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Hepcidin, Ferroportin and Hephaestin Levels in Inflammatory Bowel Disease; Are they Key Mediators of Anemia or Inflammation?

Guleryuzlu Y[1], Yilmaz N[1*], Yilmaz M[1], Demir S[1], Tosun K[2], Yilmaz UE[3], Dag I[4] and Dolapcioglu C[5]

[1]Department of Gastroenterology, Research and Training Hospital, Turkey

[2]Department of Gastroenterology, Siena College, Loudonville, NY

[3]Department of Underwater Medicine and Hyperbaric Medicine, Istanbul University, Çapa School of Medicine, Turkey

[4]Department of Biochemistry, Eyup State Hospital, Eyup, Istanbul, Turkey

[5]Department of Gastroenterology, Maltepe University School of Medicine, Maltepe, Istanbul, Turkey

***Corresponding author:**

Nevin Yilmaz,
Barisyolu Cad. Agaoglu My Country –K3B5,
Cekmekoy, Istanbul, 34782, Turkey,
E-mail: nevinylmz@yahoo.com

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Abbreviations:

ACD: Anemia of chronic disease; B12: Vitamin B12; BMI: Body mass index; CD: Crohn's disease; CDAI: Crohn's disease activity index; CRP; Serum C-reactive protein; CV: Coefficient of variation; ELISA: Enzyme linked immunosorbent assay; ESR: Erythrocyte sedimentation rate; Fpn: Ferroportin; Hb: Hemoglobin; Hepc: Hepcidin; Heph: Hephaestin; IBD: Inflammatory bowel disease; ID: Iron deficiency; IDA: Iron deficiency anemia; IRP: Iron regulatory protein; MCV: Mean corpuscular volume; PLT: Platelet cell count; RDW: Red blood cell distribution width; RI: Reference interval; SD: Standard deviation; TIBC: Total iron binding capacity; TNF: Tumor necrosis factor; TWS: Truelove-Witts' score; UC: Ulcerative colitis; WBC: White blood cell count

1. Abstract

1.1. Background: Anemia represents a significant problem in Inflammatory Bowel Disease (IBD) and diagnosis may be challenging due to inflammation. Hepcidin, ferroportin and hephaestin are novel Iron Regulatory Proteins (IRPs) involved in iron metabolism. This study aimed to identify the interactions of these proteins in IBD patients, guiding safe iron supplementation and effective treatment.

1.2. Methods: This cross-sectional study included 100 IBD patients (42 Crohn's disease, 58 ulcerative colitis) and 44 healthy controls. Blood samples were drawn from all subjects to assessment of IRPs, iron-anemia workup and the other parameters. The IRPs were analyzed using ELISA method. The patients were classified according to anemia and disease activity.

Software R statistical packages were used.

1.3. Results: Anemia was detected in 48% of patients and 79% of anemics had active diseases. All IRP levels were found significantly higher in IBDs compared to controls, regardless of anemia or disease activity ($p<0.001$, for all pair wise comparisons). In contrast to hemoglobin which was negatively correlated ($r=-0.30$, $p=0.003$), ferritin and hepcidin levels were found positively correlated with disease activity ($p<0.001$ vs $p=0.038$). The increases in serum ferroportin and hephaestin are likely to be associated with anemia due to lack of correlation between the diseases activity ($p=0.213$ vs $p=0.146$).

1.4. Conclusion: It was unlikely that hepcidin likewise ferritin were not affected by inflammation in the anemic settings. Further studies are warranted to better delineate the key role of these markers especially their bioactive forms, in iron metabolism and inflammation in IBD.

2. Introduction

Anemia represents a significant problem in patients with inflammatory bowel disease (IBD) with a reported prevalence of 15% to 75% [1]. The most common cause of anemia in IBD is iron deficiency

(ID) followed by the anemia of chronic disease (ACD) [2]. Several laboratory indices and markers such as ferritin, transferrin, transferrin saturation as well as soluble transferrin receptors can be utilized for the differential diagnosis of anemia in these patients [2]. However, the inflammation could complicate the diagnosis due to the overlap of iron-deficiency anemia (IDA) and ACD [2,3]. The possibility of these factors being affected by ongoing inflammation is also an important issue for guiding safe iron supplementation.

We have recently witnessed the discovery of novel iron regulatory proteins (IRPs) involved in iron metabolism such as hepcidin (Hepc), ferroportin (Fpn) and hephaestin (Heph). Among these, Hepc, the key regulator of systemic iron balance, is a circulatory peptide hormone mainly synthesized by the liver that coordinates the utilization and storage of iron and that prevents the release of iron into the plasma [4-7]. On the other hand, Fpn is responsible for the cellular release of iron into the plasma and allows the loading of iron to plasma transferrin [8,9]. Furthermore, Fpn is the receptor for Hepc, the binding of which leads to the inhibition of transfer of iron into plasma [10,11]. Fpn levels are under the strict control of Hepc to prevent iron overload due to excessive iron absorption [11]. Heph is a protein involved in the metabolism of iron, and probably of copper [12]. It is a copper-bound transmembrane ferroxidase responsible for the transport of dietary iron from intestinal enterocytes to the systemic circulation [12,13].

Studies examining the role of Hepc in anemia associated with IBD have been scarce in number and provided controversial results [14-18]. Until now, no studies have examined the role of Fpn and Heph in iron regulation in patients with IBD in the clinical settings. In the study, we aimed to determine the role of serum Hepc, Fpn and Heph in guiding safe iron supplementation and anemia treatment in IBD patients.

3. Patients and Methods

This cross-sectional study included a total of 144 subjects; 100 IBD patients (42 Crohn's disease (CD), 58 ulcerative colitis (UC)) and 44 age-sex matched healthy controls followed from September 2014 - February 2015 in the Gastroenterology Clinic. Diagnosis of IBD was established based on clinical, endoscopic and pathological criteria. The control group was composed of those who presented to the clinic for dyspepsia complaints without any other illnesses. Exclusion criteria were as follows: history of blood transfusion or donation, iron or vitamin intake within the last three months, presence of chronic diseases, malignancy, or any type of hematological diseases and alcohol consumption. Study protocol was approved by local ethics committee (No.2014/05). Informed consent was obtained from all participants included in the study.

3.1. Assessments

Blood samples were drawn from all subjects for study assessments including IRPs, Ironanemia work-up, basic metabolic panel and acute phase reactants. Crohn's disease activity index (CDAI) and Truelove-Witts' score (TWS) were used for the assessment of disease activity in CD and UC group, respectively [19-21]. The CDAI scores can range from 0 to ~600 and scores of < 150 has been labeled as remission, 150–219 as mildly active and scores of 220–450 as moderately active disease [19]. Based on TWS criteria UC rated as 'severe', in whom there are 6 or more stools with blood and 1 or more of hemoglobin (Hb) <10.5 g/dl, ESR >30 mm/h or CRP > 30 mg/L, fever >37.8℃, or tachycardia >90/min, as 'remission' when a stool frequency ≤ 3/day with no bleeding and all criteria are satisfied [20,21]. In the study we defined only active or remission status, severity levels were not assessed.

Enteric infections were also excluded in patients with active disease using stool amoeba antigen test, clostridium difficile toxin and stool culture, where appropriate. Anemia was defined as a hemoglobin level <13 g/dl for males and <12 g/dl for females.

3.2. Hepcidin, ferroportin and hephaestin measurements

For the measurements of these three parameters, 10 ml of venous blood was drawn from the subjects and immediately sent to the laboratory. Sera were separated through centrifugation at 3500 rpm for 10 minutes and divided into three Eppendorf tubes. Samples were kept at -80 °C until analysis. After collection of the study samples, all were analyzed for IRP levels in a single session at the biochemistry laboratory using micro- enzyme linked immunosorbent assay (ELISA) method. All serum samples were analyzed after confirmation of the absence of hemolysis according to serum index and serum potassium levels. ELISA kits for human Hepc, Fpn (SLC40A1) and Heph (Cusabio,China) were used. Following the preparation of standards and chemicals, standard and samples were placed into the wells on the plate. Then samples were allowed to change color depending on Hepc, Ferp and Heph concentrations. Following the full development of color, absorbance values of the wells were recorded using Kayto RT - 2100c Microplate ELISA reader at 450 nanometers. Concentrations were calculated using absorbance values. Values were reported in ng/mL for Hepc and Heph, and in pg/mL for Fpn. Sensitivity or lower detection limit or in other words the lowest protein concentration that could be differentiated from zero were 6.25 ng/mL, 5.8 pg/mL and 0.078 ng/mL for Hepc, Fpn and Heph, respectively. The inter (precision between assays) and intra (precision within an assay) variation coefficients (CV) of these parameters were; for Hepc CV <15% (inter and intra were the same), for Fpn <10% and% <CV% 8 and for Heph was <10% and% <CV% 8.

3.3. Hematologic, Acute Phase Reactants and Metabolic Panel Assessments

Complete blood count with differentials was measured by Siemens ADVIA 2120i hematology analyzer. Erythrocyte sedimentation rate (ESR) results were obtained by classical Westergren method. Serum albumin (Bromcresol Purple (BCP) method, reference interval (RI):3,5-5,2g/dl) and serum C-reactive protein (CRP) (Immunoturbidimetric method, RI: <0,5 mg/dl), were measured by Cobas C702 analyzer and serum iron (Ferrozine method, (RI: 33193 µg/dl) and total iron binding capacity (TIBC) (RI:125-392 µg/dl) were measured

by Cobas C502 analyzer; serum ferritin (RI:13-400 ng/ml), serum, vitamin B12 (B12) (RI: 197-771 pg/ml), and folate (RI:4,6-18,7 ng/ml) were measured by electrochemiluminescence method using Cobas E602 analyzer and transferrin saturation (range; 30-40%) was calculated. Normal reference range of red blood cell distribution width (RDW) used as 11.5-14.5%.

3.4. Statistical Analysis

ANOVA and Welch t-test were used to assess a difference between patients and controls in terms of their age, body mass index (BMI), disease duration, Hb, RDW and IRP levels.

Summary statistics of the compared values were presented as mean ± standard deviation (SD). Results were calculated separately based on anemia and disease activity. To determine whether there is any significant difference between the groups Chi-square and Fisher's Exact tests were used. Correlations between CRP and the other parameters were calculated using Pearson correlation coefficient. In order to obtain a better linear relationship, log transformation was applied on CRP values. A p-value of < 0.05 was considered statistically significant. The analyses were carried out with software R. statistical packages.

4. Results

4.1. Demographic and Clinical Characteristics

Table 1 shows the demographic and clinical characteristics of the groups along with their comparisons. There were 144 cases in the study. Forty-two (29%) were diagnosed with CD and 58 (40%) were diagnosed with UC. There were no differences (p =0.715) between the age of CD's (39.5±10.1, range 20-64 yr), UC patients (40.1±12.3, range 20-67 yr) and controls (38.1±15.1, range 19-66 yr).

Sixty (42%) of the cases were female. Fourteen (50%) patients with CD, 27 (47%) patients with UC and 19 (43%) people in the control group were female. There were no differences between the groups according to sex (p=0.404) and BMI (p =0.377).

Eighteen (%43) CD patients, 5 (9%) UC patients and 9 (21%) people in the control group were smokers. There was an association between smoking status and groups (p<0.001). There were more smokers in the CD group than others.

Twenty (48%) of the CD patients and 28 (%48) of the UC were anemic. There was no association between anemia status and disease type (p=1.000). The mean disease duration of the patients was similar; for CD; 5.4±4.8 years, range 0-20 yr and for UC patients; 4.5±3.6 years, range 0-14 yr. (p=0.296).

Crohn's patients underwent surgery more than UC patients (26% vs 2 %, p<0.001). Among CD patients, small bowel was the most common site of involvement (n=21, 50%), followed by ileocolic (n=18, 42.8%) and colonic (n=3, 7.0%). Three of the small bowel cases also have the upper system involvement. Majority of UC patients had extensive involvement (n=31, 53 %), followed by left sided disease (n=26, 44.8%) and proctitis (n=1, 1.7%).

The twenty-three (55%) CD and twenty-six (45%) UC patients were reported as in active status according to the scoring system and the ratios were similar between the groups (p=0.437). We redefined disease activity based on the CRP levels; the disease was considered active if the CRP level was ≥0.5. There were a mild differences the disease activity defined by CRP; according to results 28 (67%) of Crohn's and 24 (41%) of UC patients were in active status. This was statistically higher than UC patients (p=0.022).

The treatments of steroid and anti- tumor necrosis factor (TNF) were also significantly higher in the CD group; p=0.005 vs. 0.029, respectively. (Table 1).

Table 1: Comparison of the study groups with regards to demographic and clinical characteristics

	Controls (n=44)	Crohn's Disease (n=42)	Ulcerative Colitis (n=58)	p-value
Age, year	38.1±15.1	39.5±10.1	40.1±12.3	0.715
Sex, F/M	19/25	14/28	27/31	0.404
BMI, kg/m^2	24.5±3.9	23.5±4.6	24.6±3.9	0.377
Smoking, n (%)	9 (21%)	18 (43%)*	5 (9%)	<0.001
Anemia, n (%)	-	20 (48%)	28 (48%)	1
Disease duration, year	-	5.4±4.8	4.5±3.6	0.296
Surgery for IBD, n (%)	-	11 (26%)*	1 (2%)	<0.001
Active IBD**, n (%)	-	23 (55%)	26 (45%)	0.437
Activity IBD by CRP, n (%)	-	28 (67%)*	24 (41%)	0.022
Medication for IBD, n (%)				
Salazopyrin	-	2 (5%)	1 (2%)	0.776
5-aminosalicylic acid	-	20 (48%)	44 (76%)*	0.007
Oral steroid	-	19 (45%)*	6 (10%)	0.005
Azathioprine	-	9 (21%)	10 (17%)	0.788
Anti-TNF Metronidazole	-	4 (10%)*	0	0.029
	-	3 (7%)	0	0.072

Abbreviations: BMI; Body mass index, IBD; Inflammatory bowel disease, CRP; C-reactive protein, TNF; Tumor necrosis factor.

Age, BMI, and disease duration values are expressed as mean ± standard deviation.

* Statistically significant ($p < 0.05$, Fisher's Exact Test).

**According to scoring system.

4.2. Comparison of the Study Parameters

Table 2 and subtables show the comparison of study parameters across the groups; 2a presented comparison of the parameters in regards to anemia and inflammation, 2b was for comparison of the IRPs.

Between the groups mean corpuscular volume (MCV), white blood cell count (WBC), platelet cell count (PLT), CRP, ESR, albumin, iron, transferrin saturation and folic acid levels were found to be significantly different. ($p<0.001$ for all pair wise comparison). Except for folic acid, these differences were caused by controls, while the folic acid level was lower in the CD than in the UC and controls. In terms of TIBC and B12, no significant difference was found between the groups. (Table 2a).

As seen in Table 2b, the mean Hb and ferritin levels were found significantly lower in IBD groups than in controls. On the other hand, the mean RDW, Hepc (range 25.9-123.6 for CD and, 56-136 ng/ml for UC pts), Fpn (range 213-267 for CD and, 211-271 pg/ml for UC pts.) and Heph (range 0.371-521 ng/ml for CD and, 0.174-0.504 ng/ml UC pts) levels were significantly higher in the IBD patients than controls (Table 2b). Although there was a significant difference compared to the controls, only CRP, ESR, transferrin saturation, Hb and RDW values were out of the reference limits in the patient groups. (Table 2a-b). Likely, Hepc, Fpn and Heph levels were increased nearly to 4.5, 1.5 and 2.2 times respectively than the controls, but in contrast to the controls all three proteins remain in normal limits. (Table 2b). In the controls; Hepc (mean 10.5, range 4.9-18.3 ng/ml) and Heph (mean 0.18, range 0.11-0.229 ng/ml) levels were below the reference values except Fpn (mean 167, range 106-262 pg /ml) levels.

Table 2a: Comparison of the groups with regards to anemia and inflammatory parameters

	Contro (n=44)	Crohn's Disease (n=42)	Ulcerative Colitis (n=58)	p-value
MCV, fL	88.4±5.7*	82.2±8.3	82.6±9.3	<0.001
WBC, $x10^9$/L	6.6±1.6*	9.2±3.6	8.2±3.2	<0.001
Thrombocytes, $x10^9$/L	233±53*	381±145	346±136	<0.001
CRP, mg/L	0.2±0.2*	2.6±4.0	1.7±3.0	<0.001
ESR, mm/h	11.5±8.6*	32.9±23.0	28.1±26.1	<0.001
Albumin, g/dL	4.6±0.3*	4.0±0.7	4.2±0.5	<0.001
Serum iron, µg/dL	113.5±42.6*	44.4±29.5	51.8±34.9	<0.001
Tot.iron binding capacity,µg/dL	325±38	324±67	345±68	0.14
Transferrin saturation, %	36.4±14.0*	13.9±8.9	15.4±10.4	<0.001
Vitamin B12, ng/L	363±122	395±336	425±287	0.502
Folic acid, nmol/L	9.6±3.2	7.5±2.8*	10.5±2.8	<0.001

Abbreviations: MCV; Mean corpuscular volume, WBC; White blood cells, ESR; Erythrocyte sedimentation rate.
Data presented as mean ± standard deviation.
*Statistically significant ($p < 0.05$, Welch t-test).

Table 2b: Comparison of Crohn's and UC patients with controls in terms of hemoglobin, RDW, ferritin, hepcidin, ferroportion and hephaestin

	Controls	Crohn's Disease	Ulcerative Colitis	p-value CD vs Cont	p-value UC vs Cont
Hemoglobin, g/dL	14.9 ±1.4	12.7± 2.6*	12.4± 2.5 +	<0.001	<0.001
RDW, %	13.5±1.0	15.7±2.4*	15.9±2.5 +	<0.001	<0.001
Ferritin, ng/mL	84.0±73.2	54.3±51.7*	44.1±61.2 +	0.032	0.005
Hepcidin, ng/mL	10.5±2.8	49.5±23.5*	41.2±22.7 +	<0.001	<0.001
Ferroportin, pg/mL	167.6±31.1	246.3±14.9*	246.8±15.9 +	<0.001	<0.001
Hephaestin, ng/mL	0.18±0.03	0.41±0.04*	0.39±0.04 +	<0.001	<0.001

Abbreviations: RDW; Red blood cell distribution width, CD; Crohn's dısease, UC; Ulcerative Colitis.
Detection ranges: Hepcidin; 12.5 ng/ml -400ng/ ml., Ferroportin; 23.5 pg/ml-1500 pg/ml and Hephaestin; 0.312 ng/ml-29 ng/ ml.
Summary statistics expressed as mean ± standard deviation.
*Values of CD patients are significantly different than controls ($p < 0.05$, Welch t-test).
+Values of UC patients are significantly different than controls ($p < 0.05$, Welch t-test).

4.3. Subgroup Analysis According to Anemia and Disease Activity

Based on the previous results, subgroup evaluations were conducted to investigate why these iron binding proteins were elevated in the patient groups, whether this was due to iron deficiency or ongoing inflammation. Table 3 and subtables show comparisons of the IRP levels according to anemia (3a) and disease activity (3b-1.3b-2). Ferritin was used as a marker for both anemia and inflammatory response in the study.

4.4. Comparisons in Anemic/Nonanemic Groups

In the study, 47.8% (20/42) of Crohn's and 48% (28/58) of patients with UC were found to be anemic. The mean Hb, RDW, Hepc, Fpn and Heph levels of the anemic patients with CD were significantly different than the controls. There was no statistically significant difference between the anemic patients with CD and controls in terms of ferritin. The results for nonanemic Crohn's patients were similar except the Hb and ferritin levels. (Table 3a). There were significant differences between the anemic UC patients and controls in terms of all six parameters. (Table 3a). The results for nonanemic UC patients were similar except the Hb levels.

4.5. Comparisons in Active/Inactive Groups, according to Scoring System

There were significant differences between the active CD patients and controls in terms of all the parameters except ferritin levels. The same result applies for the active UC patients. There was a significant difference between the inactive CD patients and controls in terms of all six parameters. The same result applies for inactive UC patients. (Table 3b-1).

4.6. Comparisons in Active/Inactive Groups, According to CRP Levels

The disease is considered active if the CRP level is ≥0.5; 67 % (28/42) of Crohn's and 41% (24/58) of patients with UC were in active stage. The results were very similar to comparisons in regular activity groups (Table 3b-1) except for the comparison between CRP-inactive CD patients and controls in terms of Hb. There was no significant difference between CRP-inactive UC patients and controls. (Table 3b-2).

Table 3a: Comparison of anemic and nonanemic Crohn's and UC patients with controls in terms of hemoglobin, RDW, ferritin, hepcidin, ferroportion and hephaestin

		Controls	Crohn's Disease	Ulcerative Colitis	p-value CD vs Cont	p-value UC vs Cont
Hemoglobin, g/dL	anemic nonanemic	14.9 ±1.4	10.7±2.1* 14.5±1.2	10.4±1.8 + 14.3±1.3	<0.001 0.277	<0.001 0.074
RDW, %	anemic nonanemic	13.5±1.0	17.4±2.3* 14.2±1.1*	17.4±2.8 + 14.6±0.9 +	<0.001 0.017	<0.001 <0.001
Ferritin, ng/mL	anemic nonanemic	84.0±73.2	52.9±62.2 55.6±41.4*	35.6±66.1 52.1±56.3 +	0.087 0.048	0.005 0.038
Hepcidin, ng/mL	anemic nonanemic	10.5±2.8	51.7±24.5* 47.5±23.0*	38.1±21.5 + 44.1±23.7 +	<0.001 <0.001	<0.001 <0.001
Ferroportin, pg/mL	anemic nonanemic	167.6±31.1	245.1±17.6* 247.3±12.3*	248.9±15.1 + 244.9±16.7 +	<0.001 <0.001	<0.001 <0.001
Hephaestin, ng/mL	anemic nonanemic	0.18±0.03	0.41±0.04* 10.7±2.1*	0.39±0.02 + 10.4±1.8 +	<0.001 <0.001	<0.001 <0.001

Summary statistics expressed as mean ± standard deviation.

*Values of CD patients are significantly different than controls ($p < 0.05$, Welch t-test).

+Values of UC patients are significantly different than controls ($p < 0.05$, Welch t-test).

Table 3b–1: Comparison of active/inactive Crohn's and UC patients with controls in terms of hemoglobin, RDW, ferritin, hepcidin, ferroportion and hephaestin according to scoring system.

		Controls	Crohn's Disease	Ulcerative Colitis	p-value CD vs Cont	p-value UC vs Cont
Hemoglobin, /dL	active inactive	14.9 ±1.4	11.6±2.9* 14.0±1.2*	10.6±2.3 + 13.9±1.5 +	<0.001 0.013	<0.001 0.005
RDW, %	active inactive	13.5±1.0	16.6±2.6* 14.5±1.4*	17.2±3.1 + 14.9±1.2 +	<0.001 0.005	<0.001 <0.001
Ferritin, g/mL	active inactive	84.0±73.2	61.6±61.7 45.5±35.8*	55.5±81.4 34.9±36.7 +	0.192 0.007	0.149 <0.001
Hepcidin,g/mL	active inactive	10.5±2.8	52.7±23.9* 45.7±23.1*	47.6±29.7 + 36.0±13.1 +	<0.001 <0.001	<0.001 <0.001
Ferroportin, pg/mL	active inactive	167.6±31.1	245.7±16.4* 247.0±13.3*	249.8±15.8 + 244.5±15.9 +	<0.001 <0.001	<0.001 <0.001
Hephaestin, ng/mL	active inactive	0.18±0.03	0.42±0.04* 0.40±0.04*	0.40±0.03 + 0.39±0.05 +	<0.001 <0.001	<0.001 <0.001

Summary statistics expressed as mean ± standard deviation.

*Values of CD patients are significantly different than controls ($p < 0.05$, Welch t-test).

+ Values of UC patients are significantly different than controls ($p < 0.05$, Welch t-test).

Table 3b-2: Comparison of active/inactive Crohn's and UC patients with controls in terms of hemoglobin, RDW, ferritin, hepcidin, ferroportin and hephaestin according to CRP levels.

		Controls	Crohn's Disease	Ulcerative Colitis	p-value CD vs Cont	p-value UC vs Cont
Hemoglobin, g/dL	CRP-active	14.9 ±1.4	12.0±2.5*	11.1±2.7 +	<0.001	<0.001
	CRP-inactive		14.0±2.2	13.3±1.9 +	0.171	<0.001
RDW, %	CRP-active	13.5±1.0	16.0±2.3*	17.0±3.3 +	<0.001	<0.001
	CRP-inactive		15.0±2.5*	15.2±1.4 +	0.039	<0.001
Ferritin, ng/mL	CRP-active	84.0±73.2	56.8±55.3	66.1±81.2	0.078	0.372
	CRP-inactive		49.4±44.9*	28.6±35.8 +	0.04	<0.001
Hepcidin, ng/mL	CRP-active	10.5±2.8	50.4±25.4*	51.8±29.5 +	<0.001	<0.001
	CRP-inactive		47.8±19.9*	33.8±12.0 +	<0.001	<0.001
Ferroportin, g/mL	CRP-active	167.6±31.1	243.8±15.8*	251.6±13.8 +	<0.001	<0.001
	CRP-inactive		251.1±12.2*	243.5±16.7 +	<0.001	<0.001
Hephaestin, g/mL	CRP-active	0.18±0.03	0.41±0.03*	0.40±0.03 +	<0.001	<0.001
	CRP-inactive		0.41±0.04*	0.39±0.04 +	<0.001	<0.001

Summary statistics expressed as mean ± standard deviation.

*Values of CD patients are significantly different than controls ($p < 0.05$, Welch t-test).

+Values of UC patients are significantly different than controls ($p < 0.05$, Welch t-test).

4.5. Correlations between CRP and other six parameters in terms of IRPs

The elevation of IRPs even in the nonanemic status (Table 3a) suggested that some IRPs may increase in response to inflammation. Therefore, we looked at the correlation between the IRPs and the acute phase reactant in the study. In Table 4, the correlation coefficients between log (CRP) and Hb, RDW, ferritin, Hepc, Fpn, Heph were given for different groups. In order to obtain a better linear relationship, log transformation was applied on CRP values. According to these results in the control group, none of the parameters were correlated with CRP.

In the patient group (CD and UC together), CRP was not correlated with Fpn. There was correlation between CRP and other five parameters. Hb was negatively correlated (r=-0.30, p=0.003), (Figure 1). In the anemic group of which 72.9 % had active disease (according to CRP) and there were positive correlations between CRP with ferritin (r=0.57, p<0.001), Hepc (r=0.41, p=0.005) and Heph (r=0.32, p=0.027). In the nonanemic patients (32.6% had active diseases), there were correlations only between CRP with ferritin (r=0.29, p=0.036) and Hepc (r=0.35, p=0.012). In the active and CRP-active (67% of them were anemic) groups, there were correlations only between CRP with ferritin (Figure 2a) and Hepc (Figure 2b). In the inactive and CRP-inactive (27% were anemic) groups, none of the parameters were correlated with CRP (Table 4).

Table 4: Pearson correlation coefficient between log transformed CRP values and hemoglobin, RDW, ferritin, hepcidin, ferroportion and hephaestin.

	log(CRP)	Hephaestin	Hemoglobin	RDW	Ferritin	Hepcidin	Ferroportin
All Patients (n=100)	r	-0.30 *	0.28 *	0.38 *	0.34 *	0.02	0.21 *
	p-value	0.003	0.004	<0.001	<0.001	0.818	0.038
Controls (n=44)	r	0.16	0.16	-0.06	0.14	-0.23	-0.08
	p-value	0.34	0.341	0.713	0.383	0.16	0.591
Anemics (n=48)	r	-0.02	0.1	0.57 *	0.41 *	-0.21	0.32*
	p-value	0.887	0.511	<0.001	0.005	0.148	0.027
Nonanemics (n=52)	r	-0.01	0.06	0.29 *	0.35 *	0.24	0.14
	p-value	0.97	0.676	0.036	0.012	0.091	0.331
Actives** (n=49)	r	0.08	0.1	0.44 *	0.35 *	-0.16	0.15
	p-value	0.59	0.485	0.002	0.015	0.26	0.295
Inactives** (n=51)	r	-0.08	-0.09	0.11	0.22	0.17	0.13
	p-value	0.585	0.549	0.428	0.116	0.241	0.369
CRP-actives (n=52)	r	0.08	0.12	0.54 *	0.29 *	-0.18	0.21
	p-value	0.577	0.394	<0.001	0.038	0.213	0.146
CRP-inactives (n=48)	r	-0.01	0.08	-0.11	-0.04	0.13	0.04
	p-value	0.983	0.594	0.452	0.778	0.379	0.803

*Statistically significant (p-value < 0.05, Pearson's Correlation Analysis).

**According to scoring system. r: Correlation coefficient.

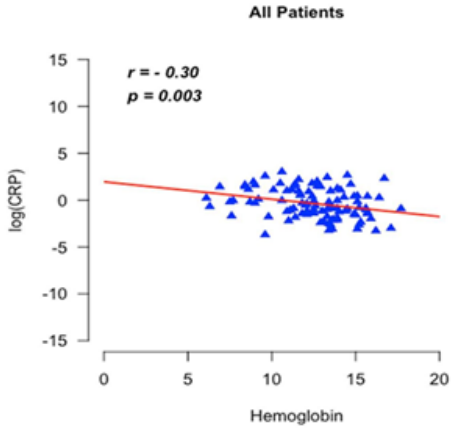

Figure 1: The correlation between CRP and hemoglobin levels in IBD (CD and UC) patients.

There is a negative correlation between CRP and hemoglobin in IBD patients Statistically significant; p-value < 0.05, Pearson's Correlation Analysis. r; Correlation coefficient.

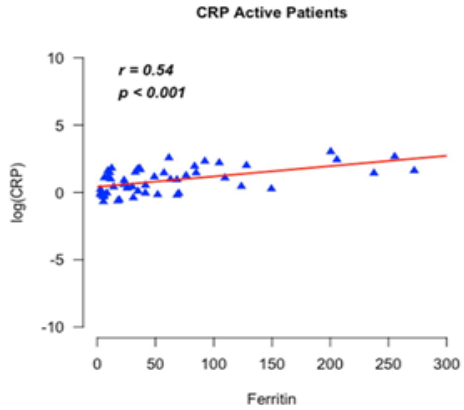

Figure 2a: The correlation between CRP and ferritin levels in active IBD (CD and UC) patients.

There was a positive correlation between CRP and ferritin levels in active IBD patients defined by CRP.

Statistically significant; p-value < 0.05, Pearson's Correlation Analysis. r; Correlation coefficient.

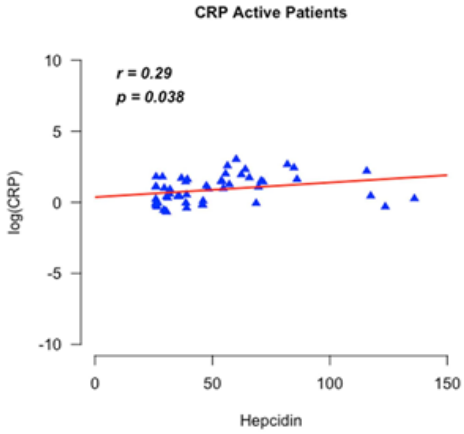

Figure 2b: The correlation between CRP and hepcidin levels in active IBD (CD and UC) patients.

There was a positive correlation between CRP and hepcidin levels in active IBD patients defined by CRP.

Statistically significant; p-value < 0.05, Pearson's Correlation Analysis. r; Correlation coefficient.

5. Discussion

One of the most frequent extra-intestinal manifestation of IBD is anemia [1,22,23] which is associated with adverse consequences in terms of the life quality and cognitive functions of patients [24]. It is also necessary to make the right decision in the treatment of these patients and to avoid the excessive loading of iron. Recent studies have focused on the diagnosis, treatment and pathophysiology of anemia in IBD, while only few studies have examined the serum levels of Hepc, which is potentially involved in the mechanisms of IBD-associated anemia. Except for tissue studies, serum levels of Fpn and Heph were not evaluated in IBD patients in this subject. Therefore, to our knowledge, our study is the first of its kind, since it involves simultaneous serum measurements of IRPs, which have roles in iron metabolism, in IBD patients.

5.1. Which type of anemia was detected?

Both IBD groups had significantly lower Hb, MCV, albumin, serum iron, and transferrin saturation and significantly higher RDW, WBC, PLT, CRP, and ESR as compared to controls, while the two IBD groups were not significantly different in terms of these parameters. However, only the CRP, transferrin saturation, Hb and RDW values were out of the reference limits in the patients. In terms of TIBC no significant difference was found between the three groups and all were in normal limits. Based on the results, inflammation induced anemia was more diagnostic for these patients. On the other hand, due to lower saturation and iron, we cannot exclude mixed type anemia (IDA and ACD). Also we suggest that UC patients were more prone to IDA compared to Crohn's, because of lower ferritin and CRP. Low folate level can be explained by proximal involvement of CD in this study.

5.2. What were the response of IRPs and ferritin?

In the present study, as reported in detail; all three IRP levels were found significantly higher in IBD patients compared to healthy controls, while there was no significant difference between CD and UC patients in this regard. In contrast to ferritin, the results were similar in these three markers for anemic and nonanemic or disease active or nonactive patients. However, ferritin in the active group was not statistically different from the controls and expected decline in the anemics was not observed. This was more pronounced on CD with higher CRP. As seen in the results, the disease was active in more than two-thirds of the anemics and suggested that increased ferritin and Hepc were caused by inflammation. The positive correlation of CRP with these two markers in the actives supported these findings. In contrast to Ferritin and Hepc, Heph showed only a weak correlation with CRP in the anemics, whereas Fpn was not affected by this condition. In other words, Heph and Fpn were more relevant with the iron deficiency or less affected by inflammation.

5.3. How these proteins functioning in IBD?

According to studies, intestinal enterocytes have important regulatory functions in the process of iron absorption [25]. Ferrous iron

(Fe+2) enters circulation via a transmembrane protein known as Fpn that is located in the basolateral site of the enterocyte and that is responsible for the extracellular transport of intracellular iron. Heph converts ferrous iron (Fe+2) to ferric iron (Fe+3) mediating the entry of iron from intestinal enterocytes to circulation in close collaboration with Fpn, and allowing iron transport after binding to transferrin. High dietary iron intake and presence of adequate iron stores within the enterocytes are associated with reduced protein synthesis of Fpn as well as Heph, leading to decreased iron absorption into circulation from enterocytes. Conversely, low dietary iron intake and depleted enterocyte iron stores causes increased synthesis of these two proteins, hence increased absorption of iron into the circulation, as shown previously [8]. The increase in Fpn and Heph in the IBD patients can be attributed to impaired iron absorption caused by chronic enterocytes injury. Inadequate iron intake and intestinal loss may also an additional cause for these patients.

How about Hepcidine ? Hepc controls the entry of iron into blood by inhibiting iron absorption at small intestine and iron release from macrophages through its effects on Fpn [26,27]. Due to inflammation, Hepc levels are elevated causing impairment of intestinal basolateral iron transport in patients with IBD. Since Fpn is suppressed by inflammation, oral iron supplementation may fail to suffice and even be contraindicated. Therefore, newer management approaches involving the use of iron and erythropoietin have been developed in IBD. [28,29].

Despite the increase in Hepc in the study, Fpn and Heph showed 1.5 to 2 fold increase in the control group. This can be explained by the mixed type of anemia or misleading of Hepc in inflammatory diseases. Compared to the controls, the patients's lower iron status (50% lower, but remained in normal limits) supports these explanations. Therefore, Hepc, such as ferritin, thought to be elevated secondary to inflammation in these patients.

5.4. What are the Results of Other Studies?

In a study by Semrin et al. [30] comparing 19 pediatric patients with active CD vs. inactive cases, the former group was found to have decreased oral absorption of iron, increased urinary Hepc, and elevated inflammatory markers. Decreased oral iron absorption exhibited a strong inverse correlation with urinary Hepc and serum inflammatory markers, while a positive correlation was reported between urinary Hepc and inflammation. Similar results were reported by Martinelli [16] et al. They found significantly higher levels of serum Hepc measured by spectrometry among 50 pediatric IBD patients as compared to 45 patients with coeliac disease and 50 controls.

Oustamanolakis P. et al. [15] also found significantly higher serum Hepc levels measured with ELISA in a total of 100 patients with IBD (49 UC and 51 CD) as compared to 102 healthy control subjects, with a positive correlation between elevated serum Hepc levels and ferritin as well as disease activity.

In a study by Arnold et al. [17] involving 61 patients with IBD and 25 healthy controls, serum Hepc levels determined by radioimmunoassay were significantly lower among IBD patients and correlated positively with IL-6 levels. In the study anemic patients with normal ferritin but low serum iron were classified as having anemia of inflammation. Unlike the others, bioactive Hepc-25 was measured in this study. Except this study [17], our results were consistent with other published reports in terms of increased serum Hepc levels and positive correlation with disease activity. If bioactive form had measured it would likely to be low, which may be another explanation of increasing Fpn and Heph in our study.

As we mentioned before, mostly Fpn and Heph studies were done at the tissue level. In a mice model, increased expression of Fpn and Heph mRNA was found in the small intestine of iron deficient animals as compared to controls [8]. Similarly, Barisani et al. reported increased Fpn and Heph levels in duodenal mucosal biopsy samples in Celiac patients with iron deficiency anemia [31].

Burpee et al. [32] also reported increased Fpn protein in the duodenal mucosal biopsy samples among anemic patients than in non-anemic patients among a cohort of 19 pediatric CD cases. Sukumaran et al. examined the expression of iron-related proteins in patients with inflammatory conditions (UC, CD and rheumatoid arthritis) versus healthy controls in duodenal mucosa [33].

In the group of patients with inflammatory diseases, gene expression of Heph was significantly higher than those in the control subjects in the duodenum; however, the difference in Fpn gene expression did not reach statistical significance. In the subgroups of patients with UC and CD, both Fpn and Heph gene expressions were upregulated in the duodenal mucosa. In addition, in patients with UC, the protein expression of Fpn in the duodenal mucosa was significantly higher. Those investigators also examined serum Hepc levels in a subgroup of UC and control patients with available serum samples, and found lower serum Hepc in UC patients than controls. As in Arnold et al's study [17], the bioactive form was measured and found to be low [33]. These latter studies are favored by increased Fpn and Heph secretion at the mucosal level in both iron deficiency and IBD patients. We obtained similar results in serum. This suggests that the measurements of Fpn, perhaps Heph, in serum are sufficient for clinical decision.

5.5. Future Therapies Targeting IRPs

As was emphasized in the literature, Hepc levels have been shown to be lower in patients with ACD who had concurrent iron deficiency than in those with ACD without ID [34]. As well known, Hepc is the key regulator of iron, and high Hepc levels cause iron blockade and anemia in chronic disease. Currently, new medications targeting Hepc is at the developing stage. Hepc antagonists may prove useful and provide advantages in the future in patients not responding to erythropoietin [35]. Also recent studies show that blockade of TNF could rescue anemia and provides a therapeutic approach in the management of anemia in IBD [36]. Future studies should ideally include more specific assays about IRPs. This will help to treat the anemia

correctly and will keep IBD patients from unnecessary iron overload.

Our study demonstrated that the patients had mixed type anemia in which weighted ACD, also directly related to disease activity. Hepc, Fpn and Heph found increased regardless of anemia or disease activity. Like ferritin, the increase in Hepc pretended secondary to inflammation, which correlated with CRP. Heph and Fpn seemed more relevant with iron deficiency and less effected by inflammation.

In conclusion; Ferritin and Hepc appear to be misleading in determining anemia type in active IBDs. To diagnosis of pure anemia, measurement of Hepc using more sensitive methods will be important in treatment decision. Fpn and Heph are clinically less important in IBD cases, since they vary depending on Hepc.

References

1. Kulnigg S, Gasche C. Systematic review: managing anaemia in Crohn's disease. Aliment PharmacolTher 2006; 24: 1507-1523.
2. Oustamanolakis P, Koutroubakis IE, Kouroumalis EA. Diagnosing anemia in inflammatory bowel disease: beyond the established markers. J Crohn's Colitis. 2011; 5: 381-391.
3. Gomollon F, Gisbert JP. Anemia and inflammatory bowel diseases. World J Gastroenterol. 2009; 15: 4659-4665.
4. Ganz T. Hepcidin--a regulator of intestinal iron absorption and iron recycling by macrophages. Best Pract Res ClinHaematol. 2005; 18: 171-182.
5. Atanasiu V, Manolescu B, Stoian I. Hepcidin--central regulator of iron metabolism. Eur J Haematol. 2007; 78: 1-10.
6. Zhao N, Zhang AS, Enns CA. Iron regulation by hepcidin. J Clin Invest. 2013; 123: 23372343.
7. Rossi E. Hepcidin--the iron regulatory hormone. ClinBiochem Rev. 2005; 26: 47-49.
8. Chen H, Su T, Attieh ZK, Fox TC, McKie AT, Anderson GJ, et al. Systemic regulation of Hephaestin and Ireg1 revealed in studies of genetic and nutritional iron deficiency. Blood. 2003; 102: 1893-1899.
9. Nemeth E. Targeting the hepcidin-ferroportin axis in the diagnosis and treatment of anemias. AdvHematol. 2010; 2010: 750643.
10. Ganz T, Nemeth E. Iron imports. IV. Hepcidin and regulation of body iron metabolism. Am J PhysiolGastrointest Liver Physiol. 2006; 290: G199-203.
11. Nemeth E, Tuttle MS, Powelson J, Vaughn MB, Donovan A, Ward DM, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. Science. 2004; 306: 2090-2093.
12. Chen H, Huang G, Su T, Gao H, Attieh ZK, McKie AT, et al. Decreased hephaestin activity in the intestine of copperdeficient mice causes systemic iron deficiency. J Nutr. 2006; 136: 1236-1241.
13. Petrak J, Vyoral D. Hephaestin-a ferroxidase of cellular iron export. Int J Biochem Cell Biol. 2005; 37: 1173-1178.
14. Dudkowiak R, Neubauer K, Poniewierka E. Hepcidin and its role in inflammatory bowel disease. AdvClinExp Med. 2013; 22: 585-591.
15. Oustamanolakis P, Koutroubakis IE, Messaritakis I, Malliaraki N, Sfiridaki A, Kouroumalis EA. Serum hepcidin and prohepcidin concentrations in inflammatory bowel disease. Eur J GastroenterolHepatol. 2011; 23: 262-268.
16. Martinelli M, Strisciuglio C, Alessandrella A, Rossi F, Auricchio R, Campostrini N, et al. Serum Hepcidin and Iron Absorption in Paediatric Inflammatory Bowel Disease. J Crohn's Colitis, 2016; 10: 566-574.
17. Arnold J, Sangwaiya A, Bhatkal B, Geoghegan F, Busbridge M. Hepcidin and inflammatory bowel disease: dual role in host defence and iron homoeostasis. Eur J GastroenterolHepatol. 2009; 21: 425-429.
18. Basseri RJ, Nemeth E, Vassilaki ME, Basseri B, Enayati P, Shaye O, et al. Hepcidin is a key mediator of anemia of inflammation in Crohn's disease. J Crohn's Colitis. 2013; 7: e286-291.
19. Best WR, Becktel JM, Singleton JW, Kern F, Jr. Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. Gastroenterology. 1976; 70: 439-444.
20. Truelove SC, Witts LJ. Cortisone in ulcerative colitis; final report on a therapeutic trial. Br Med J. 1955; 2: 1041-1048.
21. Dignass A, Lindsay JO, Sturm A, Windsor A, Colombel JF, Allez M, et al. Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 2: current management. J Crohn's Colitis. 2012; 6: 991-1030.
22. Gasche C. Anemia in IBD: the overlooked villain. Inflamm Bowel Dis. 2000; 6: 142-151.
23. Gisbert JP, Gomollon F. Common misconceptions in the diagnosis and management of anemia in inflammatory bowel disease. Am J Gastroenterol. 2008; 103: 1299-1307.
24. Wells CW, Lewis S, Barton JR, Corbett S. Effects of changes in hemoglobin level on quality of life and cognitive function in inflammatory bowel disease patients. Inflamm Bowel Dis. 2006; 12: 123-130.
25. Roberts SK, Henderson RW, Young GP. Modulation of uptake of heme by rat small intestinal mucosa in iron deficiency. Am J Physiol. 1993; 265: G712-718.
26. Ganz T. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. Blood. 2003; 102: 783-788.
27. Ganz T, Nemeth E. Hepcidin and disorders of iron metabolism. Annu Rev Med. 2011; 62: 347-360.
28. Gasche C, Dejaco C, Reinisch W, Tillinger W, Waldhoer T, Fueger GF, et al. Sequential treatment of anemia in ulcerative colitis with intravenous iron and erythropoietin. Digestion. 1999; 60: 262-267.
29. Horina JH, Petritsch W, Schmid CR, Reicht G, Wenzl H, Silly H, et al. Treatment of anemia in inflammatory bowel disease with recombinant human erythropoietin: results in three patients. Gastroenterology. 1993; 104: 1828-1831.
30. Semrin G, Fishman DS, Bousvaros A, Zholudev A, Saunders AC, Correia CE, et al. Impaired intestinal iron absorption in Crohn's disease correlates with disease activity and markers of inflammation. Inflamm Bowel Dis. 2006; 12: 1101-1106.
31. Barisani D, Parafioriti A, Bardella MT, Zoller H, Conte D, Armiraglio E, et al. Adaptive changes of duodenal iron transport proteins in celiac disease. Physiol Genomics. 2004; 17: 316-325.
32. Burpee T, Mitchell P, Fishman D, Islam S, Nemeth E, Westerman M,

et al. Intestinal ferroportin expression in pediatric Crohn's disease. Inflamm Bowel Dis. 2011; 17: 524-531.

33. Sukumaran A, James J, Janardhan HP, Amaladas A, Suresh LM, Danda D, et al. Expression of iron-related proteins in the duodenum is up-regulated in patients with chronic inflammatory disorders. Br J Nutr. 2014; 111: 1059-1068.

34. Theurl I, Aigner E, Theurl M, Nairz M, Seifert M, Schroll A, et al. Regulation of iron homeostasis in anemia of chronic disease and iron deficiency anemia: diagnostic and therapeutic implications. Blood. 2009; 113: 5277–5286.

35. Murawska N, Fabisiak A, Fichna J. Anemia of Chronic Disease and Iron Deficiency Anemia in Inflammatory Bowel Diseases: Pathophysiology, Diagnosis, and Treatment. Inflamm Bowel Dis. 2016; 22: 1198-1208.

36. Wu W, Song Y, He C, Liu C, Wu R, Fang L, et al. Divalent metal-ion transporter 1 is decreased in intestinal epithelial cells and contributes to the anemia in inflammatory bowel disease. Sci Rep. 2015; 17: 16344.