Journal of Population Therapeutics & Clinical Pharmacology

RESEARCH ARTICLE
DOI: 10.53555/jptcp.v31i6.6751

# ASSESSING THE UTILITY OF HEPCIDIN AND TOTAL IRON-BINDING CAPACITY IN DIAGNOSING IRON DEFICIENCY DURING MID-GESTATION

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## Abstract

**Objective:** This study aimed to investigate the influence of hepcidin, a key regulator of iron homeostasis, on total iron-binding capacity (TIBC) during the second trimester of pregnancy. Understanding the relationship between hepcidin and diagnostic markers, particularly acute phase markers, is crucial for the timely diagnosis and treatment of iron deficiency during gestation.
**Methods:** A cross-sectional study was conducted with 387 one-time blood samples collected from pregnant women during their second trimester at a teaching hospital in Lahore, Pakistan. Participants were aged 19-45 years, following the WHO age criteria, and had no comorbid conditions. Complete blood count, serum iron, ferritin, transferrin saturation, and TIBC were measured. Samples were categorized into three groups: non-iron deficiency (N-ID), iron deficiency (ID), and iron deficiency anemia (IDA). Hepcidin levels were determined using an enzyme-linked immunosorbent assay (ELISA) with antibody-coated 96-well plates based on the principle of antibody-antigen-enzyme-antibody complex formation. Correlations between hepcidin and other biochemical parameters were assessed.
**Results:** Hepcidin levels showed significant positive correlations with ferritin ($p<0.001$), transferrin saturation ($p<0.001$), and TIBC ($p<0.001$). Conclusion: Hepcidin and TIBC can serve as valuable markers for assessing serum iron bioavailability and diagnosing iron deficiency during the second trimester of pregnancy. Monitoring these parameters may help mitigate adverse pregnancy outcomes associated with iron deficiency.

**Keywords:** hepcidin; total iron-binding capacity; iron deficiency; anemia; pregnancy; serum iron

## 1. Introduction

Any cell life needs an iron balance. Iron homeostatic processes have evolved by reusing the iron and limiting its uptake from the atmosphere to prevent iron (Camaschella 2015). However, iron deficiency and iron deficiency anemia are frequently present during pregnancy (Rukuni et al. 2015)(Abu-Ouf and Jan 2015)(Clénin 2017). Screen-and-treat services can have a tailored, cost-effective solution to filling coverage gaps, and the proper selection of diagnostic markers is vital. (Abioye et al. 2016)There are a lot of hematological markers used for the investigation of iron deficiency and iron-deficiency anemia. It includes serum iron, ferritin, transferrin saturation, total iron-binding capacity (TIBC), hemoglobin (Hb), mean corpuscular volume (MCV), and hepcidin(Cacoub et al. 2020; Krafft et al. 2017).

Hepcidin is the younger marker in identifying and diagnosing iron deficiency and iron deficiency anemia (Hassan 2018). This small peptide, which is secreted by hepatocytes, plays an essential role in iron homeostasis. Its high and low expression results in a decrease and increase of serum iron concentrations, respectively (Lopez et al. 2016). Hepcidin levels are positively and negatively correlated with other hematological markers of ID and IDA (Manolov et al. 2015).

Being a master regulator of iron homeostasis in the body, it also has prime importance during pregnancy research has been published on hepcidin levels during pregnancy (Koenig et al. 2014) relation of the hepcidin with other hematological markers is also reported in different trimesters of pregnancy. However, during pregnancy, hematological markers show changed behavior due to iron requirements changes (Krafft et al. 2017). The diagnostic features of serum hepcidin as an iron deficiency index have not been well described, nor did the studies provide a sufficient sample of stable and iron-deficient people to assess their range of references. The susceptibility and unique significance of different iron deficiency cuts for serum hepcidin have yet to be identified (Pasricha et al. 2011). Inflammatory stimuli and increased stored iron are behind the expression of hepcidin (Sangkhae and Nemeth 2017; Sangkhae et al. 2020). Serum hepcidin is an essential indicator of iron decrease or increase level as it is declared as the master regulator of iron homeostasis (Kemna et al. 2008). The relationship between hepcidin and ferroportin is the crucial cause for this process. On the hepatocytes' surface, reticuloendothelial macrophages, placenta cells, and duodenal enterocytes are expressed ferroportin, the only known exporter of iron in mammals. As hepcidin binds to ferroportin, it internalizes, degrades, and prevents iron transmission through ferroportin (Amer, Aboelmagd, and Elshahat 2020). Screening and conclusive diagnosis of iron deficiency in pregnant women in developed countries is currently time consuming and involves the use of hemoglobin and ferritin, along with C-reactive proteins or other diagnostic markers (Zimmermann and Hurrell 2007; Zimmermann 2008; Senga, Koshy, and Brabin 2012). Few are the less intrusive laboratory studies, such as TIBC, transferrin saturation and soluble transferrin have been suggested as helpful in detecting iron deficiency. Total iron-binding capacity is said to be a negative acute phase reactant and has decrease diagnostic values (Camaschella 2015; Koperdanova and Cullis 2015; Article 2016; Pretorius and Kell 2014). Hepcidin being main regulator of iron levels has influencing relations with other diagnostic biomarkers of iron deficiency. In this research, we studied the influence of hepcidin on serum iron, transferrin saturation ferritin and total iron binding capacity in three different groups of pregnant females, i.e., Non-iron deficiency, ID, and IDA.

## 1. Methods

All the participants in this study were recruited from Govt. Kot Khawaja Saeed Teaching Hospital Lahore. Informed consent was obtained from every participant of the research, and the privacy rights are highly observed.

**Study population:** Pregnant females 396 both primigravida and multigravida were the part of studies. A total of 387 females participated after prior consent. These were divided into three groups Non-ID

that is healthy controls, ID group, and IDA group. All the females were between the age of 19-45 years.

**Lab Procedures:** All the samples were subjected to complete blood count (CBC), including hemoglobin (Hb) levels, by using an automated hematology analyzer (Sysmex-KX21, Sysmex-XP-100) three-part differential. Serum iron was measured using Fairbank et al. 1987 Stookey et al. 1970 methods while ferritin, total iron-binding capacity (TIBC), and hepcidin-25 were measured with ELISA by using antibody-coated 96-wells plates (Science glory). Standard curves for all the mentioned assays are calibrated, and then samples are performed by observing the absorbance.

**Statistical Analysis**

The ANOVA revealed that the overall means for the examined parameters hepcidin, ferritin. TIBC and TS within all groups were significantly ($P \leq 0.05$) different from each other.

However, repeated measures ANOVA (Dunnett's T3 Post Hoc test) between various groups revealed that the hepcidin in N-ID was significantly higher than the IDA and ID. However, when we see a comparison between IDA and ID, there is not a significant difference. The TIBC had a significantly lower value in group N-ID, and ID has incredibly high values. Simultaneously, the IDA is also substantially different from the other groups, and values exist between the N-ID and ID.

We use Post hoc tests as these are an essential component of ANOVA. When three group means are tested for equality using ANOVA, statistically significant findings suggest that not all of the group means are equal ("Statistical Analysis," n.d.)

The Pearson approach determines the statistical link, or connection, between the two continuous variables. As this is based on the concept of covariance, it is recognized as the best technique for quantifying the relationship between variables of interest. It reveals the size of the link, or correlation, as well as the relationship's direction ("Statistics Solutions," n.d.). On these facts Pearson approach was used to measure the correlation coefficient between various biochemical parameters is given in (Table 2). Hepcidin had a significant positive correlation with ferritin ($r= 0.163$; $P<0.001$) and TS ($r=0.169$; $P<0.001$). Ferritin had a positive correlation with TIBC ($r=0.581$; $P<0.001$) and TS ($r=0.971$; P0.001). However, hepcidin had a negative correlation with TIBC ($r=0.134$; $P<0.001$) also TIBC had a significant negative correlation with TS ($r=0.680$; $P<0.001$).

## 2. Results

TIBC ranges were defined to sub-categorize the samples for analysis. Lower and upper TIBC ranges were defined as the non-iron deficiency group's lower TIBC range was 231 μg/dL. The Upper TIBC range was 431 μg/dL in the iron deficiency group; the TIBC degree is comparatively high, which falls with the content of 329 to 729 μg/dL. In the iron-deficiency anemia group, the lower spine is 218 μg/dL and the upper range is 718 μg/dL.

In non-iron deficiency group, mean hepcidin value is 24.49 μg/L and 41.26 μg/L with the TIBC ranges of 231 to 331 μg/dL and 331 to 431 μg/dL. For the iron deficiency group the TIBC ranges are higher than the normal, while, hepcidin values are decreased in a proper manner the divided TIBC ranges groups are 329-42 μg/dL, 429-52 μg/dL, 529-62 μg/dL and 629-72 μg/dL with the mean hepcidin values of 34.77 μg/L, 23.38 μg/L, 23.30 μg/L, 13.93 μg/L and 8.12 μg/L, respectively. In the iron deficiency anemia group the TIBC ranges are 218-31 μg/dL, 318-41 μg/dL, 418-51 μg/dL, 518-61 μg/dL and 618-71 μg/dL with the mean hepcidin values of 16.04 μg/L, 12.69 μg/L, 18.27 μg/L, 16.82 μg/L and 18.11 μg/L respectively.

Ferritin mean value is 80.32 ng/ml in healthy controls, that is, N-ID group, while 22.67 ng/mL and 19.20 ng/mL in ID and IDA groups, respectively.

As for as TS is concerned, it is quite normal, which is 24.77 μg/dL, but in ID and IDA, its values are 4.52 μg/dL and 4.37 μg/dL.

**Table. 1.Results of hematological markers of iron deficiency in non-iron deficiency, iron deficiency and iron deficiency anemia groups. Values are presented as mean ± SEM.**

| Parameters | N-ID | ID | IDA |
|---|---|---|---|
| Hepcidin | 30.9036±4.70[a] | 20.4704±2.48 | 17.3347±1.90[b] |
| Ferritin | 80.3226±3.24[a] | 22.6777±1.82[b] | 19.2075±1.33[b] |
| TIBC | 327.8871±5.30[a] | 517.2479±4.95[b] | 486.7042±5.85[c] |
| TS | 24.7762±1.04[a] | 4.5209±0.39[b] | 4.3779±0.38[b] |

Different superscripts show the statistical difference between the groups.

**Table 2**: Results of the Pearson's correlation coefficient between examined biochemical parameters.

| Parameters | r square value | p value |
|---|---|---|
| Hepcidin × Ferritin | 0.163** | .0006 |
| Hepcidin × TIBC | -0.134** | .0000 |
| Hepcidin × TS | 0.169** | .0000 |
| Ferritin × TIBC | 0.581** | .0000 |
| Ferritin × TS | 0.971** | .0000 |
| TIBC × TS | -0.680** | .0000 |

**Correlation is significant at the 0.01 level (2-tailed)

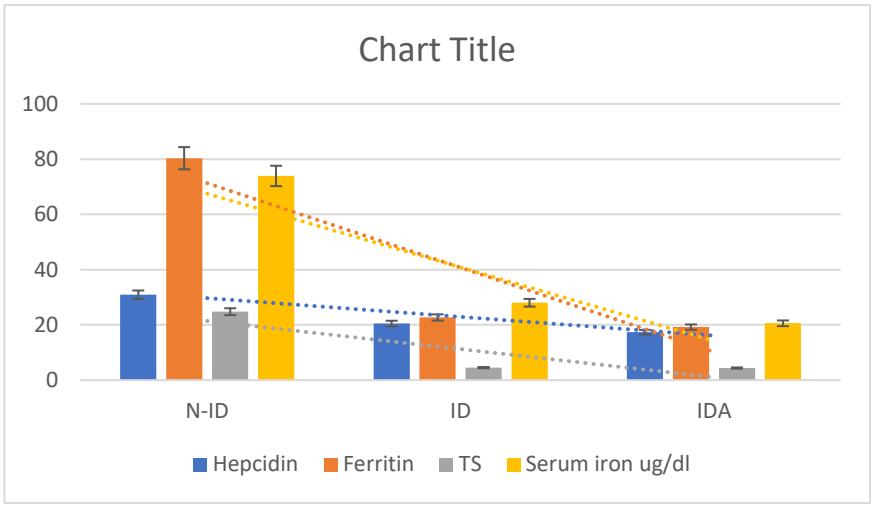


**Figure 1:** Comparison of hematological markers i.e. hepcidin, ferritin, TS and serum iron in three sub-groups of pregnant females including non-iron deficiency, iron deficiency and iron deficiency anemia.

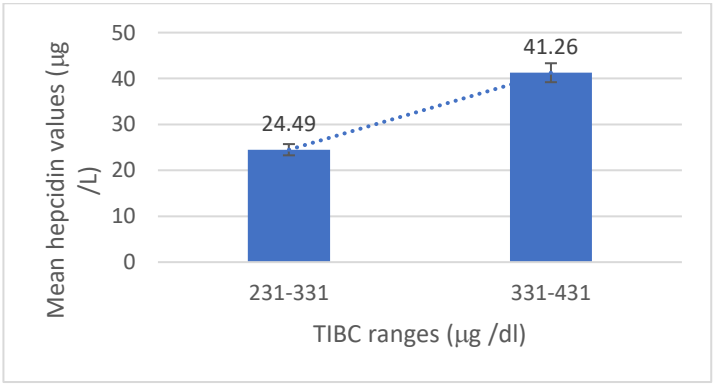


**Figure 2:** Comparison of TIBC µg/dl ranges (normal= 231-431) with mean hepcidin values of N-ID (n=64) group. N-ID samples are divided into sub-ranges of TIBC with interval of 100, and samples with mean hepcidin values are placed according to the spine.

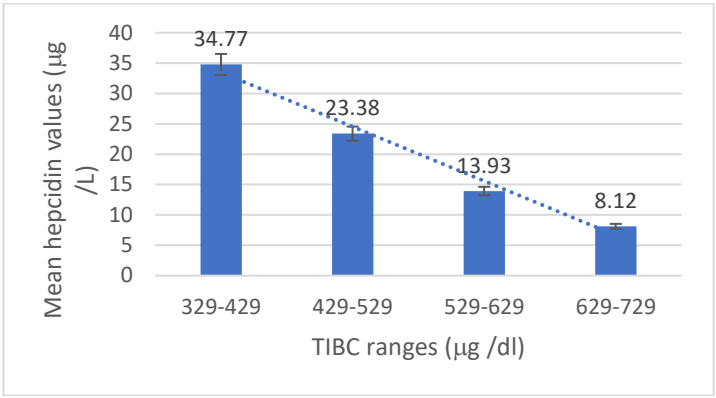


**Figure 3:** Comparison of increased TIBC µg/dl ranges (329-729) with mean hepcidin values of the ID (n=121) group. ID samples are divided into subranges of TIBC with interval of 100 and samples with mean hepcidin values are placed according to the spine.

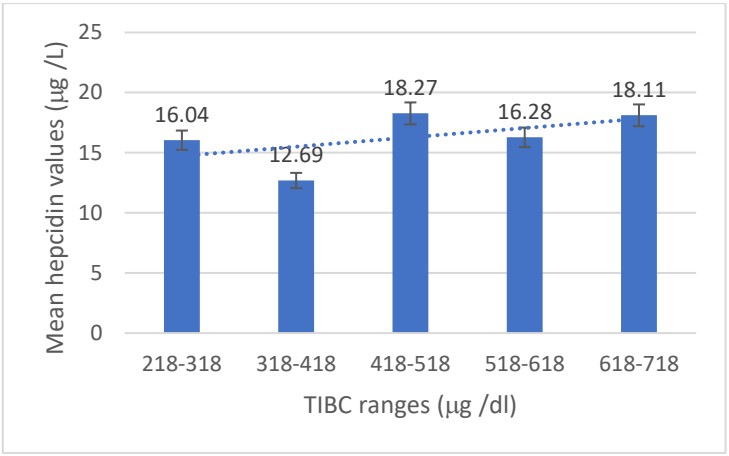


**Figure 4:** Comparison of TIBC µg/dl ranges (218-718) with mean hepcidin values of IDA (n=213) group. IDA samples are divided into subranges of TIBC with interval of 100 and samples with mean hepcidin values are placed according to spine.

## 1. Discussion:

In most of the past research, hemoglobin level was used to determine the iron state, but few studies evaluated serum ferritin as an iron status predictor (Dewey et al. 2017)(Brannon, Stover, and Taylor 2017)(Ray, Berger, and Park 2020). Serum ferritin (s-ferritin) represents the body's iron reserves and is commonly used to diagnose ID, which the World Health Organization defines as an s-ferritin 15 mg/L. (World Health Organization 2014) In our N-ID group, ferritin and transferrin saturation normal range are considered for assessment of iron status and below normal placed in ID as described in World Health Organization document (World Health Organization 2014)(Who 2011)(Daru et al. 2017).

Serum ferritin is an iron-storing protein that has long been used in humans as an indirect measure of iron deficiency anemia. Ferritin also plays a role in iron homeostasis by sequestering free iron in the middle of apoferritin (Concentration et al. 2021). We use hepcidin and total iron binding capacity along with serum ferritin and hemoglobin as indicators of ID and IDA.

Our studies observed a strong relationship between TIBC and hepcidin levels both show a positive correlation. In the N-ID group, there is an increase in the TIBC during pregnancy, reported in 2014 (Gilbert, Tawfik, and Kampfrath 2015). in a case study, this work was a stamp on Choi et al. that TIBC increased during the pregnancy (Choi, Im, and Pai 2000). Our studies found the Hepcidin mean value is directly proportional to the increase in TIBC values( Fig 3) as Hepcidin significantly increased during the first half of pregnancy (Bencaiova, Vogt, and Hoesli 2019). However, this increase is less when compared with hepcidin levels of non-pregnant females (Gambia et al. 2017).

As per authors knowledge positive correlation between hepcidin and TIBC during the second week of gestation is reported first time.

Hepcidin is involved in plasma iron regulation (Sangkhae et al. 2020); during the iron deficiency, its story may decline directly to decrease ferritin level (Gambia et al. 2017). TIBC has a negative relationship with serum hepcidin, which leads to the observation that TIBC rises in low iron conditions (Galesloot et al. 2011). Our group of iron deficiency comparison shows that TIBC and Hepcidin in a negative regression correlation strengthen the publication of A.I.Abioye et al (Abioye et al. 2020). Early studies have described a robust physiological relation between Hepcidin and the TIBC (Wray et al. 2017). The negative association between TIBC and hepcidin is consistent with existing knowledge of hepcidin's position as an iron store regulator and correlates to the reported rise in TIBC levels in iron deficiency (Kałuzna-Czyz et al. 2018). When the iron-deficiency anemia group results are considered and come in comparison with the previously discussed N-ID and ID group, the hepcidin levels are decreased (Pagani et al. 2019). It does not show a proper connection with TIBC ranges because it offers a direct relationship in few samples, while in others, it shows an indirect relation (Fig 5). Pagani et al. reported a strong connection between Hepcidin and anemia, but it has also been reported that Hepcidin expression may reduce during pregnancy. Standardization of hepcidin assay and its specificity for IDA testing is highly recommended during pregnancy (Konz, Montes-Bayón, and Vaulont 2014). Hepcidin influences TIBC in the N-ID group and ID group in positive and negative correlations, but there is no good influence in the IDA group. TIBC showed a negative correlation with TS.

Our study has few limitations self-administered questionnaires were used to collect information on BMI, use of iron supplements and any other comorbid conditions. As a result, people who are unaware that they possessed incorrect responses may be incorrectly included in the reference subset. However, since we used such a huge sample population, we anticipate that this possible misclassification has little effect on the hepcidin reference ranges.

Despite of these limitations our research has strength of including samples from rural and urban areas of the Lahore without any discrimination.

Exploring hepcidin influence on different diagnostic markers of iron deficiency will enables scientists and physicians in clinical practice all over the world to compare hepcidin concentrations and identifying guidelines for use of hepcidin assay in the diagnosis, staging, testing and treatment indications of iron disorders. Furthermore, our findings shed light on the (biochemical) correlates of serum hepcidin concentration, with TIBC being by far the most significant associate during pregnancy.

We concluded that hepcidin is the premier regulator of iron homeostasis during pregnancy. It influences females' total iron-binding capacity and can be used as a biomarker to assess serum iron bioavailability during pregnancy.

Since hepcidin was shown to be closely linked to serum iron and total iron binding capacity, changes in hepcidin levels during pregnancy may aid in the identification of iron related disorders, allowing pregnancy complications to be prevented.

Females in which iron deficiency leads to iron deficiency anemia, research on hepcidin levels during various stages of pregnancy can be used as diagnostic measure of maternal iron bioavailability during second trimester of gestation.

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## Graphical Abstract