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Using cluster analysis with principal component analysis to study the iron metabolism in polycythemia vera

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ABSTRACT

Background. Iron deficiency is a common complication in patients with polycythemia vera (PV). Unfortunately, little is known about the pathomechanisms of iron deficiency in PV. There have been no studies in the last decade documenting iron disorders in PV, despite progress in understanding the iron metabolism and new laboratory techniques measuring iron parameters.

Objectives. The aim of this study was to assess the relationships between iron metabolism parameters, haematological and biochemical factors and clinical attributes in polycythemia vera patients with the use of cluster analysis (CA) and principal component analysis (PCA).

Patients and methods. The study was performed on 60 patients (F/M 26/34) aged 38–84 (66 ± 10) years. The following parameters were determined in blood samples: hepcidin, prohepcidin, iron, total iron binding capacity (TIBC), unsaturated iron binding capacity (UIBC), ferritin, soluble transferrin receptor (sTfR), transferrin saturation (TfS), complete blood cell count, erythropoietin (Epo), uric acid, lactate dehydrogenase (LDH).

Results. The CA divided all the 17 parameters into three clusters and showed that hepcidin concentration is related to the duration of hydroxyurea therapy. PCA also revealed a positive correlation between hepcidin and therapy duration.

Conclusions. We demonstrated that CA and PCA are efficacious methods for assessing the relationship between iron metabolism parameters and clinical attributes in PV patients.

Key words: hepcidin, iron metabolism, polycythemia vera, cluster analysis, principal component analysis

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Introduction

Polycythemia vera (PV) is currently classified as one of the myeloproliferative neoplasms (MPNs) and is characterised by marrow hyperplasia with an increased number of erythrocytes as well as leukocytes and platelets in peripheral blood [1]. Several studies have shown that iron deficiency (ID) is a common disorder in polycythemia vera patients, and can significantly influence their quality of life. Iron deficiency in polycythemia vera is usually a complication of expansive erythropoiesis in addition to phlebotomy and haemorrhage [2–5]. Unfortunately, little is still known about the pathomechanisms of ID in polycythemia vera.

There have been no studies in the last decade documenting iron disorders in polycythemia vera, despite progress in understanding the iron metabolism and new

laboratory techniques measuring iron parameters. That makes the studies on iron metabolism in polycythemia vera even more interesting.

The aim of this study was to assess the relationships between iron metabolism parameters, haematological and biochemical factors and clinical attributes in polycythemia vera patients with the use of cluster analysis (CA) and principal component analysis (PCA).

Materials and methods

Patients

The study group comprised 60 patients with polycythemia vera (34 male, 26 female). The patients ranged in age from 38 to 84 years (66 ± 10). The diagnosis of

PV was based on the Polycythemia Vera Study Group Diagnostic Criteria for Polycythemia Vera and confirmed by trepanobiopsy. All the patients were followed in the haematology outpatient clinic. Physical and diagnostic examinations, consisting of complete blood cell count, uric acid concentration and lactate dehydrogenase concentration, were performed at regular intervals. The maximum patient follow-up period was 27 years, and the mean observation period was 7.4 years. Every patient from the study group was treated with hydroxyurea (HU) alone or HU with supplemental phlebotomy. The biochemical analyses were performed with blood serum samples. All procedures in the study were approved by the local ethics committee. Informed written consent was obtained after the purpose, nature, and potential risks had been explained to the subjects.

Analytical methods

The biochemical analyses were performed on blood samples. Blood samples were drawn from an antecubital vein between 8 a.m. and 10 a.m. with minimal venous stasis and then centrifuged. Serum was stored at -80°C until the analysis.

Serum hepcidin concentrations were quantified using ELISA kit (Hepcidin ELISA, EIA-4705, DRG Instruments GmbH, Germany). Levels of prohepcidin were determined by a stable ELISA (Hepcidin Prohormone ELISA, DRG Instruments GmbH, Germany).

Serum iron concentrations, values of unsaturated iron capacity (UIBC), total iron capacity (TIBC), transferrin saturation (TfS), uric acid and lactate dehydrogenase (LDH) were measured on the Architect c8000 System (Abbott Laboratories, USA).

Serum ferritin was quantified using the DRG Ferritin kit (EIA-1872, DRG International, Inc., USA). Serum soluble transferrin receptor (sTfR) concentrations were examined by an enzyme-linked immunosorbent assay (the Human sTfR ELISA, BioVendor Laboratory Medicine Inc., Czech Republic). Erythropoietin (EPO) concentrations were quantified using ELISA kit (EPO ELISA, Roche Diagnostics GmbH, Germany).

Complete blood cell count was measured on whole blood samples using ADVIA 120 (Bayer Diagnostics, USA).

Statistical analysis

All statistical analyses were performed using the software Statistica 10.0 (Stat-Soft, Krakow, Poland). Results are presented as mean \pm SD when the data demonstrated a normal distribution, or as medians (Q1–lower quartile/Q3–upper quartile) due to their being abnormally skewed of the data. All of the iron metabolism parameters, haematological and biochemical factors and clinical attributes were used in multivariate data

analysis. Ward's minimum-variance CA and PCA were used for appraisal of dependence between laboratory and clinical parameters.

Results

Table 1 shows the values of all laboratory and clinical parameters in patients with polycythemia vera.

CA divided all the laboratory and clinical parameters into three clusters (Fig. 1):

- Cluster 1 — age, duration of therapy, hepcidin, uric acid, LDH;
- Cluster 2 — sTfR, TIBC, UIBC, RBC, HGB, HCT;
- Cluster 3 — prohepcidin, EPO, ferritin, iron, TfS, MCV.

The result of the PCA is a 2D scatter plot of the cases in the space formed by the first two principal components (PC1 and PC2), which are orthogonal and uncorrelated (Fig. 2). The first two PCs explain 49.46% of the variation. PV patients form a relatively homogeneous group.

The other result of the PCA analysis (correlation circle, Fig. 3) was interpreted as follows [6]:

- when two variables are close to each other — they are strongly positively correlated;
- when two variables are on opposite sides — they are strongly negatively correlated;
- when two variables are orthogonal to each other — they are not correlated.

Discussion

Multivariate analysis captures dependencies between parameters which are often invisible in a classic two-parametric analysis. CA combines the most similar factors into clusters. In our study, the separation of clusters provides a lack of homogeneity of the examined iron parameters, other laboratory parameters and clinical features that characterise patients with polycythemia vera. CA divided all of the 17 parameters into three clusters (Fig. 1). In polycythemia vera patients, laboratory parameters assessing their iron metabolism have been classified into all three clusters. The concentration of hepcidin was included in one set with concentration of uric acid, concentration of LDH, and with such clinical parameters as age and therapy duration. The concentration of soluble transferrin receptor and values of TIBC and UIBC formed a cluster with RBC, HGB and HCT. The third cluster contained prohepcidin, ferritin, iron, transferrin saturation as well as erythropoietin concentration and values of MCV.

The interpretation of the results will be limited mainly to the parameters contained in the first cluster

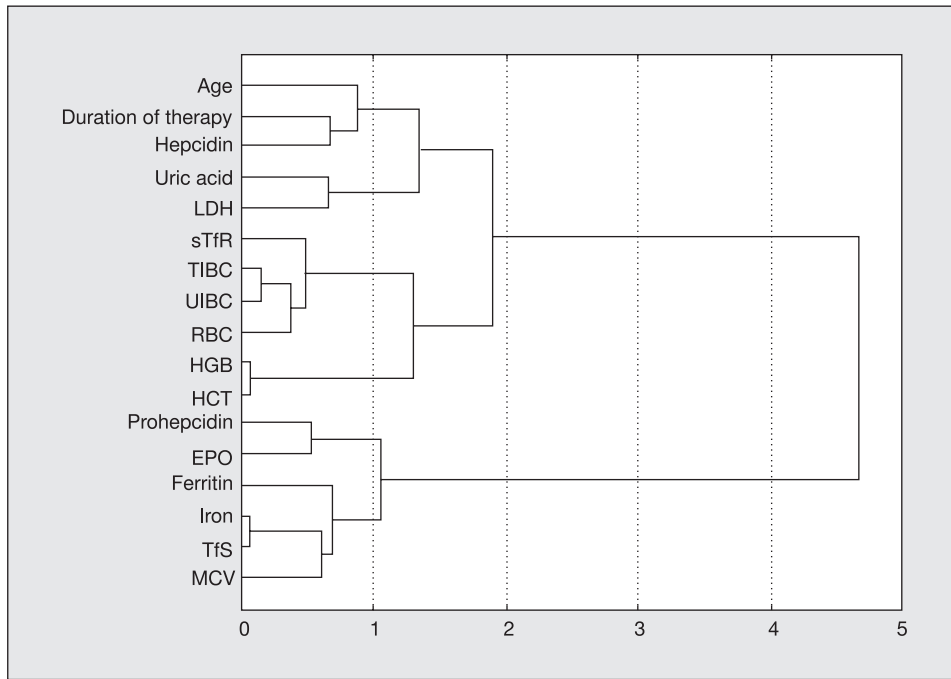


Figure 1. Clustering dendrogram obtained with Ward's method considering all laboratory and clinical attributes

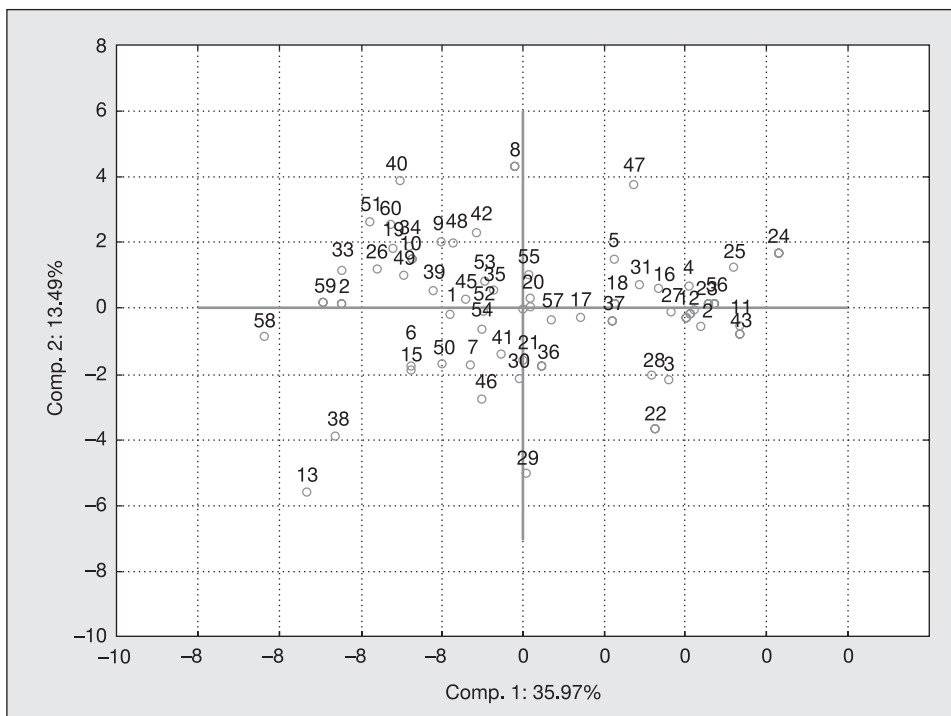


Figure 2. Scores of the first two principal components for laboratory and clinical data (the numbers are those of the PV patients included in the study)

(hepcidin, uric acid, LDH, age, duration of therapy). The main reason for such a restriction is the essential role of hepcidin in iron metabolism regulation. Hepcidin is an antimicrobial peptide hormone mainly synthesised

in the liver as an 84-amino acid prepropeptide, which is subsequently processed into a 60- to 64-residue prohepcidin peptide and then finally cleaved into 25-amino acid hepcidin [7, 8]. Hepcidin is known as the

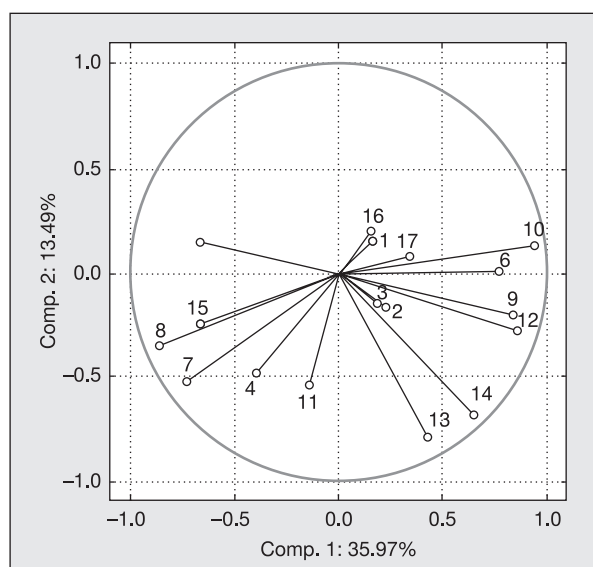


Figure 3. Projection of the laboratory and clinical variables in the two first principal components space (the interpretation was described in the ‘Results’ chapter)

main regulator of serum iron concentrations. Generally, hepcidin causes a decrease in serum iron. The mechanism of hepcidin activity depends on its interactions with its receptor ferroportin. Ferroportin is an iron exporter expressed on the surface of reticulo endothelial macrophages, hepatocytes, duodenal enterocytes and placenta cells. Hepcidin regulates post-translationally ferroportin expression. After binding to ferroportin, hepcidin is internalised and degraded in endolysosomes together with ferroportin, which in turn blocks the iron efflux from cells into plasma [9–11].

Unfortunately, a lot remains to be discovered regarding the biology and function of hepcidin. Further studies are needed to define precisely hepcidin’s role in iron metabolism, not only in physiological but also in pathological conditions. To the best of our knowledge, we are the first to evaluate hepcidin concentration in the serum of polycythemia vera patients.

CA showed a relationship between the concentration of hepcidin and duration of therapy (cluster 1, Fig. 1). PCA also revealed a positive correlation between the mentioned parameters (Fig. 3). All the PV patients in the study were on maintenance therapy with hydroxyurea (from 1–27 years), and some of them had been previously treated by phlebotomy. Therefore, we can exclude phlebotomy as the factor influencing iron status in this particular group of PV patients.

The results of our study suggest that long-term HU treatment may disturb iron metabolism homeostasis through the constant stimulation of hepcidin activity. HU is a cytotoxic and antimetabolic drug, which inhibits DNA synthesis by blocking ribonucleotide reductase [12].

Data dedicated to the impact of HU on iron status is limited. Clinical observations show that HU may decrease iron absorption from food. It has been confirmed that HU has side effects on the digestive system such as nausea, vomiting, diarrhoea, stomatitis, loss of appetite, flatulence and constipation [13]. Moreover, the hydroxamic functional group of hydroxyurea (-CO-NHOH) has the ability of strong chelation of divalent iron. After stimulation from HU, large amounts of nitric oxide are formed [14]. Nitric oxide stimulates the binding of IRP to IRE which limits ferritin mRNA translation and at the same time protects transferrin receptor mRNA from degradation [15, 16].

These findings may lead to the conclusion that HU may be treated as a potential factor involved in iron deficiency formation in polycythemia vera patients. In addition, the results of the present study confirm that many patients with PV suffer from iron deficiency. Analysis of the results showed the broad ranges of ferritin concentration values in patients with polycythemia vera — 0.48 — 188.11 ng/mL (Tab. 1). We can speculate that HU is one of the factors responsible for such a condition. Unfortunately, a comparison of those results with other papers is impossible because there have been no studies on the impact of HU on iron metabolism in PV patients.

The projection of the laboratory and clinical variables in the first two PCs space (Fig. 3) shows no correlation between hepcidin and its prohormone. Prohepcidin is a hepcidin precursor and undergoes a post-translational modification by furin to biologically active 25-amino acid hepcidin [17]. A high linkage distance between clusters 1 and 3 in CA also indicates that hepcidin is not dependent on prohepcidin located in the 3rd cluster (Fig. 1). Our results agree with other multicentre studies. No correlation between hepcidin and prohepcidin levels has been established so far [18, 19]. It should be stressed that prohepcidin concentration did not reflect the level of the active form — 25 amino acid hepcidin accurately. This may suggest the involvement of other factors responsible for the biochemical conversion of prohepcidin into hepcidin.

Regulatory mechanisms of iron status in polycythemia vera patients are unusual. Some of the findings have confirmed that the JAK2 mutation may be involved in the regulation of iron status in myeloproliferative disorders [20, 21]. The relationship between the JAK2 mutation and the regulatory axis hepcidin-ferroportin and IRP proteins remains unexplored in polycythemia vera. In physiological conditions, kinase JAK2 is involved in erythropoietin receptor signalling. It is also associated with the regulation of iron metabolism, because it phosphorylates ferroportin in response to hepcidin [22–25]. We can assume that the constitutive activation of kinase JAK2 as a result of mutations JAK2^{V617F} can lead to

Table 1. Laboratory and clinical characteristics of polycythemia vera patients

Parameter	N	X ± SD Me (Q1/Q3)	Min – Max
Iron status parameters			
Hepcidin (ng/mL)	57	103.68 (84.37/118.28)	38.46–192.36
Prohepcidin (ng/mL)	58	93.62 (80.64/105.67)	33.13–447.87
Serum iron (µg/dL)	59	82.00 (36.00/115.00)	9.00–210.00
TIBC (µg/dL)	59	330.00 (279.00/414.00)	184.00–498.00
UIBC (µg/dL)	60	233.50 (172.50/352.00)	29.00–473.00
Ferritin (ng/mL)	56	27.99 (5.81/64.62)	0.48–188.11
sTfR (µg/mL)	59	2.27 (1.48/4.66)	0.11–10.21
TfS (%)	59	26.25 (8.27/41.09)	2.04–87.87
Haematological parameters			
RBC (× 10 ⁶ /µL)	60	5.36 (4.67/6.22)	3.39–8.55
HGB (g/dL)	60	17.50 ± 2.24	12.10–22.10
HCT (%)	60	55.00 (48.10/58.75)	36.10–70.30
MCV (fL)	60	99.17 ± 12.28	69.10–125.90
Biochemical parameters			
EPO (mIU/mL)	57	12.97 (10.37/19.13)	1.99–45.23
Uric acid (mg/dL)	60	5.49 (4.53/6.96)	3.39–10.29
LDH (U/L)	60	296 (235.5/359)	149–912
Clinical parameters			
Age (years)	60	65.95 ± 10.28	38–84
Duration of therapy (years)	60	7.38 ± 6.06	1–27

uncontrolled phosphorylation of ferroportin which, in turn, disturbs the mechanisms responsible for iron efflux from cells. This process may be a signal to an enhanced prohepcidin conversion to hepcidin, resulting in increased hepcidin expression.

CA also showed relations between concentrations of uric acid, LDH and therapy duration (Fig. 1). Such results are not surprising, since elevated concentrations of uric acid and LDH are typical laboratory changes for PV patients treated with HU. Uric acid is a product of purine metabolism generated during the breakdown of nucleic acids (DNA and RNA) and ATP. In polycythemia vera, chemotherapeutic treatments, for example HU, cause a marked increase in the excretion of uric acid resulting from the nucleic acid metabolism [26, 27]. Another consequence of HU treatment is an increase in values of LDH concentration, which reflects intravascular haemolysis and tissue breakdown [27].

Conclusions

CA and PCA proved to be very useful methods in the interpretation of large amounts of laboratory and clinical

data on associations of iron metabolism parameters with other biochemical and haematological factors and clinical attributes in polycythemia vera patients.

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Conflict of interest disclosure: The authors declare no competing financial interests.

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