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Original Research Article

Evaluation of serum hepcidin as a biochemical marker in diagnosis of anemia of chronic disease

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ABSTRACT

Background: Anemia is a serious public health problem. It affects two billion people worldwide, particularly infants and young children mainly in developing countries where its etiology is multifactorial. Anemia in infancy is generally associated with impaired cognitive and behavioral development, impaired oxygen transport and a poorer prognosis in the context of many chronic diseases or chronic infections. The Present study aims to detect the serum hepcidin and ferritin levels in chronic disease anemia and correlate the values of serum hepcidin levels with their serum ferritin levels and IL-6 levels.
Methods: A total of 86 individuals were enrolled in the study. Sample for hematological evaluations were collected and estimation was carried out for biomarker estimation by ELISA method(s) using specified kit(s) procured commercially. The statistical evaluation was done using SPSS version 24.0. Analysis of variance (ANOVA) and Pearson's correlation tests were used to compare the variables and to see the correlation between the different variables.
Results: In present study, we observed statistically significant lower values of RBC count, Hbgm/dl, MCV, MCH, MCHC in ACD group than the normal group. For serum hepcidin when ROC curves and Pearson's scattered plot were made in case of ACD group; the ROC was recorded to be maximum >0.869; with a sensitivity of 84.62% and specificity 94.12% while the confidence level was 95% with an interval of 0.779 to 0.932. Further, the cutoff point determined was >72.93. Thus, the hepcidin level > 72ng/mL and above is related to the ACD. These cut off points had strong confidence interval and valuable predictive potential.
Conclusions: Serum Hepcidin can be used as a simple and cost effective diagnostic marker for identification of anemia.

Keywords: Anemia of chronic disease (ACD), IL-6, Serum hepcidin, Serum ferritin

INTRODUCTION

Anemia is a serious public health problem. It affects two billion people worldwide, particularly infants and young children mainly in developing countries where its etiology is multifactorial).[1-3] Anemia in infancy is generally associated with impaired cognitive and behavioral development, impaired oxygen transport and a poorer prognosis in the context of many chronic diseases, including HIV iron bindings or chronic infections.[4-7] Hepcidin, is a peptide hormone that is synthesized and regulated in response to iron status and the innate immune system, it evolves as the master regulator of iron metabolism, linking iron homeostasis, inflammation, infection and anemia. The molecular control of hepcidin is a part of the innate immune response to pathogens and is stimulated by IL-6, IL-22, type I interferon's, toll-like receptor (TLR) ligands, and the endoplasmic reticulum

stress response.[8] Hepcidin synthesis is reportedly stimulated by elevated plasma iron concentration, infection and/or inflammation, and is suppressed in conditions that demand increased serum iron, such as increased or ineffective erythropoiesis, hypoxia, anemia and also iron deficiency.[9-12]

A number of hepcidin studies have been conducted in adults.[13-15] Ganz et al, created a reference for plasma hepcidin concentration in adult men and women and reported a 5% to 95% range of 29 to 254ng/mL with a median 112ng/mL in men (n=65) and 17 to 286ng/mL with a median 65ng/mL in women (n=49) using the first competitive ELISA.[13] Grebenchtchikov et al, developed a new RIA (CV 4-6% range) and reported significantly higher median hepcidin concentration in men compared to women.[15] However, a study using SELDI-TOF MS did not find a significant difference in hepcidin concentrations between men and women.[14] Galesloot et al, extended these findings using a similar competitive ELISA and established age- and sex-stratified reference ranges (median, 2.5th and 97.5th percentiles) for serum hepcidin concentration using a population-based sample from the Netherlands (n = 2998).[16,17] Participants who were pregnant, had alanine aminotransferase (ALT) >50U/L, CRP >10mg/L, estimated glomerular filtration rate <60mL/min/1.73 m2, using iron supplements, anemic, or had a BMI > 30kg/m2 were not considered to be eligible for the study, however no strict definition of 'healthy' was employed.[18] Grebenchtchikov et al, and Galesloot et al, both reported diurnal variation in hepcidin levels, however their results are inconsistent. Galesloot et al, reported lower hepcidin values from blood samples obtained in the morning (before 12pm) compared to blood samples obtained between 12 and 5 pm in both men and women. In contrast median hepcidin levels were 1.83 and 1.70 times higher at 9 am compared to 4 pm in men and women respectively in a study by Grebenchtchikov et al.[15,16] Hepcidin concentrations were constant over age in men median (2.5th and 97.5th percentiles): 7.8nM (0.6 - 23.3nM) but were higher in postmenopausal compared to premenopausal women (4.1nM (0.4 - 19.7nM) in women <55 years and 8.5nM (1.2- 24.8nM) in women >55 years).[15] In another study, Galesoot et al, reported lower median hepcidin concentrations compared to Ganz et al, and found a less pronounced difference in hepcidin concentrations between men and women. These differences might be due to the fact that the 2 studies used different assays and women in the study by Galesoot et al, had a higher median age (55 years) compared to women in the study by Ganz et al (32.6 years).[13,16] Although the two studies used similar immunochemical assays, they used different antibodies highlighting the need for harmonizing hepcidin assays although the difference in absolute hepcidin values obtained by different assays precludes the comparison of hepcidin concentrations across different assays, among studies using the Ganz assay, hepcidin concentrations in infants are consistently lower compared to those in adults. These infant studies included cord blood samples from healthy term infants, neonates and preterm infants 35 days postnatal.[14,19-21]

In the present study, we determined the hepcidin to characterize normative plasma hepcidin concentrations in healthy non-anemic Indian subjects and to determine the independent effects of infection/ exposure and anemia on hepcidin concentrations. Understanding normative hepcidin levels in the selected populations will allow us to understand the hormonal regulatory mechanism that healthy subjects from developing anemia. Authors set out to measure plasma hepcidin concentrations and to characterize normative values in healthy and ACD anemia group. We used an algorithm developed of data to generate hepcidin consensus values as cut off to allow for harmonization with different hepcidin assays.

METHODS

This is a prospective analytical case control study. Patients attending the outpatient and inpatient department of the INDEX hospital were included during study period of two year from June 2014 to June 2016. Appropriate prior consent was taken from the patients and clinical details were recorded in a proforma. All investigations were done in the pathology and clinical biochemistry continue the para/ remove the tab.

laboratory of INDEX Hospital, Indore. Sample for complete blood count and biomarkers analysis were collected, coded and processed on a SYSMEX X-800i auto analyzer for hematological parameters and on ELAN 30S ELISA plate reader for Serum Hepcidin, Ferritin, IL-6 levels analysis. A total of 86 patients were evaluated, those full filling the inclusion criteria and allocated in two groups according to their RBC's indices, peripheral smear examination and serum ferritin values.

Control (Non-anaemic) were those with Hb level (mean±SD) 13.01±0.69 gm/dl and RBCs count (10^6/µl; mean±SD) 4.46±0.21 included within normal range to their respective reference age, sex and normal ferritin levels and a normal peripheral blood smear diagnosis. ACD group comprised of cases with Hb level (mean±SD) 8.65±2.88gm/dl and RBCs count (10^6/µl; mean±SD) 3.54±1.14 lower than the normal range to their respective reference age and sex and a hypochromic picture on peripheral blood smear. The biomarkers levels for both groups were analyzed, studied and compared. Patients already on iron supplements, blood transfusion in recent months and below 5 years of age (to exclude congenital conditions like thalassemia, haemochromatosis) were excluded from the study. The obtained data was tabulated using MS Excel to create a master chart. The power of study was kept at 99% and level of significance (α) at 5%. "Analysis of Variance (ANOVA)" was done to calculate the p value to compare the difference of mean of the study groups together. Post Hoc Turkey's test" was also applied for comparison of difference of mean in two study groups. The p value was calculated for each

parameter and p value <0.05 was considered to be significant. 95% CI was also calculated. "Pearson's Correlation Coefficient (r)" was determined to find out the correlation between serum hepcidin and serum ferritin, serum IL6 in studied groups. X-Y scatter plots and other graphical representation of data were done with appropriate plots and charts as needed depending on data type and distribution. AUC under ROC curves was calculated for serum hepcidin as a potential diagnostic test for detection of IDA in studied groups using Statistical Package for Social Sciences (SPSS) version 24.0 for windows and Medcalc Software.

RESULTS

The hematological parameters are summarized in Table 1 for group ACD, and normal. The patients had elevated WBC count as compared to normal group. The average mean±SD value recorded for ACD and normal group were 11.48±5.78ng/mL, and 8.15±2.22ng/mL respectively. The results well commensurate with the findings reported by chang et al.[22] In this study, the serum ferritin measured in patients of normal group was 65.67±16.76ng/mL while in case of ACD groups the serum ferritin level measured was elevated to 215.50±15.9ng/mL which was significantly higher than other groups. The calculated P value suggested significant difference (P≤0.0001). The serum hepcidin measured in patients of normal group was 54.06±15.46ng/mL while in case of ACD group the serum hepcidin level was elevated 98.36±24.29ng/mL which was significantly higher compared to other groups. The calculated P Value suggested a significant difference (P ≤0.005). The serum IL-6 measured in patients of normal group was 10.53±2.91ng/mL while in of ACD group the serum IL-6 level was elevated to 115.82±33.7ng/mL which was significantly different and highest among all the groups. The calculated P Value suggested a significant difference (P ≤0.005).

Table 1: Comparison of hematological parameters between normal and ACD groups.

Parameter	Normal group	95% CI	ACD group	95% CI	P value	Remark
WBC count (10^3/µL)(Mean±SD)	8.15±2.22	(7.3806, 8.9347)	11.48± 5.78	(9.5775, 12.7975)	0.013	NS
RBC count (10^6/µL)(Mean±SD)	4.46±0.21	(4.3862, 4.5343)	3.54±1.14	(3.2119, 3.8503)	0.000	S
Hb gm/dl (Mean±SD)	13.01±0.69	(12.7696, 13.2539)	8.65±2.88	(7.8496, 9.4580)	0.000	S
MCV (fL) (Mean±SD)	84.66±2.65	(83.7354, 85.5881)	76.29±5.29	(74.8233, 77.7689)	0.000	S
MCH(pg) (Mean±SD)	29.40±1.34	(28.9284, 29.8715)	24.52±2.39	(23.8604, 25.1933)	0.000	S
MCHC(g/dL) (Mean±SD)	34.73±1.16	(34.3314, 35.1450)	32.13±1.83	(31.6230, 32.6423)	0.000	S

Table 2: Comparison of biochemical markers between normal group and ACD groups.

Parameters (ng/mL)	Normal group	95% CI	ACD	95% CI	P value	Remark
Serum ferritin (Mean±SD)	65.67±16.76	(59.8242, 71.5257)	215.50±15.9	(58.9600, 71.9073)	0.000	S
Serum hepicidin (Mean±SD)	54.06±15.46	(48.6650, 59.4572)	98.36±24.29	(90.8842, 105.8371)	0.000	S
Serum IL6 (Mean±SD)	10.53±2.91	(9.5171, 11.5493)	115.82±33.7	(106.4399, 125.2066)	0.000	S

It is apparent that IL- 6 plays an important role in the regulation of hepcidin as reflected especially in ACD. In ACD group the elevated hepcidin concentration(s) 98.36±24.29 ng/mL was recorded. The data suggested that inflammatory cytokines could strongly stimulate hepcidin expression and this induction may be responsible for hypoferromia which may be further accompanied by inflammatory episodes. The findings could possibly explain that there is a relationship between inflammatory mediators and hepcidin in ACD group (Table 2).

Authors found an increased hepcidin concentrations in ACD subjects (P<0.0001) compared with controls. Importantly, ACD subjects had significantly higher serum hepcidin levels. Thus, the findings in the present

study coordinate well with the findings of Theur et al, (2009) however, the measured level(s) of marker differ in terms of concentration which may be due to selected method of analysis and sensitivity of kit(s) and heterogeneity of population used in the study.[23]

Receiver operating curve and scattered plot correlation curve

Iron deficiency anaemia is characterized by many abnormal laboratory features. Because none of these are unique, a small deviation from normal will detect most cases of iron deficiency (high sensitivity), but also falsely identify non-iron deficient subjects as being iron deficient (low specificity). On the other hand, a large deviation from normal will exclude most non-deficient patients (high specificity) but miss many iron deficient subjects (low sensitivity). The tradeoff shown graphically is so called receiver operating characteristic curve (ROC). These curves are constructed by plotting the sensitivity against the false positive rate (1-specificity) at various values of the analyte. The ROC curves for serum hepcidin as predictors of ACD was constructed using MedCalc software. Further, the correlation(s) between different biomarker(s) was assessed by plotting the bio exponents on X and Y axis: the scattered correlation curve so obtained was used to compare correlation coefficient and level of significance at 95% confidence level with defined confidence interval. In case of normal group, the scatter correlation curve revealed an excellent positive correlation between serum ferritin and hepcidin wherein for the sample size of 34 participants, correlation coefficient obtained on regression of curve was 0.9744, significance level P<0.0001, 95% confidence level interval 0.9489 to 0.9873. The values suggest positive correlation between the hepcidin and ferritin indicating if ferritin level may define the cut off point for anemia then hepcidin could equal and effectively be used for defining and determining ID.

Authors found a significant positive correlation between serum ferritin and hepcidin which is consistent with numerous reported studies. The serum hepcidin as well as ferritin being acute phase reactants of immune activity; are raised especially in chronic inflammatory disease hence in case ACD group the correlation was observed to be strongly positive with correlation coefficient -0.7984; significance level P <0.0001 at 95% confidence level 0.6719 to 0.8796 (Figure 1). As per proposed mechanism by Nemeth et al, this could be due to the fact that IL-6 is necessary and sufficient for hepcidin expression induction establishing that iron regulatory peptide hepcidin plays a key role in iron metabolism.[24] The study demonstrated a positive correlation between serum hepcidin and IL-6. The correlation coefficient was 0.6359 with significance level P <0.0001 at 95% confidence interval r = 0.4392 to 0.7744 inflammatory cytokines IL-6 was able to strongly stimulate hepcidin expression and as consequence such indicator could be responsible for hypoferriemia accompanied with chronic inflammatory diseases, This is in accordance with the report of kato et al, Nemeth et al.[24,25]

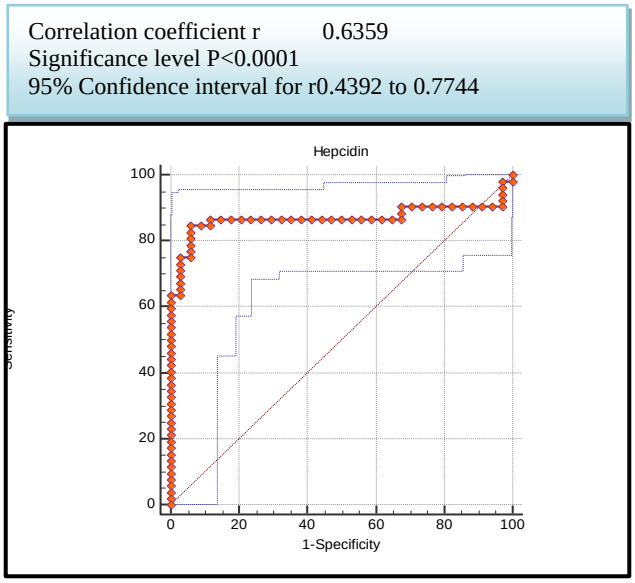

Figure 1: Receiver operating characteristic curves for serum hepcidin in ACD group.

DISCUSSION

Hepcidin a peptide and a regulator of iron metabolism is expressed in iron deficiency and also it has sensitive method of estimation i.e. immunoassay (ELISA based) was used as a marker and critically evaluated in relation to ferritin and IL-6 levels where in IL-6 being a specific cytokine expressed in chronic infection(s) inflammations.[26,27] In present study, authors observed statistically significant lower values of RBC count, Hb gm/dl, MCV, MCH, MCHC in ACD group then the normal group. In case of hepcidin when ROC curves and Pearson's scattered plot were made for ACD group; findings showed that ROC was maximum >0.869; with a sensitivity of 84.62% and specificity 94.12% while the confidence level was 95% with an interval of 0.779 to 0.932 in the case of ACD group. Further, the cutoff point determined was >72.93. Thus, the hepcidin level >72ng/mL and above is related to the ACD.

CONCLUSION

This is the pioneer study of its kind, (as per reported literature) conducted in India which is multi parameter centric and comprehensively conducted to explore the use of a biomarker i.e. hepcidin in detection and diagnosis in early stage anaemia. The available methods for iron deficiency anaemia diagnoses have their limitations as in the case of ferritin as marker it has excessive high values in chronic infections, malignancies, hepatitis, and hyperthyroidism. It is relatively less sensitive in detecting an early iron deficiency. Similarly, bone marrow aspiration is an invasive procedure, reportedly with non-patient compliance; tedious; involves high skill and

requires aseptic condition hence cannot frequently be used as a method of choice for the detection of iron deficient anaemia. It was found that hepcidin is positively correlated with IL-6 in the case of ACD means as an infection persists the iron deficiency is resulted which co-constitute a part of innate immunity and hence IL-6 as indicative marker of infection gets up regulated which correlate well with hepcidin expression (>72.93).

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Ethical approval: The study was approved by the Institutional Ethics Committee of Index Medical College, Hospital and Research Centre, Indore (M.P.), India

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