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□ ORIGINAL ARTICLE □

# Circulating Hepcidin in the Crossroads of Anemia and Inflammation Associated with Rheumatoid Arthritis

Mehmet Derya Demirag[2], Seminur Haznedaroglu[2], Banu Sancak[3], Ceyla Konca[1], Ozlem Gulbahar[3], M. Akif Ozturk[2] and Berna Goker[2]

## Abstract

**Objective** To investigate the role of circulating hepcidin, which is a homeostatic regulator of iron metabolism and a mediator of inflammation, in anemia associated with rheumatoid arthritis.

**Methods** Forty patients with rheumatoid arthritis (19 with anemia and 21 without anemia), 12 patients with iron deficiency anemia and 14 healthy adults were studied. Serum hepcidin levels were analyzed with hepcidin prohormone solid phase enzyme-linked immunosorbent assay.

**Results** The mean serum hepcidin levels were significantly higher in patients with rheumatoid arthritis with anemia compared to healthy adults and those with iron deficiency anemia. The active rheumatoid arthritis group had significantly higher mean serum hepcidin levels than the inactive rheumatoid arthritis group. In the rheumatoid arthritis group, serum hepcidin levels were positively correlated with disease activity, but inversely correlated with hemoglobin levels. The serum hepcidin level was found to be a significant predictor for hemoglobin level.

**Conclusion** Serum hepcidin levels are closely associated with disease activity in rheumatoid arthritis patients and might play a role in the pathobiology of chronic disease anemia associated with rheumatoid arthritis.

**Key words:** inflammation, anemia, hepcidin, rheumatoid arthritis, iron, red cell disorders

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## Introduction

Rheumatoid arthritis (RA) is a systemic inflammatory disease affecting mainly multiple diarthrodial joints. Numerous cytokines are involved in the pathobiology of RA. There is an imbalance in the cytokine network of RA patients. Proinflammatory cytokines such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor alpha (TNF-α) are excessively produced, however, anti-inflammatory cytokines such as IL-1Ra and IL-10 fail to respond to inflammatory status (1). Anemia of chronic disease is not infrequent during the clinical course of RA (2). Iron metabolism is impaired in chronic inflammatory diseases such as RA. Hepcidin is a liver derived small peptide hormone that functions both as a homeostatic regulator of iron metabolism and a mediator of inflammation (3-6). The aim of the present study was to assess the role of hepcidin in anemia associated with RA.

## Patients and Methods

### *Study population*

Forty RA patients, who were selected according to the ACR 1987 revised criteria (7), were included in the study. All RA patients were on one or more disease modifying antirheumatic drug (DMARD). Treatment profile is given in Table 1. RA patients were divided into two groups. Those with anemia (RA+a) (16 women, 3 men) and those without anemia (RA-a) (19 women, 2 men). Additionally, RA+a group was divided into two subgroups as RA+a with iron deficiency (ID) (10 patients, ferritin <50 mg/dL) and RA+a

[1]Department of Internal Medicine, Gazi University School of Medicine, Ankara, Turkey, [2]Section of Rheumatology, Gazi University School of Medicine, Ankara, Turkey and [3]Department of Biochemistry, Gazi University School of Medicine, Ankara, Turkey
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Correspondence to Dr. Mehmet Derya Demirag, mehmetderyademirag@yahoo.com

**Table 1. Treatment Profile in Anemic and Non-anemic RA Patients**

| n (%) | RA+a n=19 | RA-a n=21 | p |
|---|---|---|---|
| Salicylazosulfapyridine | 12 (63) | 13 (61) | 0.844 |
| Methotrexate* | 13 (68) | 14 (66) | 0.839 |
| Hydroxychloroquine | 9 (47) | 10 (48) | 0.799 |
| Leflunomide | 2 (11) | 2 (10) | 0.679 |
| Anti-TNF | 1† (5) | 1¶ (5) | 0.467 |
| 3 DMARDs | 3 (16) | 5 (24) | 0.814 |
| 2 DMARDs | 11 (58) | 8 (37) | 0.343 |
| 1 DMARD | 4 (21) | 7 (33) | 0.620 |

Anti-TNF= anti tumor necrosis factor alpha ( †Infliximab and ¶Etanercept).
* All patients who were on methotrexate have been simultaneously supported with folic acid (5-7.5 mg / week).

with non-ID (9 patients, ferritin >50 mg/dL). Disease activity score 28 joints-CRP [DAS28 (CRP)] was used for the assesment of disease activity in RA patients and DAS28 > 3.2 was considered as active disease (8). Healthy adults (HA) (11 women, 3 men) and patients with iron deficiency anemia (IDA) (11 women, 1 men) served as control groups. Hemoglobin (Hb) levels of less than 12 g/dL for females and 13 g/dL for males were taken as anemia according to the World Health Organizations (WHO) criteria in all groups (9). Patients with other diseases which may result in anemia, such as cancer, other inflammatory conditions, and folic acid or vitamin B12 deficiency were excluded in all groups. The Local Ethical Committee of Gazi University Medical Faculty approved the study protocol. Written informed consent was obtained from all study subjects.

### *Study methods*

Venous blood samples were collected from all subjects and stored at -20℃ until assayed. Serum hepcidin levels were analyzed by hepcidin prohormone solid phase enzyme-linked immunosorbent assay (IBL, Immuno-Biological Laboratories, Minneapolis). The sensitivity of the assay was 3.95 ng/mL and the intra-assay precision variation (% CV) was 4.96. The analytic recovery of hepcidin was 93.1 percent (10).

### *Statistical analysis*

The data were expressed as mean±standard deviation and median [mean±SD (median)]. Quantitative data between groups were compared using Kruskal Wallis test and if p value was less than 0.05, Mann-Whitney U test was used on each pair of groups and the p value was adjusted with Bonferroni. Therefore, a p value of less than 0.0083 was considered as statistically significant. RA subgroups were compared using Mann-Whitney U test only. The correlation of serum hepcidin levels with hemoglobin (Hb), ferritin, serum iron (SI) levels, serum iron binding capacity (SIBC), C-reactive protein (CRP) levels and DAS28 (CRP) scores were tested with Spearman correlation and partial correlation analysis. Qualitative data were tested with corrected Yates' Chi-square test. Multiple regression analyses were used for predictors and factors which were associated with Hb level. A p value <0.05 was considered as statistically significant.

## Results

Baseline characteristics of the study groups are given in Table 2. Both RA+a and RA-a groups were significantly older than IDA. IDA and RA+a groups had significantly lower Hb levels, mean corpuscular volume (MCV) and SI levels than groups of RA-a and HA. Erythrocyte sedimentation rate (ESR) was significantly higher in RA+a group when compared to groups of RA-a, IDA and HA. RA+a group had higher CRP levels when compared to RA-a group. However, this difference did not reach a statistically significant level. SIBC was significantly higher in IDA group when compared to the other groups. IDA group had significantly lower ferritin levels than RA-a group and HA.

RA+a group had significantly higher serum hepcidin levels compared to IDA group and HA (Table 2). Although serum hepcidin levels did not differ between RA+a and RA-a groups, RA+a with non-ID subgroup had significantly higher serum hepcidin levels when compared to RA-a group and HA [619±146 (598), 401±102 (391) and 341±33 (344); p values were 0.006 and 0.001, respectively]. In addition, serum hepcidin levels did not differ between RA+a with ID subgroup and IDA group [429±112 (440) and 368±53 (375), respectively, p=0.090].

The hemoglobin levels and the frequencies of the patients with anemia were similar between active and inactive RA groups. Active RA group had significantly higher serum hepcidin levels when compared to inactive RA group (Table 3).

RA+a group had higher DAS28 (CRP) scores than RA-a group, however this difference did not reach to a statistically significant level [3.3±1.1 (3.1) and 2.8±1.3 (2.6), respec-

**Table 2. Baseline Characteristics of the Study Subjects**

| Mean±SD(median) | RA+a | RA-a | IDA | HA | Overall p |
|---|---|---|---|---|---|
| **Age (years)** | 53±15(52) | 50±12(54) | 33±12(32) | 39±11(40) | <0.0001 |
| **Disease duration (years)** | 9±10(6) | 8±9(4) | - | - | 0.46* |
| **Hb (g/dL)** | 11.1±1(11.2) | 13.7±1(13.6) | 11.3±0.5(11.6) | 14.3±1.1(14.4) | <0.0001 |
| **MCV(FL)** | 81±7(81) | 88±7(88) | 75±6(77) | 87±4(86) | <0.0001 |
| **CRP (mg/dL)** | 23.1±20 (15) | 12.4±17.4 (4) | negative | negative | 0.022* |
| **ESR(mm/hour)** | 38±22(35) | 19±14(14) | 11±7(10) | 11±8(11) | <0.0001 |
| **SI (μg/dL)** | 31±26(21) | 73±53(70) | 22±12(17) | 79±37(73) | <0.0001 |
| **SIBC (μg/dL)** | 323±89(337) | 322±38(330) | 418±46(437) | 338±39(334) | <0.0001 |
| **Ferritin (ng/mL)** | 51±69(6) | 42±34(36) | 5±4(4) | 47±40(37) | 0.001 |
| **Hepcidin (ng/mL)** | 475±133(445) | 401±102(391) | 368±53(375) | 341±33(344) | 0.001 |

Overall p values are given for analysis between all groups (Kruskal-Wallis test) except for disease duration and CRP (*Mann-Whitney U test p value). **RA+a vs RA-a**; p<0.001 for Hb, 0.003 for MCV and ESR, 0.006 for SI. **RA+a vs IDA**; p=0.001 for age, <0.001 for ESR, 0.006 for SIBC and 0.005 for hepcidin. **RA+a vs HA**; p<0.001 for Hb, ESR, and hepcidin, 0.001n for SI and 0.005 for MCV.**RA-a vs IDA**; p<0.001 for Hb, MCV, SI, SIBC and ferritin, 0.001 for age. **IDA vs HA**; p<0.001 for Hb, MCV, SI and ferritin, 0.001 for SIBC. (Mann-Whitney U test was used for paired comparisons and a p value < 0.0083 was considered as statistically significant)

**Table 3. Frequencies of Anemia, Levels of Hemoglobin and Serum Hepcidin in Active and Inactive RA Patients**

| | Active RA (n=17) | Inactive RA (n=23) | p |
|---|---|---|---|
| Frequencies of anemia (%) | 58 % | 40 % | 0.40 |
| Hgb (g/dL) | 12±1.6 (11.5) | 12.4±1.6 (12.8) | 0.24 |
| Hepcidin (ng/mL) | 486±101 (449) | 393±104 (353) | 0.02 |

tively, p=0.222]. Furthermore, DAS28 (CRP) scores did not differ between RA+a subgroups (with ID and non-ID) and RA-a group [3.03±1.3 (2.9), 3.9±0.9 (4.1) and 2.9±1.3 (2.6), p values were 0.498 and 0.189, respectively].

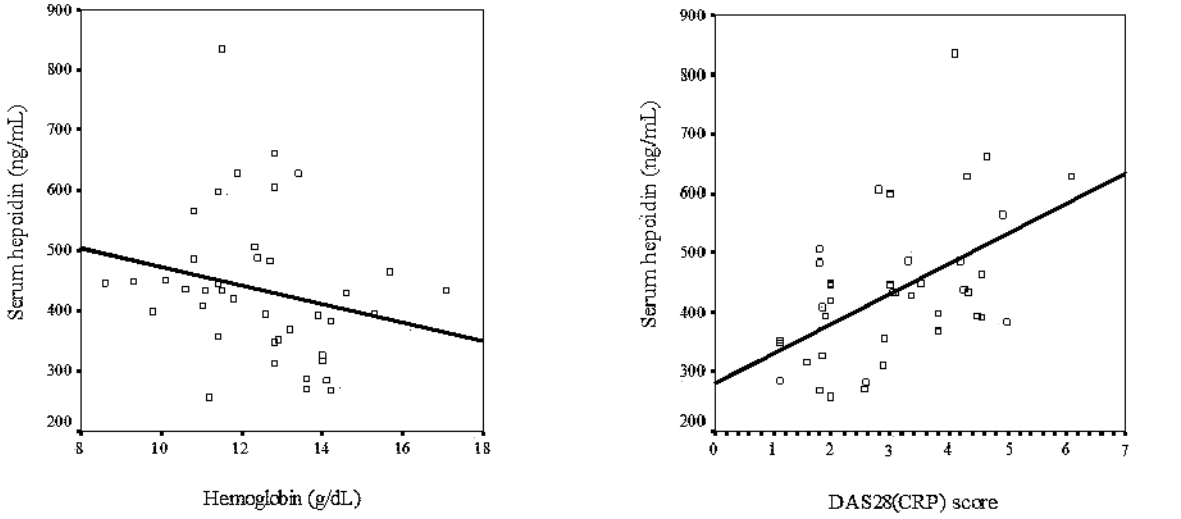
In RA patients, serum hepcidin levels were positively correlated with CRP levels and DAS28 (CRP) scores, but inversely correlated with Hb levels (Fig. 1). However, in the partial correlation analysis, the significant correlation between levels of serum hepcidin and CRP disappeared when this association was controlled by DAS28 (CRP) score (r= 0.10 and p=0.545). On the other hand, the significant correlation between serum hepcidin levels and DAS28 (CRP) scores remained statistically significant when this association was controlled by CRP (r=0.428 and p=0.007). In addition, there was no significant correlation between DAS28 (CRP) scores and Hb levels or between levels of CRP and Hb (r=-0.058, p=0.721 and r=-0.239 and p=0.137, respectively). Serum hepcidin levels did not correlate with serum ferritin, SI levels and SIBC in RA patients (all p values were >0.05). In addition, there were no significant correlations between serum hepcidin levels and other parameters in patients with IDA group and HA (all p values were >0.05).

In RA patients, multiple regression analysis showed that levels of hepcidin and SI were significant predictors for Hb level. In those relationships, serum hepcidin levels were negatively and, SI levels were positively associated with Hb levels. In those analyses, DAS28 (CRP) scores were not associated with Hb levels (Table 4).

## Discussion

Hepcidin, a small, cystein-rich peptide (11, 12), can affect both inflammation and red blood cell kinetics in health and disease. In this study, the highest circulating hepcidin levels were observed in RA patients with anemia and the lowest levels in healthy adults. In addition, patients with RA without anemia had higher circulating hepcidin levels compared to those with IDA. While the differences between groups of RA+a and IDA as well as groups of RA+a and HA were statistically significant, there were no statistically significant differences between the other groups.

The effects of inflammation, particularly the role of cytokines, on hepatocyte hepcidin mRNA expression has been previously investigated. Nemeth et al (3) demonstrated that IL-6 and lipopolysaccharide induced hepcidin mRNA expression in human hepatocytes, but IL-1 and TNF-α had no




A. Serum hepcidin levels were inversely correlated with Hb levels ( r = - 0.364 and p = 0.021)

B. Serum hepcidin levels were positively correlated with DAS28(CRP) scores ( r = 0.477 and p = 0.002)

**Figure 1.** **The correlations between circulating hepcidin and other parameters in the RA group. A. Serum hepcidin levels were inversely correlated with Hb levels (r=-0.364 and p=0.021). B. Serum hepcidin levels were positively correlated with DAS28 (CRP) scores (r=0.477 and p=0.002).**

**Table 4.** **Predictors for Hemoglobin Levels in RA Patients**

| | β coefficient | T value | p |
|---|---|---|---|
| **DAS28(CRP) score** | 0.294 | 1.519 | 0.142 |
| **Ferritin** | 0.203 | 1.202 | 0.242 |
| **Serum Iron** | 0.459 | 2.730 | 0.012 |
| **Hepcidin** | -0.631 | -3.203 | 0.004 |

such effect. Prolonged exposure (after 24 hours) to IL-1α also induced hepcidin mRNA expression. However, the investigators speculated that this effect was an indirect stimulus and was probably associated with stimulation of IL-6 induced by IL-1α. In another study, Lee at al (13) showed that hepcidin transcription was stimulated not only by IL-6 but also by IL-1α and IL-1β in murine hepatocytes. In addition, this study demonstrated that indirect stimulation of IL-6 by IL-1 was not the case. Because, IL-$6^{-/-}$ cells could not have produced IL-6 and Lee et al demonstrated that both form of IL-1 strongly stimulated hepcidin expression in IL-$6^{-/-}$ cells (13). IFN-β inhibited hepcidin transcription in both studies (3, 13). All of these data suggest that hepcidin plays an important role in inflammatory conditions. The role of hepcidin particularly comes into prominence in anemia of inflammation. In our study, we demonstrated that RA+a patients had higher serum hepcidin levels than in HA and IDA group. These findings are compatible with results of Nemeth et al (3). Nemeth et al evaluated urinary hepcidin excretion in their subjects. IL-1 and IL-6, which induce hepcidin production, also have an important role in pathogenesis of RA. RA patients have higher plasma levels of IL-1β and IL-6 than controls (14, 15).

In the present study, we found that CRP levels and DAS 28 (CRP) scores did not differ between groups of RA+a and RA-a. Subgroups of RA+a (with ID and non-ID) had similar DAS28 (CRP) scores when compared to RA-a group. Although, serum hepcidin levels were not statistically different between RA+a and RA-a groups, RA+a with non-ID subgroup had significantly higher serum hepcidin levels when compared to RA-a group, while RA+a with ID did not. In addition, serum hepcidin levels were not different between RA+a with ID subgroup and IDA group. All of these data demonstrated that serum hepcidin might play an important role in anemia associated with RA even if the groups had similar disease activity. At first sight, this result might be interpreted that the increase in hepcidin levels is independent of disease activity. However, further analysis demonstrated that hepcidin levels were positively correlated with disease activity and negatively correlated with Hb levels, while neither CRP levels nor DAS28 (CRP) scores was correlated with Hb levels. In addition to these results, active RA patients had significantly higher hepcidin levels when compared to inactive RA patients although Hb levels and frequencies of anemia were similar. These results suggest that disease activity is not an adequate marker for prediction of

anemia. This hypothesis was supported by multiple regression analysis performed with all RA patients showing that the hepcidin level was a significant predictor for Hb level while DAS28 (CRP) score was not.

In this study, IDA group had significantly higher SIBC than other groups. IDA group also had significantly lower serum ferritin levels than RA-a group and HA. In addition, IDA group had significantly lower MCV and SI levels than RA-a group and HA. Pure iron deficiency anemia is hypochromic-microcytic in character and, in this type of anemia, SI and ferritin levels are low, and SIBC is high (16). Classically, chronic disease anemia is associated with low SI and SIBC and high or normal serum ferritin levels (17). In the present RA patients, SI status was consistent with this classical data, however SIBC was not significantly different when compared to HA. Iron dynamics during the complicated course of RA seems to be chaotic. Chronic disease anemia may not only be normochromic-normocytic it may also have hypochromic-microcytic or normocytic features (18, 19). Our RA+a group had significantly lower SI levels and MCV compared to RA-a group and HA. However, in our study, only the IDA group showed microcytic features. Vreugdenhil et al have shown that the anemia was normochromic-normocytic in 60% and hypochromic-normocytic in 30% of those with chronic disease anemia (19). Differentiation between iron deficiency and chronic disease anemia can sometimes be difficult, especially when they both coexist. Iron deficiency is not infrequent in anemic RA patients. Vreugdenhil et al (19, 20) have also demonstrated that frequency of iron deficiency detected by stainable bone marrow were over 50% and 52% in anemic RA patients. Indeed, our RA+a patients had very low mean serum ferritin levels. Because of their heterogenous iron status, the anemic RA group was divided into two subgroups. Our results demonstrated that RA+a without ID subgroup had significantly higher serum hepcidin levels when compared to RA-a group and HA. Furthermore, serum hepcidin levels did not differ between RA+a with ID subgroup and IDA group. These data suggest that hepcidin is an important pathogenetic marker in pathobiology of anemia in RA patients without iron deficiency. On the other hand, it is known that hepcidin is an important cause of hypoferremia in anemia of inflammation (4, 5, 21, 22). In our study, in addition to hepcidin, we found that the serum iron level was a significant predictor for Hb level in all RA patients. In humans, experimental hepcidin increase, induced by IL-6 infusion, is accompanied by a decrease in SI levels (21). In addition, serum hepcidin levels are rapidly down-regulated by IL-6 blockage (22). Hepcidin also inhibits iron absorbtion from the intestine in mice, contributing to hypoferremia (4, 5). The other two pathogenetic mechanisms of hepcidin in chronic disease anemia are that hepcidin inhibits iron mobilization from macrophages in the reticuloendothelial system (6) and could inhibit erythroid colony formation in the bone marrow (23).

It should not be forgotten that chronic disease anemia is a complex phenomenon and hepcidin is not the single molecule playing part in this condition. Numerous cytokines, particularly TNF-α, which does not induce hepcidin m-RNA (3), can play an important role as well (3, 24-26) and the coexistance of iron deficiency may further complicate the process. In conclusion, our findings suggest that hepcidin is strongly associated with disease activity in RA patients and might play a significant role in the pathobiology of chronic disease anemia associated with RA. Future studies to determine serum levels of hepcidin at different time points during the clinical course of RA patients, preferably in the active and the remission periods of the disease after effective therapies will be needed to confirm our results. Moreover, inflammatory cytokines which can induce hepcidin synthesis, such as IL-6, and serum erythropoietin levels should also be investigated concurrently to determine the exact mechanisms underlying the contribution of hepcidin in the crossroad of anemia and inflammation associated with RA.

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