## ORIGINAL PAPER

# Dilemma: Correlation Between Serum Level of Hepcidin and IL-6 in Anemic Myeloma Patients

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## ABSTRACT

**Introduction:** Anemia occurs in 60% to 80 % of patients with newly diagnosed myeloma multiplex (MM). The cause of anemia in MM is probably multi factorial and involved among the others hepcidin and some cytokines, especially interleukine-6. Anemia in MM is one of the risk factor used in Durie-Salmon classification for staging and prognostic score. Treatment options are set according to this score with most significant impact on survival. **Aim:** To estimate baseline level of serum hepcidin, IL-6 and iron metabolism markers in anemic MM patients, possible role of hepcidin and its interaction with IL-6. **Methods:** 27 patients with newly diagnosed MM were enrolled in this observational, prospective study and age, gender matched 60 healthy controls. Erythrocyte count, hemoglobin, serum hepcidin, interleukin-6, iron, ferritin and transferrin were measured. **Results:** Anemia was diagnosed in 70% of MM patients. Serum hepcidin was significantly higher in MM group (55.5 ng/mL) than in control 5.9 ng/mL (p=0000). In myeloma patients serum IL-6 was 3.59 pg/mL, anemic 3.80 pg/mL, non-anemic 0.33 pg/mL, without significant difference. It was not found significant correlation between hepcidin and IL-6 in anemic myeloma patients. **Conclusion:** High level of hepcidin probably causes anemia in MM but its high expression is not due only to IL-6.
**Keywords:** multiple myeloma, hepcidin, anemia, interleukin-6.

## 1. INTRODUCTION

Multiple myeloma (MM) is plasma cell neoplasm derives from malignant transformation of B cells, plasmocytes (1). Anemia in MM is probably multifactorial, as some patients also have renal insufficiency and low endogenous erythropoietin levels, some show an apoptotic effect of myeloma cells on red blood cell precursors (2). The most of research in this field indicated that predominant cause of anemia in MM patients is impaired iron utilization which is cardinal characteristic of anemia inflammation also called anemia chronic diseases (ACD) (3).

Majority of MM patients develops anemia either at the beginning of the disease or during treatment. In pathogenesis of ACD are involved hepcidin and some cytokines, especially interleukine-6 (2). Hepcidin is small peptide hormone composed of 25 amino acids synthesized in hepatocytes (3, 4) and involved in iron metabolism. Hepcidin binds to ferroportin, iron exporter on cell membrane and induces internalization and degradation of ferroportin, resulting in retention of iron in enterocytes, macrophages and hepatocytes (3, 5). Overproduction of hepcidin results in reductions of serum iron available for erythropoiesis and causing iron restricted anemia (6). High concentration of hepcidin was found in serum and urin of MM patients (2, 3, 6). Since hepcidin was discovered only 16 years ago there is a need for clinical research to establish it's role in iron hemostasis in various diseases including myeloma. Previous studies focused on role of cytokines,

especially IL-6 in pathogenesis, not only of anemia but also of myeloma (7). IL-6 induces hepcidin synthesis resulting in iron sequestration (8). Recent studies suggest that there are more cytokines which contribute to anemia in ACD (9, 10).

It is essential to understand cause of anemia in myeloma and possible role of hepcidin in this process, because ACD is one of risk factors used in Durie-Salmon classification for staging and prognostic score. Treatment options are set according to this score with most significant impact on survival.

## 2. AIM

This study aimed to analyze baseline level of serum hepcidin, IL-6 and iron metabolism markers in MM patients and to estimate the role of hepcidin and its interaction with IL-6 in anemia in MM patients.

## 3. PATIENTS AND METHODS

The study was prospective, descriptive observational, conducted at Clinic of Hematology of Clinical Center of Sarajevo University (CCU), from September 2012 until January 2015. Study was approved by CCU Sarajevo Ethical committee and Medical faculty Ethical committee and conducted according to criteria of Helsinki declaration. In the study were included 27 newly diagnosed MM patients both sexes, age 18 and above. Patients which did not have sufficient data according to diagnostic algorithm at time of diagnosis were excluded.

Control group included 60 healthy volunteers, matching age and gender without clinical signs of inflammation, anemia or malignancy. All participants, patients and control group were informed about research plan and procedures and gave written consent. Diagnostics and staging was done according to clinical algorithm, anamnestic and clinical data, laboratory findings, bone marrow biopsy and bone marrow smear according to World Health Organization classification from 2008 (11). Bone marrow biopsy was analyzed at Clinical pathology and cytology at CCU Sarajevo by hematopathologist. Staging was done using standard radiological methods: skeletal X ray and abdominal ultrasound at Clinic of Radiology at CCU Sarajevo. Hematologist did bone marrow smear. Performance status was assessed using Karnofsky performance status scale. Stage was determined according to Durie Salmon stage and International staging system for MM.

Samples for laboratory analysis, including hepcidin, were collected from cubital vein from 6.30 to 9.30 a.m.. Serum samples were stored at -70 degrees after coagulation and centrifuge for 5 minutes at 5000. Hematological and biohumoral parameters were done using standard methods in Central laboratory of CCU. Serum hepcidin was determined at Center for cytogenetics and molecular medicine at Medical faculty of Sarajevo University using Enzyme-linked immunosorbent assay-ELISA. Hepcidin-25 kit was used (DRG Instruments GmbH, Germany). DRG hepcidin test is based on competition of endogenous hepcidin-25 from sample and hepcidine-25 conjugated with biotin for binding site of murine mono-

clonal antibody at pits of microplates. After incubation at room temperature, unbound conjugate is washed with streptidine-peroxidase enzyme. Enzyme substrate is added in every pit, which results in blue color after incubation period, which afterwards turns yellow. Intensity of color is inversely proportional to hepcidine-25 level using STAT FAX 2100 USA reader at wavelength 450 nm, test range: 0-80 ng/ml. (12). IL6 in serum was determined using ELISA Quantikine®ELISA, Human IL-6 Immunoassay at Clinical immunology, CCU Sarajevo.

### Statistical analysis

IBM SPSS program, 21.0 version was used to analyze data. Most parameters were significantly different from normal (Shapiro-Wilk test), with one or more extreme values. Mann Whitney test was used to determine significance in variables in between groups while Mood's Median test compared tree or more groups. Categorical variables were expressed as counts and percentages and Chi-square test and Fisher test compared these variables. Linear regression analysis analyzed relationship between variables and Pearson's correlation coefficient was calculated. Spearman correlation coefficient was used for distribution which was significantly different from normal. $p$ value < 0.05 was considered statistically significant.

## 4. RESULTS

Median age of MM patients was 60 (40-88 years) and 24 of them were over 60 years of age, 70% male. Anemia was diagnosed in 70% MM patients. Erythrocyte count in anemic group was 3.18 x10 $^{12}$/L, significantly less (p=0.002) then in non-anemic (4.36 x 10$^{12}$/L). Hemoglobin concentration was statistically significantly lower in anemic (98 g/L) to non-anemic group (139 g/L) (p 0.000). Hematocrit was significantly lower in anemic (0.28) than non-anemic (0.41) group (p=0.002). Mean corpuscular volume (MCV) was 93 fL for anemic group, 91 fL in non-anemic group. Serum iron in anemic MM patients was 14,7 µmol/L, ferritin 325 ng/mL and UIBC 26.7 µmol/L and in non-anemic group respectively 10.7 µmol/L, 245.8 ng/mL, 24.2 µmol/L, (p= 0.255; p=0.335; p=0.136). In anemic group ferritin was above range (4-240 ng/mL). Serum hepcidin in MM group was 45.5 ng/mL (1.79-104.80) and in control group 5.9 ng/mL (p=0000). In anemic patients serum hepcidin was 45.50 ng/mL (1.70-104.80) and non-anemic 35.20 ng/mL (13.40-59.70) with no significant difference (0.280). Serum IL-6 in MM patients was 3.59 pg/mL (0.22-33.96),

| | Non-anemic n=6 | Anemic n=21 | p = * |
|---|---|---|---|
| Erythrocyte (10$^{12}$/L) | 4.36 (3.60-4.99) | 3.18 (1.65-4.23) | 0.002 |
| MCV (fL) | 91 (85-99) | 93 (79-106) | 0.319 |
| Hemoglobine (g/L) | 139 (124-152) | 98 (53-130) | 0.000 |
| Hematocrit (%) | 0.41 (0.31-0.44) | 0.28 (0.15-0.38) | 0.002 |
| Fe (µmol/L) | 10.7 (6.9-17.5) | 14.7 (5.5-30.6) | 0.255 |
| UIBC (µmol/L) | 34.2 (29.2-43.3) | 26.7 (7.7-63.1) | 0.136 |
| Ferritin (ng/mL) | 245.8 (80.9-332.3) | 325.0 (65.7-1227.3) | 0.335 |
| Transferin (g/L) | 2.4 (1.8-3.6) | 2.2 (0.9-3.8) | 0.559 |
| IL-6 (pg/mL) | 2.85 (0.22-33.96) | 3.80 (0.33-29.70) | 0.923 |
| Hepcidin (ng/mL) | 35.20 (13.40-59.70) | 45.50 (1.70-104.80) | 0.280 |

Table 1. Serum hepcidin and IL-6, hematological and iron metabolism parameters in anemic and non-anemic MM patients

in anemic 3.80 pg/mL (0.33-29.70) and non-anemic 2.85 pg/mL (0.22-33.96) (0.923 ) (Table 1).

Results are showed as median values (min-max), * p<0,05 statistically significant. It was found no significant correlation of serum hepcidin and iron, UIBC and transferrin (p=0.556, p=0.834, p=0.693) respectively in entire MM group. Strong inverse correlation was found between hepcidin and hemoglobin (p=0.012) in MM patients (Figure 1).



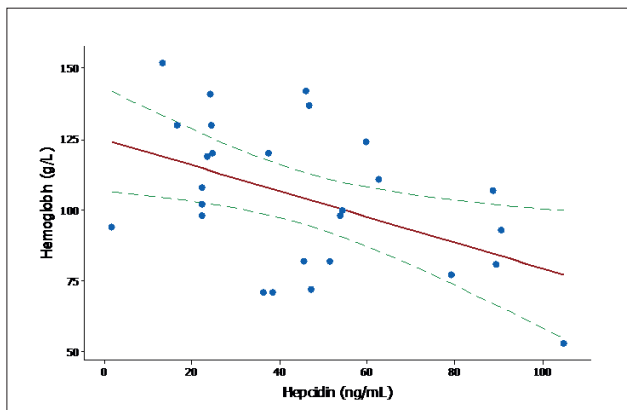Figure 1. Correlation of serum hepcidin and hemoglobin in MM patients

Regression equation, Pearson correlation coefficient (r) and *p*-value: Hemoglobin (g/L) = 125,0–0,4558 Hepcidin (ng/mL); r= -0,475, *p*=0,012. Strong inverse correlation was found between hepcidin and hematocrit (p=0.014) in entire MM patients. Serum ferritin in MM patients was above normal range. Serum hepcidin and ferritin showed strong positive correlation, marking increase of ferritin with increase of hepcidin (p=0.000) (Figure 2).
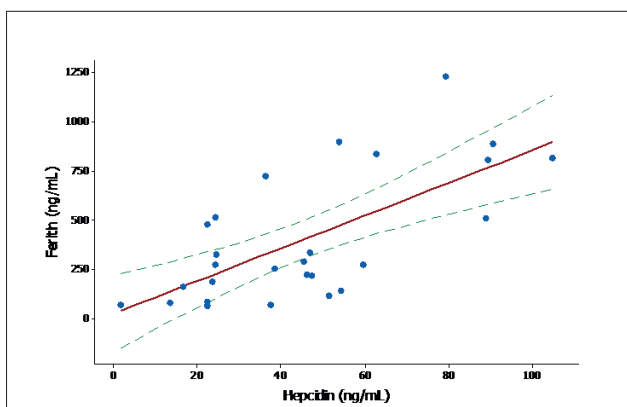


Figure 2. Relation of hepcidin and ferritin in MM patients

Regression equation, Pearson correlation coefficient (r) and *p*-value: Ferritin (ng/mL) = 23,35 + 8,305 Hepcidin (ng/mL); r=0,682, *p*=0,000. No significant correlation between serum IL-6 and hepcidin (p=0.587) was found in MM group as well as in both anemic (p=0.999) and non-anemic groups (p=0.166) MM group (Figure 3).

Regression equation, Pearson coefficient of correlation (r) i *p*-value: 0 non-anemic: hepcidin (ng/mL) = 25,37 + 0,8642 IL-6 (pg/mL); r=0,645, *p*=0,166; 1 anemic: hepcidin (ng/mL) = 46,22–0,0015 IL-6 (pg/mL); r=0,000, *p*=0,999.
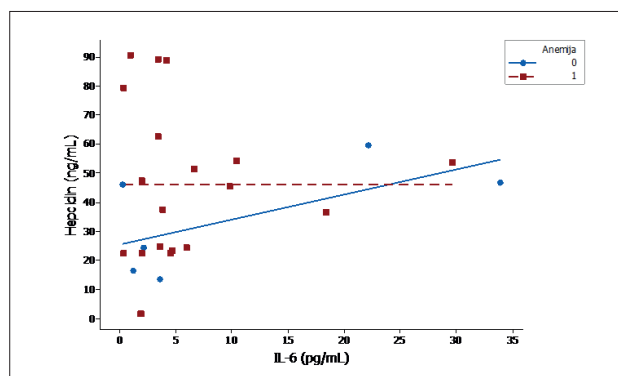


Figure 3. The correlation of hepcidin and IL-6 in anemic and non-anemic MM patients

## 5. DISCUSSION

At time of diagnosis, 70% of our patients were anemic, which confirms Birgegards (13) data, 85.3%. Age of our anemic patient was 60, which is in agreement with previously reported data (14). 70 % of our patients were male patients, similar to Haraguchti's study (15), 56% male patients, average age of 66 indicating that myeloma is disease occurring in older age mainly in men. Most of the patients in our study were in advanced stage III (51%) according to ISS, corresponding Katodritou's (14) and Haraguchi's data (15).

Anemia in our patients was normocytic (MCV 93fL). Mean Hgb 98 d/L is in accordance with data of Katodritou's (14) (Hgb 9.7 g/dL), Meas's (6) (Hgb 10 g/dL) and Sharma Hgb 10.1 g/dL (2). Iron level in anemic group was 14.7 μmol/L, ferritin 325 ng/mL and UIBC 26.7 μmol/L. These data indicated presence of normochromic normocytic anemia. Normal serum iron and elevated ferritin saturation above referral range indicates intracellular deposition of iron. These data imply anemia of chronic disease in accordance with Katodritou's data (14) serum ferritin of anemic patients 793 ng/mL (256-1997 ng/mL). Similar data was published by Maes (6).

The serum hepcidin in our MM patients, 45,5 ng/mL (1.70 ng/mL–104.80 ng/mL) was significantly higher (p= 0.000) than in control group 5.9 ng/mL (1.70 ng/mL–22.4 ng/mL). In anemic myeloma group serum hepcidin was 45.5 ng/mL (min 1.70 ng/mL–max 104.80 ng/mL), 20% higher to non anemic group, 35.2 ng/mL (13.40 ng/mL-59.70 ng/mL) without significant difference (p=0.280). In relation to control group, anemic MM patients had significantly higher value of serum hepcidin (p=0.000). Our data are supported by previous study (14) even though their serum hepcidin in anemic MM patients were higher, 236 ng/mL, (28 ng/mL-1038 ng/mL) .

Sharma's analysis of 44 anemic MM patients found significantly elevated values of urin hepcidin comparing to healthy control (p<0.0001) supporting our study (2). Strong inverse correlation of hepcidin and hemoglobin (p=0.012) and hematocrit (p=0.014) found in our study indicate that rise of hepcidin induces anemia. Katodritou's clinical study also found significant inverse correlation of hepcidin and hemoglobin (p=0.01) (14) in MM patients. Sharma's study found inverse correlation of hepcidin and stage of anemia (p=0.014) (2) in MM pa-

tients. These data support thesis that rise of hepcidin has crucial role in development of anemia in MM (16). Elevated serum hepcidin and ferritin, normal serum iron and MCV, low hemoglobin in MM patients indicate anemia of chronic disease.

In vitro research showed that hepcidin retains iron within cells, enterocytes, hepatocytes and macrophages. In clinical practice, this is registered as elevated level of serum ferritin (5, 16, 17). Our study found strong relation of hepcidin and serum ferritin in myeloma patients. This supports claims that raise of hepcidin induces elevation of serum ferritin, indicating the retention of iron within RES cells, reported by Katodritou (14) who found significant positive correlation of serum hepcidin and ferritin (p<0.009). Serum IL-6 value in myeloma patients was 3.59 pg/ml, in anemic 3.80 pg/mL and non-anemic 2,86 pg/ml without significant differences between anemic and non-anemic patients, but since this was clinical study, numerical difference of 1 unit (normal range 0-14 pg/mL) might impact on development of anemia, even though other cytokines may influence this process (9). Our study did not show correlation of IL-6 and hemoglobin as Solary's data (18). IL-6 is thought to be major mediator of hepcidin secretion, which during inflammation causes reduction of serum iron and transferrin saturation (5). It seems that IL-6-hepcidin link is important and that hepcidin is major mediator of hypoferremia in inflammation (3). Recent studies suggest that there are more cytokines which contribute to anemia in ACD (9, 10, 19). Hepcidin plays specific role in development of anemia lymphoproliferative diseases. Anemia may be result of IL-6 secretion or influenced by some other cytokines. BMP-2 may play significant role, besides IL-6, in multiple myeloma (6). BMP-2 might be specific for multiple myeloma or it has synergism with IL-6 in this setting, causing hepcidin secretion.

Development of anemia in myeloma is very complex. Besides IL-6, hyper production of hepcidin in liver is probably mediated by BMP-2 (9). Our data support this, since we found elevated serum hepcidin with normal IL-6 levels in MM patients.

There are still unanswered questions on exact role of cytokines in regulation of hepcidin production in patients with myeloma multiplex besides IL-6. Does hepcidin impact myeloma cells growth and their survival enabling influx of iron in tumor cells and stroma cells? Large prospective study may give some insight into this problem.

## 6. CONCLUSION

Anemia in myeloma patients was normocytic, normochromic and had all characteristics of ACD. High level of hepcidin probably causes anemia but it's high expression is not only due to effect of IL-6. Other mechanisms independent of IL-6 may impact this. High level of hepcidin and significant negative correlation of hepcidin, hemoglobin and hematocrit indicate significant role of hepcidin in development of anemia in myeloma patients.

Significant negative correlation between serum hepcidin and ferritin indicates that hepcidin causes influx of iron into cells where it remains bound to ferritin.

- Conflict of interest: none declared.

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