ORIGINAL ARTICLE

SERUM HEPCIDIN-25: A PREDICTOR OF RESPONSE TO ORAL IRON THERAPY.

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ABSTRACT

Background: Discovery of hepcidin, and its receptor in year 2000 has revolutionized iron metabolism studies. Measurement of serum hepcidin, may supplement existing investigational parameters of iron status in anemias. The aim of the study is to estimate baseline hepcidin level and evaluate it as a predictor of response after treatment of iron deficiency anemic with oral iron.

Methods: This was a purposive follow up study; involving 102 patients aged 18 years and above selected from medical outpatient departments of a tertiary care hospital of Karachi. Baseline CBC, iron studies, and serum hepcidin-25 were measured. Patients of IDA were treated with oral ferrous sulphate 200mg thrice daily for 8 weeks. Repeat CBC on follow up visit assessed response, defined as increment in hemoglobin level by >1gm/dl post treatment. Logistic regression analysis was applied and receiver operating characteristics curves were plotted to find utility and identify hepcidin cutoff values as a predictor for response.

Results: Mean baseline serum hepcidin-25 was 37.34±22.60 ng/mL. Regression models showed for every one unit increase of hepcidin, the odds of hemoglobin improvement decreased by (1-0.972)=0.028gm/dl, serum hepcidin-25 by itself significantly predicted improvement in hemoglobin level P=<0.05. The area under the curve for hepcidin compared with post treated hemoglobin was 0.73 (95% CI: 0.63-0.83). For predicting response to therapy, hepcidin cutoff value of 40ng/mL, had sensitivity of 68%, specificity of 63%, positive predictive value of 85% and negative predictive value of 38%. Hepcidin quartiles were also constructed compairing 4th quartile parameters with 1st quartile parameters.

Conclusion: Hepcidin 25 is a fair predictor for response of iron therapy in IDA patients; baseline hepcidin of 40ng/ml predicts treatment response.

KEY WORDS: Iron deficiency, Anemia, Parameters, Tablet Ferrous Sulphate 200mg, Serum Hepcidin-25, ELISA, Bio Predictor For Response, Cutoff Value, Logistic Regression.

INTRODUCTION

Iron deficiency (ID) is a prelatent phase in iron deficiency anemia (IDA), prolonged or untreated ID progresses to IDA the commonest anemia, affecting two billion people worldwide[1-3]. Prevalence of IDA in South Asia is 43% -45%, with adverse health and economic consequences[4]. Treatment of IDA includes, dietary adjustments, iron enrichment of food and iron therapy[1]. Patients unresponsive to oral iron treatment (OIT) are advised intravenous iron preparations[5].

Iron levels are exquisitely maintained in body, preventing iron toxicity[6]. Previous studies[7-9] prove serum hepcidin-25 (SH) as master iron regulatory hormone, influencing iron absorption from enterocytes and its release in circulation. Initially produced in hepatocyte, as 84 amino acid preprohepcidin, and finally cleaved to 25 amino acid SH.[10,11] Transmembrane receptor of hepcidin, Ferroportin is located at basolateral surface of enterocyte, macrophage, hepatocyte, and placental cells, they are the only known iron exporter channels[12]. Hypoxia, inflammation and iron status regulate hepcidin. Erythropoiesis inhibits hepcidin expression[13, 14].

Lemos et.al; provided data for hepcidin as a potential biomarker in IDA patients, thus SH levels may supplement complete blood count (CBC) and iron profile parameters in confirming IDA diagnosis, and appears to have lead over other novel indicators of

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iron e.g. soluble transferrin receptor (sTfR), zinc protoporphyrin (ZPP), [15] reticulocyte hemoglobin content (CHr), and percentage of hypochromic red blood cells (%HRBC) [6, 16, 17]. Transferrin saturation percentage (TSAT%) shows diurnal variation and is influenced by inflammatory cytokines, nutritional status and liver disease, while ferritin, being an acute phase reactant may not effectively predict iron status, thus conventional haematological iron markers have limitations[18]. Interestingly, hepcidin levels are increased in anemia of chronic disease (ACD) and decreased in IDA[19]. Previously, hepcidin in ACD setting has been extensively researched, recently human and murine studies emerged on hepcidin in IDA setting [20, 21].

No local studies, on SH as a biopredictor exists[22, 23]. The objective of study was to assess baseline hepcidin level in newly diagnosed IDA cases and to evaluate usefulness of SH as a predictor of response to OIT, therefore saving the medication expense and time of both physician and patient, minimizing economic burden of anemia on human resource and health budgets of resource challenged countries.

METHODS

This was a purposive study, involving 102 patients, of 18 years and above, approved by Ziauddin University Ethical Review, and Research and Advocacy Committees. Clinically suspected patients of anemia were selected from medical OPD of Ziauddin hospital, Karachi. Written and informed consent was taken from patients at interview and clinical exam by researcher. Screening for IDA was by CBC, performed on automated hematology analyzer (Beckman coulter LH 500 Hematology Analyzer, USA) and iron study parameters (serum iron and Total Iron Binding Capacity estimation performed by calorimetric method and Serum Ferritin by immunoturbidimetric method on Roche Hitachi P-800 automated chemistry analyzer Germany, TSAT% was measured by calculating, serum iron levels/TIBCx100)[24]. Diagnosed IDA patients were further subjected to SH level (ELISA kit, MyBioSource USA, detection range of kit was 4.69-300ng/ml). Sampling duration was October 2013 -August 2014.

Patients, were included in study when; baseline hemoglobin(Hb) was<13gm/dl in men, <12gm/dl in women, hematocrit percent (Hct %) < 45% in men, and < 35% in women, mean cell volume(MCV)<80 fl, mean concentration of hemoglobin (MCH) <27pg, and mean concentration of hemoglobin content (MCHC) < 31gm/dl, serum ferritin <15 ng/ml, serum iron< 40ug/dl, transferrin saturation (TSAT%) <15%, and total iron binding capacity (TIBC) of >388ug/dl.

Patients with all other forms of anemia, alcoholism, chronic blood loss, thalassemia major and trait, obesity (based on BMI) and pregnancy[25] were excluded from study. Ferrous sulphate 200 mg was prescribed thrice daily, for eight weeks. Patients were reminded for medicine and follow up by telephonic and SMS alerts. At follow up, CBC was reperformed to check response to treatment, defined as increment in Hb values by >1gm/dl after 8 weeks of OIT.

STATISTICAL ANALYSIS

Data entry and analysis was done on IBM SPSS version 20. Descriptive analysis for all the variables was done. For categorical and numerical variable, mean and standard deviation was calculated. To check baseline hepcidin as a biomarker, logistic regression was applied and odds ratio calculated. The efficiency of hepcidin as a bio predictor was evaluated using null and final model. After data was fitted to the logistic regression model, insignificant variables were taken out methodically and the model was modified, until model had variables having significant explainable effect on the response variable. Serum hepcidin quartiles were constructed to quantify scatter and fourth quartile was compared with first quartile. Receiver operating characteristics (ROC) curve was calculated for hepcidin levels as a predictor for response. The curves were assessed to identify suitable hepcidin cutoffs. The area under the curve for ROC curves (AUCROC) was created by non-parametric method. Sensitivity, specificity, positive predictive value (PPV), negative predicted value (NPV) of hepcidin as a bio predictor was determined for possible cutoff value of hepcidin, P value less than 0.05 was considered statistically significant.

RESULTS

Table 1 gives the descriptive characteristics of study participants before treatment. The mean age, Hb level, RBC count and SH level was 29.05(14.94) years, 9.20(1.51) gm/dl, 4.17(0.70) millions/ml3 and 37.34(22.60) ng/mL respectively. All parameters of iron study were observed to be lower than normal range. Table 2, shows the comparison of red cell indices before and after OIT, with statistically significant improvement p = < 0.0001 in all parameters. Table 3 shows hepcidin quartiles levels and other serological parameters along with frequency. Hepcidin level was higher in fourth quartile as compared to first quartile, as well as better levels of ferritin, serum iron and TSAT% yet patients failed to increase their Hb levels comparable to patients in first quartile, validating that high hepcidin level prevents iron absorption.

The predictability of baseline SH, as a bio predictor for iron response was tested by logistic regression analysis, also called a logit model, used to model log odds of the dichotomous outcome (improvement in Hb after OIT; yes or no), as a linear combina-

tion of the predictor variables. A complex model, including all demographic variables such as age, gender, height, sex, weight, ethnicity, marital status along with iron profile parameters and hepcidin was initially fit on the data. All demographic and iron profile variables were statistically insignificant except for hepcidin (p=0.031). A second model was then fit where the impact of hepcidin alone was measured on the odds of improvement via OIT.

The second model was a good fit with a decrease in likelihood ratio of 8.026 for a loss of 1 degree of freedom (p-value = 0.0046) compared to the null (intercept-only) model. Indicating that hepcidin was statistically significant as a predictor of response to iron therapy. The model predicted 0.029 gm/dl decrease in the log-odds of Hb improvement thus, for every one unit increase in hepcidin, the odds of Hb improvement decreased by (1-0.972-0.028 gm/dl).

Cutoff value of hepcidin that best separates Hb improvement from no improvement by OIT was calculated by plotting ROC curve (Fig1). Based on the data set, hepcidin cutoff point of 40ng/mL was selected as it minimizes the number of false positives and false negatives and has Sensitivity of 68%, Specificity of 63%; PPV of 85% and NPV of 38%. The alternate value of 20ng/mL was also evaluated for comparison, and ruled out as undesirable, because the Sensitivity decreased to 32% while the specificity increased to 100% (all cases that did not improve were classified correctly). Out of 78 patients who showed response to OIT, hepcidin cut off value of 40 ng/mL picked up n=53 (68%)-true positives but failed to pick n=25 (32%)–false negatives, and out of 24 patients who showed no response to OIT, hepcidin cut off value of 40ng/mL picked up n=15 (63%)-true negatives and failed to pick n=9 (39%) patients who showed no response to OIT. Area under the curve was 0.73 or 73% (95% CI: 0.63, 0.83), demonstrating that the cutoff value is a fair discriminator of hemoglobin improvement. Diagnostic odds ratio (DOR) shows the usefulness of a diagnostic test, and the DOR for cutoff value 40 ng/mL is 3.53, demonstrating that hepcidin is 3.53 times more likely to predict improvement in Hb levels after treatment relative to not being able to do so. The DOR is greater than one so the test is discriminating correctly, which is favorable. The discriminating power for all models was in acceptable range.

Limitations of this study were small sample size, C reactive protein and α1 acid glycoprotein was not evaluated due to budget limitations. Erythrocyte Sedimentation Rate was available for some patients only, (although high levels of ferritin and white blood count were checked to rule out ACD and inflammation, none of the patients had high levels). Collection time could not be specified for iron study to patients for logistics reasons.

Table 1: Descriptive parameters, of study participants (n=102)

Bio Parameters	**Mean (±S.D)**
Age (yrs)	29.05 (±14.94)
Weight (kg)	54.46 (±13.36)
Height (cm)	158.19 (±7.92)
BMI (kg/m^2)	21.86 (±4.86)
CBC parameters	
Homoglobin (gm/dl)	9.20 (±1.51)
Hematocrit (%)	29.31 (±4.75)
RBC (x10E12/L)	4.17 (±0.70)
MCV (f1)	70.31 (±8.29)
MCH (pg)	22.10 (±3.32)
MCHC (gm/dl)	30.91 (±1.66)
Iron studies parameters	
Ferritin (ng/ml)	12.52 (±10.81)
Serum iron (ug/dl)	26.35 (±14.52
TIBC (ug/dl)	392 (±97.30)
UIBC (ug/dl)	370 (±90.13)
TSAT (%)	6.90 (±3.69)
Serum hepcidin-25 level	
Hepcidin (ng/mL)	37.34 (±22.60)

RBC=red blood cell; MCV=mean corpuscular volume; MCH=mean corpuscular hemoglobin; MCHC=mean corpuscular hemoglobin concentration; TIBC= total iron binding capacity; UIBC= unsaturated iron binding capacity; TSAT%= transferrin saturation percentage.

Table 2: Comparison of pre and post treatment CBC

Parameters (units) (SD)	Pre treatemt values	Post treatemt values	Hb* Difference
Hb (gm/dl)	9.20 (±1.51)	11.50 (±1.39)	2.15**
Hct (%)	29.31 (±4.75)	35.32 (±4.03)	5.68**
RBT (x10E12/L)	4.17 (±0.70)	4.63 (±0.62)	0.46**
MCV (fl)	70.31 (±8.29)	78.08 (±6.78)	7.69**
MCH (pg)	22.01 (±3.32)	25.13 (±3.00)	3.12**
MCHC (gm/dl)	30.91 (±1.66)	31.95 (±1.95)	1.05**

*: Hb, hemoglobin
** P value =.0001

Table 3: Table for Hepcidin quartiles

Variables Mean (Std.Deviation)	First quartile n=25	Second quartile n=26	Third quartile n=26	Fourth quartile n=25
Serum hepcidin (ng/mL)	**13.96(±3.51)**	**26.95(±3.98)**	**40.78(±3.55)**	**66.34(±15.89)**
Hb pretreatment (gm/dl)	8.82(±1.48)	9.22(±1.23)	9.47(±1.37)	9.29(±1.92)
Hb posttreatment	11.71(±1.08)	11.77(±1.52)	11.37(±4.27)	11.13(±1.57)
Hb improvement	2.89	2.55	1.9	2.01
Ferritin (ng/mL)	10.45(±10.66)	11.98(±11.72)	11.84(±8.22)	15.86(±12.16)
Serum Iron (ug/mL)	23.04(±12.09)	23.84(±10.15)	30.11(±20.65)	28.36(±12.52)
TIBC (ug/mL)	405.72(±85.87)	381.88(±102.00)	379.46(±117.04)	402.80(±82.25)
T.SAT (%)	6.02(±3.51)	6.71(±3.23)	7.56(±4.27)	7.29(±3.70)

Hb= hemoglobin; TIBC = total iron binding capacity; TSAT%= transferrin saturation percentage.

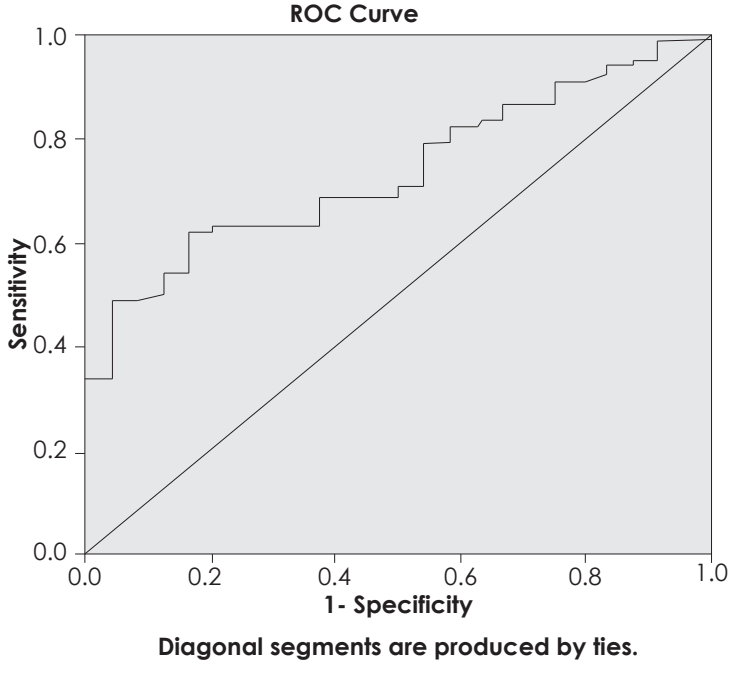

Figure 1: ROC curve for Hepcidin as independent and response as dependent variable

DISCUSSION

In this follow up study of IDA patients, recruited through internal medicine OPD of a tertiary care hospital, we measured baseline hepcidin levels and evaluated the performance of SH concentration as a biopredictor to diagnose responsiveness to OIT. Our mean baseline hepcidin value in IDA patients was higher than that reported by Naqvi et.al; [23].

Ramesh kumar reported mean hepcidin levels of 33.6±5.5ng/ml in 5out of 218 IDA patients[27]. Finding of Ramesh kumar was almost similar to our study; however the sample size was only 5 and cannot be generalized for whole population. Previously done studies in western region report mean hepcidin levels in IDA patients to be lesser than normal reference range[11,18]. Reasons for a higher mean baseline hepcidin levels in IDA patients in our study as compared to levels reported in western studies may be due to different, ELISA kit (MyBioSource), hepcidin quantification method e.g. mass spectrometry; environmental pollution, circadian rhythm[28] and genetic makeup[11,18].

Hepcidin level of >20ng/mL as a cut off value predicts unresponsiveness OIT with sensitivity of 41.3%, specificity of 84.4%, PPV of 81.6% and NPV of 46.3% [29]. Our AUC for hepcidin was 0.73 [95% CI: 0.63, 0.83] with post treated Hb, and this moderately predicted response to OIT.

Pasricha et.al; reports, in a study for SH as diagnostic test of ID in female blood donors of premenopausal age, AUCROC curves for SH compared with ferritin <15 ng/mL and with sTfR-F index>3.2 as 0.87(0.82, 0.92) in 59 ID donors and 0.89 (0.84, 0.93) in 53 ID donors respectively. Furthermore, Hepcidin cutoff value of <8ng/mL, had sensitivity and specificity of 41.5% and 97.6%. However when cutoff value increased to <18ng/mL sensitivity and specificity was 79.2% and 85.6% respectively[18]. Pasricha et.al; obtained different result as he included non-anemic ID patients, who donated blood three times a year, however, in our study chronic blood loss was excluded. Furthermore, erythropoiesis, due to increased blood donation, may inhibit hepcidin production, and thus lower SH levels[14].

Hepcidin has been evaluated as a modest predictor of dietary iron bioavailability in n=4 subjects after oral iron dose of 3.8 and 60 mg of iron for fourteen days[30], our study also demonstrated SH as a modest bio predictor of Hb response to OIT.

Concurrent to other studies this study recommends that severely anemic patients have high hepcidin levels; such patients may have a high propensity of failure to OIT[29]. This may warrant earlier and serial follow up and change in course of therapy at an earlier time. However, it needs to be determined if these patients are better candidate for earlier initiation of intravenous iron therapy.

Future endeavors for hepcidin may involve its use as a point of care instrument for diagnosing IDA or iron status of patient in a developing country setting. Further research is warranted as normal reference range for hepcidin has not been standardized yet. Additionally, lack of automated analyzer limits clinical application of serum hepcidin for now.[19]

CONCLUSION

Serum hepcidin-25 moderately predicts response to oral iron therapy in IDA patients, hemoglobin improvement after 8 weeks of iron therapy was less compared to individual with low hepcidin. This indicates, an inverse relation exists between hepcidin levels and improvement in hemoglobin levels.

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