Role of Hepcidin in Anemia of Chronic Disease in Rheumatoid Arthritis

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Abstract

Objective   Anemia of chronic disease is a frequent consequence in rheumatoid arthritis and is associated with major clinical and patient outcomes. The present cross-sectional study explored the role of hepcidin (HEP) in anemia of chronic disease in rheumatoid arthritis by studying its relationships with markers of anemia, iron metabolism, inflammation, and erythropoiesis.

Methods   Blood samples from anemic ($n = 43$) and nonanemic ($n = 43$) rheumatoid arthritis patients were analyzed for markers of anemia (hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cells distribution width, and reticulocyte hemoglobin), iron metabolism (iron, total iron binding capacity, ferritin, transferrin saturation, soluble transferrin receptor), inflammation (erythrocyte sedimentation rate, C-reactive protein, and interleukin 6), and erythropoiesis (erythropoietin and HEP). Correlation analysis was used to identify relationships between HEP and all other variables. Principal component analysis was used to identify common underlying dimensions representing linear combinations of all variables.

Results   HEP had statistically significant mostly moderate-to-large correlations with markers of anemia (0.30–0.70, all $p < 0.01$), small correlation with markers of iron metabolism and markers of inflammation ($r = 0.20$–0.40, all $p < 0.01$), and moderate correlations with markers of erythropoiesis. Principal component analysis revealed two underlying components (factors) capturing approximately 50% of total variability. Factor 1 comprised mainly of markers of anemia, iron metabolism, and erythropoiesis and was related to "erythrocyte health status," while factor 2 comprised mainly markers of inflammation and iron metabolism and was related to "acute phase reactants." HEP was the only variable demonstrating substantial loadings on both factors.

Conclusions   HEP is related to markers of anemia, iron metabolism, inflammation, and erythropoiesis. In addition, when all variables are "reduced" to a minimum number of two "latent" factors, HEP is loaded on both, thus underlying its pivotal role in the complex interaction of the erythropoietic response in inflammation-induced anemia and/or functional iron deficiency.

Keywords
► principal component analysis
► iron
► functional iron deficiency
► dimension reduction

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune inflammatory disorder that affects multiple joints and is characterized by a number of extra-articular manifestations such as rheumatoid nodules, vasculitis, lung or heart disease, anemia of chronic disease (ACD), and peripheral neuropathy. In fact, ACD has been identified as a predictor of radiographic progression as well as an indicator of active clinical or subclinical inflammatory state.[1,2] In addition, anemia in RA may contribute to secondary manifestations such as fatigue, reduced mobility, cardiovascular conditions, and increased hospitalization.[3,4] Thus, in order to provide an index of the patient's level activity and progression, the DAS28 composite score has been proposed.[5] However, although hemoglobin levels are considered an index of system inflammation, they are not included in the disease activity scores (DAS), while more "traditional" markers of inflammation such as erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) are included.[6,7] ACD in RA has a multifacet pathogenesis that implicates changes in iron metabolism, impairment of erythropoiesis, downregulation of response to erythropoietin (EPO), or most likely a combination of all the above.[8] Thus, an index of anemia implicated in iron metabolism, erythropoiesis, and inflammation could provide more global information regarding the complex interaction of homeostatic markers of erythropoiesis/iron metabolism and mediators of inflammation.[9-13] In this regard, hepcidin (HEP) has received increased attention.[9,14] In this complex interplay of anemia, inflammation, and iron metabolism, HEP (along with EPO) is considered a mediator of hematopoiesis, but its production is also influenced by markers of inflammation such as interleukin-6 (IL-6) and tumor necrosis factor alpha.[15] Typically, HEP binds to cell membrane-localized ferroportin and initiates the internalization and degradation of ferroportin that results in rapid iron sequestration into specialized storage cells and iron retention in the duodenal epithelium.[16]

Thus, the aim of the present cross-sectional observational study was to assess the relationships of HEP with markers of anemia, iron metabolism, inflammation, and erythropoiesis.

Methods

Patients

Forty-three consecutive unselected RA patients with ACD (age = 69.0 [interquartile range, IQR: 14] years, weight = 65.7 ± 10.9, height = 1.63 ± 0.07 m, and disease duration = 18.0 [IQR: 16]) and 43 age- and gender-matched RA patients (age = 69.0 [IQR: 14] years, weight = 74.3 ± 10.0, height = 1.62 ± 0.07 m, and disease duration = 18.0 [IQR: 15]) without anemia were studied. All patients fulfilled the American College of Rheumatology criteria for RA[6] and all patients were taking disease-modifying antirheumatic drugs (DMARD). Coexisting morbidities that were of higher prevalence in the anemic group were hypertension, diabetes, impaired thyroid function, extra-articular RA, and gastrointestinal symptoms, while there was no difference for chronic obstructive pulmonary disease and osteoporosis. Anemia

was defined as hemoglobin (Hb) ≤ 11.5 g/dL for women and ≤ 13.5 g/dL for men. Exclusion criteria included pregnancy or lactation, blood diseases (i.e., thalassemia and sickle cell anemia), active gastrointestinal bleeding or a history of gastrectomy, a history or presence of malignancy, chronic renal failure (serum creatinine ≥ 1.6 mg/dL), cirrhosis, alcoholism, coexistence of serious illness (i.e., heart or lung disease and chronic infection), blood transfusion, or treatment with iron, vitamin B_{12}, or EPO in the last months before entry in the study. In addition, patients were excluded in case of anemia associated with the use of DMARDs such as megaloblastic anemia associated with methotrexate or anemia by decreased erythrocyte production in bone marrow due to azathioprine. There was no difference between groups for corticosteroids, immunosuppressive, or biological inhibitors. Ethical approval for patient participation was obtained from the local University Hospital Review Board for experiments involving human subjects. Written informed consent was provided by all participants.

Clinical and Laboratory Assessment

Demographic characteristics, markers of anemia, iron metabolism and inflammation, as well as mediators of erythropoiesis were collected in all participants on their outpatient visit. In addition, all patients underwent a complete physical examination to evaluate articular and extraarticular manifestations of the disease. Blood samples were collected in the morning, after an overnight fast and serum was separated by centrifugation at 1500 g for 15 min. Markers of anemia (Hb, mean corpuscular volume [MCV], mean corpuscular hemoglobin [MCH], mean corpuscular hemoglobin concentration [MCHC], red cells distribution width [RDW], and reticulocytes hemoglobin [RETHb]) were measured with an automated hematology analyzer (Sysmex XE-500; Sysmex, Japan). For the markers of iron metabolism serum iron (IRON) and total iron binding capacity (TIBC) were determined with the Beckman AU 5800 analyzer (Beckman Coulter Inc., United States), while transferrin saturation (TFsat) was calculated as the ratio of IRON/TIBC[17]; ferritin (FER) and soluble transferrin receptor (sTFr) were measured with the Beckman DxI 800 analyzer (Beckman Coulter Inc.). Regarding markers of inflammation, ESR was measured with the Alifax Test 1 analyzer (Alifax SrL, Italy), CRP was measured with the Beckman Coulter Immage 800 analyzer (Beckman Coulter Inc.), and IL-6 was measured with the Siemens Immulite 2000 analyzer (Siemens Healthcare Diagnostics Inc., Germany). Finally, regarding the mediators of erythropoiesis, EPO was measured with the Beckman DxI 800 analyzer (Beckman Coulter Inc.) and HEP levels were determined with the DRG Hepcidin 25 HS ELISA immunoassay (DRG Instruments GmbH, Germany). DAS were derived on all patients using a four-variable, composite DAS28, based on 28 joint evaluations and the ESR.

Statistical Analyses

Correlations between HEP and markers of anemia, iron metabolism, inflammation, and erythropoiesis were assessed by Spearman's rank correlation coefficient (± 95% confidence interval). The magnitude of correlations was

assessed according to Cohen's criteria: trivial (< 0.1), small (0.11–0.3), moderate (0.31–0.5), large (0.51–0.70), very large (0.71–0.90), and almost perfect (> 0.90).[18] The level of significance was set at the 0.05 level.

In addition, we performed data reduction between the measured parameters using principal component analysis (PCA) with either orthogonal (varimax) or oblique (direct oblimin) rotation on the above 17 variables.[19] PCA is an exploratory mathematical procedure that tries to explain the maximum amount of total variance in a correlation matrix by transforming the original variables into linear components in a multidimensional space with a common eigenvector, thereby reducing the data complexity by a common extent of cumulative variability.[19] Thus, sets of variables are "reduced" in to a short list of factors (i.e., components) each one representing "latent" variable. The strength of the association between each of the original variables and the newly derived factor(s) is quantified by using loadings (ranging from –1 to 1, similar to correlation coefficients). Loadings of parameters > 0.4 are considered to be relevant for the respective dimension; that is, a variable with a loading < 0.4 does not associate appreciably with the respective factor.[19] Based on the similarities between the variables that load the most on a factor, a "theme" can be ascribed to that particular factor representing a common underlying dimension associated with the respective combination of variables.[19] The PCA method does not include a formal testing for significance; in addition given the sample size of our study, we elected to limit our analysis to the extraction of 2 factors.[20]

All statistical analyses were performed with IBM SPSS Statistics v.23 (Armonk, New York, United States).

Results

HEP had statistically significant mostly moderate negative relationships with markers of anemia with the exception of RDW that was positive (►Table 1). In addition, HEP correlated negatively with all markers of iron metabolism with the exception of a positive correlation with sTFr (►Table 1). Finally, HEP had small-to-moderate positive correlations with all markers of inflammation and erythropoiesis (►Table 1).

All markers of anemia loaded significantly on factor 1 of the PCA. All markers of inflammation loaded on factor 2 (►Table 1). Some markers of iron metabolism loaded significantly on factor 1 (IRON, TFsat, sTFr), while the rest (TIBC, FER) loaded significantly on factor 2 (►Table 1). All markers of erythropoiesis (HEP, EPO) loaded on factor 1; HEP had also a significant loading on factor 2 (►Table 1). ►Fig. 1 represents the loadings of every single variable on both factors in rotated space as well as the unique combination of markers of anemia, iron metabolism, inflammation, and erythropoiesis that "reduces" to the corresponding factors. HEP is the only variable that contributes to both factors.

Discussion

The present cross-sectional observational study aimed to clarify relationships between HEP and a comprehensive set of variables covering markers of anemia, iron metabolism, inflammation, and erythropoiesis. Our results revealed that in a group of anemic and nonanemic RA patients, HEP is correlated (to varying magnitudes) to markers anemia, iron metabolism, inflammation, and erythropoiesis (►Table 1). This is in accordance with previous studies

Table 1 Spearman correlation coefficients (90% CI) between HEP and the markers of anemia, iron metabolism, inflammation, and erythropoiesis

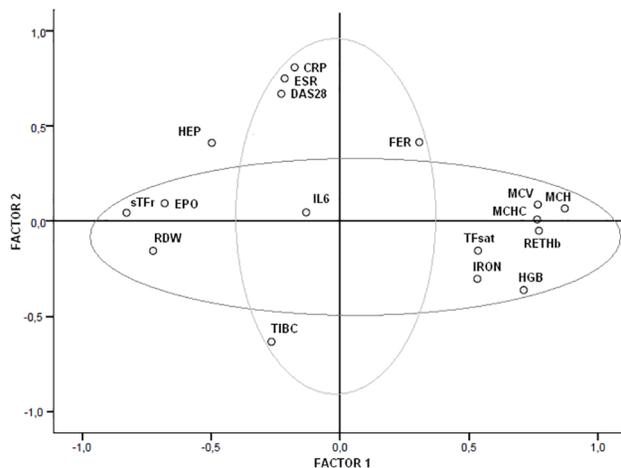|	Markers of anemia					
	Hb	MCV	MCH	MCHC	RETHb	RDW
HEP	0.73 (0.65; 0.78) very large	0.27 (0.06; 0.45) small	0.35 (0.15; 0.53) moderate	0.31 (0.13; 0.47) moderate	0.29 (0.10; 0.45) small	0.31 (0.10; 0.48) moderate
	Markers of iron metabolism					
	IRON	TIBC	FER	TFsat	sTFr	
HEP	0.27 (0.07; 0.49) small	0.20 (0.03; 0.43) small	0.05 (0.17; 0.27) trivial	0.22 (0.01; 0.43) small	0.41 (0.22; 0.58) moderate	
	Markers of inflammation					
	ESR	CRP	IL-6	DAS28		
HEP	0.15 (0.10; 0.38) Small	0.10 (0.15; 0.35) unclear	0.13 (0.11; 0.35) unclear	0.24 (0.04; 0.48) small		
	Markers of erythropoiesis					
	EPO					
HEP	0.41 (0.24; 0.56) moderate					

Fig. 1 Component plot of the extracted factors in rotated space. CRP, C-reactive protein; DAS28, disease activity score 28; EPO, erythropoietin; ESR, erythrocyte sedimentation rate; FER, ferritin; Hb, hemoglobin; HEP, hepcidin; IL-6, interleukin 6; IRON, iron metabolism serum iron; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; RDW, red cells distribution width; RETHb, reticulocytes hemoglobin; TIBC, total iron binding capacity; sTFr, soluble transferrin receptor; TFsat, transferrin saturation.

demonstrating similar associations between HEP and anemia markers[21-25] or iron metabolism[25-29] however, others have reported no correlation.[23,30-32]

In order to better understand the dynamics of this complex correlation matrix, we performed data reduction using PCA where linear combinations of the original variables were "reduced" to single factors.[19] Our PCA analysis revealed that regarding the factor 1, explaining approximately 1/3 of total variance, variables that had significant loadings were all markers of anemia, markers of iron metabolism (IRON, TFsat, and sTFr), and erythropoiesis (EPO and HEP). Anemic state in chronic disease is influenced by the complex interaction between inflammation, erythropoiesis, and iron metabolism[15,33-35] however, it is not clear to what extend anemia is merely a marker of disease severity and progression or a causative factor that specifically impacts on the underlying inflammatory process by the progression of joint destruction.[15,34] Inflammation-induced cytokines activate HEP production (IL-6) and increased levels of HEP induce a state of "functional iron deficiency"[33] however as the disease progresses low, IRON levels may actually exert inhibitory effects on HEP production.[15] On the other end of the spectrum low IRON availability further activates erythropoiesis, while EPO may act regulatory effects on HEP.[15] This loop of inflammation-induced erythropoiesis-iron availability-anemia is depicted in factor 1 of our PCA analysis.

We propose that the longitudinal monitoring of these markers may be useful in patient evaluation as the disease progresses through time. For example, MCH and MCHC directly measure Hb content per red blood cell (RBC) and RETHb provides direct information regarding the iron supply to red-cell precursors.[36] In addition, it has been demonstrated that, at least in the early stages of RA, the

upregulation of IL-6 suppresses the maturation of erythrocytes in bone marrow, which leads to the immature erythrocytes entering the circulation, resulting in the changes of RDW level.[37,38] Furthermore, it has been proposed that RDW can be influenced not only by anemia but also from inflammation.[39] Similarly, sTFr has been proposed as a marker reflecting iron status, namely to identify functional iron deficiency in RA.[40] HEP had the lowest loading on factor 1, which is not unexpected given the cascade of events that span from the level of stimulation of these markers to the level of "disturbed" iron status at the single RBC.[15] Therefore, the combination of the above variables is considered to measure different aspects of some common underlying dimension just as suggested by our PCA.[19] Given that most variables that had significant loading on factor 1 are associated with the individual erythrocyte level (MCH, MCHC, RETHb, sTFr), it can be concluded that factor 1 represents a global index of "RBC health status."

Our PCA analysis further demonstrated the existence of second (independent) factor explaining approximately 16% of total variance. Variables having substantial loadings were markers of inflammation (CRP and ESR), clinical activity (DAS28), another subset of iron metabolism (TIBC and FER), and finally HEP. Given that TIBC actually reflects transferrin levels,[41,42] both TIBC and FER are also considered acute phase protein along with CRP.[43,44] In fact TIBC as a surrogate of transferrin had a strong negative loading on factor 2, while FER and CRP had a positive loading that demonstrates their role as "negative" and "positive" acute phase protein, respectively.[45] Albeit weak, HEP did have a meaningful loading on factor 2 also that may underscore its function in inflammation[14] and also justify the small correlations between HEP and inflammation markers (►**Table 1**). In this regard, our results are in agreement with previous studies demonstrating that HEP represents the link between the "crossroad" of anemia and inflammation,[23,25,35,46,47] although not clinical disease activity[28,48] however, given that neither ESR nor CRP is included in the composite activity analysis,[7] it is not surprising that DAS28 had also substantial loading on factor 2. Thus, it can be concluded that factor 2 represents a global index of "acute phase reactants."

The pattern that emerged from our analysis is that the majority of variables (MCH, sTFr, MCHC, RETHb, RDW, Hb, EPO, MCV, TFsat, IRON) loaded highly on "RBC health status" and considerably less on "acute phase reactants," while the opposite was true for the remaining set of variables (CRP, ESR, DAS28, TIBC, FER) (►**Table 2**). The exception was HEP that presented similar loadings on both factors. On one hand from a physiological/clinical perspective, this may represent the pivotal role of HEP in the interaction between anemia and inflammation outlined above.[14,23,35,47] On the other hand from a "statistical" perspective, if a variable has similar-sized loadings across two or more factors this could be due to the factors representing similar constructs (that is being correlated) or that is not a good index distinguishing those constructs.[19] Given that the two rotations produced similar solutions, "RBC health status" and "acute phase reactants" are likely independent dimensions; this is further supported

Table 2 Loading of each variable on the extracted factors

	Component	
Variable	1	2
MCH	0.845	
sTFr	0.825	
MCHC	0.764	
RETHb	0.750	
RDW	0.726	
Hb	0.715	
EPO	0.690	
MCV	0.606	
TFsat	0.556	
IRON	0.549	
Hepcidin	0.504	0.410
IL-6		
CRP		0.809
ESR		0.751
DAS		0.674
TIBC		0.642
FER		0.426

Abbreviations: CRP, C-reactive protein; DAS, disease activity score; EPO, erythropoietin; ESR, erythrocyte sedimentation rate; FER, ferritin; Hb, hemoglobin; IL-6, interleukin 6; IRON, iron metabolism serum iron; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; RDW, red cells distribution width; RETHb, reticulocytes hemoglobin; TIBC, total iron binding capacity; sTFr, soluble transferrin receptor; TFsat, transferrin saturation.

by the fact that no other variable exhibited substantial loadings (> 0.4) on both factors. Thus, the possibility remains that HEP cannot distinguish well either "RBC health status" or "acute phase reactants." However, given that both loadings of HEP were above the cutoff value of 0.4, it provides some evidence in favor of its diagnostic potential.[14,49] Thus, it can be concluded that HEP can be viewed as an index of "RBC health status" in the presence of inflammation.[50]

The present findings should be interpreted in view of potential limitations. The cross-sectional design limits any causal inferences and is inherent in all observational studies. In addition, PCA is used as an exploratory technique and the corresponding loadings do not provide biological associations. Furthermore, PCA analysis limits conclusions to the sample collected and generalization of results requires cross-validation of the factor structure in a different sample. In this regard, PCA was used to generate future hypotheses, mainly regarding the diagnostic potential of HEP in a complex state of various degrees of inflammation, anemia, and iron availability.

In conclusion, we demonstrated that HEP shows significant relationships with markers of anemia, iron metabolism, inflammation, and erythropoiesis in a population of anemic and nonanemic RA patients. Furthermore, reducing the combination of these markers onto two "latent" factors describing "RBC health status" and "acute phase reactants," HEP demonstrated significant loading on both factors. However, despite the pivotal role of HEP in these relationships approximately half of total data variability remained unexplained. Larger studies are needed to identify more "latent" variables than can potentially explain more variability and/or ascribe more physiological roles on HEP.

Abbreviations: CI, confidence interval; CRP, C-reactive protein; DAS28, disease activity score 28; EPO, erythropoietin; ESR, erythrocyte sedimentation rate; FER, ferritin; Hb, hemoglobin; HEP, hepcidin; IL-6, interleukin 6; IRON, iron metabolism serum iron; MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; RDW, red cells distribution width; RETHb, reticulocytes hemoglobin; TIBC, total iron binding capacity; sTFr, soluble transferrin receptor; TFsat, transferrin saturation.

Conflict of Interest
None.

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