the input data.

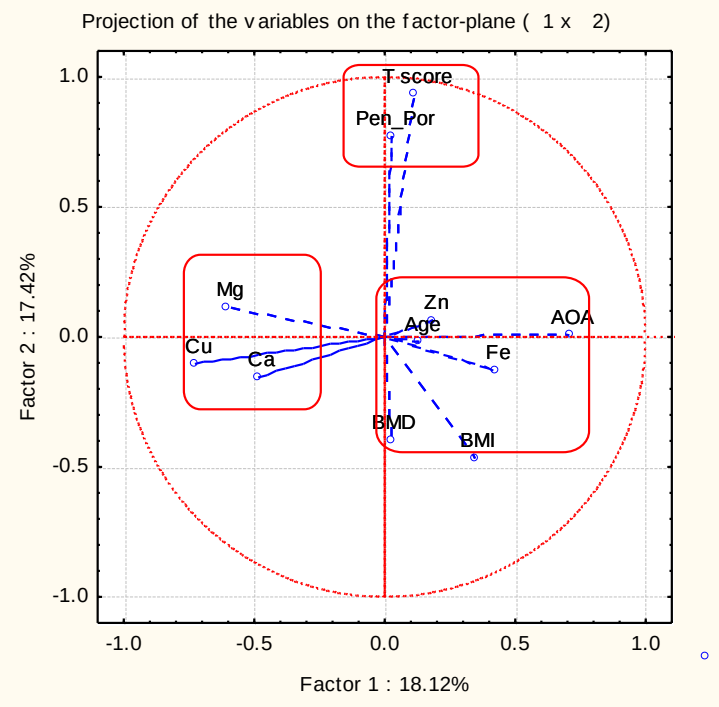


Figure 7. Diagram of factor loadings for the plane of the first two components from principal component analysis

Figure 8 shows the diagram of the factor results. The three groups (patterns) of patients are well separated.

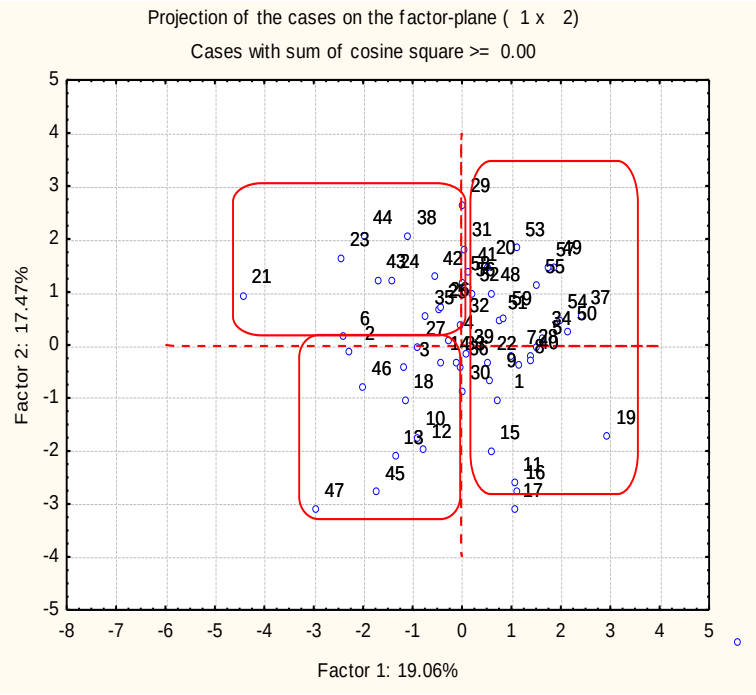


Figure 8. Diagram of factor scores for the first two principal components

5. Multivariate statistical analysis for determining the relationship between hip BMD, Cu, Mg, Zn and AOA in patients and controls

The input matrix for this specific case is of size [54 x 9], comprising 54 subjects (patients and controls) described by 9 variables. Cluster analysis and principal component analysis were used for data interpretation.

In hierarchical clustering of the variables (Age, Cu, Mg, Zn, AOA, T-score, BMI, BMD, and disease/control type), 3 statistically significant clusters were formed:

- Cluster 1 (Mg, Zn, Cu) – illustrates the influence of mineral composition
- Cluster 2 (AOA, T-score, Age) – reflects the influence of AOA and age factors.

- Cluster 3 (BMI, BMD, disease/control type) – represents the effect of body indices and disease type.

Figure 9 below depicts the hierarchical dendrogram for clustering 54 subjects (patients with hip osteopenia and osteoporosis and controls).

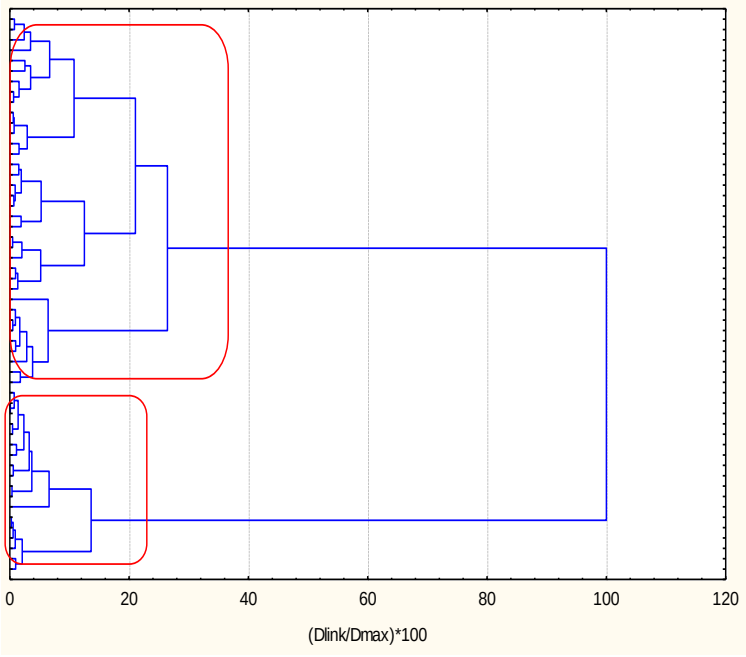


Figure 9. Hierarchical diagram for clustering 54 subjects

Two clusters are formed. The small cluster consists of 18 subjects (numbers 37 – 54). It is composed of all control subjects. The large cluster includes 36 subjects numbered 1 – 36 (all patients with the disease), with 19 of them having code 1 (osteopenia) and 17 with code 2 (osteoporosis).

An important stage in the study is identifying the variables responsible for distinguishing the two clusters (controls and patients). Figure 10 presents a graph of the mean values of each variable (standardized output data) for each of the identified clusters. Specific characteristics (descriptors) for the control cluster and the patient cluster can easily be identified.

The cluster of control subjects is characterized by low values of mineral components (magnesium, zinc, copper), AOAs, age, and high values of T-score, disease code (code 3 for controls), and body indices. Conversely, the cluster of patients diagnosed with osteopenia or osteoporosis at the hip is characterized by high values of mineral components (magnesium, zinc, copper), AOAs, age, and low values of T-score, disease code (codes 1 and 2 for patients), and body indices. This is a logical result, suggesting that the disease affects older individuals with reduced BMD values.

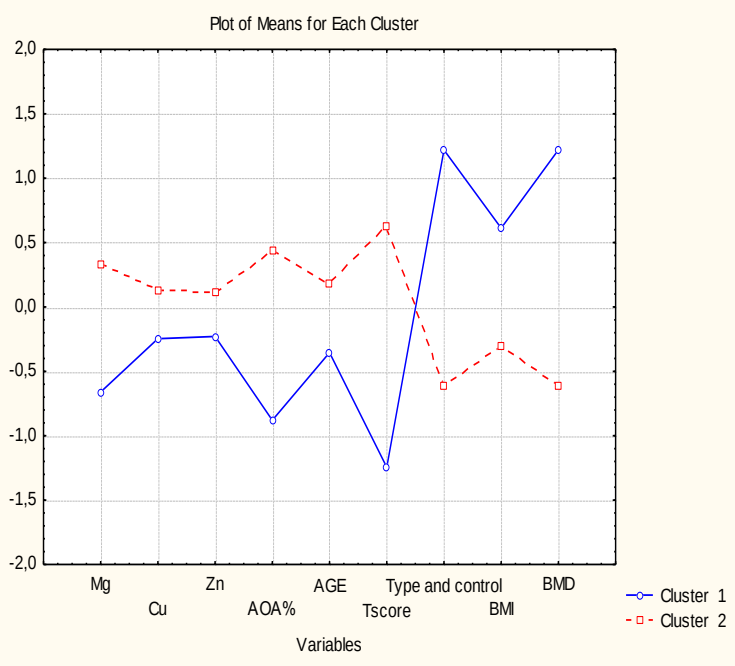


Figure 10. Graph of mean values (standardized output data) for each variable for each identified cluster (Cluster 1 - controls, blue color; Cluster 2 - patients, red color).

In the next stage of chemometric data processing, factor analysis was conducted to determine the latent factors responsible for the structure of the data. Table 6 presents the factor loadings for identifying hidden factors.

Three latent factors explain over 70% of the total variation in the system and can be interpreted as statistically significant.

The first latent factor explains over 35% of the total variation and includes high factor loadings for variables related to body indices and the disease codes for both controls and patient types. This factor can be termed as the 'body indices factor,' defining the influence of BMI and BMD on the nature of the health issue.

Table 6. Factor loadings

Factor loadings (loadings marked are >0,7)			
Variables	Factor - 1	Factor - 2	Factor - 3
Mg	-0,266	**-0,778**	-0,228
Cu	-0,080	**-0,743**	-0,265
Zn	0,007	**-0,867**	0,220
AOA%	-0,379	0,015	**0,768**
AGE	-0,418	0,242	**0,705**
T-score	-0,214	-0,182	**0,914**
Type and control	**0,778**	0,420	0,113
BMI	**0,703**	0,004	0,496
BMD	**0,933**	0,099	0,148
Expl.Var %	*36.4*	*20.2*	*16.8*

The second hidden factor explains over 20% of the total variation and can conditionally be termed as the "mineral composition factor," as it is associated with significant values of the three chemical (mineral) components in the list of variables.

The third latent factor, explaining over 15% of the total variation in the system, is associated with age characteristics, AOAs, and the T-score variable. It can conditionally be referred to as the 'age factor'.

The graphical representation of the roles of the three identified latent factors can be presented (on the plane of Factor 1/Factor 2) as follows (Fig. 11):

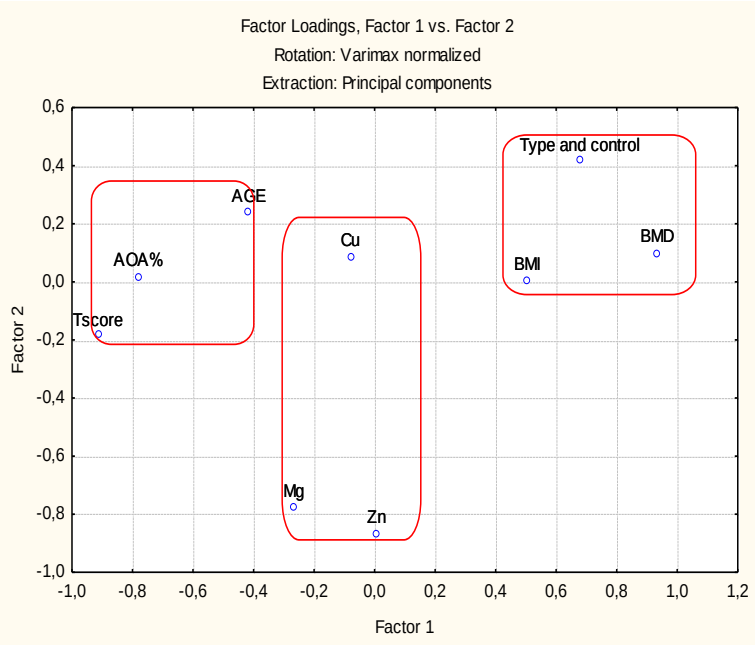


Figure 11. Graph for identifying hidden factors

Further application of PCA to demonstrate the projections of variables and subjects on the plane of Factor 1/Factor 2 Reaffirmed the results discussed earlier in full (Fig. 12 and Fig. 13).

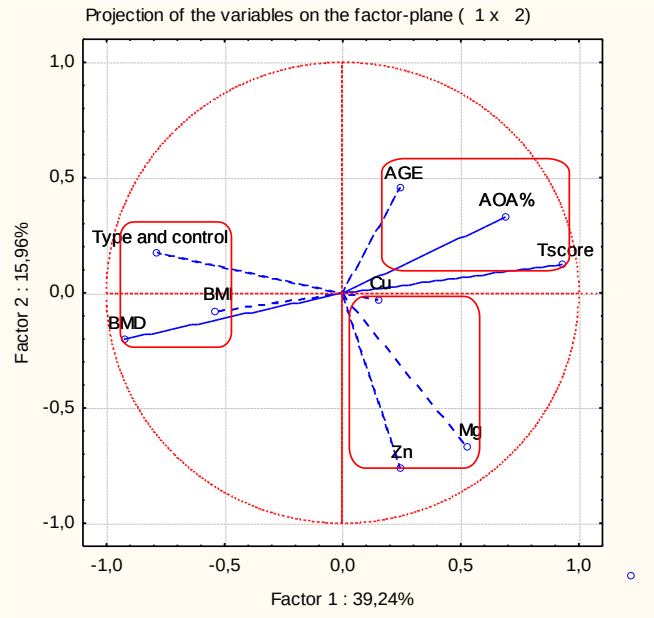


Figure 12. Projection of variables onto the plane of Factor 1/Factor 2.

The three factors clearly separate with their respective factor loadings for significant variables for each factor.

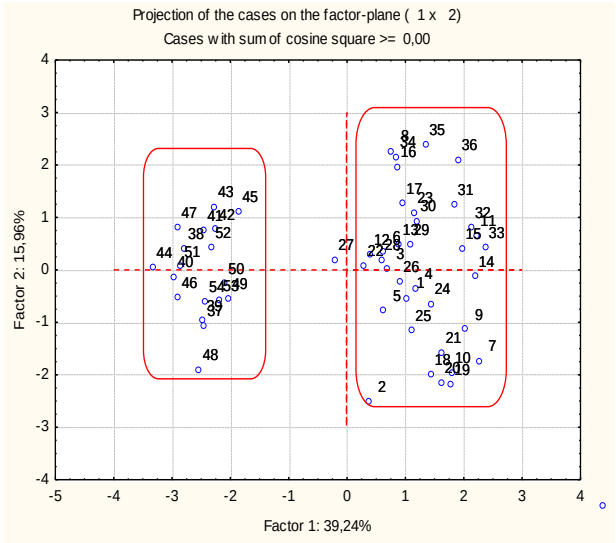


Figure 13. Projection of the subjects onto the same plane.

The two clusters distinctly differentiate based on similarity – patients with the disease (subjects 1 – 36 on the right side of the graph) and controls (numbers 37 – 54 on the left side). The only disputed object is number 27, which is very close to the group of patients but formally could also belong to the controls (despite being diagnosed with hip osteoporosis, it unexpectedly shows a high BMD value closer to that of control subjects rather than patients).

6. Multivariate statistical analysis for determining the relationship between BMD of the lumbar spine, Cu, Mg, Zn и AOA in patients and controls

In this case, the input matrix has dimensions of 64 subjects (patients with lumbar vertebral osteoporosis and controls), characterized by 9 variables (the same as in cases of hip osteoporosis), or [64 x 9]. The input data are standardized and subjected to both cluster and factor (principal component analysis) analysis.

Once again, three clusters emerge with almost identical composition as in the cases of hip osteoporosis. There is only one distinction – the position of zinc. Formally, it belongs to the cluster with participants AOAs, age, and T-score, but by choosing a different level of cluster significance, it could also be assigned to the cluster with copper and magnesium (mineral factor). The latter option seems more logical to us and we will use it in interpretation.

Thus, we have three clusters of variables that correspond closely to the identified patients with decreased hip bone density. The first includes the mineral components, the second is an 'age cluster,' and the third is the cluster of body indices and disease code.

Cluster 1 (Mg, Zn, Cu) – “mineral” cluster

Cluster 2 (AOA, T-score, age) – influence of AOA and age cluster

Cluster 3 (BMI, BMD, disease type and controls) – cluster of body mass indices and type of problem

In the following Figure 14, the hierarchical clustering dendrogram for 64 subjects (patients with osteoporosis and controls) is presented.

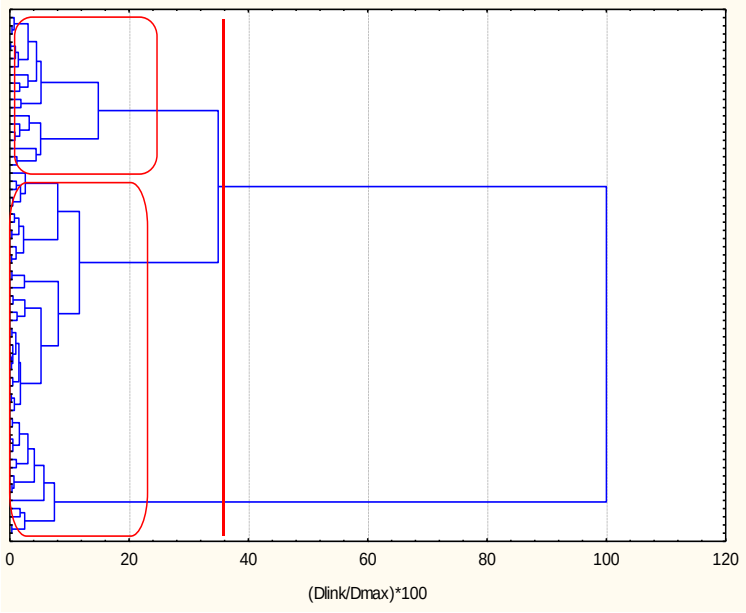


Figure 14. Hierarchical dendrogram for clustering 64 subjects

Two significant clusters are formed:
Large cluster with 49 subjects (object numbers 1 – 49, all with code 2 – osteoporosis)
Small cluster with 15 subjects (object numbers 50 – 64, all with code 3 – controls)
The result is analogous to that for patients with hip disease. A clear distinction between controls and patients with the disease has been accomplished. It is logical to expect that this separation is due to the same specific factors as in the cases of hip issues.
Figure 15 depicts a graph of the mean values of each variable (standardized output data) for each of the identified clusters. Specific characteristics (descriptors) for the control cluster and the patient cluster can easily be determined.

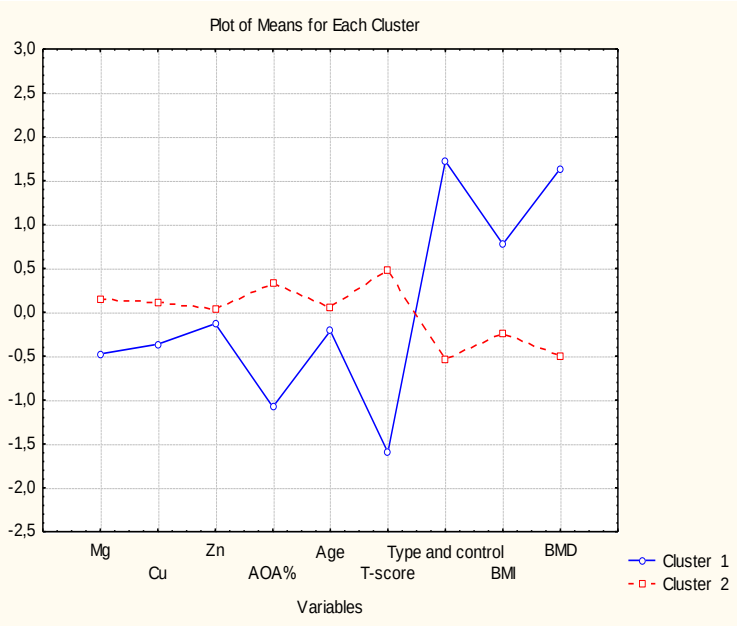


Figure 15. Graph of mean values (standardized output data) for each variable for each identified cluster (Cluster 1 - controls, blue colour; Cluster 2 - patients, red colour).

The cluster of control subjects is characterized by low values of mineral components (magnesium, zinc, copper), AOAs, and age, and high values of T-score, disease code (code 3 for controls), and body indices. In contrast, the cluster of patients diagnosed with osteopenia or osteoporosis at the hip is characterized by high values of mineral components (magnesium, zinc, copper), AOAs, age, and low values of T-score, disease code (code 2 for patients), and body indices. This is a logical result, suggesting that the disease affects older individuals with reduced BMD values.

The only minor differences compared to cases with patients and controls from the 'hip' subjects are related to the zinc levels and age data. Here, the zinc levels for both clusters are almost identical, which also defines the disputed position of the 'zinc' variable in clustering. The mean values for the 'age' variable are very close between controls and diagnosed patients. In 'hip' cases, controls are significantly younger than patients. In this sense,

zinc and age are not suitable descriptors for separating controls and patients diagnosed with vertebral osteoporosis.

In the next stage of chemometric data processing, factor analysis was conducted to determine the latent factors responsible for the structure of the data. Table 7 presents the factor loadings for the identified latent factors.

Three latent factors explain nearly 75% of the total variation in the system and can be interpreted as statistically significant.

The first latent factor explains over 35% of the total variation and includes high factor loadings for variables related to body indices and the disease codes for both controls and patient types. This factor can be tentatively named the "body indices factor," as it defines the influence of BMI and BMD on the nature of the health issue.

Table 7. Factor loadings

Factor loadings (Varimax normalized) (loadings marked are > ,7)			
Variables	Factor - 1	Factor - 2	Factor - 3
Mg	0,187	**-0,782**	0,322
Cu	0,013	**-0,848**	-0,233
Zn	0,106	**0,832**	0,110
AOA%	0,048	0,512	**0,722**
Age	-0,263	0,085	**0,775**
T-score	0,010	-0,045	**0,937**
Type and control	**-0,908**	0,229	-0,027
BMI	**-0,542**	-0,027	0,142
BMD	**-0,931**	0,116	0,029
Expl.Var %	*38.5*	*19.7*	*16.2*

The second hidden factor explains almost 20% of the total variation and can tentatively be called the 'mineral composition factor,' as it is associated with significant values of the three chemical (mineral) components in the variable list.

The last (third) latent factor, explaining over 15% of the total variation in the system, is associated with age characteristics, AOAs, and the T-score variable. It can tentatively be called the “age factor”.

These three hidden factors are responsible for the structure of the data in the studied system and can be summarized as “bone density”, “mineral composition”, and “age”.

The graphical representation of the projection of variables and subjects in the plane of Factor 1/Factor 2 (Figures 16 and 17), resulting from principal component analysis, convincingly illustrates the achieved results in conjunction with other chemometric approaches.

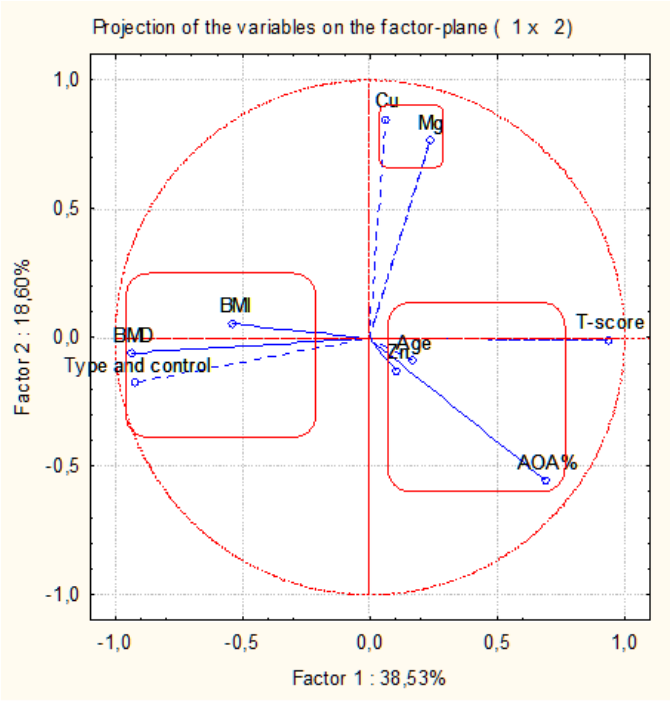


Figure 16. Projection of variables onto the plane of Factor 1/Factor 2.

The three latent factors are clearly separated with their respective factor loadings for significant variables for each factor. The specific positions of the variables zinc and age are also confirmed.

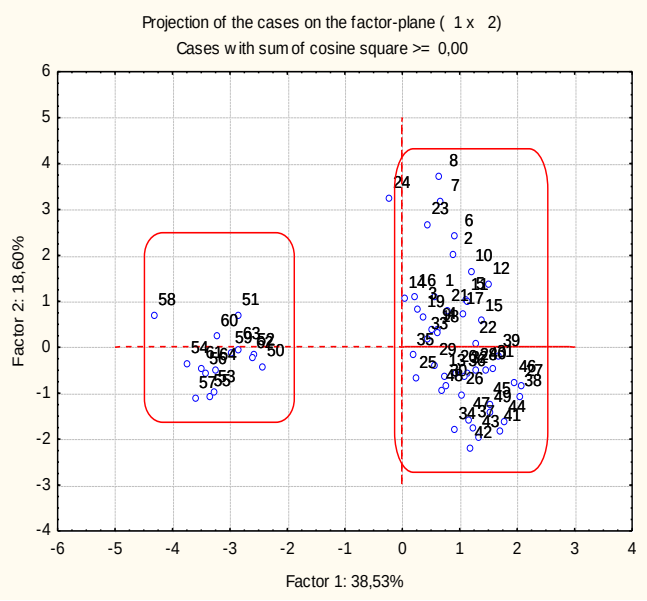


Figure 17. Projection of the subjects onto the same plane.

The two clusters are clearly delineated – patients with the disease (subjects 1-49 on the right side of the graph) and controls (numbers 50-64 on the left side). The only contentious object is number 24, which is very close to the patient group but formally could also belong to the control group (despite being diagnosed with vertebral osteoporosis, it unexpectedly shows a high value for copper, which aligns it closer to the copper levels of controls rather than patients).

7. Multivariate statistical analysis for determining the relationship between the decrease in BMD and questionnaire data related to menopause, cigarette use, coffee consumption, and dairy products in patients with osteoporosis

In this study, the input matrix includes 49 patients with osteoporosis and 9 variables, 5 of which are related to clinical indications, and the remaining 4 are results from a survey related to the duration of menopause and the use of cigarettes, coffee, and dairy products. Instead of the BMD variable, there

is a new ΔBMD – a reduction in bone density (the difference between the mean bone density of the controls and the bone density of the patients). The aim is to explore the relationship between the reduction in bone density (ΔBMD) and the survey responses to the given questions.
After hierarchical clustering of the variables, two well-defined clusters are formed:
Cluster 1 (AOA, age, years of menopause, BMI, dairy products)
Cluster 2 (T-score, BMD, smoking, coffee consumption)
Even at this stage of separating the variables into similarity patterns (clusters), it can be concluded that external factors (included in the survey) are in some manner related to bone density: smoking and frequent coffee consumption directly decrease bone density, while the other two factors (absence of menstruation and daily intake of dairy products) indirectly influence the reduction of bone density through their direct effect on AOA and BMI, and through the age variable.

Table 8 presents the factor loadings for the two identified latent factors that determine the structure of the data for the analysed system.

Table 8. Factor loadings

Factor loadings (Varimax normalized) Extraction: Principal components (loadings marked are > 0.7)		
Variables	Factor - 1	Factor - 2
AOA%	0,200	**0,751**
Age	0,160	**0,750**
T-score	**0,758**	0,461
BMI	-0,286	**0,736**
Δ BMD	**0,790**	0,493
menopause	-0,050	**0,720**
smoking	**0,773**	-0,142
coffee	**0,710**	-0,374
dairy	-0,213	**0,784**
Expl.Var %	39.6	27.4

The results of the principal component analysis thoroughly affirm the relationships identified in the hierarchical clustering. The two latent factors explain about 70% of the total variation of the system, with the first factor conditionally named "factor of direct influence on bone density change" (explaining nearly 40% of the total variation) and the second "factor of indirect influence on bone density" (explaining almost 30% of the total variation). Figure 18 shows the planar projection of the factor loadings, where the discovered relationships between the survey factors and the variables related to bone density are clearly visible once again.

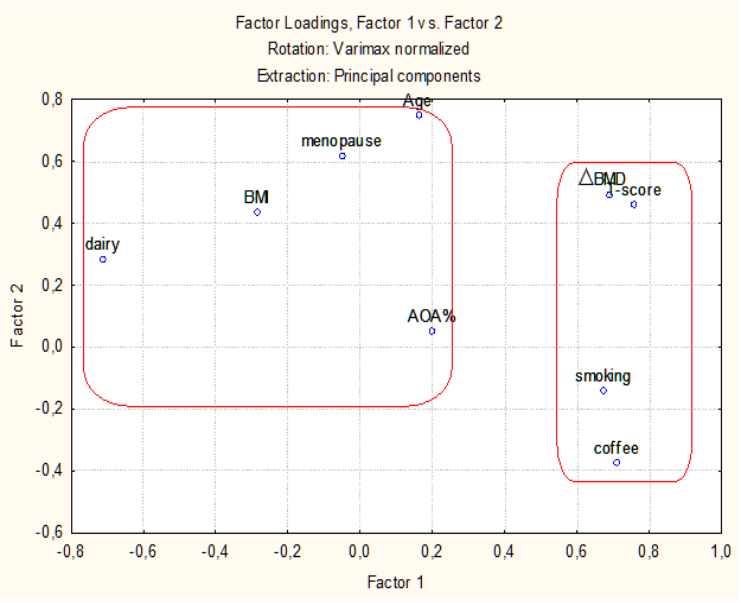


Figure 18. Projection of variables in the plane of Factor 1/Factor 2.

From the chemometric analysis performed, conclusions can be drawn regarding the similarities among the patients' profiles. Generally, the group is homogeneous in regards to the type of disease, but nevertheless, it is possible to identify 4 patterns (clusters) among the subjects (Fig. 19).

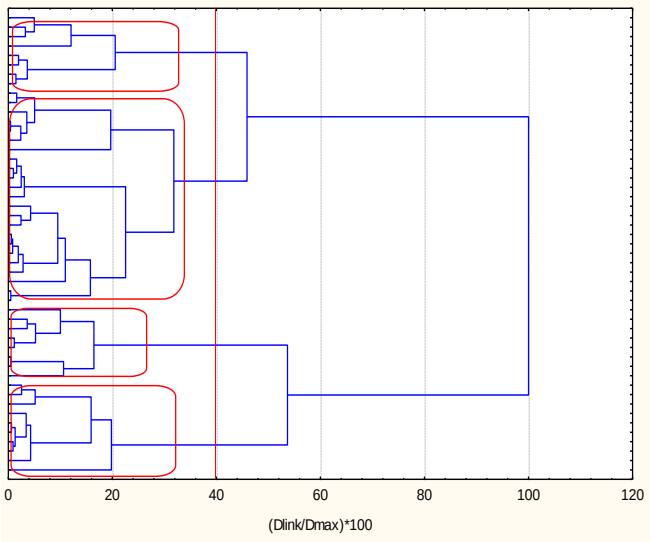


Figure 19. Hierarchical dendrogram for clustering 49 subjects

Figure 20 shows a graph of the mean values of each variable for each of the identified similarity patterns.

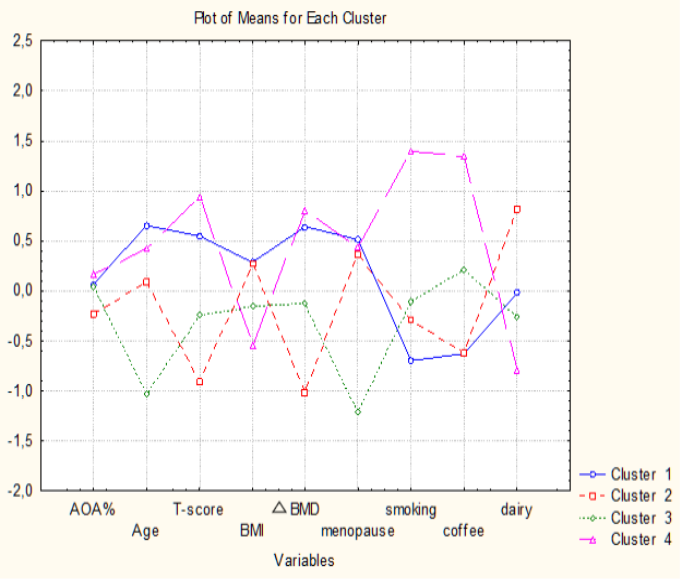


Figure 20. Mean values (standardised data) for each variable and for each identified cluster.

Type 1 patients (Cluster 1): relatively low bone density despite long-term menopause, with low consumption of cigarettes and coffee and moderate consumption of dairy products, with a relatively high age value - a pattern of "stable" patients;
Type 2 patients (Cluster 2): least reduced bone density and long-term menopause, of older age, with low consumption of coffee but significant consumption of dairy products; smokers - a pattern of patients without established health habits;
Type 3 patients (Cluster 3): moderate reduction in bone density, relatively young with the fewest years since menopause, significant consumption of coffee and cigarettes, and moderate consumption of dairy products - a pattern of "problematic" patients with potential deterioration in health condition;
Type 4 patients (Cluster 4): with the most significant reduction in bone density, strong dependence on coffee and cigarettes, and no daily consumption of dairy products - a pattern of the most significantly "at-risk" patients.
This particular classification, despite lacking quantitative measurements, allows for detailed diagnosis of osteoporosis patients based on dietary habits and attitudes towards harmful habits akin to domestic "addictions."

8. Multivariate statistical analysis for determining the relationship between decreased BMD in patients with different localisations of osteopenia and osteoporosis, and concentrations of Ca, Cu, Mg, Zn, AOA

The input matrix in this case is sized [84 x 9]. The matrix combines cases with reduced bone density in the hip (36 cases) and cases with reduced bone density in the lumbar vertebrae (48 cases). Thus, there are a total of 84 objects, using 9 variables as descriptors of the condition: Ca, Cu, Mg, and Zn levels, AOA (antioxidant activity), T-score, BMI (body mass index), age, and ΔBMD (change in bone mineral density).
After hierarchical clustering based on variables, three major clusters were formed: C1 (Ca, Mg, Cu); C2 (Zn, AOA, Age, BMI); C3 (T-score, ΔBMD).

The first cluster suggests a "pattern of chemical impact". Within this cluster, the relationship between chemical descriptors related to the medical condition of reduced bone density is depicted. Thus, it represents a chemical clustering link with the patients' condition.

The second cluster is a "pattern of physical impact" and demonstrates the associated impact of indicators such as Zn and AOA, as well as BMI and Age.

The third cluster is a "pattern of diagnostic impact," demonstrating the logical connection between the T-score condition indicator and the reduction in bone density (ΔBMD).

While Cluster 2 demonstrates the logical connection between the patient's age and the T-score condition indicator - a diagnostic cluster based on age - Cluster 3 shows the related effects of AOA, BMI, and BMD indicators - a cluster related to physical conditions.

Figure 21 depicts the hierarchical dendrogram illustrating the division of all 84 cases.

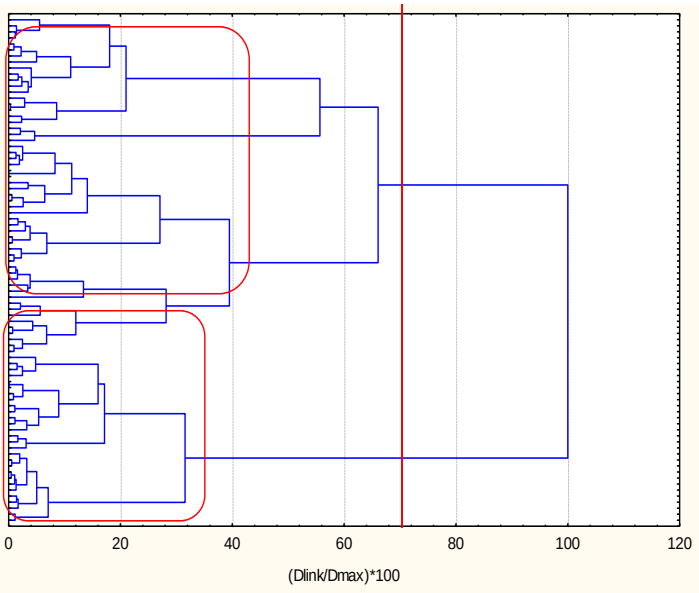


Figure 21. Hierarchical dendrogram for clustering 84 cases (objects).

Two main clusters can be identified. For convenience, the members of each cluster are presented in Tables 34 and 35 as results of K-means clustering based on the a priori hypothesis of the existence of 2 clusters (likely "hip" and "spinal" similarity patterns).

Clustering variables using K-means reveals a similar separation as observed in hierarchical grouping.

- ✓ Cluster 1 consists of 38 cases (1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 20, 21, 22, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 37, 38, 39, 41).

This cluster predominantly contains cases with reduced bone density in the hip (34 out of 36 cases of hip osteoporosis; four cases of lumbar osteoporosis are misclassified into the "hip" cluster, which is an acceptable result). Therefore, Cluster 1 can be termed as a "hip bone density reduction" pattern with over 90% correctly classified cases.

- ✓ Cluster 2 consists of 46 cases (35, 36, 40, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72,73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84).

The second cluster can be termed a "lumbar bone density reduction" pattern, as it includes 44 cases with lumbar localization of the disease (4 cases are missing due to being misclassified into Cluster 1, and 2 cases from the hip cases are incorrectly added to the cluster). The correct classification rate is nearly 90%, which is a very good result. Figure 22 plots the average values for each variable for each identified cluster.

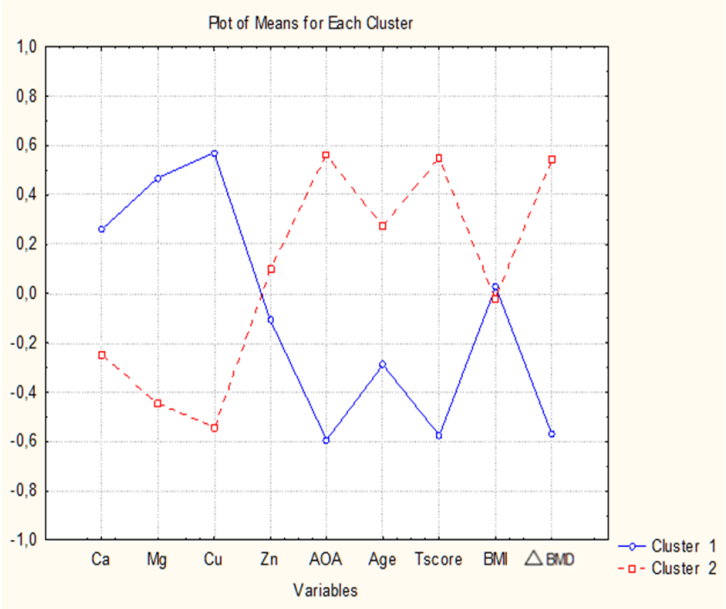


Figure 22. Graph of mean values for each variable for each cluster.

It is evident that both clusters (blue - "hip" pattern and red - "spinal" pattern) are clearly separated. Cluster 1 is characterized by higher values of the "chemical impact" variables (Ca, Mg, Cu) and lower values of the "physical impact" variables (AOA, Age) and the "diagnostic" variables (ΔBMD). For Cluster 2, the opposite trend is observed. The Zn levels and BMI values are not significant for differentiating between the two clusters.
Table 9 presents the factor loadings for the 3 identified latent factors responsible for the data structure.

Table 9. Factor loadings

Factor loadings (Varimax normalized). Significant loadings are marked in bold.			
Variables	Factor - 1	Factor - 2	Factor - 3
Ca	**-0,678**	-0,091	0,326
Mg	**-0,657**	0,009	-0,597
Cu	**-0,790**	-0,145	0,089
Zn	0,259	-0,035	-0,462

AOA	0,422	0,066	**0,882**
Age	0,054	0,051	**0,660**
T-score	0,206	**0,941**	0,055
BMI	0,046	-0,269	0,239
ΔBMD	0,127	**0,948**	0,113
Expl.Var%	29.9	22.1	18.8

Three latent factors explain over 70% of the total variance of the system. Generally, they align closely with the conclusions made above.

The first latent factor, explaining nearly 30% of the total variance, could be tentatively labelled as the "chemical factor" (due to high loadings for Ca, Mg, and Cu). The second factor (explaining over 22% of the variance) could tentatively be termed the "diagnostic factor" because of the high loadings and the relationship between the T-score and ΔBMD variables. Lastly, latent factor 3 (tentatively the "physical factor") reveals the relationship between age and AOA. The variables Zn and BMI show statistically insignificant loadings and have minimal influence on the structure of the dataset.

Figure 23 depicts a two-dimensional plot of the distribution of factor loadings in the plane of Factor 1 versus Factor 2.

In this case, there is no evidence of a relationship between AOA and T-score; instead, AOA is associated with age.

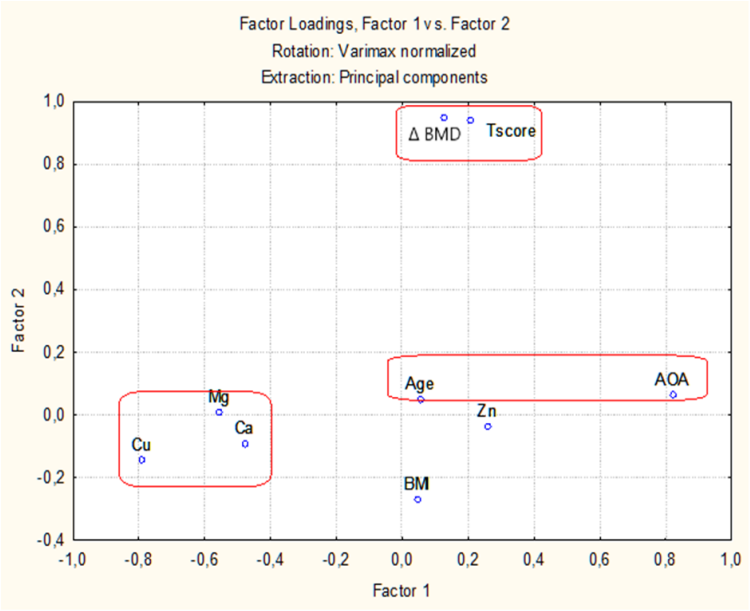


Figure 23. Factor loadings plot (F1 / F2)

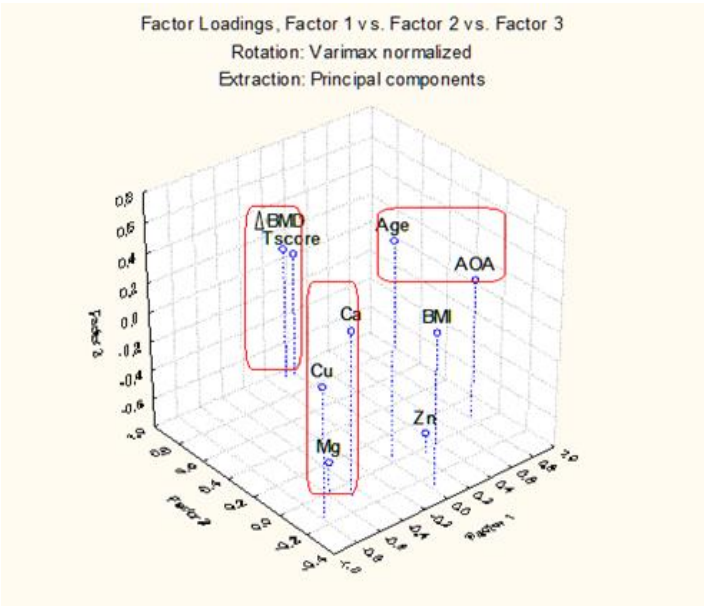


Figure 24. 3D plot of factor loadings under the given conditions

CONCLUSION

From our research, we can conclude that patients with reduced bone density exhibit higher values of RSA, elevated concentrations of copper and zinc, and increased Cu/Zn ratio compared to the control group.

The increase in serum levels of copper and zinc in patients with reduced bone density may be attributed to the degradation of copper and zinc, which are components of enzymes involved in bone metabolism. This degradation is triggered by elevated levels of ROS within cells in the context of osteoporosis. The elevated concentration of copper ions in the serum initiates secondary radical processes that further increase its AOA. Patients with normal bone metabolism maintain lower serum concentrations of Cu and Zn because their intracellular function remains undisturbed. The decreased production of additional radicals explains the lower serum AOA observed in the control group.

These findings confirm the combined impact of free radicals and redox-active metals, such as copper, on both AOA and BMD.

The multivariate statistical analysis we conducted highlighted the relationship between BMD, the levels of biogenic elements, and AOA.

SUMMARY OF FINDINGS

- When determining the concentrations of Cu, Zn, and Mg in blood serum, we obtained higher mean values in patients with disrupted bone metabolism, particularly noticeable in patients with osteopenia.
- The Cu/Zn ratio proves to be an important marker for the severity of the condition.
- The increase in total antioxidant activity levels in the blood serum of osteoporosis patients is higher compared to controls and osteopenia patients.
- The correlation analysis defined stochastic relationships between bone density, magnesium, AOA, T-score, BMI, and age.
- A regression model was developed for establishing the relationship between bone density, body mass index and AOA, which can be used for

easy diagnostic prediction of bone density reduction from readily available descriptors.

- The summarised results from the chemometric analysis define the following primary factors that determine the structure of the experimental data:

A factor responsible for forming a "pattern of diagnostic impact," including the descriptors T-score and ΔBMD; a factor associated with reduced bone density status and forming a "pattern of mineral content effect," accounting for the roles of Ca, Mg, and Cu descriptors; a factor related to overall health status, revealing relationships among BMD, BMI, AOA, Age, Fe, and Zn descriptors, forming a "pattern of patient's physical condition."

CONTRIBUTIONS

1. An original marker for the degree of osteoporosis and osteopenia has been proposed, utilizing the ratio of Cu/Zn concentrations instead of traditional clinical analyses of serum concentrations of the two components.

2. An adequate regression model has been developed for the relationship between patient bone density and the independent parameters AOA and BMI, enabling straightforward and rapid prediction of bone density values based on known regressor values.

3. Based on results from one of the chemometric analyses involving cluster and factor analysis (patients without a control group), three conditional phenotypes of patients with reduced bone density (osteopenia or osteoporosis as the primary diagnosis) have been identified: Phenotype 1: Patients with the most negative impact of osteoporosis and significant metabolic disturbances; Phenotype 2: Patients affected by early-stage osteoporosis; Phenotype 3: Patients with typical osteopenia.

4. Chemometric analysis also allowed the identification of specific descriptors for each of the identified phenotypes. This facilitates the diagnostic procedure and pre-classification of the patient into one of the phenotypes.

SCIENTIFIC PUBLICATIONS RELATED TO THE DISSERTATION

1. **Sv. Asenova,** P. Andreeva-Gateva. Prevention of Osteoporosis through Micronutrients - Contemporary Pathophysiological Aspects. General Medicine, 2020; 22, (2):66-72. ISSN 1311-1817, Web of Science (CABI); Scopus, Q4; SJR $_{2020}$ − 0.111
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Acknowledgments

Thank you to everyone who contributed to the completion of this dissertation work:

- To my academic supervisor, Assoc. Prof. Radka Tomova, D.Chem., for her support and guidance throughout;
- To my scientific consultant, Assoc. Prof. Bisera Atanasova, MD, from the Clinical Laboratory of University Hospital "Alexandrovska," for her assistance and support in the clinical and laboratory aspects of the dissertation;
- To Dr. Rodina Nestorova from the Rheumatology Centre "Sveta Irina" - Sofia, for invaluable assistance in patient selection and providing the samples for investigation.
- To Assoc. Prof. Nadya Hristova, PhD, and to Assistant Professor Liliya Atanasova, PhD, from the Department of Medical Physics and Biophysics, Medical University of Sofia, for their support and trust in me.
- To the Research Department at Medical University Pleven for the opportunity and guidance provided.
- To Professor Angelina Stoyanova, D.Chem, Head of the Chemistry Department, and to all colleagues from the department for their support and constructive comments and remarks during the discussion of the project dissertation.
- To all my close friends, relatives, and loved ones for their patience.