doi.org/10.36721/PJPS.2021.34.6.SUP.2347-2355.1

Serum hepcidin response as a marker of iron deficiency during second trimester of pregnancy: A multicenter cohort study in Lahore

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Abstract: Iron deficiency diagnosis is a key health priority during pregnancy. The precise determination of indicators is needed for the evaluation of iron deficiency. In the present study, we investigated the diagnostic features of hepcidin concentration as an iron deficiency assay during the second trimester of pregnancy. We collected 401 venous blood samples of pregnant females from 4 separate birthing hospitals. All the females were within 13-26 weeks of their pregnancy and without any comorbid conditions. The complete blood count, total iron binding capacity, ferritin, serum iron and serum hepcidin were determined. The women were categorized as being non-iron deficient (N-ID), iron deficient (ID), or with iron deficiency anemia (IDA). The mean hepcidin values for examined groups were, i.e., non-iron deficiency was 31.45±4.70 (μg/L), iron deficiency 20.47±2.48 (μg/L) and iron deficiency anemia was 17.33±1.90 (μg/L). The N-ID's hemoglobin mean levels were 13.05±0.10g/dL, ID 12.66±0.05g/dL and the IDA 8.11±00.12g/dL. In this article variations in hepcidin levels between N-ID, ID and IDA women are uncovered and it is reported that the lower hepcidin levels diagnosed in IDA are closely linked to hemoglobin in Pakistani women. Hence it is concluded that hepcidin can be a valuable marker in identifying iron deficiency and iron deficiency anemia during the second trimester of pregnancy, according to the Pearson's correlation data.

Keywords: Hepcidin, pregnancy, iron deficiency, iron deficiency anemia, Lahore.

INTRODUCTION

Iron deficiency is the most drastic and earnest dietary issue in the world. Iron is required for the proper functioning of all cells due to its functions in oxygen transport, enzymatic activity and electron transport are all important. Iron is required by cells with high metabolic rate and are more prone to dysfunction during iron deficiency (Georgieff, 2020a). Women of reproductive age are affected by iron deficiency all over the developing world. It is also commonly present in the industrialized world, with substantial prevalence among menstruating women (Clénin, 2017). Socioeconomic situation, parity, age and food can all influence the amount of iron stored in women of reproductive age. (Mawani and Aziz Ali, 2016). Severe iron deficiency can cause iron deficiency anemia (IDA) (DeLoughery, 2017).

Iron deficiency occurs due to increased body iron needs during pregnancy. During pregnancy, iron requirements increase considerably as the mother's blood volume rises and the fetus grows and develops. Physiological iron needs rise significantly throughout pregnancy to promote fetoplacental growth and maternal pregnancy adaption. Iron needs vary during the three trimesters of pregnancy. Because menstruation ceases during the first trimester, the requirements (estimated at w0.8mg/d) are lower than before pregnancy. As the pregnancy progresses, maternal RBC mass rises, placental and fetal development accelerates, resulting in a 4-6 mg/d increase in physiologic iron needs in the third trimester (Bothwell, 2000a). Iron deficiency in pregnant women can be caused by reduced iron intake, greater iron requirements or pathologically decreased absorption (Camaschella, 2019). In pregnant women, iron deficiency is linked to adverse pregnancy outcomes such as increased maternal sickness, preterm and intrauterine growth restrictions, anemia, decreased birth weight and shortened gestation (Ali *et al*., 2020; Georgieff, 2020b). Iron deficiency in pregnant and breastfeeding mothers has been linked to poor cognitive development in their children (Rees *et al*., 2020). Iron deficiency is the most common cause of anemia during pregnancy because due to insufficient iron, less amount of hemoglobin is produced. The prevalence of anemia in pregnancy is >20% in many countries of the world. That's why it is considered a particular health issue in women, especially during pregnancy (Garzon *et al*., 2020). In developing nations, women of reproductive age suffer from anemia, which is a major public health issue. Anemia has been linked to higher maternal age and parity, poor socioeconomic status, low education and nutritional status in Pakistani women. There is a need to enhance women's diets and overall health (Ali *et al*., 2021). IDA leads to countless mortality, maternal bleeding, reduced educational performance in vulnerable communities and reduced productivity (Kuang *et al*., 2020). In developing countries, anemia is also found in 56% of pregnant females. IDA is conceded as a public health concern,

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**Corresponding author:* e-mail: dr.amina@ucp.edu.pk especially for women of reproductive age (Habib *et al.*, 2018a).

According to National Nutritional Survey-2018 in Pakistan, around 41.7% of women of reproductive age are anemic, with a slightly more significant proportion in rural settings (44.3%) than in urban settings (40.2%) (Pakistan, 2019). Research is done in rural regions of Pakistan, 77% of reproductive-aged women are anemic, with 7.8%, 48.7% and 20.8% classed as severely, moderately, and slightly anemic, respectively.

Different approaches are used to investigate and detect iron deficiency and iron deficiency anemia (Ning and Zeller, 2019). Hemoglobin, serum iron, ferritin, transferrin saturation and hepcidin are the commonly used tests in diagnosing ID and IDA among pregnant women. During pregnancy, the iron pathophysiology dramatically changes due to a rise in maternal red cell mass, sustaining placental and fetal developments and compensating for possible blood loss during childbirth. However, the normal daily iron absorption is just 1-5mg (Abioye *et al.*, 2020; Benson *et al.*, 2021). Iron needs must rise significantly during pregnancy. To promote fetoplacental growth and maternal pregnancy adaptations, including expanded needs for erythropoiesis. To satisfy iron needs, both dietary iron absorption and iron mobilization from stores improve, a process that is heavily reliant on iron-regulatory hormone hepcidin (Fisher and Nemeth, 2017b; Mazgaj *et al.*, 2021). Maternal adjustments such as the fetus and placental growth increase body iron requirement during pregnancy. This mechanism mainly depends on the hepcidin iron regulating hormone (Sangkhae *et al.*, 2020). This tiny peptide, released by hepatocytes, is necessary for iron homeostasis. Its high and low expression levels cause serum iron concentrations to fall and rise accordingly (Lopez *et al.*, 2016).

Hepcidin, the newly emerging iron hormone suppression, enhances iron release to maximize the plasma iron by using absorptive enterocytes and recycling macrophages (Camaschella, 2019). The assessment of serum concentrations of hepcidin has been hypothesized as an additional method for predicting and controlling iron supplementation requirements (Zaritsky *et al.*, 2010). The relationship of hepcidin to other hematological indicators has also been found. However, during pregnancy, hematological indicators exhibit altered behavior due to alteration in iron needs (Krafft *et al.*, 2017).

Based on hepcidin levels, anemias may also be classified as anemias with low and high hepcidin. IDA may be due to prolonged high hepcidin levels, which results in a low supply of iron for erythropoiesis by blocking iron absorption (Pagani *et al.*, 2019). Screening and conclusive diagnosis of iron deficiency in pregnant women take time and require the use of hemoglobin and ferritin and C-reactive protein or other diagnostic markers (Zimmermann and Hurrell, 2007; Zimmermann, 2008; Senga *et al.*, 2012). Serum hepcidin is a crucial biomarker of iron decrease or increase since it is the fundamental and main regulator of iron homeostasis (Kemna *et al.*, 2008). Hepcidin levels as an index of iron deficiency during pregnancy have been reported in few studies. Whether hepcidin and hemoglobin, measurements can reliably differentiate non-iron deficiency, iron deficiency, and iron deficiency anemia during 13-26 weeks of pregnancy in women from Lahore, Pakistan.

MATERIALS AND METHODS

Study population

This study examined the biochemical markers of iron deficiency, including the recently investigated marker hepcidin. Pregnant females registered at the Gynecology and Obstetrics Outpatients' Departments of different hospitals (two public sector hospitals and one private, public partnership) were recruited during their regular visit. The selected hospitals were Government. Kot Khawaja Saeed Teaching Hospital, Government Said Mitha Teaching hospital and Shalamar Institute of Health Sciences. A specially designed study proforma was used to record the demographic and other variables under study.

Sample size

Sample size was calculated using Rao soft © 2004 sample size calculator (Saadatian *et al.*, 2012) with a margin of error of 3% and the confidence level was 95%. The recommended sample size was 376.

Inclusion criteria

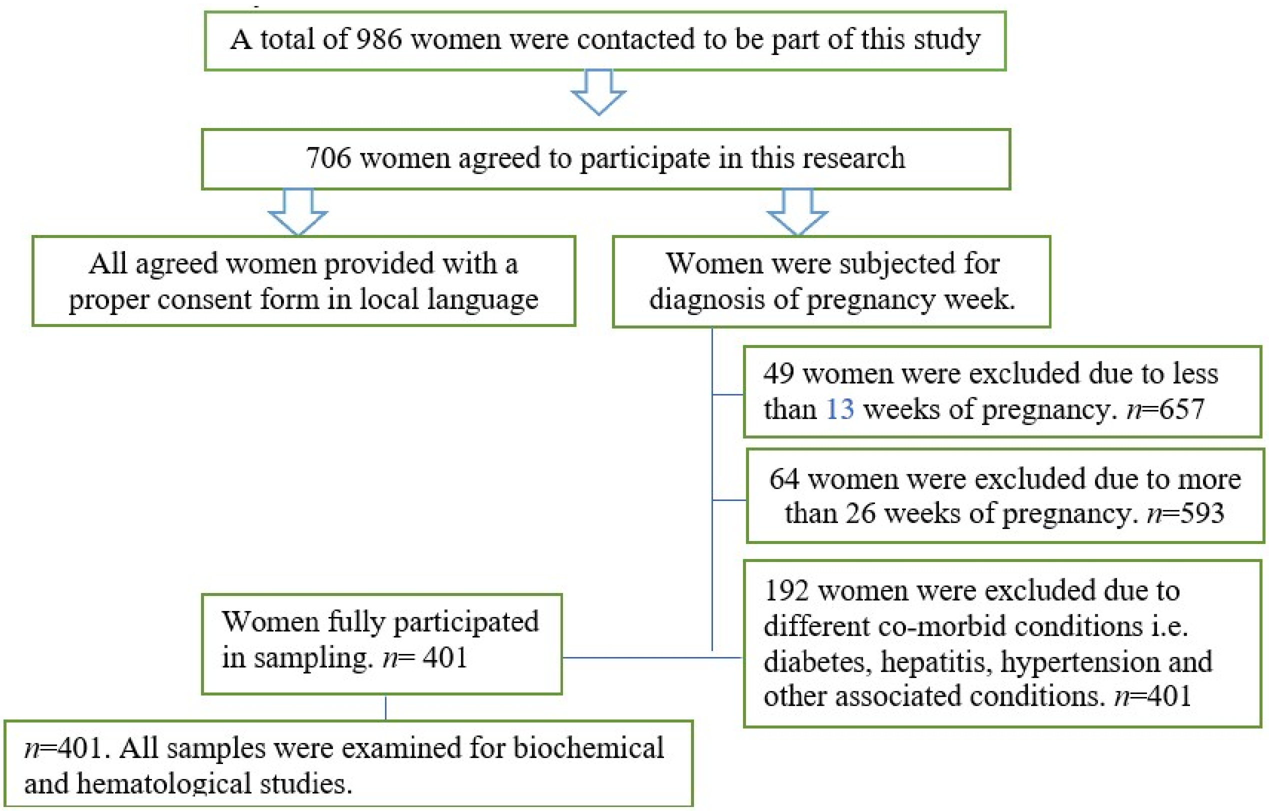
Pregnant females in the 19-40 years of age group, both primigravida and multigravida, which were in the 2nd trimester of their pregnancy, were recruited all the females participating gave prior consent, and the sampling was conducted after informed consent was completed.

Exclusion criteria

Pregnant females taking iron supplements from 1st trimester of pregnancy or diagnosed with diabetes, hepatitis, hypertension, ectopic pregnancy, or any other comorbid condition were excluded from the study. Females with twin and IVF pregnancies were also excluded.

Sample collection

Blood (5ml) was withdrawn from each enrolled participant by a trained phlebotomist. Of this, 2ml of blood was placed into CBC vials, and the remaining 3ml transferred into gel vials for serum separation. The samples were transported from hospitals to the Center for Advanced Research in Life Sciences (CARLS) in a cold chain. The separated serum after centrifugation was stored at -20^0C until analysis.



Complete Blood Count (CBC.): All the samples were analyzed for CBC within 6 hours of collection on an automated hematology analyzer called Sysmex-KX21, Sysmex-XP-100 three-part differential. The examined parameters were hemoglobin, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC). According to WHO recommendations, all biochemical values were recorded after comparing with reference ranges for the Pakistani population.

Fig. 1: Scheme for selection of study participants. *n*= 401.

ELISA Assay

Serum iron detection for each sample was done on an auto-chemistry analyzer (Wu, 2016; Stookey, 1970). Total iron-binding capacity, serum ferritin and hepcidin were determined using ELISA kits and antibody-coated 96-wells microplates. Sample coagulation at room temperature for 10-20 minutes was followed by centrifugation for 20 minutes at a speed of 2000-3000 r.p.m. Then, removed the supernatant and used it for the detection of serum ferritin. The FE ELISA Kit (PRS-00610hu) was used to measure the level of ferritin by using purified human FE antibody to coat microtiter plate wells, making solid-phase antibody, then adding FE to wells, and combined FE antibody. After washing thoroughly, the antibody-antigen-enzyme-antibody complex became an antibody-antigen-enzyme-antibody complex with HRP labeling. When substrate solution was added, it turned blue. HRP enzyme-catalyzed reactions were terminated by adding a sulphuric acid solution, and the color change was measured spectrophotometrically at 450nm. The concentration of FE in the samples was then calculated by comparing their optical density (OD) to the reference curve. To detect serum hepcidin, a highly sensitive (HS) ELISA kit, Science glory Hep-25 (PRS-00760hu), was used, a highly sensitive enzyme immunoassay for quantitative *in vitro* diagnostic determination hepcidin in serum. Standard curves for all the mentioned assays were calibrated and unknown values were determined.

Ethical approval

The study followed the Declaration of Helsinki principles and was approved by the Human Research Ethics Committee of the University of Central Punjab, Lahore via letter-number UCP/FLS/2020/136.

STATISTICAL ANALYSIS

The statistical analysis was done by using the ANOVA T3 Post Hoc Test. Averages of the biochemical parameters were examined for statistically meaningful relationships with hepcidin levels. Pearson's correlation coefficients were determined among the initial study of

401 women to investigate the relationship between hepcidin and indices of iron stores ferritin, serum iron, TIBC and hemoglobin (Laflamme, 2010; Marković *et al*., 2005). Because of these variables and gestational age, all groups were substantially ($P \leq 0.05$) different in characterizing hepcidin concentrations. All the groups were significantly ($P \leq 0.05$) different from describing hepcidin concentrations due to these factors and gestational age. SPSS 20.0 software was used for all the statistical analyses.

RESULTS

We observed differences in hepcidin levels in N-ID pregnant women compared to ID and IDA in our samples during the second trimester of pregnancy. A reduction in hepcidin levels in iron deficiency anemia is closely related to hemoglobin, HCT, MCV and MCHC (table 2).

The ANOVA revealed that the overall means for the iron parameters, hepcidin, hemoglobin, HCT, RBC, MCV, MCH and MCHC within all groups (N-ID, ID and IDA) were significantly ($P \leq 0.05$) different from each other. However, repeated ANOVA (Dunnett's T3 Post Hoc Test) measures between various groups revealed that the hepcidin in N-ID was significantly higher than the IDA and ID. In contrast, these groups had no significant difference between them.

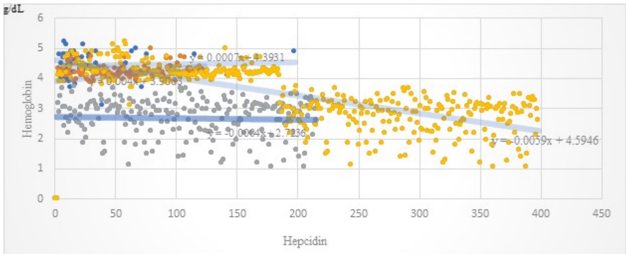


Fig. 2: Correlation between hepcidin and hemoglobin.

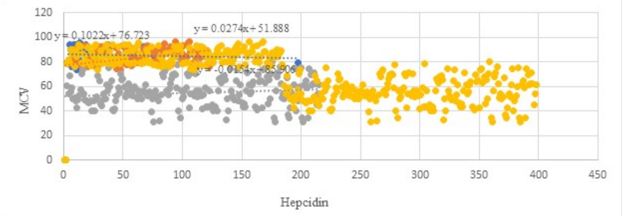


Fig. 3: Correlation between hepcidin and MCV

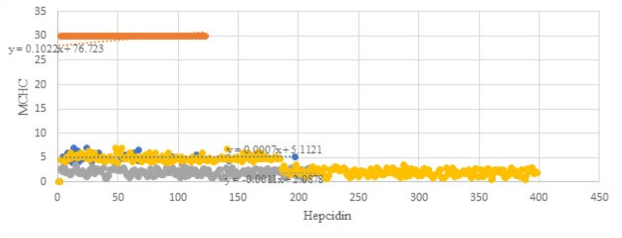


Fig. 4: Correlation between hepcidin and MCHC.

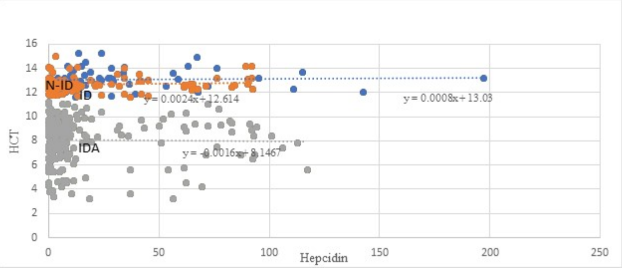


Fig. 5: Correlation between hepcidin and HCT.

The result of correlation coefficient between various iron parameters is given in table 3. Hepcidin had a significant positive correlation with MCH ($r=0.997$; $P<0.001$), Hemoglobin had a positive correlation with HCT ($r=1.00$; $P<0.001$); RBC ($r=0.794$; $P<0.001$) and MCHC ($r=0.999$; $P<0.001$), HCT with RBC ($r=0.794$; $P<0.001$), RBC with MCHC ($r=0.784$; $P<0.001$) also MCV with MCHC ($r=0.848$; $P<0.001$). But MCV had a negative correlation with MCH ($r=-0.38$; $P<0.001$) also MCH had a significant negative correlation with MCHC ($r=-0.44$; $P<0.001$).

The hepcidin with hemoglobin, HCT, RBC, and MCHC, RBC with MCH had a non-significant positive correlation. Simultaneously, the hepcidin with MCV, the hemoglobin with MCV and MCH, HCT with MCV and MCH, RBC with MCV, MCV with MCH and MCH with MCHC non-significant negative correlation.

The recorded demographic characteristics were age, parity, gestational age, educational and employment status. The participants' frequency and the percentage are in table 1. The mean values of biochemical parameters for N-ID, ID and IDA groups showed a significant difference expressed in table 2.

DISCUSSION

Iron is required for cell growth and differentiation. It also plays a vital role in hemoglobin synthesis, neurotransmission, cardiopulmonary and immune functions (Frise and Robbins, 2021; Litton and Lim, 2019). Iron needs to be increased during pregnancy as its use start from the 1st trimester and it increases till the 3rd trimester of pregnancy (Habib *et al*., 2018b; Anjum *et al*., 2015; Abu-Ouf and Jan, 2015). It has been seen in many studies that iron deficiency is becoming more common among pregnant women (Habib *et al*., 2018a). Pakistani women have information and a good mindset, but they do not put it into practice. Every woman must highlight the need for an iron-rich diet and supplementation throughout the prenatal period (Habib *et al*., 2018a).

Biochemically iron status is assessed by many serum-based parameters and these mainly focus on indicating the deficiency states of iron (Krafft *et al*., 2017). Iron deficiency is precisely divided into three phases. The first

Table 1: Demographic characteristics of participants

Age	Number	Percent
>20	23	5.7%
21-30	129	32.16%
31-40	171	42.64%
<40	78	19.4%
Educational Status		
No formal education	221	55.11%
Formal education	180	44.88%
Employment Status		
Un-employed	92	22.94%
Formal employment	113	28.17%
Informal employment	196	48.87%
Parity		
Primiparity	117	29.17%
Multiparity	284	70.82%
Gestational Age		
13-19 weeks	210	52.36%
20-26 weeks	191	47.63%

Table 2: Biochemical parameters for non-iron deficiency, iron deficiency, and iron-deficiency anemia (Results expressed as mean +/- SD (*n*=401).

	Groups		
Parameters	N-ID	ID	IDA
Hepcidin µg/L	31.45±4.70^{a}	20.47±2.48^{b}	17.33±1.90^{b}
Hemoglobin g/dL	13.05±0.10^{a}	12.66±0.05^{b}	8.11±0.12^{c}
HCT. %	39.16±0.31^{a}	37.99±0.16^{b}	24.35±0.37^{c}
RBC. x10^{6}/µL	4.41±0.05^{a}	4.21±0.01^{b}	2.70±0.04^{c}
MCV fL	85.42±0.75^{a}	84.42±0.54^{a}	55.35±0.75^{b}
MCH. %	8.04±1.20^{a}	30.0±0.00^{b}	30.0±0.00^{b}
MCHC.	5.13±0.08^{a}	4.82±0.04^{b}	1.98±0.04^{c}

Different superscript shows the statistical difference between the groups.

one is Storage depletion which has lower blood ferritin levels than predicted. Ferritin is the body's iron storage form, and low ferritin levels are the first warning when the body's iron reserves are depleted. The second stage is mild deficiency during this transport iron (also known as transferrin) declines.

Even if hemoglobin levels remain normal, this is frequently followed by a decline in the size of red blood cells. Iron deficiency anemia is the last stage. Hemoglobin levels begin to fall in this stage which may be characterized as IDA based on additional blood tests. The red blood cells are fewer in number, smaller and contain less hemoglobin at this stage (Stages of iron deficiency). Iron deficiency anemia is a combined diagnosis based on ferritin and hemoglobin levels because erythropoiesis is generally sustained until the later stages of iron insufficiency, anemia is the outcome of iron insufficiency (Pratt and Khan, 2015).

Serum ferritin (SF), transferrin saturation, and transferrin receptor (sTfR), as well as erythrocyte protoporphyrin, are serum-based indicators those are used to measure iron status biochemically. Iron status interpretation is typically based on a combination of numerous variables. These markers may pose difficulties in national nutritional surveys as well as clinical practices (Krafft *et al*., 2017). This study used hepcidin, hemoglobin and hematocrit as markers of iron status during pregnancy (Mendoza *et al*., 2021). We mainly focus on hepcidin as a marker of ID and IDA in different groups of pregnant females (Pagani *et al*., 2019). As a marker of iron deficiency, Hepcidin shows significant correlations with other biochemical features like hemoglobin and hematocrit (Kuang *et al*., 2020; Abioye *et al*., 2020). Hepcidin, as a direct facilitator of iron absorption and recycling, gives direct insight into iron physiology, specifically how oral iron is used. Iron intake and utilization can be predicted using hepcidin (Zimmermann *et al*., 2009). The WHO directed

Table 3: Result of Pearson's correlation coefficient between examined biomarkers of non-iron deficiency, iron deficiency anemia group. An overall correlation score is also expressed.

Parameters	N-ID	ID	IDA	Overall
r Score				
Hepcidin × Hemoglobin	0.038^{NS}	0.044^{NS}	-0.024^{NS}	0.106*
Hepcidin × HCT	0.038^{NS}	0.044^{NS}	-0.024^{NS}	0.106*
Hepcidin × RBC	0.066^{NS}	0.045^{NS}	-0.021^{NS}	0.112*
Hepcidin × MCV	-0.095^{NS}	0.045^{NS}	-0.008^{NS}	0.094^{NS}
Hepcidin × MCH	0.997**	--	--	0.062^{NS}
Hepcidin × MCHC	0.039^{NS}	0.069^{NS}	0.023^{NS}	0.131**
Hemoglobin × HCT	1.00**	1.00**	1.00**	1.00**
Hemoglobin × RBC	0.794**	1.00**	0.999**	0.994**
Hemoglobin × MCV	-0.025^{NS}	0.856**	0.601**	0.868**
Hemoglobin × MCH	-0.016^{NS}	--	--	-0.399**
Hemoglobin × MCHC	0.999**	0.921**	0.716**	0.937^{NS}
HCT × RBC	0.794**	1.00**	0.999**	0.994**
HCT × MCV	-0.025^{NS}	0.856**	0.601**	0.868**
HCT × MCH	-0.016^{NS}	--	--	-0.399**
HCT × MCHC	0.999**	0.921**	0.716**	0.937^{NS}
RBCs × MCV	-0.187^{NS}	0.855**	0.597**	0.856**
RBCs × MCH	0.021^{NS}	--	--	-0.412**
RBCs × MCHC	0.784**	0.920**	0.714**	0.934**
MCV × MCH	-0.38**	--	--	-0.379**
MCV × MCHC	0.848**	0.838**	0.417**	0.848**
MCH × MCHC	-0.44**	--	--	-0.441**

**Correlation is significant at the 0.01 level (2-tailed), *Correlation is significant at the 0.05 level (2-tailed) and NS for non-significant, -- is no correlation found between the parameters.

the Hb concentration <110gI^{-1} during pregnancy, but it also mentioned that physiological fall might affect Hb level and usually during the second trimester which was the time we considered for the sampling of pregnant women (Pavord *et al*., 2020). The Hb levels declined during the pregnancy in all three groups, as shown in table 1. The parallel biomarker hepcidin was synthesized in the liver, but it controls the systemic iron homeostasis (Koenig *et al*., 2014). Hepcidin levels are thought to be reduced during pregnancy to ensure higher iron bioavailability for both the mother and the fetus, according to research. Pregnant women with undetectable blood hepcidin transferred more iron to their fetus than women with detectable hepcidin, demonstrating that maternal hepcidin influences iron bioavailability to the fetus in part. Inflammatory conditions, such as preeclampsia, malaria infection and obesity, on the other hand, were linked to greater hepcidin levels during pregnancy (Koenig *et al*., 2014). Hepcidin levels are decreased during blood loss, iron deficiency and iron deficiency anemia (Ganz, 2013; Benson *et al*., 2020). During the second and third trimesters of pregnancy, hepcidin levels decrease; this may be due to increased absorption of dietary iron, and it also promotes the release of iron from stores (Sangkhae *et al*., 2020, Fisher and Nemeth, 2017a; Gambia *et al*., 2017). Our experimental work manifested the hepcidin decline in ID and IDA.

Decrease in newborn iron storage, placental difficulties, intrauterine developmental retardation, premature birth the possibility of drop in maternal blood reserves after birth and the necessity for transfusion in situations of severe blood loss are all hazards linked with iron deficiency anemia during pregnancy. Other risks include cardiac stress, anemia symptoms, a prolonged hospital stay, and a decrease in neonatal iron storage. As a result, early detection and treatment of iron deficiency are important (Breymann, 2002, Reveiz *et al*., 2007, Savajols *et al*., 2014, Api *et al*., 2015). The diagnosis of iron deficiency in pregnant women at various gestational stages would identify the risk group of anemic women and select the treatment options (Aringazina *et al*., 2020).

The main strength of our study findings to detect iron deficiency during pregnancy and gives its comparison with anemia and non-iron deficiency. It is not a single disorder based on its etiology, pathogenesis, and clinical hematology. Iron deficiency anemia is the most prevalent form of anemia during pregnancy, accounting for 80-90 percent of all cases (Aringazina *et al*., 2020). In Pakistan, a country with fewer resources of medical facilities, diagnostics with new and more markers, i.e., hepcidin, is an amenity. According to our research findings, serum hepcidin outperforms hemoglobin, serum iron, serum ferritin, TS, and TIBS to measure ID and IDA as in Zaman *et al* (2021). As a result of our studies, it is obvious that the use of hepcidin as a diagnostic tool for iron status has sparked attention. The significance of hepcidin as an indicator of iron deficiency in the second trimester of pregnancy has not been known in Pakistani women.

Our research has few limitations: Our sample's inclusion just entailed pregnant women without any comorbid conditions. Iron deficiency is associated with preeclampsia, gestational diabetes, and obesity (Amil *et al*., 2020; Kim *et al*., 2021). We couldn't include women with these conditions in sampling. Planning this cross-sectional study method would not allow for the investigation of the causation of the complex etiology of iron deficiency.

Despite these limitations with respect to the Pakistani population, this is the first research of authors knowledge which focus on investigating hepcidin along with other hematological markers of iron deficiency during the second trimester of gestation considering three groups of pregnant women, i.e., N-ID, ID, and IDA. Study of hepcidin in all three trimesters of pregnancy by doing follow-up sampling and associations of these levels with cord blood samples are recommended for future studies.

Future research is required to investigate the relationship between hepcidin levels in pregnancy and pregnancy-related problems. It is necessary to do research on early pregnancy hepcidin levels because raised or extremely low hepcidin levels might be utilized as an early diagnostic sign of maternal iron bioavailability.

CONCLUSION

We found differences in hepcidin level among non-ID pregnant women compared to ID and IDA during the second trimester of pregnancy in a cohort of women at Lahore. Biomarkers for iron deficiency including hemoglobin, MCV, HCT and MCHC were also studied.

A decrease in hepcidin level in IDA is directly linked with hemoglobin during 2nd trimester of gestation. Hepcidin level can predict iron levels in Pakistani women and can be used as a diagnostic biomarker for N-ID, ID, and Iron deficiency anemia. Hepcidin levels may enhance the targeting of iron supplementation programs in resource-constrained regions; however, the high cost of hepcidin may limit its application.

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