ORIGINAL ARTICLE

Serum prohepcidin and other iron metabolism parameters in elderly patients with anemia of chronic disease and with iron deficiency anemia

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KEY WORDS

anemia of chronic disease, iron deficiency anemia, prohepcidin

ABSTRACT

INTRODUCTION   The diagnosis of iron deficiency anemia (IDA) in the context of the anemia of chronic disease (ACD) in elderly patients is often difficult due to the existence of many disorders. Recent studies have shown that hepcidin measurement (combined with the existing diagnostic methods) may possibly help in the differential diagnosis of IDA and ACD.

OBJECTIVES   The aim of the study was to evaluate the differential diagnostic value of serum prohepcidin in elderly patients with IDA and ACD.

PATIENTS AND METHODS   The study included 65 individuals aged 65 years or more: 26 patients with ACD, 13 patients with IDA, and 26 age-matched controls. Prohepcidin, ferritin, soluble transferrin receptor, erythropoietin, and interleukin 6 (IL-6) were measured using the commercially available enzyme-linked immunosorbent assay kits. Complete blood count, total iron-binding capacity (TIBC), and iron, transferrin, and C-reactive protein (CRP) levels were assayed using the standard laboratory methods.

RESULTS   Prohepcidin concentrations were similar in patients with ACD (196.59 ng/ml) compared with those with IDA (230.16 ng/ml) ($P = 0.35$). Patients with ACD had significantly lower levels of TIBC compared with those with IDA ($P < 0.0001$). Serum ferritin concentration in patients with ACD was almost 20-fold higher compared with those observed in patients with IDA ($P < 0.0001$). CRP and IL-6 concentrations in patients with ACD were significantly higher compared with those with IDA.

CONCLUSIONS   The results of the study indicate that serum prohepcidin has limited value in the differential diagnosis of IDA and ACD in elderly patients.

INTRODUCTION   Anemia of chronic disease (ACD) and iron-deficiency anemia (IDA) are common hematological conditions present in elderly patients.[1-3] They are a risk factor for cardiovascular diseases, contribute to fatigue, and negatively affect cognitive and physical functions as well as the quality of life.[4] ACD is a typically micro- or normocytic, normochromic anemia associated with chronic infections, chronic noninfectious inflammatory diseases, and malignancies. The pathogenesis of ACD is multifactorial. It involves impaired erythropoietin-dependent erythropoiesis caused by proinflammatory cytokines (interleukin 6 [IL-6], C-reactive protein [CRP], and tumor necrosis factor-α), impaired iron mobilization, and shortened red blood cell survival.[4-8] IDA develops, in the most part, due to chronic blood loss from the gastrointestinal tract, caused by aspirin or other nonsteroidal anti-inflammatories, ulcers, colon cancer, or diverticulosis.[3] Other causes of chronic blood loss or iron deficiency due to poor nutrition in this patient group are less likely.[9-12] In the elderly population, severe IDA may also affect the production of other proteins containing Fe^{2+}, such as cytochromes, myoglobin, catalase, and peroxidase.[13]

IDA is also frequently diagnosed in patients with chronic heart failure.[14]

The diagnosis of IDA within the context of ACD in elderly patients is often difficult due to the presence of multiple abnormalities.[15] The diagnosis of ACD is usually made on the basis of a low serum iron concentration, low or normal total iron-binding capacity (TIBC), low transferrin saturation, and increased ferritin combined with the presence of chronic disease. The diagnosis of IDA includes measurement of serum iron, transferrin saturation, and serum ferritin. However, the reference diagnostic criteria for these parameters are not available for this population.[16]

The standard treatment of IDA requires the administration of iron therapy to rebuild iron stores, while the administration of iron in ACD may even worsen the underlying disease. For this reason, an accurate assay, with high specificity and sensitivity, for the differential diagnosis of ACD and IDA in elderly patients is extremely important.

In 2000, hepcidin was found to be a link between the immune system and the transport of iron to and from the storage pool.[17,18] Circulating hepcidin binds to ferroportin (iron exporter present in the membrane of macrophages, hepatocytes, and enterocytes), inducing receptor--ligand internalization and lysosomal degradation in the cytoplasm.[19,20] As a result, hepcidin regulates iron absorption in the small intestine as well as iron release from the reticuloendothelial macrophages.[19,20] In 2002, Nicolas et al.[21] discovered that the gene encoding hepcidin was regulated in response to anemia, hypoxia, and inflammation. Studies indicate that IL-6 and other inflammatory mediators may increase hepcidin concentrations in human serum through the JAK2/STAT3 signaling pathway.[22-24]

Elderly persons are often affected by a chronic mild proinflammatory state characterized by elevated levels of proinflammatory markers, including IL-6 and CRP. There is a hypothesis that this mild inflammatory state may cause a chronic elevation of circulating hepcidin and, consequently, develop anemia. A link between hepcidin and ACD has been shown by Fleming and Sly.[5] Hepcidin assays may facilitate the diagnosis of IDA and ACD, probably in combination with the existing diagnostic methods.

The aim of this study was to evaluate the differential diagnostic value of serum prohepcidin in different causes of anemia in elderly patients.

PATIENTS AND METHODS The study group consisted of 39 patients (17 men and 22 women) aged between 65 to 91 years (mean age, 78.92 ±8.32 years) admitted to the Department and Clinic of Geriatrics of the Collegium Medicum in Bydgoszcz, Poland, with the diagnosis of anemia (World Health Organization definition). Based on the physical examination and such parameters as serum iron, ferritin, TIBC, and transferrin saturation (according to the algorithm proposed by Balducci),[25] patients were divided into 2 subgroups: group 1 including

26 patients (67%) with ACD, and group 2 including 13 patients (33%) with IDA. The exclusion criteria were chronic kidney disease and cancer.

The control group consisted of 26 healthy non-obese elderly volunteers (11 women, 15 men) between 65 and 88 years of age (mean age, 68.46 ±7.47). The exclusion criteria were chronic inflammatory diseases, anemia, undernutrition, cancer, rheumatic disorders, inflammatory bowel diseases, endocrine diseases, and use of steroid or nonsteroidal anti-inflammatory drugs. All persons who took any vitamin and mineral supplements were also excluded from the study.

Peripheral blood samples were taken after the diagnosis of anemia had been established but prior to the administration of treatment.

All procedures in the study were approved by the local ethics committee. Informed written consent was obtained after the purpose, nature, and potential risks were explained to subjects.

Analytical methods Levels of prohepcidin were determined by a stable enzyme-linked immunosorbent assay (ELISA) (Hepcidin Prohormone ELISA, DRG Instruments GmbH, Germany). Serum iron concentrations and TIBC were measured using the Architect c8000 System (Abbott Laboratories, United States). Serum ferritin was quantified using the DRG Ferritin kit (EIA-1872, DRG International Inc., United States). Serum soluble transferrin receptor (sTfR) concentrations were examined by an ELISA (Human sTfR ELISA, Bio-Vendor Laboratory Medicine Inc., Czech Republic). CRP concentrations in serum were determined using BNTMII Systems (Dade Behring, Marburg, Germany). IL-6 concentrations were determined using the IL-6 kit (Bender MedSystems GmbH Vienna, Austria). Plasma erythropoietin (EPO) was determined by the EPO ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany). Complete blood cell count was analyzed using the Sysmex XE-2100 (Roche Diagnostics GmbH).

Statistical analysis All statistical analyses were performed using the Statistica 8.0 software (Stat-Soft, Cracow, Poland). The normal distribution of the variables was evaluated using the Shapiro-Wilk test. Parametric data were expressed as mean, standard deviation, minimum and maximum. Nonparametric parameters were presented by median and percentile values. An independent sample t test for parametric continuous variables and the Mann-Whitney U test for nonparametric continuous variables were used to compare the difference between the 2 groups of patients. The relationships between the examined parameters were determined by the Spearman's rank-order correlation. A P value of less than 0.05 was considered statistically significant.

RESULTS The subgroups (ACD vs. IDA) did not differ in terms of age (TABLE 1). There were nonsignificant differences between the subgroups in the body mass index and body fat percentage

TABLE 1 Hematological indices in patients with anemia of chronic disease and iron deficiency anemia

Parameter	Study groups		Control group	P
	anemia of chronic disease I (n = 26)	iron deficiency anemia II (n = 13)	no anemia III (n = 26)	
age, y	79.78 ±8.27 (65.83–91.33)	79.00 ±8.11 (62.75–91.25)	68.46 ±7.47 (65.17–88.33)	NS (I vs. II) NS (I vs. III) NS (II vs. III)
BMI, kg/m²	26.30 ±5.61 (18.38–45.71)	28.28 ±4.81 (24.02–39.64)	31.44 ±4.11 (23.95–38.50)	NS (I vs. II) 0.002 (I vs. III) NS (II vs. III)
%FM, %	29.39 ±10.52 (7.60–44.25)	31.46 ±7.63 (17.58–41.85)	39.54 ±5.97 (29.30–48.15)	NS (I vs. II) 0.0008 (I vs. III) 0.004 (II vs. III)
HGB, g/dl	10.28 ±1.60 (7.00–12.9)	10.16 ±1.85 (7.10–12.90)	14.07 ±1.34 (12.30–16.90)	NS (I vs. II) <0.0001 (I vs. III) <0.0001 (II vs. III)
HCT, %	32.34 ±4.74 (22.30–40.40)	32.98 ±6.38 (20.90–41.70)	42.00 ±3.37 (36.10–50.70)	NS (I vs. II) <0.0001 (I vs. III) 0.0001 (II vs. III)
MCV, fl	91.52 ±4.99 (81.90–99.40)	82.88 ±7.32 (68.70–94.10)	92.49 ±4.22 (85.50–101.5)	0.003 (I vs. II) NS (I vs. III) 0.0003 (II vs. III)
RBC, 10⁶/µl	3.54 ±0.54 (2.43–4.31)	4.01 ±0.85 (2.23–5.21)	4.55 ±0.50 (3.78–5.63)	NS (I vs. II) <0.0001 (I vs. III) 0.028 (II vs. III)
WBC, 10³/µl	10.79 ±3.52 (6.10–20.58)	7.16 ±2.19 (3.05–10.48)	5.85 ±1.13 (3.80–8.33)	0.003 (I vs. II) <0.0001 (I vs. III) 0.032 (II vs. III)

Results are reported as mean ± standard deviation (range).

Abbreviations: BMI – body mass index, HCT – hematocrit, HGB – hemoglobin, MCV – mean corpuscular volume, NS – nonsignificant, RBC – red blood cells, WBC – white blood cells, %FM – body fat percentage

(%FM). %FM was significantly higher in the control group compared both with patients with ACD and those with IDA (TABLE 1).

The hematological parameters are shown in TABLE 1. As expected, hemoglobin, hematocrit, and erythrocyte count in both subgroups were significantly lower than in the control group. However, there was no difference in these parameters between patients with IDA and those with ACD. Moreover, patients with ACD had the highest white blood cell count among all groups. The mean corpuscular volume (MCV) in patients with IDA was significantly lower than that observed in patients with ACD and controls. There was no significant difference in the MCV between patients with ACD and controls (91.52 fl vs. 92.49 fl; $P = 0.06$).

We demonstrated that serum prohepcidin concentrations were significantly lower in elderly patients with anemia (both ACD [196.59 ng/ml] and IDA [230.16 ng/ml]) compared with controls (292.18 ng/ml) (TABLE 2). However, no significant difference was observed between the subgroups of patients with IDA and those with ACD ($P = 0.3456$). Moreover, the 2 subgroups did not differ significantly in serum iron, sTfR, and EPO. In both subgroups, serum iron concentrations were significantly lower compared with the control group. Patients with ACD had significantly lower levels of TIBC compared with patients with IDA and controls. The serum ferritin concentration in ACD patients (186.33 ng/ml) was almost 20-fold higher compared with patients with IDA (9.18 ng/ml, $P < 0.0001$) and about 4-fold higher than in the control group (42.62 ng/ml, $P < 0.0001$) (TABLE 2). Furthermore, CRP and IL-6 concentrations in the group of patients with ACD were significantly higher compared with patients with IDA and controls (TABLE 2).

There was a significant positive correlation between prohepcidin and ferritin in patients with ACD ($r = 0.55$, $P < 0.03$). In patients with IDA, there was a negative correlation between prohepcidin and IL-6 ($r = -0.58$, $P = 0.047$) and a positive correlation with WBC ($r = 0.6573$, $P = 0.02$). In the control group, the serum prohepcidin level did not correlate with any hematological and iron parameters or inflammatory markers.

DISCUSSION The differential diagnosis of IDA and ACD in elderly patients is often difficult because of the lack of good differentiating biochemical indicators (with high sensitivity and specificity). Each of the routine biochemical markers

TABLE 2 Iron metabolism parameters in patients with anemia of chronic disease and iron deficiency anemia

Parameter	Study groups		Control group	P
	anemia of chronic disease I (n = 26)	iron-deficiency anemia II (n = 13)	no anemia III (n = 26)	
prohepcidin, ng/ml	196.59 165.4; 231.6	230.16 198.4; 241.1	292.18 257.8; 335.8	NS (I vs. II) 0.0002 (I vs. III) 0.0277 (II vs. III)
iron, µg/dl	22.55 16.55; 37.20	30.75 13.35; 38.95	81.60 70.00; 92.00	NS (I vs. II) <0.0001 (I vs. III) <0.0001 (II vs. III)
sTfR, µg/ml	2.27 1.47; 3.03	2.29 1.77; 6.39	1.56 1.11; 2.03	NS (I vs. II) NS (I vs. III) 0.0165 (II vs. III)
TIBC, µg/dl	171.41 130.20; 212.00	309.01 274.64; 333.51	257.00 229.0; 337.8	<0.0001 (I vs. II) <0.0001 (I vs. III) NS (II vs. III)
ferritin, ng/ml	186.33 109.01; 332.76	9.18 2.24; 24.51	42.62 31.66; 75.50	<0.0001 (I vs. II) <0.0001 (I vs. III) 0.0002 (II vs. III)
EPO, U/l	12.89 7.48; 17.28	8.54 4.79; 33.06	4.91 3.76; 8.64	NS (I vs. II) 0.0009 (I vs. III) NS (II vs. III)
CRP, mg/l	80.75 45.15; 124.0	5.50 3.19; 7.40	3.19 3.00–5.00	<0.0001 (I vs. II) <0.0001 (I vs. III) 0.047 (II vs. III)
IL-6, pg/ml	12.20 1.95; 15.99	0.17 0.00; 2.19	0.10 0.00; 1.11	0.018 (I vs. II) <0.0001 (I vs. III) NS (II vs. III)

Results are reported as medians (Q1; Q3) – 25th and 75th percentiles.

Abbreviations: CRP – C-reactive protein, EPO – erythropoietin, IL-6 – interleukin 6, sTfR – soluble transferrin receptor, TIBC – total iron-binding capacity, others – see **TABLE 1**

(such as transferrin saturation, CRP, ferritin, or transferrin receptor) has disadvantages, especially in the context of the older population.[10,26-28] In contrast to the increased hepcidin levels in ACD,[22,23,28,29] the classic IDA in humans is associated with low hepcidin expression.[30,31] This makes hepcidin a potential marker discriminating between IDA and ACD.

In the present paper, we examined the usefulness of measuring prohepcidin levels in the differentiation of IDA from ACD and compared prohepcidin with the traditional parameters of the iron status. We also analyzed the associations between prohepcidin levels and hematological, biochemical, and inflammatory parameters in both types of anemia. Our data showed that circulating prohepcidin levels in patients with ACD were similar to those observed in the group of patients with IDA, and the difference between the groups was not statistically significant.

Our findings are in line with the recent observations of Dallalio et al.,[32] who reported that hepcidin levels, measured by Western blotting, were approximately the same in the 3 different types of anemia. However, another study, which analyzed urinary hepcidin levels,[31] reported contradictory results. The authors analyzed 2 types of

anemia. The first group consisted of patients with ACD and inflammation diagnosed on the basis of a clinical interview and elevated ferritin levels. The second group included patients with sideropenic anemia. The urinary concentration of hepcidin in patients with anemia of inflammation was higher compared with individuals with IDA.[31] The discrepancies between the results could be due to the wide range of diagnostic methods used for the quantification of hepcidin and prohepcidin in different biological materials.

The analysis of the correlations provided interesting results. The positive correlation between prohepcidin and ferritin ($r = 0.5536$, $P = 0.0323$) was observed in the group of patients with ACD. This finding is consistent with those reported by Małyszko et al.,[29] Dallalio et al.,[32] Nemeth et al.,[31] and Ferrucci et al.[33] and seems to confirm the postulated role of hepcidin in the pathomechanism of ACD and inflammation. As expected, our comparative analysis revealed that patients with ACD had significantly higher levels of ferritin, CRP, and IL-6 compared with patients with IDA. These findings are consistent with the observations by Artz et al.[34] In the group of patients with IDA, we observed a strong positive correlation between serum prohepcidin and leukocyte count ($r = 0.66$,

P = 0.02). However, it should be noted that WBC in this subgroup was in the reference range. Research findings and clinical observations suggest that patients with iron deficiency are more susceptible to infections due to decreased immunity. Iron plays an important role in the maturation, activation, and proliferation of lymphocytes.[35,36] Peyssonnaux et al.[37] revealed that bacteria-activated macrophages and granulocytes are capable of hepcidin synthesis. The correlation between prohepcidin and leukocyte count observed in our study confirms the significant role of prohepcidin in nonspecific immunity.[17,18,38] Furthermore, an inverse correlation between prohepcidin and IL-6 (r = –0.58, *P* = 0.047) was observed in the group of patients with IDA. These findings differ from the results of biological and human studies, which have revealed hepcidin to be type II acute-phase protein.[21,31,39-44]

Ferrucci et al.[33] examined the association between urinary hepcidin levels, proinflammatory markers, and anemia in older persons. The authors found that IL-6 and CRP levels were associated with anemia and low iron status. However, they were not associated with higher urinary hepcidin.[33] Moreover, the authors suggested that increased hepcidin synthesis occurs only in overt inflammation.[33] On the basis of the presented findings, it can be concluded that the regulation of hepcidin expression in the analyzed types of anemia is mediated by different mechanisms. Interestingly, both in ACD and IDA, the hepcidin level did not correlate with the indices of iron status (that is, iron, erythropoietin, saturation of transferrin), which are important determinants of hepcidin regulation.

It should be noted that the majority of studies dealing with the regulation of hepcidin expression were conducted on in-vitro cell cultures or in animal models.[21,31,42-44] Only few clinical studies addressing the problem in question did not analyze the levels of hepcidin expression in real clinical conditions, but rather in experimentally induced states.[43,45,46] Moreover, in view of the recent findings on the relationship between the degree of body adiposity and elevated expression and serum level of hepcidin,[47-50] future studies should consider the potential effect of the nutritional status on circulating hepcidin levels. It is likely that the coexistence of obesity and anemia can limit the value of hepcidin in the differential diagnosis of anemia.

Numerous studies demonstrated that ferritin is more useful than other parameters in differentiating IDA form ACD. According to our study, ferritin levels were almost 20-fold higher in ACD compared with IDA. Ferritin, together with TIBC, IL-6, and CRP, provides the most useful information on the size of the iron pool as well as the stage of the inflammatory process. The major drawback of measuring serum ferritin to identify IDA is that the normal levels may be observed despite iron deficiency in numerous clinical conditions that are common in hospitalized and elderly patients (for example, infections, rheumatic disorders, malignancy, and other chronic illnesses). Thus, ferritin is of limited diagnostic value in elderly patients.

The sTfR assay has been suggested as a sensitive test for iron deficiency in older people. The sTfR concentration increases with iron deficiency but is not affected by acute or chronic inflammatory diseases.[10,26-28] However, Chua et al.[28] and Wians et al.[51] showed that sTfR is comparable to ferritin in diagnosing IDA in elderly patients. Our study also showed no usefulness of sTfR in the differential diagnosis of anemia in this patient group. Plasma sTfR concentrations in patients with ACD were similar (and did not show any statistical difference) to the concentrations observed in patients with IDA. Moreover, it has been demonstrated that both serum ferritin and sTfR increase with age. This limits the use of these parameters as iron status markers in elderly patients.[10,26,27]

The results of our preliminary study do not support the assumption that prohepcidin can be a useful laboratory parameter in the differential diagnosis of ACD and IDA in elderly patients. However, we cannot exclude that the active hormone, hepcidin, demonstrates such a discriminatory potential. An improved assay that measures serum hepcidin is now available[40] and should be used to confirm these results. In addition, future studies should be conducted on a larger study group. Moreover, in some cases, ACD and IDA may coexist. For this reason, including the third subgroup with coexisting IDA and ACD would help to answer the question of whether prohepcidin/hepcidin is useful in the differentiation of IDA from ACD and IDA/ACD.

In conclusion, the results of our study indicate that serum prohepcidin has limited differential diagnostic value and does not help distinguish between IDA and ACD in elderly patients.

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ARTYKUŁ ORYGINALNY

Stężenie surowiczej prohepcydyny oraz innych parametrów gospodarki żelazowej u pacjentów w wieku podeszłym z niedokrwistością chorób przewlekłych oraz niedokrwistością z niedoboru żelaza

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SŁOWA KLUCZOWE

niedokrwistość chorób przewlekłych, niedokrwistość z niedoboru żelaza, prohepcydyna

STRESZCZENIE

WPROWADZENIE   Rozpoznanie niedokrwistości z niedoboru żelaza (*iron deficiency anemia* – IDA) w kontekście niedokrwistości chorób przewlekłych (*anemia of chronic disease* – ACD) u pacjentów w wieku podeszłym jest często trudne ze względu na istnienie wielu zaburzeń. Najnowsze badania wykazują, że oznaczenie hepcydyny (w połączeniu z istniejącymi metodami diagnostycznymi) może prawdopodobnie pomóc w diagnostyce różnicowej IDA i ACD.

CELE   Celem badania była ocena przydatności surowiczej prohepcydyny w diagnostyce różnicowej IDA oraz ACD u pacjentów w podeszłym wieku.

PACJENCI I METODY   Do badań zakwalifikowano 65 osób w wieku ≥65 lat: 26 pacjentów z ACD, 13 pacjentów z IDA oraz 26 osób w podobnym przedziale wiekowym jako grupę kontrolną. Za pomocą dostępnych komercyjnych testów immunoenzymatycznych mierzono stężenie: prohepcydyny, ferrytyny, rozpuszczalnego receptora transferyny, erytropoetyny i interleukiny 6 (IL-6). Dodatkowo przy użyciu standardowych metod laboratoryjnych oznaczono morfologię krwi, całkowitą zdolność wiązania żelaza (*total iron-binding capacity* – TIBC) oraz poziom żelaza, transferyny i białka C-reaktywnego (CRP).

WYNIKI   Stężenie prohepcydyny u pacjentów z ACD (196,59 ng/ml) było podobne do stężenia obserwowanego u pacjentów z IDA (230,16 ng/ml) (p = 0,35). Pacjenci z ACD mieli znacząco niższy poziom TIBC niż pacjenci z IDA (p <0,0001). Stężenie ferrytyny u pacjentów z ACD było prawie 20-krotnie większe w porównaniu z wartościami uzyskanymi w grupie chorych z IDA (p <0,0001). Stężenie CRP i IL-6 u pacjentów z ACD było istotnie większe niż u chorych z IDA.

WNIOSKI   Wyniki badań wskazują na ograniczoną wartość surowiczej prohepcydyny w diagnostyce różnicowej IDA i ACD u osób w podeszłym wieku.