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Anemia in Hodgkin's Lymphoma: The Role of Interleukin-6 and Hepcidin

Stefan Hohaus, Giuseppina Massini, Manuela Giachelia, Barbara Vannata, Valentina Bozzoli, Annarosa Cuccaro, Francesco D'Alo', Luigi Maria Larocca, Reinier A.P. Raymakers, Dorine W. Swinkels, Maria Teresa Voso, and Giuseppe Leone

ABSTRACT

Purpose

Cytokines play a pivotal role in Hodgkin's lymphoma (HL). Because interleukin-6 (IL-6) induces expression of hepcidin, one of the principal regulators of iron metabolism, we studied the contribution of hepcidin in anemia in HL at diagnosis.

Patients and Methods

Plasma samples from 65 patients with HL were analyzed for hepcidin levels using a combination of weak cation exchange chromatography and time-of-flight mass spectrometry; cytokine levels were analyzed using enzyme-linked immunosorbent assays and parameters of iron metabolism and acute-phase reaction.

Results

Hepcidin plasma levels were significantly higher in HL patients when compared with controls, independent of the presence of anemia ($P = .001$). In the subset of patients with anemia, hepcidin levels inversely correlated with hemoglobin levels ($P = .01$). Analyzing parameters of iron metabolism, hepcidin levels showed a positive correlation with ferritin ($P < .001$) and an inverse correlation to iron and iron-binding capacity. Hepcidin strongly correlated to IL-6 levels ($P < .001$) but not to IL-10 or thymus and activation-regulated cytokine (TARC)/chemokine (C-C motif) ligand 17 (CCL17) levels. In a multivariate regression analysis, IL-6 and fibrinogen levels were independently associated with hepcidin. Higher hepcidin levels were observed in patients with more aggressive disease characteristics: stage IV disease ($P = .01$), presence of B symptoms ($P = .03$), and International Prognostic Score > 2 ($P = .005$).

Conclusion

Our findings suggest that in HL, hepcidin is upregulated by IL-6. Elevated hepcidin levels result in iron restriction and signs of anemia of chronic inflammation, although hepcidin-independent mechanisms contribute to development of anemia in HL.

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INTRODUCTION

Anemia is a presenting symptom in approximately 40% of patients with Hodgkin's lymphoma (HL). It is more frequently observed in advanced stages and is usually associated with B symptoms such as fever, night sweats, and weight loss. In general, the anemia is normochromic and normocytic and is usually mild, with hemoglobin (Hb) levels between 10 and 12 g/dL.

The anemia of chronic disease is seen in a wide variety of inflammatory states including acute systemic inflammatory response syndrome, chronic infections, inflammatory disorders, and some cancers.^{1,2} This iron-refractory anemia is characterized by low serum iron (hypoferremia), reduced iron-binding capacity, and subnormal transferrin saturation,

while bone marrow iron is relatively preserved. Traditional biochemical iron indicators (eg, serum iron, ferritin, transferrin saturation) are of only limited use because of the distorting effects of inflammation on their levels. Serum ferritin levels, although useful indicators of iron status in patients without underlying chronic disorders, increase in patients with inflammatory diseases. Ferritin levels have been reported to be elevated in patients with HL, in particular in advanced stages and during disease progression.^{3,4}

Studies in humans and mice suggest that the iron-regulatory hormone hepcidin is the principal mediator of anemia of chronic disease and/or inflammation.⁵⁻¹¹ Hepcidin is a liver-produced acute-phase peptide whose overproduction leads to iron-limited erythropoiesis. Hepcidin binds to the

From the Institute of Hematology and Institute of Pathological Anatomy, Catholic University S. Cuore, Rome, Italy; University Medical Center Utrecht, Utrecht; and Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands.

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Corresponding author: Stefan Hohaus, MD, Istituto di Ematologia, Università Cattolica S. Cuore-Roma, L.go A. Gemelli, 1, Rome, Italy 00168; e-mail: stefan.hohaus@rm.unicatt.it.

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cell membrane iron exporter ferroportin and induces its internalization and degradation, thus decreasing iron release from macrophages and enterocytes. Inflammatory cytokines increase the expression of hepcidin,^{12,13} leading to decreased absorption of iron from the intestine, and block the release of iron from the reticuloendothelial system and the liver.¹⁴ Infusion of interleukin-6 (IL-6) in human volunteers resulted in increased excretion of urinary hepcidin and development of hypoferrmia.¹² IL-6 is a potent inducer of hepcidin expression through a signal transducer and activator of transcription 3–dependent transcriptional mechanism.^{15–17} In addition, macrophages express hepcidin in response to microbial stimulation.¹⁸ A pathogenic cascade for the development of anemia of inflammation has been proposed that leads from IL-6 to hepcidin to hypoferrmia and, as a consequence, to anemia of inflammation.⁷

The inflammatory response surrounding the Hodgkin's and Reed-Sternberg cell is part of HL as the cell of origin itself. In the interactions between the neoplastic Hodgkin's and Reed-Sternberg cells and the reactive cells of the microenvironment, there are high levels of cytokines, such as IL-6, IL-10, and the chemokine thymus and activation-regulated cytokine (TARC) among others.¹⁹ Local production of these cytokines results in elevated systemic levels in the peripheral blood, and these cytokines are responsible for the development of systemic symptoms and laboratory abnormalities that are correlated with disease prognosis.^{20–22} IL-6 is among the cytokines most strongly associated with anemia.²⁰ We were interested in the contribution of hepcidin to the pathogenesis of anemia of HL and its relation to other cytokines important in the biology of the disease, including IL-6, IL-10, and TARC.

PATIENTS AND METHODS

Patient Characteristics

Our analysis included 65 patients (median age, 35 years; range 15 to 83 years; 36 females and 29 males) diagnosed with HL between March 2004 and May 2009 and observed at the Institute of Hematology of the Catholic University of Rome. Thirteen patients had stage IV disease, but only five patients showed bone marrow infiltration. Of these five patients, four patients were anemic, but only one patient had signs of reduced hematopoiesis as a result of bone marrow infiltration. Additional patient characteristics, including the International Prognostic Score (IPS),²³ are detailed in Table 1. Peripheral blood samples were obtained at the time of initial diagnosis, and samples were collected early in the morning. All parameters of iron metabolism were determined in the central laboratory of the Catholic University. A group of 24 healthy individuals (median age, 41 years; range 18 to 63 years; 13 females, 11 males) was used as a control.²⁴ Informed consent was obtained from patients and controls according to institutional guidelines, and blood sample collection was approved by our institutional ethical committee.

Enzyme-Linked Immunosorbent Assay for Plasma Levels of IL-6, IL-10, and TARC

IL-6, IL-10, and TARC levels were determined in pretreatment plasma samples, which had been stored at -70°C and thawed for the first time, by a sandwich enzyme-linked immunosorbent assay, according to the manufacturer's instructions (Human IL-10 DuoSet and Human CCL17/TARC DuoSet, R&D Systems, Minneapolis, MN; Human IL-6 US, BioSource International, Camarillo, CA).

Hepcidin-25 Quantification

Plasma hepcidin measurements were performed in July 2009 (testing laboratory: Hepcidinanalysis.com, Nijmegen, the Netherlands) by a combination of weak cation exchange chromatography and time-of-flight mass spectrometry (TOFMS). An internal standard (synthetic hepcidin-24; Peptide

Table 1. Associations Between Patient Characteristics and Hemoglobin, Hepcidin, and IL-6 Levels

Variable	No. of Patients	Hemoglobin (n = 65)		Hepcidin (n = 59)		IL-6 (n = 55)		TARC (n = 57)	
		g/dL	P	nmol/L	P	ng/mL	P	ng/mL	P
Age, years			.02		.03		.05		.98
< 45	43	12.4		6.5		0.7		3,575	
> 45	22	11.2		10.4		4.1		4,529	
Sex			.001		.06		.5		.02
Female	36	11.3		6.9		2.1		5,223	
Male	29	12.9		9.4		1.3		2,271	
Stage			.04		.01		.02		.37
I-III	50	12.4		6.3		0.6		3,671	
IV	13	10.8		13.7		5.4		3,434	
B symptoms			< .001		.03		.003		.07
No	40	12.8		5.9		0.6		2,999	
Yes	23	10.9		10.4		2.1		5,829	
Bulky disease			.21		.85		.32		.04
No	46	12.3		7.8		1.2		3,033	
Yes	17	11.5		7.2		0.6		6,806	
IPS*			< .001		.005		.002		.59
Low-risk	49	12.7		6.2		0.6		4,045	
High-risk	14	9.9		12.2		3.0		3,444	

NOTE. P values were determined by Mann-Whitney rank sum test. Abbreviations: IL-6, interleukin-6; TARC, thymus and activation-regulated cytokine; IPS, International Prognostic Score. *The IPS was calculated according to Hasenclever and Diehl.²³ IPS scores 0-2 were considered as low-risk and ≥ 3 as high-risk.

International, Louisville, KY) was used for quantification (www.hepcidinanalysis.com).^{25,26} Peptide spectra were generated on a Microflex LT matrix-enhanced laser desorption/ionisation TOFMS platform (Bruker Daltonics, Billerica, MA). Plasma hepcidin-25 concentrations were expressed as nmol/L. The lower limit of detection of this method was 0.5 nmol/L; average coefficients of variation were 2.7% (intranun) and 6.5% (inter-run). The median reference level of plasma hepcidin-25 is 4.2 nmol/L (range, 0.5 to 13.9 nmol/L).²⁴

Statistical Analysis

Wilcoxon signed rank test was used for two-sample comparisons of hepcidin plasma levels between patient and control groups or according to dichotomized patient characteristics. Comparisons between controls and patients were also performed using analysis of covariance models including sex, age, and ferritin levels as covariates. Since the distribution of plasma parameters in our population revealed a departure from normality that was mitigated using a logarithmic transformation, the log of the concentration was used for testing purposes. However, untransformed values were used for reporting results. Plasma parameters were analyzed both as continuous variables following logarithmic transformation and as dichotomous variables using the upper limit of normal of controls as a cutoff point. Correlations between the various blood parameters were calculated by linear regression models. A multivariate logistic regression model was used to examine the relationship between the dependent variable (hepcidin plasma level) and potential predictor variables, including Hb, IL-6, ferritin, and fibrinogen levels. Stepwise backward elimination of nonsignificant parameters was used to obtain the best model. Computations were performed using STATA 10.0 software (STATA, College Station, TX).

RESULTS

Anemia and Patient Characteristics in HL

We studied 65 patients with HL at diagnosis. Anemia, defined as Hb concentration < 12 g/dL, was present in 30 patients, and it was

mild (Hb between 10 and 12 g/dL) in 20 patients. Anemia was normocytic and normochromic in 19 patients, while it was microcytic in 11 patients. Associations of anemia with patient characteristics are presented in Table 1. As expected, Hb concentration was lower in female patients, in patients older than age 45 years, and in patients with B symptoms, stage IV disease, and a higher IPS score (> 2).

Hepcidin Levels and Anemia in HL Patients

Hepcidin levels in 59 patients were determined in plasma samples taken at diagnosis by using a combination of weak cation exchange chromatography and TOFMS. The mean hepcidin level in HL patients was 7.9 nmol/L, ranging from below the detection limit of 0.5 nmol/L in three patients to 27.4 nmol/L, and was significantly higher than that of a group of 24 non-anemic normal controls (mean, 2.77 nmol/L; range, 0.5 to 8.2 nmol/L; $P = .001$). The difference between HL patients and controls remained significant after including age and sex and adjusting for ferritin levels ($P = .002$), which may influence hepcidin levels in healthy controls. Strikingly, when comparing controls with non-anemic HL patients, hepcidin was still significantly higher in HL ($P = .02$; Fig 1).

Patients older than age 45 years had higher hepcidin levels ($P = .03$), and there was a borderline significance for higher hepcidin levels in males compared with females ($P = .06$; Table 1). In anemic patients, hepcidin levels inversely correlated with Hb values ($r = -0.43$; $P = .02$; Fig 2), while there was no correlation in the absence of anemia.

Hepcidin and Iron Metabolism in HL

Data on iron metabolism were available for 39 patients. The median serum iron and total iron-binding capacity in HL patients

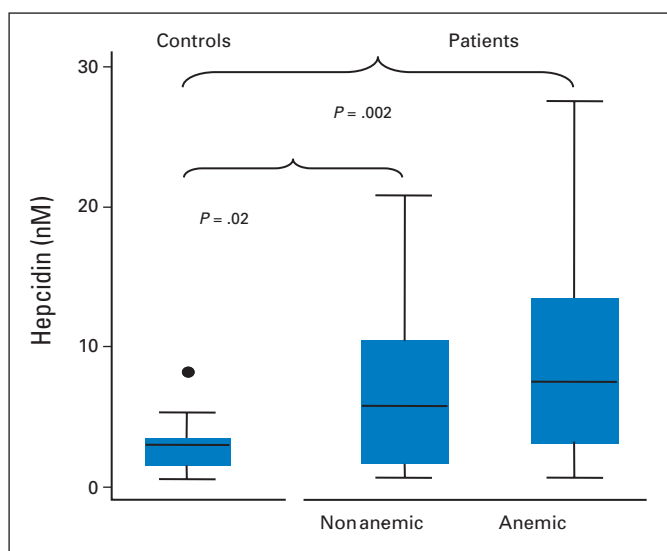


Fig 1. Box plots of hepcidin plasma concentrations in healthy controls ($n = 24$) and patients with Hodgkin's lymphoma (HL) at diagnosis ($n = 59$). Patients with HL were divided into a non-anemic group with hemoglobin levels > 12 g/dL ($n = 30$) and an anemic group with hemoglobin levels < 12 g/dL ($n = 29$). The upper border of the box indicates the upper quartile (75th percentile), while the lower border indicates the lower quartile (25th percentile), and the horizontal line in the box indicates the median. The vertical lines are the whiskers indicating the upper and lower adjacent values. Both anemic and non-anemic patients had significantly higher levels of hepcidin when compared with controls ($P = .002$ and $.02$, respectively), while the difference between anemic and non-anemic patients was not statistically significant ($P = .18$).

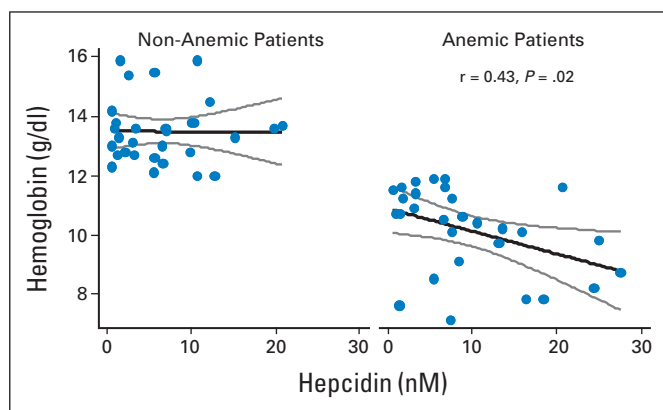


Fig 2. Correlation between hemoglobin and hepcidin in Hodgkin's lymphoma (HL) patients. Scatter plots with linear regression lines (black lines) and 95% CIs (gray lines) showing significant inverse correlation between hemoglobin and hepcidin levels in anemic HL patients ($r = -0.43$; $P = .02$) but not in non-anemic patients ($r = 0.04$; $P = .8$).

were $33 \mu\text{g/dL}$ (range, 9 to $183 \mu\text{g/dL}$) and $233 \mu\text{g/dL}$ (range, 154 to $366 \mu\text{g/dL}$), respectively, and were lower than the normal range (40 to $150 \mu\text{g/dL}$ for iron and 250 to $425 \mu\text{g/dL}$ for total iron-binding capacity). Ferritin levels showed a wide variation with a median of 90 ng/mL and a range between 7 and 7,500 ng/mL. Only three patients had iron deficiency anemia, defined by Hb levels < 12 g/dL and microcytosis and ferritin levels < 12 ng/mL.

Looking at parameters of iron metabolism, there was a strong correlation between hepcidin and ferritin ($r = 0.62$; $P < .001$) and an inverse correlation with iron and iron-binding capacity ($r = -0.42$, $P = .009$; and $r = -0.43$, $P = .02$, respectively; Fig 3). After adjustment for multiple testing, the correlation between hepcidin and parameters of iron metabolism, but not iron itself, maintained statistical significance (Table 2).

Correlation of Hepcidin With Levels of IL-6 and Other Markers of Inflammation

Because hepcidin is an acute-phase reactant during infection and/or inflammation, we examined the relationship between hepcidin and inflammatory cytokines and other proteins of the acute-phase reaction. Among these, ferritin, as described above, and fibrinogen correlated with hepcidin (Table 2).

IL-6 concentration was studied in 55 patients. It was below the detection limit of the assay (0.01 ng/mL) in 15 patients, while the mean concentration was 2.4 ng/mL ($\pm 6.15 \text{ ng/mL}$) in the remaining 40 patients. IL-6 correlated with several laboratory parameters and clinical characteristics as detailed in Tables 1 and 2. In particular, IL-6 inversely correlated with Hb values ($r = -0.49$; $P < .001$). We found a highly significant correlation between levels of hepcidin and IL-6 ($r = 0.55$; $P < .001$; Fig 4), whereas there was no significant correlation with IL-10 or TARC levels, arguing against a role for these cytokines in hepcidin upregulation in HL patients.

Because several parameters indicative for anemia of inflammation were associated with hepcidin levels, we next performed a multivariate analysis to predict hepcidin levels. We included Hb values and the significant parameters from univariate analysis (Table 2): IL-6 as the most relevant cytokine, ferritin as the most important parameter for iron metabolism, and fibrinogen levels as the parameter for acute-phase reaction. Using a stepwise backward regression analysis, Hb and

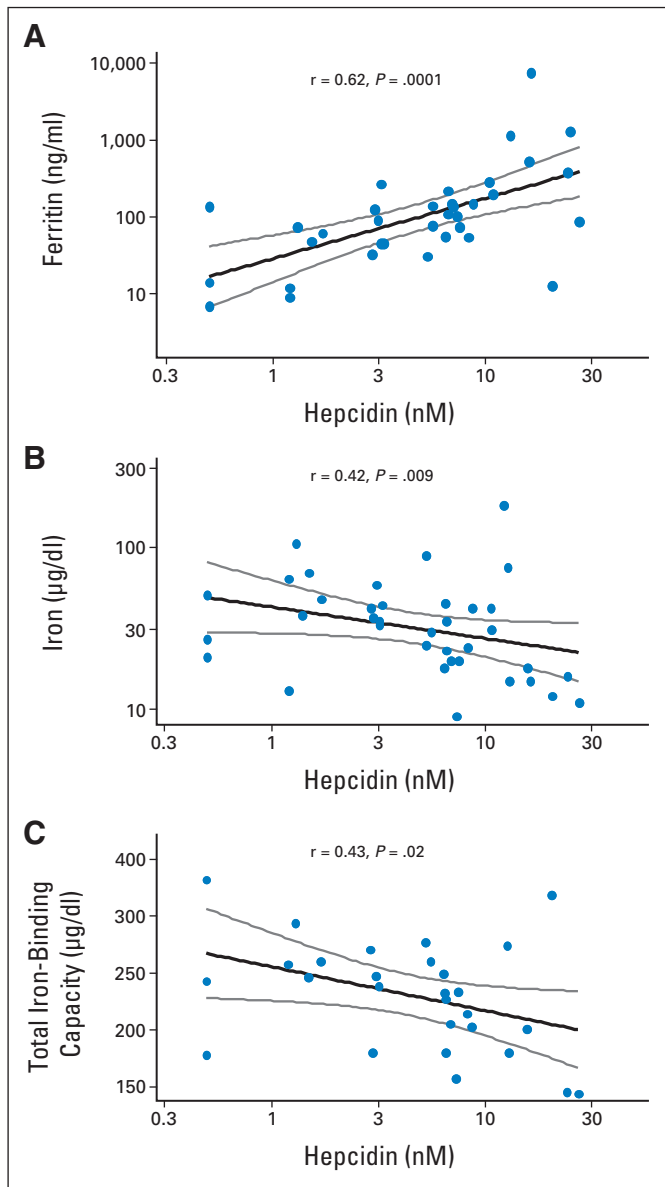


Fig 3. Correlations between hepcidin and parameters of iron metabolism. Scatter plots with linear regression (black lines) and 95% CIs (gray lines) show significant correlations between hepcidin and (A) ferritin, (B) iron, and (C) total iron-binding capacity.

ferritin levels were excluded, while IL-6 and fibrinogen independently correlated with hepcidin ($P = .03$ and $P = .001$, respectively). Rerunning the model with only IL-6 and fibrinogen confirmed the results obtained by the backward selection technique (Appendix Table A1, online only).

Hepcidin and Clinical Characteristics

We analyzed for associations of hepcidin levels with clinical characteristics with known prognostic impact. Higher hepcidin levels were observed in patients with stage IV disease ($P = .01$), in the presence of B symptoms ($P = .03$), and in patients with an IPS score ≥ 3 ($P = .005$; Table 1).

DISCUSSION

Our study shows that hepcidin plasma levels are increased in patients with HL. Hepcidin levels strongly correlate with changes typical for inflammation and Hodgkin's disease activity, such as increased IL-6 levels. Increased hepcidin was also found in non-anemic patients, whereas in anemic patients, there was a striking inverse correlation with the severity of anemia. It correlated with changes in iron metabolism that are typical for inflammation and acute-phase reaction, such as elevated ferritin, lower iron, reduced total iron-binding capacity, and higher fibrinogen levels. This is in line with the role of hepcidin as a gatekeeper for iron homeostasis, inhibiting the cellular efflux of iron through ferroportin, resulting in iron being trapped in macrophages and iron-absorbing enterocytes and reducing the availability of iron for erythropoiesis.

Iron redistribution is a hallmark of anemia of chronic disease. Although some cases of anemia can be attributed to iron deficiency, in our case series, there were only three patients with iron-deficiency anemia; the vast majority of patients with HL who are anemic present with anemia of chronic disease. Bone marrow infiltration as a confounding factor for development of anemia is rare in HL and was present in only five patients in our case series, with only one patient having extensive bone marrow infiltration in the biopsy that could explain anemia.

Given its high prevalence, anemia in HL is an interesting model for studying cytokine-mediated anemia. Experiments in mice and clinical observations have demonstrated that anemia of chronic disease is characterized by a complex interaction between the components of inflammation and indicate that IL-6 seems to be the principal cytokine inducing iron-restricted erythropoiesis and anemia.^{2,12,27-30} IL-6 knockout mice fail to produce hepcidin in response to inflammatory challenges.^{12,27} During inflammation, IL-6 alone can rapidly induce hepcidin synthesis and corresponding hypoferrremia.¹² In a patient presenting with hemochromatosis and an auto-inflammatory disease, there was clear evidence for the IL-6–hepcidin axis in the development of hypoferrremia and anemia of inflammation.³¹ In cell culture experiments, IL-6, but not IL-1 or tumor necrosis factor alpha (TNF- α), induced hepcidin mRNA expression in human hepatocytes.³² In myeloma patients, plasma IL-6 could induce hepcidin while no effects for TNF- α or interleukin-1 beta (IL-1 β) were observed.²⁹ In this line, we observed a strong association between IL-6 and hepcidin in HL, while IL-10 or TARC did not play a significant role.

Because IL-6 levels more strongly correlated with development of anemia than hepcidin levels did, other IL-6–associated mechanisms than hepcidin-mediated iron restriction are also probably involved in the induction of anemia in HL. Administration of recombinant human IL-6 induces a rapid-onset, dose-dependent, progressive anemia that is quickly reversible after the cessation of therapy, with the characteristics of a dilutional anemia driven by marked increases in plasma volume.^{33,34} Impairment of proliferation and differentiation of erythroid progenitors could also contribute to IL-6–mediated anemia. IL-6 itself does not suppress erythropoiesis and has no demonstrable direct effect on the proliferation of hematopoietic progenitors.³⁵ It is even a potent megakaryopoietic growth factor and may be the humoral mediator of reactive thrombosis accompanying inflammatory states. Suppression of erythropoiesis may be due to other acute-phase

Table 2. Associations Between Hepcidin, Hemoglobin, and IL-6 Levels, and Parameters of Iron Metabolism and Acute-Phase Reaction

		Hepcidin		Hemoglobin		IL-6		Ferritin		Iron		Fibrinogen		Albumin		
Hepcidin	ρ	1.0														
	No.	59														
Hemoglobin	ρ	-0.237	1.0													
	No.	59	65													
	P	1.0														
IL-6	ρ	0.534*	-0.512*	1.0												
	No.	49	55	55												
	P	.0016	.0014													
Ferritin	ρ	0.612*	-0.002	0.589*	1.0											
	No.	35	38	29	38											
	P	.0020	1.0	.016												
Iron	ρ	-0.420	0.400	-0.370	-0.19	1.0										
	No.	37	39	31	33	39										
	P	.2	.26	.85	1.0											
Fibrinogen	ρ	0.524*	-0.344	0.407	0.47	-0.550*	1.0									
	No.	53	59	50	36	36	59									
	P	.0012	.16	.07	.08	.01										
Albumin	ρ	-0.10	0.601*	-0.470*	-0.12	0.195	-0.02	1.0								
	No.	50	54	45	32	35	49	54								
	P	1.0	<.001	.024	1.0	1.0	1.0	1.0								

NOTE. Spearman rank correlation coefficients were calculated for each pair of variables; ρ values are given in the first line of each cell, No. indicates the number of observations, and P indicates the significance level. Significance levels were adjusted for multiple testing using the Bonferroni correction.

Abbreviation: IL-6, interleukin-6.

*Correlations with P values < .05.

reactants induced by IL-6. Reduced expression of transferrin receptors, impaired binding affinity of transferrin receptors, and inadequate erythropoietin production and response may contribute to the defect of erythropoiesis in the anemia of chronic disease.^{2,36-38}

Hepcidin levels were higher in HL patients with advanced-stage disease and B symptoms. A similar association of elevated hepcidin levels with advanced-stage disease has been reported for patients with non-Hodgkin's lymphoma and myeloma.^{29,39} Moreover, Ukarma et al³⁹ observed higher hepcidin levels in anemic patients with hematologic tumors than with solid tumors. The association of hepcidin with unfavorable prognostic factors in our HL study raises the question of whether hepcidin may have a role as a biomarker. Anemia and IL-6 levels are well-known prognostic markers in HL,^{20,23,40,41} and future

studies might address the relationship of IL-6, anemia, and hepcidin with prognosis in HL and whether these correlations will correct after therapy.

Elevated hepcidin levels in HL may also open a perspective for antihepcidin or anti-inflammatory therapy in reversing the anemia of inflammation in this disease. Administration of an anti-IL-6 receptor antibody tocilizumab in two patients with multicentric Castleman's disease rapidly lowered serum hepcidin levels and resulted in a prompt Hb increase even without iron administration.⁴²

In conclusion, we propose the following scenario: HL disease activity is associated with production and release of IL-6 into the systemic circulation, which stimulates the overproduction of hepcidin as an acute-phase reactant in the liver. Elevated levels of hepcidin in HL correlate with iron restriction and contribute to anemia. However, elevated hepcidin levels do not appear sufficient to induce anemia. Other hepcidin-independent mechanisms induced by pro-inflammatory cytokines, in particular IL-6, leading to anemia are likely to be involved.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

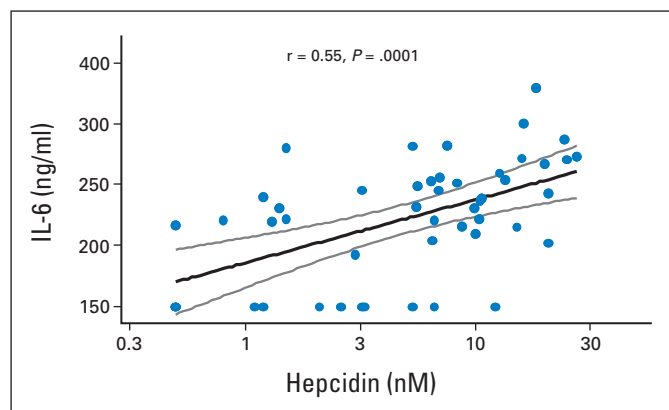


Fig 4. Correlation between hepcidin and cytokines. Scatter plot with linear regression (black line) and 95% CIs (gray lines) shows significant correlation between hepcidin and interleukin 6 (IL-6).

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AUTHOR CONTRIBUTIONS

Conception and design: Stefan Hohaus, Giuseppina Massini, Manuela Giachelia, Maria Teresa Voso, Giuseppe Leone
Financial support: Giuseppe Leone
Provision of study materials or patients: Stefan Hohaus, Giuseppina Massini, Manuela Giachelia, Barbara Vannata, Valentina Bozzoli, Annarosa Cuccaro, Francesco D'Alo', Luigi Maria Larocca

Collection and assembly of data: Stefan Hohaus, Giuseppina Massini, Manuela Giachelia, Barbara Vannata, Valentina Bozzoli, Annarosa Cuccaro, Francesco D'Alo'
Data analysis and interpretation: Stefan Hohaus, Giuseppina Massini, Reinier A.P. Raymakers, Dorine W. Swinkels, Maria Teresa Voso
Manuscript writing: Stefan Hohaus, Giuseppina Massini, Luigi Maria Larocca, Reinier A.P. Raymakers, Dorine W. Swinkels, Maria Teresa Voso, Giuseppe Leone
Final approval of manuscript: Stefan Hohaus, Giuseppina Massini, Manuela Giachelia, Barbara Vannata, Valentina Bozzoli, Annarosa Cuccaro, Francesco D'Alo', Luigi Maria Larocca, Reinier A.P. Raymakers, Dorine W. Swinkels, Maria Teresa Voso, Giuseppe Leone

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