Hepcidin and iron-related parameters in pregnancy: how do they correlate?

DISSERTATION

Submitted to

THE TAMILNADU DR. MGR MEDICAL UNIVERSITY

In partial fulfillment for the degree

DOCTOR OF MEDICINE

IN

BIOCHEMISTRY - BRANCH XIII

MAY 2022

University Registration Number: 201923201

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CERTIFICATE

This is to certify that the study titled "**Hepcidin and iron-related parameters in pregnancy: how do they correlate?**" is the bonafide work of Dr. Arthur Amit Suryakumar, who conducted it under the guidance and supervision of Prof. Dr. Molly Jacob, Professor of Biochemistry, Christian Medical College, Vellore. The work in this dissertation has not been submitted to any other university for the award of a degree.

Dr. Molly Jacob,

Professor,

Department of Biochemistry,

Christian Medical College, Vellore.

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Dr. Joe Varghese

Professor and Head,

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Christian Medical College, Vellore.

Dr. Anna B. Pulimood,

Principal, Christian Medical College, Vellore.

DECLARATION

I hereby declare that the investigations, which form the subject matter of this study, were conducted by me under the supervision of Prof. Dr. Molly Jacob, Professor of Biochemistry, Christian Medical College, Vellore.

Dr. Arthur Amit Suryakumar,

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PLAGIARISM CERTIFICATE

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ABSTRACT

Background of study

In pregnancy, there is an increased requirement for iron to meet maternal and fetal demands. How exactly the placenta takes up iron from the maternal blood and transfers it to the fetus is not clear. Hepcidin is the central regulator of systemic iron homeostasis. Its levels in maternal blood have been shown to decrease in pregnancy to enable uptake of iron by the fetus. The underlying mechanism for such decreases is not known. It is also not clear to what extent maternal iron status during pregnancy influences that of the fetus.

Aims

The aim of this study is to study haematological and iron-related parameters, and a marker of inflammation, in blood from pregnant women and in the cord blood of their babies, and to determine how these parameters correlate with each other and with outcomes of pregnancy.

Methodology

Primigravidae, with uncomplicated pregnancies, who attended the antenatal clinics of Unit 4 of the Department of Obstetrics and Gynaecology, at Christian Medical College (CMC), Vellore, India, were the subjects of the study. They were recruited in their first trimester, after obtaining informed consent. Blood

samples were collected from them. At the time of delivery, a sample of cord blood was also obtained.

Haematological parameters (haemoglobin and mean corpuscular volume) and parameters linked to iron were estimated in the blood samples collected. The latter consisted of concentration of serum ferritin, serum iron and total iron-binding capacity (TIBC). Transferrin saturation was calculated from values of serum iron and TIBC. Concentrations of serum C-reactive protein (a marker of systemic inflammation) were also estimated. Correlations were determined between maternal and fetal parameters studied.

Results

Twenty subjects were recruited for the study. Their mean age was 25 years (SD 3.21), gestational age at delivery was 39.6 weeks (SD 0.98), hemoglobin level was 12.01 gm/dL (SD 1.14) and MCV was 82.27 fL (SD 1.14). Mean maternal serum iron was 74 µg/dL (SD 29.30), ferritin was 29.72 ng/mL (SD 30.48), TIBC was 383.3 µg/dL (SD 51.90) and transferrin saturation was 20.19 % (SD 9.03). The mean weight of the babies delivered was 3.07 kg (SD 0.31).

In cord blood, the mean hemoglobin value was 14.92 g/dL (SD 2.16) and MCV was 111.2 fL (SD 4.59). Mean serum iron was 114.19 µg/dL (SD 24.54), serum ferritin was 131.73 ng/dL (SD 53.89), TIBC was 273.42 µg/dL (SD 50.69) and transferrin saturation was 42.51% (SD 8.92).

In the maternal samples, serum iron correlated significantly and positively with serum ferritin and transferrin saturation, and negatively with TIBC. Maternal serum hepcidin correlated significantly and positively with maternal serum iron and transferrin saturation. Iron-related parameters in cord blood also correlated with one another and with some hematological parameters. Maternal total iron binding capacity (TIBC) correlated significantly and positively with cord blood haemoglobin. Maternal mean corpuscular volume (MCV) correlated significantly and positively with cord blood mean corpuscular volume (MCV).

Conclusion

Maternal serum hepcidin in the first trimester correlated significantly and positively with maternal serum iron and transferrin saturation. Work is ongoing to determine if this is so in the other 2 trimesters as well, and also in cord blood.

REVIEW OF LITERATURE

OVERVIEW OF IRON METABOLISM

Iron is a trace element that is required for many biological processes in humans. It is a transition metal which has two important properties: the ability to exist in 2 oxidative states, viz. ferrous (Fe^{2+}) and ferric (Fe^{3+}), and to form coordinate complexes. Based on the first property, it can accept and donate electrons, enabling it to play a critical role in enzymatic catalysis of redox reactions in the body (Vogt et al., 2021). Based on the second property, iron is present in heme, a tetrapyrrole coordinate complex found in haemoglobin (for oxygen transport), myoglobin (for oxygen storage), cytochrome P450 monooxygenases, cytochromes in the electron transport chain and in ribonucleotide reductase (Hoffbrand et al., 2016; V.W. Rodwell et al., 2018; Vogt et al., 2021). Iron is, therefore, a very important part of metabolism (Hoffbrand et al., 2016).

The body contains about 3 to 4g of iron. It is distributed in haemoglobin (2500 mg), ferritin (1000 mg), myoglobin and various enzymes (300 mg) and transferrin (3-4 mg) (Rodwell et al., 2018), As shown in Figure 1, large majority of iron required for erythropoiesis is provided by recycling of iron present in hemoglobin in senescent RBCs by the splenic macrophages. This constitutes

about 20-25 mg of iron every day. Iron is highly conserved in the body; only a small amount of iron is lost by desquamation of skin and cells lining the gastrointestinal tract (1-2 mg per day). Women in the pre-menopausal age group lose additional iron in menstrual blood. Iron lost from the body is replaced by intestinal absorption of dietary iron.

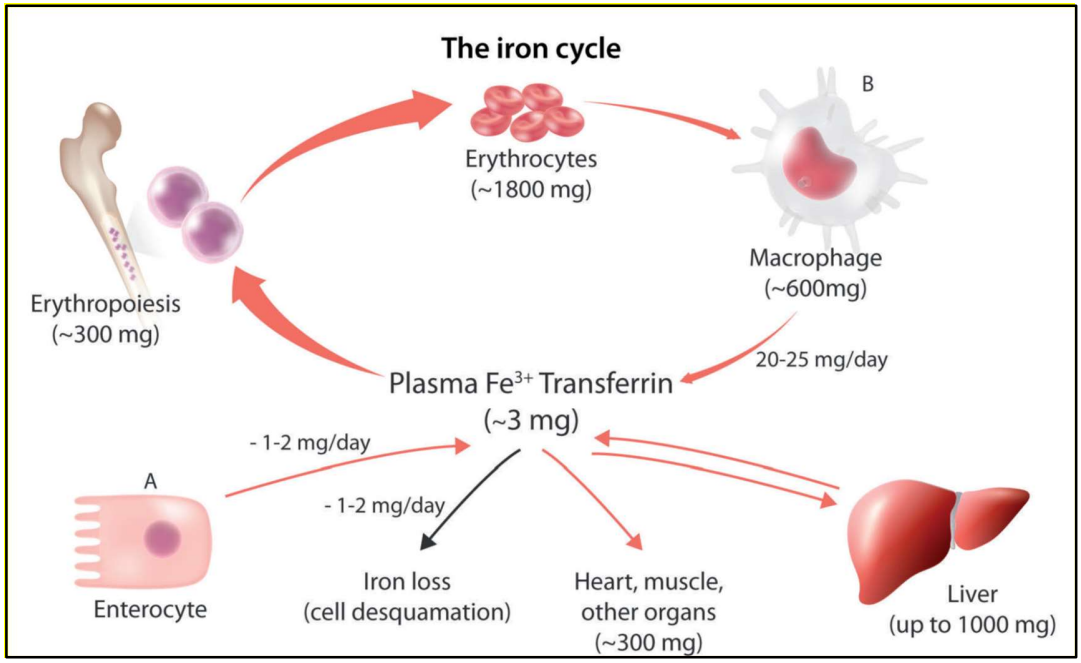

Figure 1: Iron cycle in the human body. Source (Camaschella et al., 2020)

Iron absorption

As there are no physiologically regulated mechanisms for excretion of iron from the body, the regulation of iron homeostasis primarily occurs at the level intestinal absorption. (Hoffbrand et al., 2016). Iron content in the earth's crust is ~4% and most plant and animal foods contain significant amounts of iron. However, all of it is not available for absorption (Hoffbrand et al., 2016). It is established that a major cause of anaemia is dietary iron deficiency (Vogt et al., 2021; WHO, 2011a).

The iron in the diet is complexed with proteins. It is released from the protein by the action of hydrochloric acid and proteolytic enzymes secreted by the stomach and small intestine. This results in the release of heme from heme-containing proteins, and non-heme iron from other sources(Barrett et al., 2019; Hoffbrand et al., 2016)

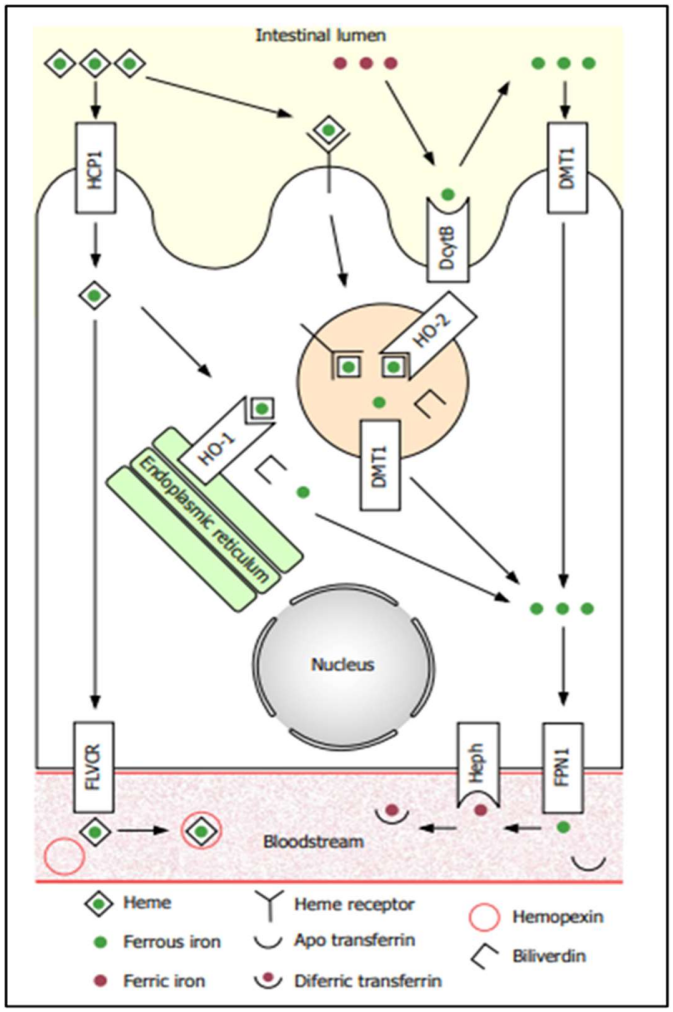

Figure 2 Heme and non- heme iron transport across the enterocytes. From the luminal side of the enterocyte, heme is taken up via HCP1 (heme carrier protein -1). Heme may also be taken up by clathrin-coated pits contianing heme binding receptors. Inside the enterocyte, heme degradation is catalyzed by the enzyme HO-1(heme oxygenase-1). Alternatively, it can enter the circulation through the transporter present on the basolateral membrane, FLCVR (Feline leukemic virus receptor). Non-heme iron in the intestinal luman is converted from Fe^{3+} to Fe^{2+} form by DcytB (Duodenal cytochrome B). Fe^{2+} is then taken up by DMT 1 (Divalent metal transporter protein 1) into the enterocyte. Subsequently, iron can exit to the circulation through FPN1 (ferropotin 1). Fe^{2+} is then converted to Fe^{3+} by HepH (Hephaestin) and binds to carrier protein in blood, transferrin**. Source: (West and Oates, 2008)**.

Factors affecting iron absorption are (Hoffbrand et al., 2016):

1. Content of iron in the diet
2. Iron bioavailaibilty
3. Body iron status

The absorption differs depending on how iron is found in the diet – as heme and in non-heme forms. Heme is present abundantly in animal proteins and is absorbed efficiently by enterocytes. As shown in figure 1, there are two known mechanisms by which heme iron is absorbed. Firstly, heme receptor mediated endocytosis and secondly via heme transporters (Hooda et al., 2014; West and Oates, 2008). In contrast, non-heme iron is usually present in the diet in its ferric (Fe^{3+}) form and is poorly bioavailable. It must be converted to its ferrous (Fe^{2+}) form before it can be absorbed. This is done by duodenal cytochrome b, a ferri-reductase found on the luminal surface of enterocytes. The ferrous form of iron is taken up into the enterocyte by divalent metal transporter 1(DMT 1), which is also found on the luminal surface of enterocytes (Victor W. Rodwell et al., 2018).

Inside the enterocyte, heme-bound iron is released from heme by the action of heme oxygenase. The iron released can be stored or transferred out of the cell. Iron is stored by binding to ferritin. Ferritin is a globular protein complex of 474kDa molecular weight, with 24 subunits. It can hold about 3000 to 4000 ferric atoms. On the other hand, ferrous iron may be transported across the basolateral membrane and released into the blood, via ferroportin. Ferroportin is the only known iron exporter in mammalian cells. This step is regulated by a peptide hormone, hepcidin, which is produced by the liver and is the central regulator of systemic iron homeostasis (Victor W. Rodwell et al., 2018). Once exported, ferrous iron is converted to its ferric form by the membrane-bound, copper-containing ferroxidase, haephestin.

Iron in circulation

In the circulation, ferric iron binds with high affinity to the plasma protein, transferrin. Transferrin is produced in the liver and exists in two forms in blood: apo-transferrin (not bound with ferric atoms) and holo-transferrin (bound to two ferric atoms)(Kennelly et al., 2018; Victor W. Rodwell et al., 2018)

Iron uptake by cells

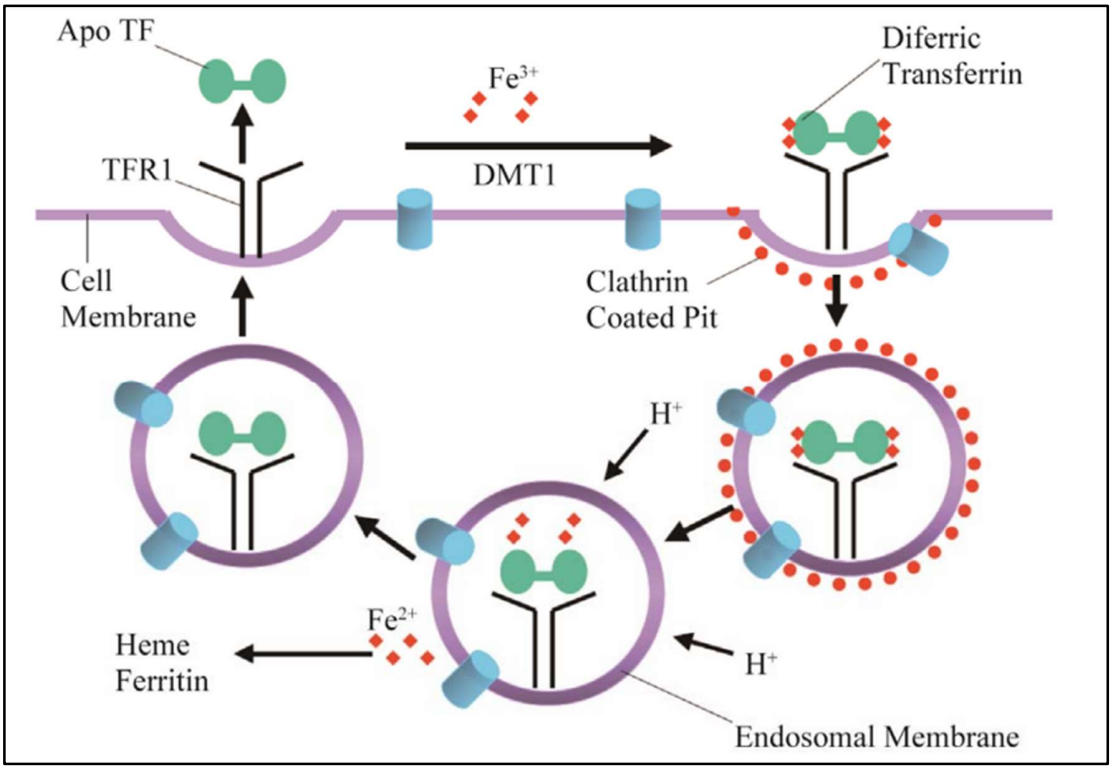

Figure 3 Transferrin cycle. Holo-transferrin binds with transferrin receptor 1 (TfR1) which is present on the surface of cells in clathrin-coated pits along with DMT1. The membrane invaginates to form an endosome with transferrin bound to TFR1 and DMT1 on the membrane walls. The pH in the endosome becomes acidic; this causes the release of iron from transferrin. The iron is released into the cytoplasm from the endosome through DMT1. Following this, the endosome is recycled back to the cell surface where transferrin dissociates from TfR1 and is released into the circulation. **Source : (Elliott and Head, 2012)**

Iron is taken up from transferrin by almost all cells of the body. However, large majority of transferrin-bound iron is taken up by the erythroid precursors of the bone marrow where it is used for the synthesis of haemoglobin. Holo-transferrin is taken up by cells via a membrane-bound receptor called transferrin receptor 1 (TfR1). As shown in figure 3, once transferrin binds to its receptor, it is endocytosed and forms an endosome within the cell. Iron is released from the transferrin when the pH inside the endosome becomes acidic. It exits the endosome via DMT1 on the endosomal membrane. The apo-transferrin, still bound to the transferrin receptor, is recycled to the cell surface, where the two separate; the receptor remains on the cell surface and apo-transferrin is released into the blood. (Victor W. Rodwell et al., 2018).

Recycling of iron from senescent erythrocytes by macrophages

Macrophages present in the spleen and liver form the reticuloendothelial system. They phagocytose senescent and damaged erythrocytes. In the phagocytic vesicle formed, heme released from haemoglobin is degraded to biliverdin, carbon monoxide and iron by the enzyme heme oxygenase. The iron that is released is transported out of the vesicle by DMT1 on the vesicular membrane. Iron is subsequently either stored as ferritin or transported out of the macrophage by ferroportin. The iron released from macrophages is in its Fe^{2+}

form. Ceruloplasmin, a plasma protein with ferroxidase activity, converts it into Fe^{3+}. The ferric form then binds with transferrin and is available cells to be taken up via the transferrin cycle.(Victor W. Rodwell et al., 2018)

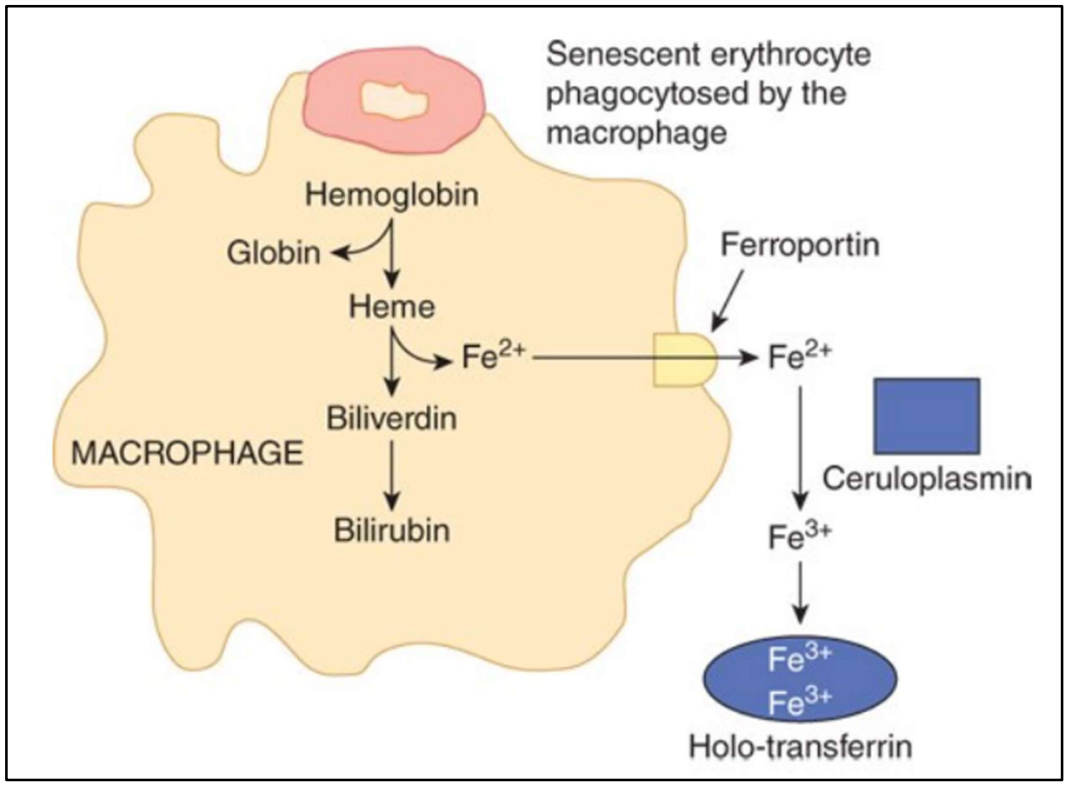

Figure 4 Iron recycling: Macrophages phagocytose senescent and damaged red blood cells in the circulation. The haemoglobin released is degraded to release globin and heme. The heme is further degraded by heme oxygenase to give ferrous (Fe2+) iron and biliverdin; biliverdin is subsequently converted to bilirubin. Ferrous (Fe2+) iron is released into circulation by ferroportin. Ceruloplasmin is a ferroxidase that converts Fe^{2+} to Fe^{3+}. Ferric iron is transported in blood bound to transferrin. **Source: (Victor W. Rodwell et al., 2018)**

Hepcidin – central regulator of systemic iron homeostasis

Regulation of iron homeostasis in the body is of great importance. Higher than normal levels in the body can lead to toxicity. This is because iron can take part in the Fenton reactions, resulting in production of free radicals, which can damage tissue (Backa et al., 1993; Jomova et al., 2012; Park et al., 1987; Winterbourn, 1995). If iron levels are abnormally low in the body, it can lead to iron-deficiency anemia.

Liver produces hepcidin, which is the chief regulator of systemic iron homeostasis. It is known to bind to ferroportin on the surface of cells, triggering the internalization and degradation of ferroportin. This results in decreased release of iron from the enterocytes and macrophages into the blood, resulting in hypoferremia. (Nemeth et al., 2004b; Victor W. Rodwell et al., 2018)

The term “hepcidin” has 2 parts, with corresponding meanings; hep- (of hepatic origin) and -cidin (possessing anti-microbial activity)(Ganz, 2003a; Park et al., 2001). It is made up of 25 amino acids (Krause et al., 2000) and is encoded by the *HAMP* gene.

Hepcidin is up-regulated by iron overload and inflammation, and down-regulated by erythropoiesis and hypoxia (Zhao et al., 2013). Hepcidin has been shown to regulate iron absorption in the gut, iron release from the macrophages andiron storage in the liver.(V.W. Rodwell et al., 2018) The mechanisms involved are summarized in Figure 5.

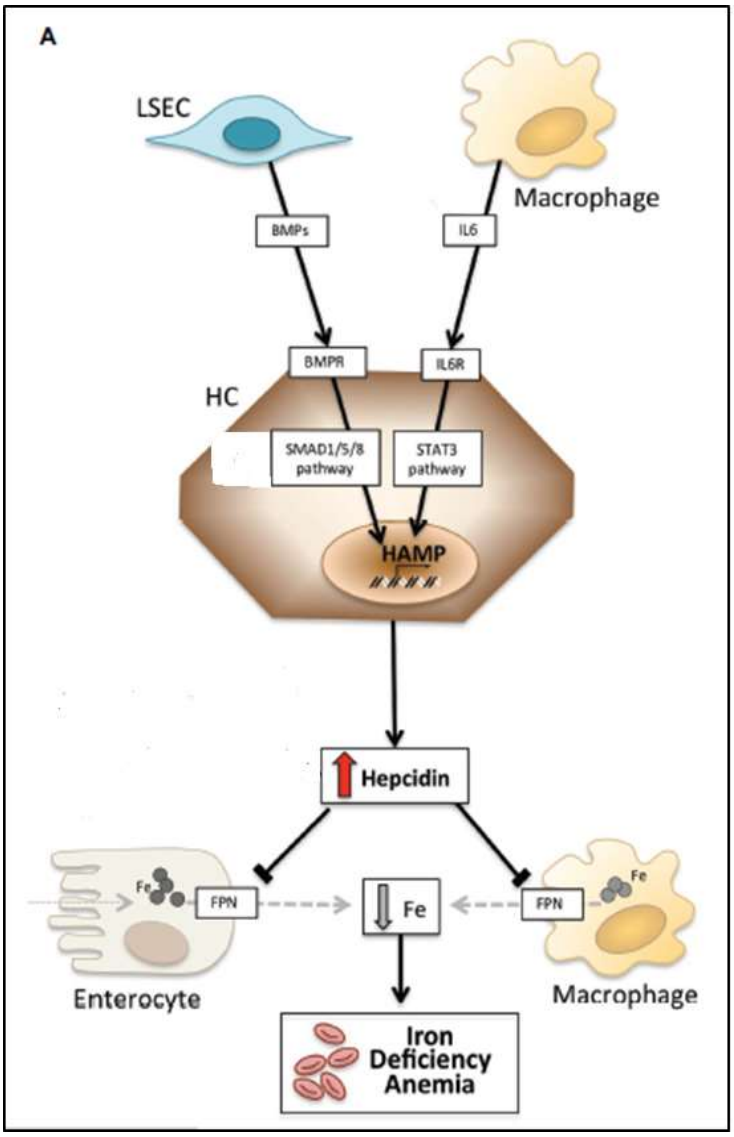

Figure 5: Regulation of hepcidin: Sinusoidal endothelial cells (LSEC) produce bone morphogenetic proteins (BMP2 and BMP6) that bind to bone morphogenetic receptor (BMPR) on the plasma membrane of hepatocytes to phosphorylate and activate intracellular SMAD proteins. SMAD proteins translocate to the nucleus where it induces hepcidin *(HAMP*) gene transcription. Pro-inflammatory macrophages release interleukin 6 (IL6), which binds to interleukin 6 receptors (IL6R). IL6R activates the STAT3 pathway and activates transcription of *HAMP* gene. **Source: (Pagani et al., 2019)**

Cellular iron homeostasis

Intracellular iron homeostasis is achieved mainly by the iron regulatory element (IRE) / iron regulatory protein (IRP) system that regulates cellular iron uptake, storage and export (Hentze et al., 2004).

There are 2 orthologous RNA-binding iron regulatory proteins - IRP 1 and IRP 2. They bind to cis-regulatory hairpin structures known as iron response element (IRE) present on the untranslated regions (UTRs) of mRNA. The IREs are found either in the 5' or 3' UTR of the mRNAs coding for some of the most critical genes involved in iron homeostasis, including ferritin, transferrin receptor, divalent metal transporter 1, ferroportin, erythroid specific form delta – aminolevulinic acid synthase, and hypoxia inducible factor- 2 α. (Ec, 1993; Muckenthaler et al., 2008).

The binding of IRP to IRE present on the 3'IRE on mRNA results in its stabilization and increase in half-life of the mRNA. This eventually results in increased protein translation. Transferrin receptor 1 (TfR1) is a classic example of a gene with multiple IREs in its 3'UTR. On the other hand, binding of IRP to 5'IRE results in translation arrest. This is classically seen in ferritin mRNA which has a highly conserved IRE in its 5' UTR.

The two IRPs, IRP 1 and IRP 2, are regulated by iron (and other factors) by distinct mechanisms as shown below.

Functioning of the IRP in various states		
Type & state	**Iron replete state**	**Iron-deficient state**
IRP 1(Dupuy et al., 2006; Walden et al., 2006)	In the presence of iron, IRP1 forms a 4Fe-4S cluster that converts it to a cytosolic aconitase that is unable to bind to IRE.	In the absence of iron, the cluster of (4Fe-4S) is lost. In this form, IRP 1 binds to IRE in the mRNA.
IRP 2(Takahashi-Makise et al., 2009)	In the presence of iron, FBXL5 (Ubiquitin ligase) ubiquitinates and induces proteasomal degradation of IRP2.	In the absence of iron, FBXL5 activity decreases, thus increasing the levels of IRP 2

As shown in Figure 9, in the absence/deficiency of intracellular iron, the IRPs are active and bind to 3'-IRE of TfR1 and DMT-1 mRNA resulting in their stabilization and increased translation. The net result would be increased uptake

of iron in order to increase intracellular iron levels. On the other hand, binding of IRPs to the 5'-IREs results in the inhibition of translation of the following mRNA:

1. Ferritin – L
2. Ferritin – H
3. Ferroportin
4. Mitochondrial aconitase
5. Erythroid ALA-synthase (ALAS2)

This results in increased storage of iron (ferritin) and increased itestinal absorption of iron (via ferroportin). In erythroid cells, there is IRP-dependent control of amino levulenic acid synthase- 2 (Alas-2). This is the rate limiting enzyme in heme synthesis (Cooperman et al., 2005; Melefors et al., 1993).

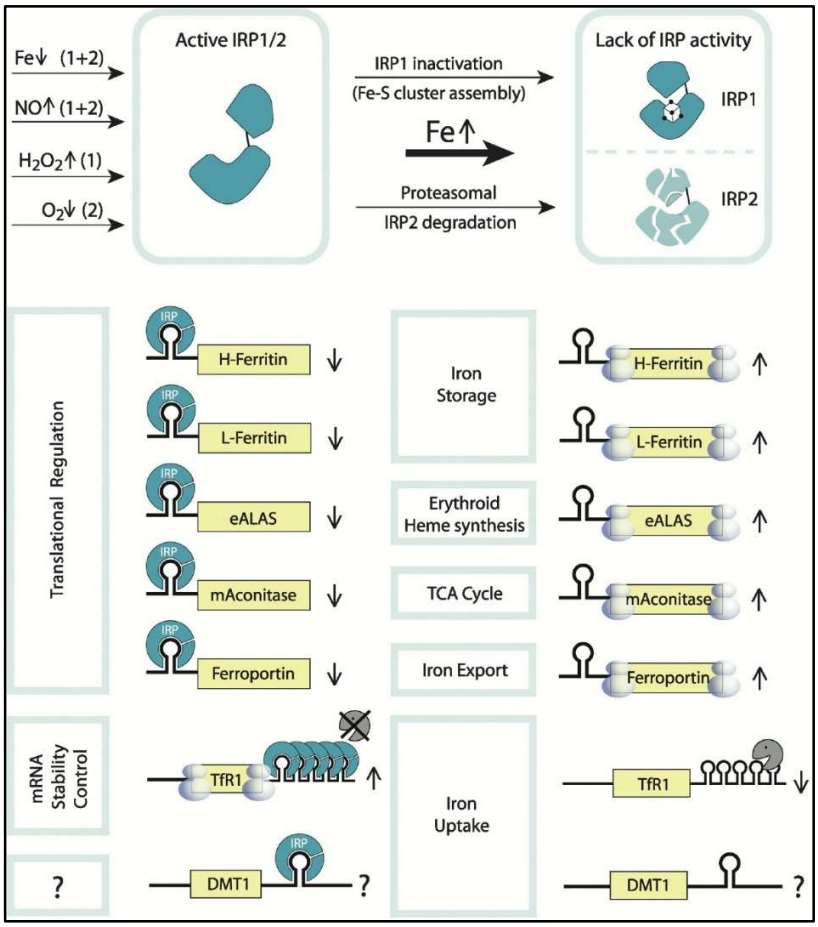

Figure 9 : IRP 1 and IRP2 regulate the cellular iron homeostasis.

In the presence of iron, IRPs are stabilized and bind to IREs present in mRNA of specific genes. Genes that have 5'IREsm such as ferritin, ferroportin, eALAS etc. are subjected to translational arrest, while those with 3-IREs, such as TfR1, are stabilized. The IRPs themselves are regulated by iron, nitric oxide (NO), hydrogen peroxide (H2O2) and hypoxia. **Source : (Hentze et al., 2004)**

IRON METABOLISM IN PREGNANCY

Physiologic changes in pregnancy

One of the most important physiological changes that occurs during pregnancy is the increase in blood volume (Cunningham et al., 2018a; Koller, 1982). This is achieved primarily by increasing the plasma volume. The increase in blood volume is essential during pregnancy for the following reasons (Cunningham et al., 2018a) –

1. To maintain blood flow for the enlarged uterus and its hypertrophied vasculature
2. To supply nutrients for the rapidly growing fetus and placenta
3. It protects the mother and the fetus against the effect of impaired venous return in supine or erect position due to the pressure of the gravid uterus on the abdominal venous system
4. It protects against hypovolemia due to blood loss associated with parturition

Iron requirements during pregnancy

The increase in plasma volume in pregnancy is associated with an increase in erythropoiesis to prevent dilutional anaemia. Therefore, iron requirement increases to meet the demand for the increased erythropoiesis (Bencaiova et al., 2019). In addition, iron requirements increase significantly during pregnancy to

meet the demands of the placenta and the growing fetus (Table 1 and Figure 5). The requirement for iron in each trimester varies, depending on the physiological needs of the mother and on her stores of iron (Chełchowska et al., 2016). When the increased demand for iron during pregnancy is not met, it often results in anaemia (Scholl, 2011).

In non-pregnant women, obligate losses of iron occur via menstruation and via the skin, gut, and urine. In pregnancy, menstruation does not occur. Hence, the losses to be compensated during pregnancy are losses from the skin, gut and in urine. Obligatory losses via these routes in a pregnant woman is <0.8 mg/d in a 55-kg woman (230 mg/pregnancy)(Bothwell,2000).

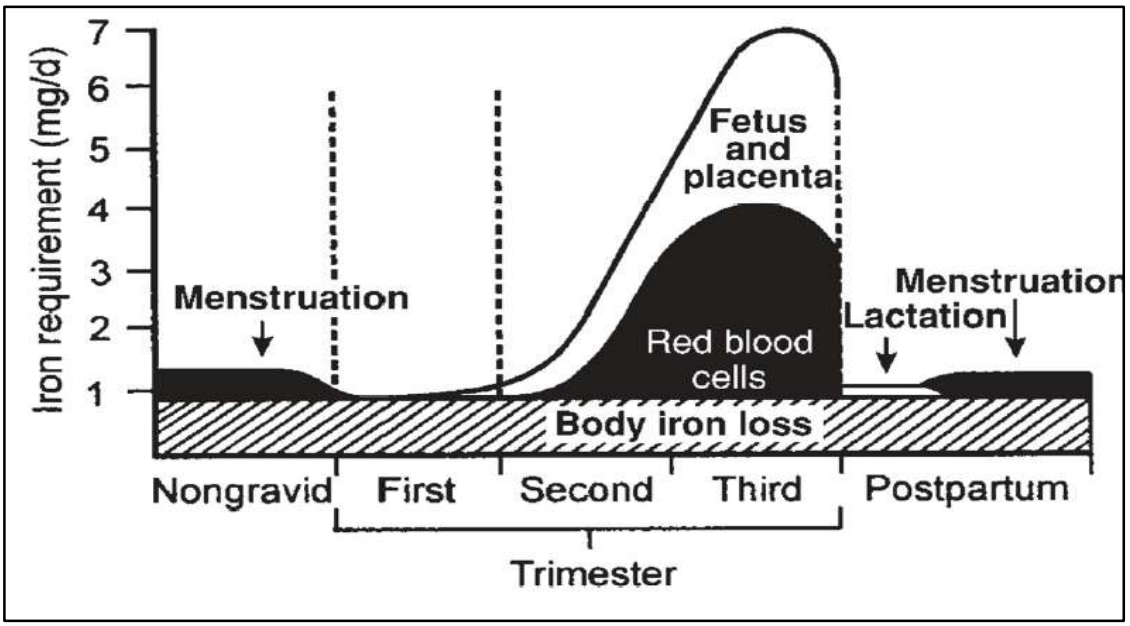

Figure 6 Estimated iron requirements during pregnancy in a 55-kg woman. In a non-pregnant menstruating female, the iron requirement is approx. 1.5 mg/day. In the first trimester of pregnancy, iron requirements are low due to the absence of menstrual blood loss. As pregnancy progresses into second trimester and third trimester, the iron requirement increases from 1 mg/day to 7 mg/day to meet the demands for increased erythropoiesis and growing fetus and placenta. **Source: (Koenig et al., 2014)**

Table 1 (reproduced from Bothwell, 2000)

Iron requirement in pregnancy	**Amount In mg**
Total need in pregnancy	
Fetus	270
Placenta	90
Expansion of red cell mass	450
Obligatory basal losses	230
Maternal blood loss at delivery	150
Total	1190
Net iron requirement in pregnancy	
Contraction of maternal red blood cell mass	- 450
Absence of menstruation during pregnancy	- 160
Total	- 610
	1190 – 610
Net total	580

Mechanism of iron transfer across the placenta

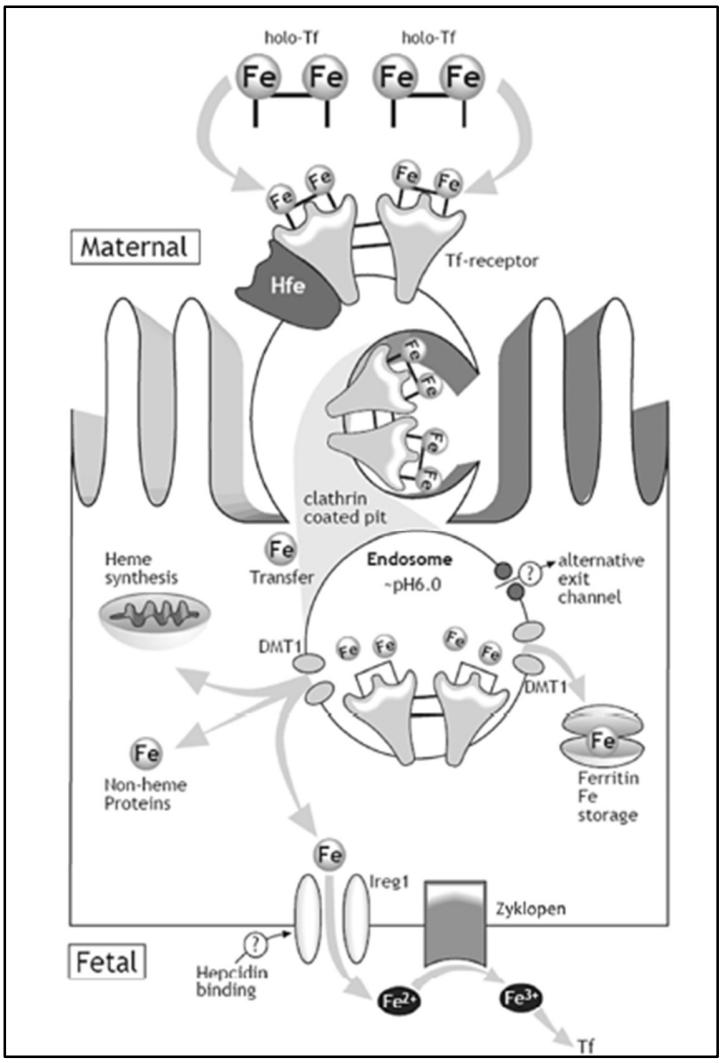

Figure 7 Placental iron transfer: Holo-transferrin in maternal blood binds to transferrin receptors on the maternal aspect of the syncytiotrophoblast and is endocytosed by the cell forming an endosome. Iron is released from transferrin when the pH becomes acidic in the endosome. The released iron exits the endosome through DMT1. Iron is then exported into fetal circulation via ferroportin which is present on the fetal aspect of placental synctiotrophoblast. The released Fe^{2+} is converted into $Fe^{3+}$by the placental ferroxidase, zyklopen. **Source: (Koenig et al., 2014)**

Iron from the maternal blood is transferred to the fetal circulation via mechanisms similar to those in other cells (Figure 7). Holo-transferrin is taken up via the transferrin cycle that takes place in syncytiotrophoblasts (Koenig et al., 2014; Sangkhae and Nemeth, 2019). Iron is then transferred to the fetal circulation by ferroportin (Ireg1) (Koenig et al., 2014). Iron is loaded into fetal transferrin after is oxidized to its ferric form by a ferroxidase, called zyklopen. (Figure 7)

Anaemia in pregnancy

Anaemia is a global problem, with iron-deficiency anaemia being the commonest cause for it (WHO, 2011.). It has been estimated that 40.1 % of pregnant mothers in the world suffer from iron-deficiency anaemia (Stevens et al., 2013; WHO, 2016.). India contributes significantly to this burden (National Family Health Survey 4 report 2015-2016; Rai et al., 2018; Rammohan et al., 2011).

Pregnant women are particularly vulnerable to anaemia because of the increased demands for iron in pregnancy. The additional amount of elemental iron needed during pregnancy is about 1g. Demands for iron increase with increasing

gestational age (Ganz, 2003b). Supplementation of iron is often required to meet the increased demands in pregnancy.

Several parameters in blood can be used to determine the iron status of the body. These include haemoglobin serum ferritin, serum iron, TIBC, transferrin saturation, RBC porphyrins, soluble transferrin receptor, and RBC morphology (Goddard et al., 2011; Victor W. Rodwell et al., 2018). Haemoglobin levels in blood decrease when there is a deficiency of iron which is required for the synthesis of heme.(Victor W. Rodwell et al., 2018).

Serum iron is a measure of iron present in the blood that is bound to transferrin. It decreases in iron-deficient states (Burtis and Bruns, 2014; Victor W. Rodwell et al., 2018). Ferritin is the intracellular storage form of iron in the body. Levels of ferritin in the serum is widely held to reflect body iron stores. (Victor W. Rodwell et al., 2018). The total iron binding capacity (TIBC) of blood indicates how much iron can be bound to transferrin (Victor W. Rodwell et al., 2018). It is increased in iron-deficiency anaemia which is characterized by elevated transferrin levels in blood. Transferrin saturation is an indication of the extent to which the total iron-binding capacity of transferrin is saturated. In normal conditions, ~30% of the binding sites on transferrin have iron bound to them.

The amount of iron bound to transferrin decreases in iron-deficient states and increases in iron-overloaded states (Victor W. Rodwell et al., 2018). RBC porphyrins are a measure of red cell protoporphyrins; an increase in levels of red cell protoporphyrin indicates iron deficiency (Victor W. Rodwell et al., 2018).

When iron levels decrease in cells, the number of transferrin receptors on their surfaces increases to enable it to take up more iron. Some of these receptors are cleaved and found in blood as soluble transferrin receptor (sTfR). Approximately 80 to 90% of TfR are present on erythropoietic precursor cells. Hence, the sTfR is proportional to the TfR on erythroid precursor cells, where they increase markedly in iron-deficient states (Berlin et al., 2011; Gupta and Abbi, 2003; Victor W. Rodwell et al., 2018; Y, 2003). RBC morphology can also indicate iron deficiency, when they appear as hypochromic and microcytic cells (Ford, 2013; Kumar et al., 2012).

Table 2: Diagnostic criteria for anemia (WHO, 2011a)

Pregnant women	**Haemoglobin value (g/dL)**
No anaemia	11 or higher
Anaemia Mild Moderate Severe	 10.0-10.9 7.0-9.9 Lower than 7.0

Table 3: Diagnostic criteria for iron deficiency (WHO, 2020)

	Iron deficiency	
	Apparently healthy individuals	**Individuals with infection or inflammation**
Infants & young children	<12	<30
Pregnant women	<15 (first trimester)	-
Adults (20 to 59 years)	<15	<70

Table 4: Reference ranges for iron-related parameters in normal adult (reproduced from Adamson, 2018**)**

Parameters	**Normal**	**Negative iron balance**	**Iron-deficient erythropoiesis**	**Iron-deficiency anaemia**
Marrow iron stores	1+ to 3+	0 to 1 +	0	0
Serum ferritin (µg/L)	50-200	<20	<15	<15
TIBC (µg/dL)	300-360	>360	>380	>400
Serum iron (µg/dL)	50-150	-	<50	<30
Transferrin saturation (%)	30-50	-	<20	<10

Table 5: Reference ranges for iron-related parameters in pregnancy (reproduced from Cunningham et al., 2018b**)**

Parameter	**Non-pregnant**	**First trimester**	**Second trimester**	**Third trimester**
Haemoglobin (mg/dL)	12-15.8	11.6-13.9	9.7-14.8	9.5-15.0
Mean corpuscular volume (MCV) (fL)	79-93	81-96	82-97	81-99
Serum iron (µg/dL)	41-141	72-143	44-178	30-193
Serum ferritin (µg /L)	10-150	6-130	2-230	0-116
Transferrin iron binding capacity (TIBC) (µg/dL)	251-406	278-403	Not reported	359-609
Transferrin saturation (%)	22-46	Not reported	10-44	5-37

Hepcidin in pregnancy

Maternal hepcidin

As we have seen above, there is increased physiological demand of iron in pregnancy to support feto-placental development and other maternal changes during pregnancy. The iron requirement during the pregnancy is not uniform. We have also seen that the iron requirement in the first trimester is <0.8 mg/d due to the absence of menstrual cycles. The need of iron requirement increases 1.0 mg/d in 2nd trimester to 7 mg/d in the 3rd trimester(Thomas H Bothwell, 2000)

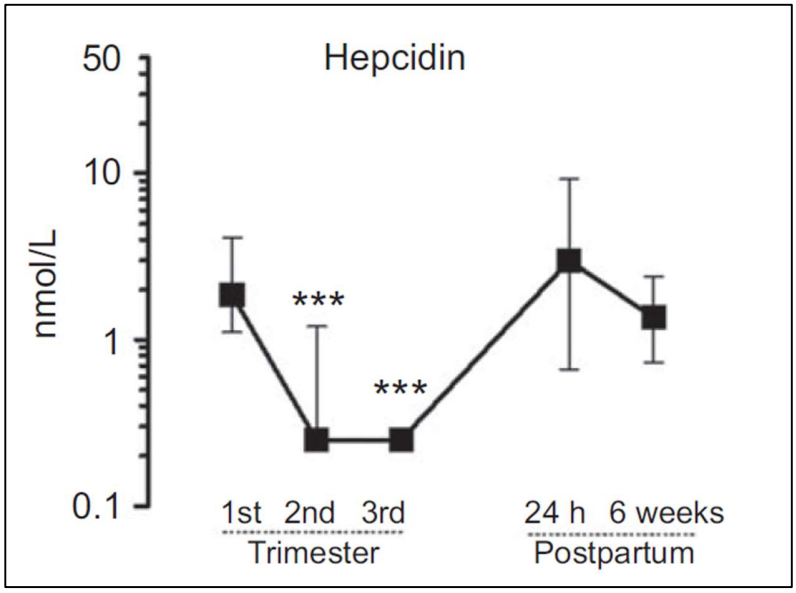

Figure 10: Serum hepcidin concentrations during the pregnancy. Shown as medians with the interquartile range. The asterisks represent the significance in relation to first trimester values, ** p < 0.001, *** p < 0.0001 **Source :Reproduced from van Santen et al., 2013**

Normal hepcidin values during pregnancy reported by various studies is given below (Koenig et al., 2014):

The hepcidin values given by various studies are listed below:	
	Maternal hepcidin findings in serum
Van Santen et al., 2011	4.2 nmol/L (0.5–13.9) nmol/L
Finkenstedt et al., 2012	1st trimester: median = 16 ng/mL (4–97) 2nd trimester: median = 11 ng/mL (6–36) 3rd trimester: median = 9.5 ng/mL (1–43), $p < 0.001$
Van Santen et al., 2013	Median 2.0 nmol/L (range < 0.5–12.3 nmol/L) 15–19 weeks gestation: 1.85 nmol/L (1.10–4.10) 20–25 weeks gestation: 0.25 nmol/L (0.25–1.20) 29–35 weeks gestation: 0.25 nmol/L or undetectable 24 h postpartum: 3.0 nmol/L (0.66–9.22) 6 weeks post-delivery: 1.35 nmol/L (0.73–2.40)

Young et al., 2012	At time of admission to hospital after onset of labour Median = 9.30 ng/mL (range not provided in publication)

In order to meet the increasing iron requirements, there needs to be more intestinal absorption and iron recycling from macrophages than in the non-pregnant state(Ganz, 2003a). This is made possible by suppression of the iron regulatory hormone, hepcidin. In pregnant state, hepcidin suppression may be due to increased erythropoiesis (due to plasma dilution) and increased iron utilization by the feto-placental unit. However, the exact mechanisms involved are not clearly known (Fisher and Nemeth, 2017; Young et al., 2012).

It is seen that regulation of hepcidin during pregnancy is similar to that seen in the non-pregnant state, except that the thresholds are set at a lower level. In previous studies, maternal hepcidin was shown to positively correlate with serum ferritin and transferrin saturation throughout pregnancy. It also correlated inversely with soluble transferrin receptors and hemoglobin. This shows that

these regulatory pathways are intact and functional during pregnancy (Fisher and Nemeth, 2017).

Hepcidin levels are generally low or undetectable in maternal blood during the third trimester with the decline starting in the beginning of second trimester (Koenig et al., 2014; van Santen et al., 2013).It has been argued that there is gradually increasing iron deficiency during the course of the pregnancy and that this may explain hepcidin suppression. However, iron replete pregnant women are also seen to have suppressed hepcidin.

It is known that the pregnancy is a mild inflammatory condition in the initial first trimester as the implantation process is considered as an open wound in the endometrium. However, in studies done on healthy pregnant women, it is shown that serum hepcidin markers did not correlate with inflammatory markers. This suggests that the mild inflammation associated with normal pregnancy does not affect maternal hepcidin (Dekel et al., 2014; Schulze et al., 2008)

There is increase in hepcidin concentration in obese pregnant women and preeclamptic individuals compared to normal pregnant women which did not

interfere with the haematological and iron parameters in the mother or newborn (Toldi et al., 2010). This suggests us that mild elevation of hepcidin concentration during pregnancy does not cause a significant decrease in iron absorption or recycling.

The advantage of measuring hepcidin is that it provides an integrated picture of iron handling in the body (Bah et al., 2017). Hepcidin levels reflect the net effect of the various factors that impact upon hepcidin gene transcription (Sangkhae and Nemeth, 2017). Therefore, serum hepcidin is a useful marker for detecting iron deficiency during early pregnancy even before anaemia is detected (Rehu et al., 2010). Abioye et al., in their study found hepcidin and haemoglobin to be the best predictors of iron deficiency and iron deficiency anaemia in pregnancy women.

Since hepcidin is induced by inflammatory cytokines, especially interleukin 6 (IL-6), it reduces the availability of iron causing iron restricted erythropoiesis in inflammatory conditions resulting in anaemia of inflammation. If hepcidin is elevated, then it can differentiate anaemia of inflammation from iron deficiency anaemia (Abioye et al., 2020).

Fetal Hepcidin

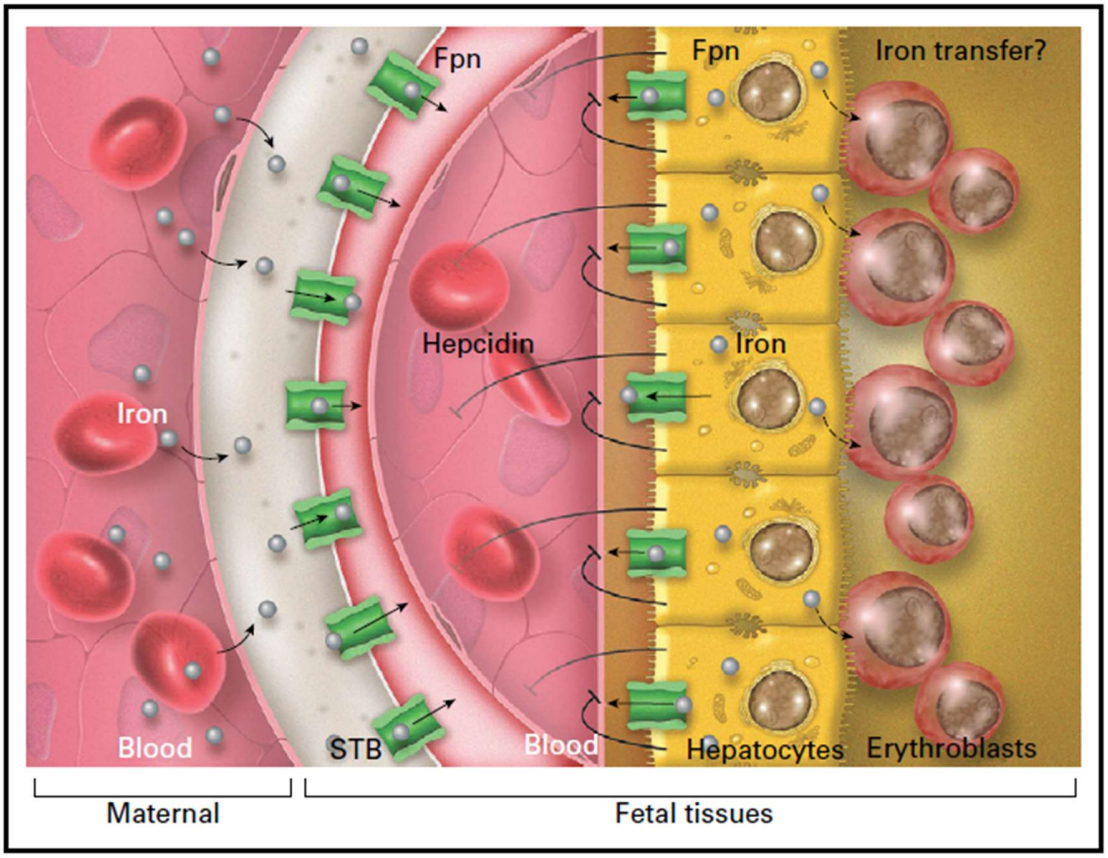

Figure 11: Action of fetal hepcidin: Hepcidin secreted from the fetal liver primarily acts on the ferroportin (fpn) present on the fetal hepatocytes. Regulation of hepatocyte ferroportin plays a role in the supply of iron to the developing erythroid progenitor cells in the fetal liver. **Source : (Ganz, 2020)**

Very little is known about the physiological role of fetal hepcidin and regulation of hepcidin expression in the fetal liver. It has been shown that fetal hepcidin, at physiological levels, does not have a significant effect on ferroportin expression in the syncytiotrophoblasts, suggesting that it may not play a major role in regulation of maternofetal iron transport across the placenta (Ganz, 2020; Kämmerer et al., 2020). On the other hand, supraphysiological levels of fetal hepcidin (as seen in Tmprss6-deficinet and hepcidin-overexpressing mouse embryos) down-regulates placental ferroportin, resulting in iron deficiency in the fetus (Sangkhae et al., 2020).

The fetal liver is a major site of erythropoiesis in the growing fetus. Hepcidin, which is secreted by the fetal liver, is thought to have an autocrine/paracrine effect on fetal hepatocytes. It is hypothesized that it may play a key role in supplying iron to rapidly proliferating erythroblasts in the liver by regulating the levels of ferroportin expression in both the erythroblasts and hepatocytes (Ganz, 2020; Kämmerer et al., 2020). However, further work is essential to confirm this and provide evidence for the physiological role of fetal hepcidin.

SCOPE OF STUDY

It is not totally clear whether maternal iron status during pregnancy correlates with that of the fetus. This information is important because it is generally assumed that improving maternal iron status would also improve iron status in the fetus. It is not clear whether this assumption is true. As far as is ascertainable, there is no data from Indian subjects on this aspect. This study attempts fill these lacunae in the literature. It is envisaged that the results of this study will provide insights into links between maternal hepcidin and fetal iron status.

THE STUDY

BACKGROUND TO THE STUDY

In pregnancy, requirements for iron are increased to meet maternal and fetal demands. Mechanisms involved in the uptake of iron from the maternal blood, its handling by the placenta and its subsequent transfer to the fetus are not clear. Hepcidin (the key regulator of systemic iron homeostasis) in the maternal circulation has been postulated to play a role in such processes. Its levels have been shown to decrease in pregnancy to enable uptake of iron by the fetus, and to return to normal levels post-partum. The mechanism that underlies such decreases is not known. There is little known on whether fetal hepcidin regulates placental iron uptake and transfer. It is also not clear to what extent maternal iron status during pregnancy influences that of the fetus. As far as ascertainable, there is no data on Indian subjects on these aspects.

HYPOTHESIS

Haematological and iron-related parameters in maternal blood, during pregnancy, correlate with the same parameters in cord blood.

AIM

The aim of this study was to estimate hematological and iron-related parameters in blood from pregnant women and in the cord blood of their babies and to determine how these parameters correlate with each other and with outcomes of pregnancy.

OBJECTIVES

(a) To estimate iron-related and haematological parameters, and a marker of inflammation, in blood from pregnant women and in the cord blood of their babies

(b) To determine how these parameters correlate with each other and with outcomes of pregnancy

MATERIALS

Equipment used

1. Table-top refrigerated centrifuge - MPW R 350, MPW Med Instruments, Poland
2. Freezer (-70°C) – Cryo Scientific Systems Pvt Limited, Chennai, India
3.

Materials used

1. Vacutainer tubes for blood collection (BD Biosciences, Plymouth, UK)
2. Micro centrifuge tubes 1.5mL and micro tips - Tarsons Products Private Limited, Kolkata, India

METHODS

The study was approved by the Institutional Review Board (IRB) at Christian Medical College (CMC), Vellore, India (IRB minute number 13367, dated 02.09.2020) (approval letter in Appendix I).

Subjects

In this study, primigravidae who attended the antenatal clinics of Unit 4 of the Department of Obstetrics and Gynecology at Christian Medical College (CMC), Vellore, India, were recruited in their first trimester, after obtaining informed consent. They were followed up through pregnancy, until delivery.

Inclusion criteria:

1. Primigravidae (between the ages of 18 and 35) in their first trimester
2. Those with singleton uncomplicated pregnancies
3. Those who planned to deliver their babies in CMC, Vellore
4. Those willing to give consent to be part of the study

Exclusion criteria:

1. Those with multiple pregnancies
2. Those who developed any complication of pregnancy
3. Those who were considered by the obstetric team to have a high-risk pregnancy

Eligible subjects were screened, according to the inclusion and exclusion criteria listed above. Informed consent was sought from those who were found to meet these criteria and who were willing to participate in the study.

Informed consent

An information sheet about the study was provided to each willing subject. This was provided in 3 languages – English, Tamil and Hindi. Each subject chose the language that she was most conversant with. The investigators also explained the details of the study to each potential subject and answered any questions she or her attendant relative had. Written informed consent was obtained from those who were willing to participate. The form for this is shown in Appendix II.

Data collected

Once recruited into the study, basic and relevant demographic data was collected from each subject. Relevant past medical history, including gynecological and obstetric history, was obtained. Any supplements that the subjects were on at that point was noted from the hospital record and from the history. Subjects were followed up throughout pregnancy until delivery. The extent of subjects' compliance with iron supplements prescribed throughout pregnancy was documented during antenatal visits.

After recruitment, a blood sample was collected from each subject. The sample was used for estimation of haematological parameters (haemoglobin and mean corpuscular volume), iron-related parameters (serum iron, ferritin and hepcidin, total iron-binding capacity, transferrin saturation) and C-reactive protein (a marker of inflammation). At the time of delivery, a sample of cord blood was obtained to estimate the same parameters, as listed above. Outcomes of pregnancy were documented.

Sample size

A sample size calculation was done, based on a publication by Simavli et al., 2014. The mean hepcidin level reported in healthy pregnant women in this publication was 7.8 ng/mL, with a standard deviation of 3.4 ng/mL. With a precision of 1 ng/mL and 95% confidence interval, the sample number that was calculated, based on these figures, was 44 subjects. The sample size calculation was done with nMaster 2.0. The formula that was used is shown below.

Single mean - estimating the population mean - absolute precision	
Standard deviation of hepcidin	3.4
Absolute precision	1
Desired confidence level (%)	95
Required sample size	44

***Formula*:**

$$n = \frac{z^2_{1-\alpha/2}\sigma^2}{d^2}$$

Where,

σ : Standard deviation

d : Precision

1- α/2 : Desired Confidence level

Sample collection

A fasting sample of blood (about 6 mL) was collected from each subject, The patient was followed up, during subsequent antenatal visits, till delivery. At the time of delivery, a cord blood sample was collected.

The blood samples collected were used for estimation of haematological parameters (haemoglobin and mean corpuscular volume) and iron-related parameters (serum iron, ferritin, total iron-binding capacity, and transferrin saturation) and C-reactive protein (a marker of systemic inflammation)

Processing of blood samples

Blood samples collected in the red tube were allowed to clot and then centrifuged at 2500 relative centrifugal force (RCF) for 10 minutes to obtain serum; this was done within 3 hours of collection of blood.

Storage of blood samples

The serum obtained was collected and stored at -70ºC, till analyses was carried out. On the days of the analyses, the samples were thawed to room temperature, and used for estimation of serum iron, TIBC, ferritin, hepcidin, and CRP.

Estimations carried done in the diagnostic laboratory of the Department of Clinical Biochemistry, CMC, Vellore

1. ***Estimation of serum iron***

Reference intervals:

Males: 60 to 160 µg/dL

Females: 40 to 145 µg/dL

Sample matrix: Serum

Instrument: Roche - Cobas c 702 modular analyzers

Principle: Guanidine/ ferrozine spectrophotometric method

Guanidine released the ferric ions bound to transferrin, which was present in the sample. Hydroxylamine reduced ferric iron to its ferrous form. A purple-colored complex was formed, when ferrozine reacted with the ferrous form of iron. The intensity of the color obtained was measured at 560 nm, using a

spectrophotometer, and was directly proportional to the concentration of iron in the sample.

2. Estimation of serum ferritin

Reference intervals

Males: 22 to 322 ng/mL

Females: 10 to 291 ng/mL

Sample matrix: Serum

Instrument: Chemiluminescence immune assay (CLIA): SIEMENS - Atellica immunoassay

Principle: double sandwich immunoassay

In the reagent there were 2 ferritin antibodies. A goat polyclonal anti-ferritin antibody labeled with acridinium ester was the first antibody, and a mouse monoclonal anti-ferritin antibody was the second antibody. These were in the solid phase, coupled to paramagnetic particles. The system detected relative light units (RLU), which was in directly proportional to the amount of ferritin present in the sample.

3. Estimation of total iron-binding capacity (TIBC)

Reference intervals:

Male: 300 to 400 µg/dL

Female: 250 to 350 µg/dL

Sample matrix: Serum

Instrument: Roche-Cobas 8000 c 702 modular analyzer

Principle: At alkaline pH, a known amount of Fe^{+3} was added to the sample. These Fe^{+3} ions bound to the transferrin in the sample. Unbound ions were measured by a spectrometric methos using ferrozine (described under estimation of serum iron). The value of unbound iron was subtracted from total amount of iron added. This gave the value for unsaturated iron-binding capacity (UIBC).

UIBC = (Amount of ferrous iron added) – (Amount of unbound ferrous iron)

TIBC was calculated by adding serum iron concentration to the UIBC.

4. Calculation of transferrin saturation (TSAT)

The reference range for transferrin saturation is 30 - 40 %

TSAT = (Serum iron / TIBC) x 100

5. Estimation of hs-CRP

Reference interval: < 3mg/L

Sample matrix: Serum

Instrument: SEIMENS- IMMULITE 2000

Principle: Solid-phase chemiluminescent immunometric assay (CLIA)

There were 2 phases in this assay.

1. Solid phase: anti-ligand coating on the beads
2. Liquid phase: murine monoclonal antibody to CRP was attached to the anti-ligand, rabbit polyclonal anti-CRP antibody conjugated with alkaline phosphatase in buffer

A diluted serum sample was added to the reagent and incubated for 30 minutes. The reaction chamber was washed. The CRP bound to the anti-CRP murine antibody was retained. Unbound antibodies were removed.

CRP in the sample formed a double sandwich complex. A chemiluminescent substrate was then added to the tube. The intensity of photons released was measured and was directly proportional to the CRP concentration in the sample.

Estimation carried out in the Department of Biochemistry, CMC, Vellore

1. ***Estimation of hepcidin***

Sample matrix: Serum

Reagents used: Intrinsic Hepcidin IDx™ ELISA

The following reagents and materials were provided in the kit.

Hepcidin-25 mAb coated wells (96-well plate)

Hepcidin-25 standard

Hepcidin-25 control 1,

Hepcidin-25 control 2

Biotinylated hepcidin-25 tracer

Streptavidin-HRP conjugate

TMB substrate

Stop solution

Sample diluent

Polypropylene (PP) 96 microwell plate covers

Wash buffer

Principle:

This was a competitive binding assay. The kit contained a monoclonal antibody (mAb) that bound with high affinity to the N-terminus of hepcidin-25. The antibody had low affinity to N terminus of isomers of hepcidin–25. It was a competitive binding assay between hepcidin-25 in the sample and a biologically active biotinylated human hepcidin-25 tracer, for a fixed number of high-affinity anti-hepcidin-25 N-terminal-specific mAb binding sites.

Procedure:

The monoclonal antibody (mAb) was coated on the microwells in the plate. The hepcidin-25 standard provided, hepcidin-25 controls (controls 1 and 2), and patients' samples were added to the wells. They were incubated with biotinylated hepcidin-25 tracer for 60 minutes. This biotinylated hepcidin-25 tracer competed with native or reference hepcidin for a fixed number of N-terminal specific antibody-binding sites. The amount of tracer bound that was able to bind to the antibody decreased with increasing concentrations of hepcidin in the patients' samples. The unbound tracer was washed out. The streptavidin-horseradish peroxidase (HRP) conjugate was added to the wells next and incubated for 30 minutes. The wells were then washed to remove unbound streptavidin-HRP. The substrate (3,3',5,5'-tetramethylbenzidine) was

then added and incubations carried out for 15 minutes. The reaction was stopped by addition of the stop solution provided. The absorbance of the solutions in each well was measured at 450nm, using a microwell plate reader. The data obtained was recorded. All reactions were carried out in duplicate.

Estimations carried done in the diagnostic laboratory of the Department Transfusion Medicine, CMC, Vellore

1. ***Estimation of hemoglobin***

Reference interval:

Males: 13 to 17 g/L

Females: 11to 15 g/L

Sample Matrix: Blood anticoagulated with EDTA

Analytical Method: Photometric measurement on automated cell counter (transmittance 525 nm).

2. ***Estimation of mean corpuscular volume (MCV)***

Reference interval: 80 to 100 fL

Sample matrix: Blood anticoagulated with EDTA

Analytical Method: Derived from RBC histogram

Statistical analyses

The Shapiro–Wilk test was used to assess the distribution of data obtained. Normally distributed data was expressed as means and standard deviations. Correlations were determined among the various parameters, using Spearman's correlation coefficients. A p value of < 0.05 was taken to be statistically significant. All the analyses were carried out using Statistical Package for the Social Sciences (SPSS) software version 21 (SPSS Inc., Chicago, IL, USA).

RESULTS

The circumstances of the pandemic made recruitment of subjects extremely challenging. Patients were lost to follow up, as they did not come for antenatal care due to restrictions imposed by the pandemic. Several subjects recruited also finally did not manage to come to CMC for their delivery because of difficulties in transportation and travel, due to the pandemic. In view of this, it was possible to follow up only 20 subjects for the purpose of this thesis. There were also financial constraints encountered and delays in procurement of reagents for estimation of hepcidin. The various estimations had to be done on 2 samples of blood, for each subject recruited. These were the maternal blood sample at time of recruitment and the cord blood sample at the time of delivery. Hence, hepcidin estimations were carried out only on the maternal samples collected.

Table 1: Clinical characteristics of patients in the study (n=20)

Characteristics of subjects	
Age (years)	25 (± 3.21)
Height (meters)	1.58 (± 0.06)
Weight at the time of recruitment (kg)	54.49 (± 9.05)
Body mass index (BMI) (kg/m^2)	21.78 (± 3.28)
Gestational age at recruitment (weeks)	9.92 (± 2.60)

Values shown indicate means (± SD)

Table 2: Outcomes of delivery

Parameters	
Gestational age at delivery (weeks)	39.6 (± 0.98)
Weight of baby at delivery (kg)	3.07 (± 0.31)
Placental weight (gm)	590.3 (± 103.12)
Length of baby (cm)	49.2 (± 1.67)
1 min APGAR score	9 (± 0.00)
5 min APGAR score	10 (± 0.00)
Mean APGAR	9.5 (± 0.00)

Values shown indicate means (± SD)

Table 3: Haematological, iron-related parameters and an inflammatory marker in maternal blood (n = 20)

Parameters	
Hemoglobin (g/dL)	12 (± 1.14)
Mean corpuscular volume (fL)	82.3 (± 6.75)
Serum iron (µg/dL)	74.36 (± 29.30)
Serum ferritin (µg/L)	29.72 (± 30.48)
Transferrin iron binding capacity (TIBC) (µg/dL)	383.26 (±51.90)
Transferrin saturation (%)	20.19 (± 9.03)
Serum hepcidin (ng/mL)	11.12 (± 6.11)
CRP (mg/dL)	6.73 (± 8.65)

Values shown indicate means (± SD)

As shown in table 3, values for haemoglobin and MCV were within reference ranges, showing that the patients in the study were not anaemic at recruitment. Serum iron and serum ferritin values were also within reference ranges and indicate that the recruited subjects were iron-sufficient.

Table 4: Haematological, iron-related parameters and an inflammatory marker in cord blood (n = 17)

Parameters	
Hemoglobin (g/dL)	14.92 (± 2.16)
Mean corpuscular volume (fL)	111.15 (± 4.59)
Serum iron (µg/dL)	114.19 (± 24.54)
Serum ferritin (µg/L)	131.735 (± 53.89)
Transferrin iron binding capacity (TIBC) (µg/dL)	273.42 (± 50.69)
Transferrin saturation (%)	42.51 (± 8.92)
hs-CRP (mg/dL)	<0.02 (± 0.00)

Values shown indicate means (± SD)

Haematological parameters in cord blood samples were studied for 17 subjects only, as 3 samples had clotted due to over-filling of the collection tubes. In the cord blood samples, mean values for haemoglobin and mean corpuscular volume (MCV) were within the reference ranges (15.0 to 24.0 g/dL and 99 to115 fL respectively, for age- 0 to 30 days) (Kliegman and Geme, 2019), indicating that the newborns were not anaemic. Mean values for serum iron and ferritin were also within reference values (22-184 µg/L for all ages) (Kliegman and Geme, 2019) and the WHO cutoff of <12 µg/L (0-23 months) for iron deficiency) (WHO, 2020), indicating that the newborns were iron- sufficient. CRP levels were undetectable in the cord blood, showing absence of inflammation.

Correlation analyses (by use of Spearman's correlation coefficient)

Table 5: Correlations among parameters in maternal blood

Maternal blood sample (n=20)		Serum iron	Serum ferritin	TIBC	Tf-sat	hs-CRP	Serum hepcidin	Haemoglobin	MCV
Serum iron	Correlation coefficient	1.000	.633**	-.530*	.976**	-.250	.523*	.266	.328
	Sig. (2-tailed)		.003	.016	.000	.288	.018	.257	.157
Serum ferritin	Correlation coefficient	.633**	1.000	-.490*	.655**	.219	.414	.294	.330
	Sig. (2-tailed)	.003		.028	.002	.354	.069	.208	.155
TIBC	Correlation coefficient	-.530*	-.490*	1.000	-.637**	.139	-.399	-.147	-.255
	Sig. (2-tailed)	.016	.028		.003	.558	.081	.537	.278
Tf-sat	Correlation coefficient	.976**	.655**	-.637**	1.000	-.204	.494*	.303	.366
	Sig. (2-tailed)	.000	.002	.003		.389	.027	.195	.112
hs-CRP	Correlation coefficient	-.250	.219	.139	-.204	1.000	.037	-.037	-.076
	Sig. (2-tailed)	.288	.354	.558	.389		.877	.877	.750

Serum hepcidin	Correlation coefficient	.523*	.414	-.399	.494*	.037	1.000	.059	.281
	Sig. (2-tailed)	.018	.069	.081	.027	.877		.806	.231
Haemoglobin	Correlation coefficient	.266	.294	-.147	.303	-.037	.059	1.000	.081
	Sig. (2-tailed)	.257	.208	.537	.195	.877	.806		.733
MCV	Correlation coefficient	.328	.330	-.255	.366	-.076	.281	.081	1.000
	Sig. (2-tailed)	.157	.155	.278	.112	.750	.231	.733	

Serum iron correlated significantly and positively with serum ferritin, transferrin saturation, and serum hepcidin and negatively with TIBC. Serum ferritin correlated significantly and positively with transferrin saturation. TIBC correlated significantly and negatively with transferrin saturation. Serum hepcidin correlated significantly and positively with serum iron and transferrin saturation.

Table 6. Correlations among parameters in cord blood (CB)

Cord blood sample		Serum iron	Serum ferritin	TIBC	Tf-sat	Haemo globin	MCV
Serum iron	Correlation coefficient	1.000	.042	.326	.575**	-.156	.544*
	Sig. (2-tailed)		.860	.161	.008	.550	.024
	N	20	20	20	20	17	17
Serum ferritin	Correlation coefficient	.042	1.000	-.327	.152	-.684**	.155
	Sig. (2-tailed)	.860		.159	.523	.002	.554
	N	20	20	20	20	17	17
TIBC	Correlation coefficient	.326	-.327	1.000	-.505*	.416	.067
	Sig. (2-tailed)	.161	.159		.023	.097	.799
	N	20	20	20	20	17	17
Tf-sat	Correlation coefficient	.575**	.152	-.505*	1.000	-.336	.327
	Sig. (2-tailed)	.008	.523	.023		.187	.200
	N	20	20	20	20	17	17

Haemoglobin	Correlation coefficient	-.156	-.684**	.416	-.336	1.000	.044
	Sig. (2-tailed)	.550	.002	.097	.187		.866
	N	17	17	17	17	17	17
MCV	Correlation coefficient	.544*	.155	.067	.327	.044	1.000
	Sig. (2-tailed)	.024	.554	.799	.200	.866	
	N	17	17	17	17	17	17

In cord blood, serum iron correlated significantly and positively with transferrin saturation and mean corpuscular volume (MCV). Serum ferritin correlated significantly and negatively with haemoglobin. TIBC correlated significantly and negatively with transferrin saturation.

Table 7 Correlations among parameters in maternal and cord blood

		Cord blood sample					
First trimester maternal blood		**Serum iron**	**Serum ferritin**	**TIBC**	**Tf-sat**	**Haemo globin**	**MCV**
Serum iron	Correlation coefficient	.031	.146	.262	-.242	-.144	-.064
	Sig. (2-tailed)	.897	.539	.264	.303	.582	.807
	N	20	20	20	20	17	17
Serum ferritin	Correlation coefficient	.010	.328	.029	.013	-.352	.103
	Sig. (2-tailed)	.967	.158	.905	.957	.166	.694
	N	20	20	20	20	17	17

TIBC	Correlation coefficient	.093	-.412	.114	.005	.598*	-.177
	Sig. (2-tailed)	.696	.071	.633	.982	.011	.498
	N	20	20	20	20	17	17
Tf-sat	Correlation coefficient	-.005	.185	.223	-.237	-.177	.017
	Sig. (2-tailed)	.985	.435	.344	.315	.498	.948
	N	20	20	20	20	17	17
hs-CRP	Correlation coefficient	-.150	.182	-.187	.151	.087	-.222
	Sig. (2-tailed)	.529	.442	.431	.525	.740	.392
	N	20	20	20	20	17	17
Serum hepcidin	Correlation coefficient	.138	.233	-.219	.389	-.018	.184
	Sig. (2-tailed)	.563	.323	.354	.090	.944	.480
	N	20	20	20	20	17	17

Haemoglobin	Correlation coefficient	.008	-.367	.255	-.141	.053	-.093
	Sig. (2-tailed)	.972	.112	.278	.554	.840	.722
	N	20	20	20	20	17	17
MCV	Correlation coefficient	.168	.069	-.003	.100	.028	.585*
	Sig. (2-tailed)	.479	.772	.990	.675	.914	.014
	N	20	20	20	20	17	17

Cord blood haemoglobin correlated significantly and positively with maternal TIBC. Cord blood MCV (mean corpuscular volume) significantly and positively correlated with maternal MCV

DISCUSSION

In the present study, primigravidae with uncomplicated pregnancies were recruited in their first trimester and a sample of blood collected. They were followed up till delivery, at which time point a sample of cord blood was also obtained. Haematological parameters (haemoglobin and mean corpuscular volume) and parameters linked to iron were estimated in the blood samples collected. The latter included serum ferritin, serum iron and total iron-binding capacity (TIBC). Transferrin saturation was calculated from values of serum iron and TIBC. Concentrations of serum C-reactive protein (a marker of systemic inflammation) were also estimated. Outcomes of pregnancy were documented. Correlations were determined between maternal and fetal parameters studied.

Only primigravidae were recruited in the present study to avoid the effects of previous pregnancies on iron homeostasis in the subjects. The subjects delivered at term. The babies born had normal birth weights, indicating they had developed appropriately in utero. Their APGAR scores were 9 and 10, at 1 and 5 minutes respectively, showing that the babies did not have any untoward incident during birth.

The mean value for maternal haemoglobin in the study was 12 g/dL (± 1.14). The women in the study, thus, did not have anaemia (WHO, 2011b). They were also found to be iron-sufficient, based on their mean serum ferritin level being 29.72 μg/L (± 30.48). The WHO has set a value of <15 μg/L of ferritin in the first trimester for the diagnosis of iron deficiency (WHO, 2020).

In the present study, serum CRP levels were estimated to detect the presence of systemic inflammation. CRP is an acute phase protein and, hence, is useful as a marker of inflammation. It plays a role in the innate immune system (Peisajovich et al., 2008). In inflammatory conditions, pro-inflammatory mediators are released from various immune cells (Kany et al., 2019). One such pro-inflammatory mediator is interleukin 6 (IL-6), released from macrophages, Th cells, and fibroblasts (Turner et al., 2014). IL-6 triggers the JAK/STAT signaling pathway and triggers transcription of hepcidin. Thus, inflammation induces hepcidin and causes its levels in blood to increase (Nemeth et al., 2004a; Wrighting and Andrews, 2006). This increase in hepcidin decreases the availability of iron for invading microorganisms (Barton and Acton, 2019). Pregnancy is an inflammatory state, especially in the first trimester, because inflammation plays a role in implantation (Dekel et al., 2014). In the present study, serum hepcidin did not show any significant correlation with serum CRP.

Van Santen et al (2013) have reported similar findings. It is possible that IL-6 may be a better inflammatory marker to be used to look for correlations with hepcidin (Suega and Widiana, 2019). This was, however, not estimated in the present study.

Hepcidin is the central regulator of systemic iron homeostasis (Nemeth and Ganz, 2009). Its levels are suppressed in pregnancy; this increases the availability of iron for uptake by the placenta (Fisher and Nemeth, 2017). In pregnancy, the requirement for iron is increased to meet maternal and fetal needs. Decreases in hepcidin during pregnancy enables increased intestinal absorption of iron and recycling of iron from macrophages. This makes iron available for the fetus (Koenig et al., 2014).

Studies have estimated hepcidin in blood in pregnant women, using different methodologies. Finkenstedt et al. (2012) reported a median value of 16 ng/mL (range reported was between 4 and 97 ng/mL) for serum hepcidin in the first trimester, as estimated by mass spectrometry. The study by van Santen et al., (2013) also used mass spectrometry to measure serum hepcidin concentrations; they did this across the three trimesters. They reported that levels decreased from the first trimester to the second trimester, to reach undetectable limits by

the third trimester. Hedengran et al., (2015) used a liquid chromatography system for estimations of hepcidin and reported that hepcidin was suppressed in pregnant women on iron supplementation, with the suppression occurring between 13- 20 weeks and 21-28 weeks. On the other hand, Simavli et al. (2014) have reported that there was no decrease in serum hepcidin levels during pregnancy, and that there were no associations between serum hepcidin levels and iron-related parameters in maternal blood. They used an enzyme-linked immunosorbent assay (ELISA) (DRG Instruments, Marburg, Germany) for the estimation of serum hepcidin. Bah et al., 2017 studied pregnant women with singleton fetuses with no accompanying complication of pregnancy, who were on iron supplementation. They also estimated hepcidin levels in blood, using an ELISA (Bachem, Peninsula Laboratories International), at different gestational ages and showed that hepcidin concentrations declined after the first trimester. They suggested that serum hepcidin may be useful as a marker of iron deficiency in pregnancy, if it is undetectable in serum. In a study by Guo et al., (2019) on Chinese primigravidae, the mean serum hepcidin level in the first trimester (measured by an ELISA [Intrinsic Lifesciences]) was reported to be 36.4 ng/mL (SD 27.6). This value is higher than the value reported in the present study (mean serum hepcidin level 11.12 ng/mL [± 6.11]), which also estimated serum hepcidin using the same commercially available ELISA (Intrinsic Life Sciences, USA). The reason for this difference in observations is not clear. The

Chinese primigravidae had a higher mean hemoglobin level than that in the present study (13.22 g/dL [SD 9.0] vs 12 g/dL [SD 1.14]). Their mean serum ferritin value was also higher than that in the present study (31.6 ng/mL vs 29.72 ng/mL). These may be important factors that may contribute to differences seen in the values for serum hepcidin, as hepcidin is regulated by iron stores in the body (Nemeth and Ganz, 2009).

From the published studies listed above, it is clear that there are marked differences in the values reported for serum hepcidin in pregnancy. The various studies have used different assays to measure serum hepcidin. Currently, assays for serum hepcidin have not been standardized. Efforts are on-going to harmonize the assays available, with standardization as the ultimate goal (Girelli et al., 2016). Hence, it is difficult to make direct comparisons of the results of the various studies that have used diverse methodologies.

The mean cord blood haemoglobin in the present study was 14.92 g/dL (SD 2.16). Several studies have suggested reference ranges for this parameter. These are summarized in the table below.

Concentrations of cord blood haemoglobin reported in studies done in different countries		
Country	**Authors**	**Haemoglobin value (g/dL)**
Greece (n=2000)	Katsares et al., (2009)	8.8 (SD 2.9)
Taiwan (n=5602)	Chang et al., (2011)	11.2 (SD 1.5)
Nigeria (n=130)	Adewumi et al., (2014)	Male =13.27 (SD 1.60) Female =13.32 (SD 1.61)
Sudan (n=500)	Elgari and Waggiallah, (2014)	14.35 (SD 1.55)
Chennai, South India. (n=120)	Suman, (2015)	14.9 (SD 1.7)
Pakistan (n=316)	Pasha et al., (2015)	15.4 (SD 1.9)
Nepal (n=210)	Basnet et al., (2016)	15.24 (SD 1.96)
Ethiopia (n=139)	Angelo et al., (2021)	15.8 (SD 1.64)

This table shows that there is a wide variation in mean values reported for cord blood hemoglobin. The value reported in the present study falls among the various values reported.

The lower reference limit for serum ferritin, set by the World Health Organization, for the age group 0-23 months (for apparently healthy babies) is 12 μg/L. The cut-off value for serum ferritin in the presence of infection or inflammation is 30 μg/L (WHO, 2020). A value below the lower reference limit is interpreted as indicating iron deficiency. The mean cord blood ferritin in the present study was 131.735 μg/L (± 53.89), indicating that the newborns were iron-sufficient. Cord blood CRP concentrations were undetectable, indicating an absence of inflammation.

In the present study, maternal iron and haematological parameters were found to correlate with one another, as has been shown in earlier studies (Bencaiova et al., 2019; Finkenstedt et al., 2012; Means, 2020; van Santen et al., 2013; Young et al., 2012). Studies have shown that serum hepcidin concentrations in the first trimester of pregnancy correlated positively with values for serum ferritin and transferrin saturation. It has also been reported to correlate negatively with the soluble transferrin receptor-ferritin index (sTfR/log ferritin ratio) and haemoglobin concentrations, indicating that hepcidin regulation by iron and erythropoiesis is not disturbed in pregnancy (Bah et al., 2017; Rehu et al., 2010; Schulze et al., 2008; van Santen et al., 2013; Young et al., 2012).

Transferrin receptors on the surface of cells help them take up iron from transferrin in blood (Rodwell, 2018). They are highly expressed on erythroid precursors cells (Ponka and Lok, 1999). Truncated forms of the receptor are shed into blood and referred to as soluble transferrin receptor (sTfR). When there is increased erythropoiesis, the number of transferrin receptors on erythroid precursor cells increases, with increased numbers being released into the circulation. The concentration of circulating sTfR also increases in the presence of iron deficiency, before anemia is apparent (Beguin, 2003; R'zik and Beguin, 2001; Shih et al., 1990). Its levels are, however, not elevated in anaemia of inflammation (Punnonen et al., 1997).

Serum ferritin levels are an indicator of body iron stores ((Rodwell, , 2018)). When there is negative iron balance, serum ferritin levels decrease and sTfR level increases (Skikne, 2008). The ratio between them gives a good indication of iron status in the body. The sTfR- ferritin index (sTfR/log ferritin ratio) has been suggested to be a better indicator of iron-deficiency anaemia, than serum ferritin and sTfR individually (Skikne et al., 2011). A ratio greater than 2 has been taken to be an indicator of iron deficiency (Speeckaert et al., 2010).

A significant positive correlation has been reported between concentrations of serum ferritin and hepcidin in pregnancy (Finkenstedt et al. 2012) The present study did not show such a correlation. Van Santen et al., (2013) showed that maternal serum hepcidin levels correlated with iron-related parameters in blood, but not with inflammatory markers. In the present study, maternal serum hepcidin was correlated significantly and positively with maternal serum iron and transferrin saturation, but not with the inflammatory marker, CRP.

It has been hypothesized that fetal hepcidin influences the release of iron from the placenta into the fetal circulation (Donker et al., 2021). In the present study, no correlation was found between maternal serum hepcidin and fetal iron levels and haematological parameters. It is not clear whether maternal hepcidin plays a direct part in regulation of placental iron transport and fetal uptake (Cao and Fleming, 2016; Hedengran et al., 2015). Declines in hepcidin levels in maternal blood have been shown to occur from the second trimester onwards, to increase iron availability for needs of the growing fetus (Bah et al., 2017; van Santen et al., 2013). Hepcidin concentrations in blood have been shown to reach undetectable levels in the third trimester, when the requirement for iron is maximal (Fisher and Nemeth, 2017).

It would have been useful to study correlations of second and third trimester maternal blood parameters with cord blood parameters. This is likely to have given a better understanding of how maternal and fetal hepcidin play a role in iron metabolism in pregnancy. However, in the present study, it was not possible to obtain blood samples in the second and third trimester of pregnancy. The circumstances of the pandemic made recruitment of subjects extremely challenging. Patients were lost to follow up, as they did not come for antenatal care due to restrictions imposed by the pandemic. Several subjects recruited also finally did not manage to come to CMC for their delivery because of difficulties in transportation and travel, due to the pandemic. In view of this, it was possible to follow up only 20 subjects for the purpose of this thesis. There were also considerable financial constraints to carrying out additional estimations. For example, there were delays in procurement of reagents for estimation of hepcidin. The various estimations had to be done on 2 samples of blood, for each subject recruited. These were the maternal blood sample at time of recruitment and the cord blood sample at the time of delivery. Because of financial constraints and limited supply of reagents (due to disruptions in supply systems as a result of the restrictions imposed by the pandemic), hepcidin estimations were carried out only on the maternal samples collected. All the above are limitations of the present study.

CONCLUSIONS

Maternal serum hepcidin in the first trimester correlated significantly and positively with maternal serum iron and transferrin saturation. It did not correlate with any parameters in cord blood. Maternal MCV correlated with fetal MCV. Work is ongoing to determine if this is so in the other 2 trimesters as well, and in cord blood.

LIMITATIONS

The calculated sample size was not achieved, due to the circumstances of the pandemic.

It was also not possible to obtain blood samples in the second and third trimesters of the subjects in the study. This would have provided more information on the parameters of interest.

It was not possible to estimate hepcidin in the cord blood samples due to lack of reagents because of disruptions in the supply chain.

Estimation of serum IL-6 would have been a better marker of inflammation.

It was also not possible to obtain a maternal blood sample at the time delivery.

Multivariate analyses would be also need to be done necessary to reliably elucidate any relationships that exist among the parameters studied. It had not been possible to do this.

All these short-comings are being addressed as the study is continued.

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APPENDIX I – Letter of approval from the Institutional Review Board

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Dr. B.J. Prashantham, M.A., Dr. Min (clinical)
Director, Christian Counselling Center
Chairperson, Ethics Committee

Dr. Anna Benjamin Pulimood, MD., Ph.D.,
Chairperson, Research Committee, Principal

Dr. Suceena Alexander, MD., DM., FASN.,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

February 16, 2021

Dr. Arthur Amit Suryakumar,
PG Demonstrator
Department of Biochemistry,
Christian Medical College,
Vellore – 632 002.

Sub: Fluid Research Grant: New Proposal:
Hepcidin and iron-related parameters in pregnancy: how do these correlates?

Dr. Arthur Amit Suryakumar (Emp. No. 21795), Biochemistry, Dr. Molly Jacob (Emp. No. 14509), Biochemistry, Ms. Nikhitha Mariya John (Emp. No. 81929), Dr. Joe Varghese (Emp. No. 20405), Biochemistry, Dr. Manisha Beck (Emp. No. 31294), Obstetrics and Gynecology, Dr. Thenmozhi Mani (Emp. No. 32347), Biostatistics.

Ref: IRB Min. No. 13367 [OBSERVE] dated 02.09.2020.

Dear Dr. Arthur Amit Suryakumar,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Suceena Alexander, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Suceena Alexander
Secretary (Ethics Committee)
Institutional Review Board

Dr. Suceena Alexander, MD, DM, FASN.
Secretary (Ethics Committee)
Institutional Review Board
Christian Medical College,
Vellore - 632 002, Tamil Nadu, India.

Cc: Dr. Molly Jacob, Professor, (Emp. No. 14509), Biochemistry

Ethics Committee Blue, Office of Research, I Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284508 Fax: 0416 – 2262788 E-mail: research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., Dr. Min (clinical)
Director, Christian Counselling Center
Chairperson, Ethics Committee

Dr. Anna Benjamin Pulimood, MD., Ph.D.,
Chairperson, Research Committee, Principal

Dr. Suceena Alexander, MD., DM., FASN.,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Dr. Rohin Mittal	MS, DNB	Professor, Department of General Surgery, CMC Vellore	Internal Clinician
Mrs. Ida Nirmal	MSc Nursing	Professor, Addl Deputy Dean, College of Nursing, CMC, Vellore	Internal, Nurse
Mrs. Rebecca Sumathi Bai	MSc Nursing	Professor and Head of Specialty Nursing7, CMC Vellore	Internal, Nurse
Dr. HS. Asha	MBBS, DNB	Professor, Department of Endocrinology, CMC Vellore	Internal Clinician
Mrs. Sophia V	M.Sc Nursing	Addl. Deputy Dean CMC, Vellore	Internal, Nurse
Mrs. Nirmala Margaret	MSc Nursing	Addl. Deputy Nursing Superintendent, College of Nursing, CMC, Vellore	Internal, Nurse
Dr. Rekha Pai	MSc, PhD	Associate Professor, Pathology, CMC, Vellore	Internal, Basic Medical Scientist
Dr. John Jude Prakash	MBBS, MD,	Professor, Clinical Virology, CMC, Vellore	Internal, Clinician
Dr. Winsely Rose	MD (Paed)	Professor, Paediatrics, CMC Vellore	Internal, Clinician
Dr. Premila Abraham	M.Sc. Ph.D	Professor, Department of Biochemistry, CMC, Vellore	Internal Clinician

We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of Withdrawals for the study entitled: "Hepcidin and iron-related parameters in pregnancy: how do these correlates?. Please send copies of this to the Research Office (research@cmcvellore.ac.in).

Ethics Committee Blue, Office of Research, I Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284508 Fax: 0416 – 2262788 E-mail: research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., Dr. Min (clinical)
Director, Christian Counselling Center
Chairperson, Ethics Committee

Dr. Anna Benjamin Pulimood, MD., Ph.D.,
Chairperson, Research Committee, Principal

Dr. Suceena Alexander, MD., DM., FASN.,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Name	Qualification	Designation	Affiliation
Dr. Anna B. Pulimood	MD, PhD	Principal, Chairperson-Research Committee, IRB, CMC, Vellore	Internal, Clinician
Dr. B. J. Prashantham	MA (Counseling Psychology), MA(Theology), Dr. Min (Clinical Counselling)	Chairperson, Ethics Committee, IRB. Director, Christian Counseling Centre, Vellore	External, Social Scientist
Dr. Suceena Alexander	MD., DM., FASN	Secretary – (Ethics Committee), IRB, Addl. Vice Principal (Research), Professor of Nephrology, CMC, Vellore	Internal Clinician
Dr. Sathish Kumar	MD, DCH	Professor, Child Health, CMC, Vellore	Internal, Clinician
Ms. Grace Rebekha	M.Sc., (Biostatistics)	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert
Dr. Ratna Prabha	MBBS, MD (Pharma)	Associate Professor, Clinical Pharmacology, CMC, Vellore	Internal, Pharmacologist
Rev. Rainard Pearson	BA., B. Th., M. Div.,	Sr. Chaplin, CMC, Vellore.	Internal, Social Scientist
Dr. Anuradha Rose	MBBS, MD, MHSC (Bioethics)	Associate Professor, Community Health, CMC, Vellore	Internal, Clinician
Dr. Barney Isaac	DNB (Respiratory Diseases)	Associate Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Dr. Joe Varghese	MBBS, MD Biochemistry	Professor, Department of Biochemistry	Internal Clinician
Dr. Balu Krishna	MBBS MD DNB DMRT	Professor, Department of Radiotherapy, CMC Vellore	Internal Clinician

Ethics Committee Blue, Office of Research, I Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
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February 16, 2021

Dr. Arthur Amit Suryakumar,
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Sub: Fluid Research Grant: New Proposal:
Hepcidin and iron-related parameters in pregnancy: how do these correlates?

Dr. Arthur Amit Suryakumar (Emp. No. 21795), Biochemistry, Dr. Molly Jacob (Emp. No. 14509), Biochemistry, Ms. Nikhitha Mariya John (Emp. No. 81929), Dr. Joe Varghese (Emp. No. 20405), Biochemistry, Dr. Manisha Beck (Emp. No. 31294), Obstetrics and Gynecology, Dr. Thenmozhi Mani (Emp. No. 32347), Biostatistics.

Ref: IRB Min. No. 13367 [OBSERVE] dated 02.09.2020.

Dear Dr. Arthur Amit Suryakumar,

The Institutional Review Board (**Blue**, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Hepcidin and iron-related parameters in pregnancy: how do these correlates?" on September 02, 2020.

The Committee reviewed the following documents:

1. IRB Application Form
2. Signature Page
3. Patient information Sheet and Informed Consent (English, Tamil, Hindi)
4. Cvs. of Drs. Amit, Joe, Manisha, Molly, Nikhitha, Thenmozhi.
5. No.of Documents 1 – 4.

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on September 02, 2020 in the New IRB Room, Christian Medical College, Vellore 632 004.

Ethics Committee Blue, Office of Research, I Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284508 Fax: 0416 – 2262788 E-mail: research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., Dr. Min (clinical)
Director, Christian Counselling Center
Chairperson, Ethics Committee

Dr. Anna Benjamin Pulimood, MD., Ph.D.,
Chairperson, Research Committee, Principal

Dr. Suceena Alexander, MD., DM., FASN.,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

The Institutional Ethics Committee expects to be informed about the progress of the project, Any **adverse events** occurring in the course of the project, any **amendments in the protocol and the patient information / informed consent**. On completion of the study you are expected to submit a copy of the **final report**. Respective forms can be downloaded from the following link: http://172.16.11.136/Research/IRB_Polices.html in the CMC Intranet and in the CMC website link address: http://www.cmch-vellore.edu/static/research/Index.html.

Fluid Grant Allocation:

A sum of 3,00,000/- INR (Rupees Three Lakh Only) will be granted for 2 years. 1,50,000/- INR (Rupees One Lakh fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 1,50,000/- INR (Rupees One Lakh fifty Thousand only) each will be released at the end of the first year as 2 nd Installment.

Yours sincerely,

Dr. Suceena Alexander
Secretary (Ethics Committee)
Institutional Review Board

Dr. Suceena Alexander, MD.,DM.,FASN.
Secretary (Ethics Committee)
Institutional Review Board
Christian Medical College,
Vellore - 632 002, Tamil Nadu, India.

IRB Min. No. 13367 [OBSERVE] dated 02.09.2020 5 of 5

APPENDIX -II

Informed consent form

Study title: Hepcidin and iron-related parameters in pregnancy: how do these correlate?

Subject's name: **Date:**

Hospital number:

Date of birth:

I confirm that I have read and understood the contents of the information sheet provided for the above study and have had the opportunity to ask Dr. Arthur Amit Suryakumar I had. [ ]

I understand that my participation in the study is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal right being affected. [ ]

I understand that my participation in the study, a blood sample of 6 mL will be collected from me. [ ]

I understand that if I agree to participate in the study, 6 mL of blood from the umbilical cord will be collected, after delivery of my baby. [ ]

I understand that these will not affect my health or that of my baby in a foreseeable way[ ]

I understand that the blood samples collected will be used only for research purposes. If there is any sample remaining after this study is completed, I give permission for the samples to be stored and used for related studies in the future. [ ]

I understand that the investigator of this study, the ethics committee and regulatory authorities will not need my permission to look at my health records both in respect of

the current study and any further research that may be conducted in relation to it, even if I withdraw from the trial. I agree to this access.

[ ]

I agree to take part in the above study of my own free will. [ ]

Subject’s name and signature/thumb impression (with date):

Witness’s name and signature/thumb impression (with date) and address:

Investigator’s name and signature (with date):

HEPCIDIN AND IRON RELATED PARAMETERS IN PREGNANCY: HOW DO THESE CORRELATE?

INFORMATION SHEET FOR STUDY PARTICIPANTS

Date:

Department of Biochemistry & Department of Obstetrics and Gynaecology (Unit 4), Christian Medical College, Vellore

You are invited to participate in a study, conducted by the Department of Biochemistry and the Department of Obstetrics and Gynaecology (Unit 4), Christian Medical College, Vellore, to see what influences iron being taken up by the baby from the mother's blood. This is being done to help doctors understand better how this happens.

For this study, we require 6 ml of blood sample to be collected from each person in the study, at 3 points during antenatal visits (once each in the first, second and third trimesters). We also require 6 ml of cord blood immediately after delivery. We would like to ask if you are willing to provide these samples for the purpose of this study. Collection of blood will not cause harm to you or to your baby's health and will be done at the same time that blood is taken for other routine tests, as far as possible.

The samples collected will be used only for research purposes. If there is any sample left over after this study is completed, we would like to request you for permission to store it and use it for related research studies in the future.

All information you give us will be kept confidential. You may not benefit directly by participation in the study. However, if you are willing to provide the required samples, you will contribute to improving understanding of how the baby acquires iron from the mother.

If you are not willing to participate in the study, you are free to say so. It will not affect the treatment you will receive in the hospital. Participation in the study does not entitle you to concession or any other special treatment. You have to pay for all the routine tests that the doctor might ask you to do as part of your antenatal health check-up, however you need not pay for the tests done as a part of this study.

If you have further queries, please contact us, using one of the numbers given below.

Contact persons

Dr. Arthur Amit Suryakumar, PG demonstrator, Department of Biochemistry, CMC, Vellore 632002. Contact phone number: 9381388414

Ms. Nikhitha Mariya John, Junior Research Fellow, Department of Biochemistry, CMC, Vellore 632002. Contact number: 9791152307

Dr. Molly Jacob, Professor, Department of Biochemistry, CMC, Vellore,
Contact number: 0416 – 2284267

Dr. Manisha Beck, Professor, Unit 4, Department of Obstetrics and Gynaecology, CMC, Vellore
Contact number: 0416 - 2286185

Dr. Joe Varghese, Professor, Department of Biochemistry, CMC, Vellore, Contact number: 0416 – 2284267

உயிர்வேதியியல் துறை, மகப்பேறியல் துறை, கிறித்துவ மருத்துவக்கல்லூரி,

வேலூர்- 632002

கர்ப்பத்தில் ஹெப்சிடின் மற்றும் இரும்பு சம்பந்தப்பட்ட அளவுருக்கள் : இந்த அளவீடுகள் எவ்வாறு தொடர்புடையது?

ஆய்வில் பங்கேற்பவருக்கான தகவல் படிவம்

தேதி :

உயிர்வேதியியல் துறை மற்றும் மகப்பேறியல் துறை, கிறித்துவ மருத்துவக்கல்லூரி, வேலூர் நடத்தும் ஆராச்சியில் நீங்கள் பங்கேற்க அழைக்கப்படுகிறீர்கள் . இந்த ஆய்வு, தாயின் இரத்தத்திலிருந்து குழந்தையால் இரும்பு எடுக்கப்படுவதைப் பார்க்க. இது எவ்வாறு நிகழ்கிறது என்பதை மருத்துவர்கள் நன்கு புரிந்துகொள்ள உதவும் வகையில் செய்யப்படுகிறது.

இந்த ஆய்வுக்கு கர்ப்பம் காலத்தில் 6 மில்லி இரத்தம் மூன்று முறை (முதல், இரண்டாவது மற்றும் மூன்றாவது மூன்று மாதங்களில் ஒரு முறை)மற்றும் பிரசவம் முடிந்த உடனடியாக 6 மில்லி தண்டு (Cord blood) இரத்தமும் தேவைப்படுகிறது. இந்த ஆய்வின் நோக்கத்திற்காக இந்த மாதிரிகளை வழங்க உங்கள் விருப்பத்தை நாங்கள் கேட்கின்றோம்

இரத்தத்தில் உள்ள இரும்பு சத்து சார்ந்த பரிசோதனைகளை செய்ய இது தேவைப்படும். இந்த இரத்தம் அளிப்பதால் தஙகளுக்கோ, குழ்ந்தைக்கோ எந்த பாதிப்பும் இல்லை. இந்த ஆய்வில் பங்கேற்பதால் தங்களுக்கு எவ்விதமான அதிக செலவும் ஆகாது இந்த ஆய்வில் பங்கேற்பதனால் தங்களுக்கு நேரடியான பயன் எதுவும் இருக்காது. ஏனினும் தாங்கள் இந்த ஆய்வில் பங்கேற்பதனால் கருவில் இருக்கும் குழந்தை எவ்வாறு இரும்பு சத்தினை தாயிடம் இருந்து பெற்றுக் கொள்கிறத என்பதை தெரிந்து கொள்ள உதவும். தங்களிடம் இருந்து பெறப்பட்ட இரத்தம் ஆராய்சிக்காக மட்டுமே பயன்படுத்தப்படும். மீதமாகும் இரத்தம் சேகரிக்கவும் பிற்காலத்தில் இது சார்ந்த ஆராய்சிக்காக பயன்படுத்தவும் அனுமதி தருமாறு கேட்டுக்கொள்கிறோம்.

இந்த ஆய்வுக்காக பெறப்படும் தங்களது தகவல்கள் பாதுகாக்கப்படும். இந்த ஆய்வின் முடிவு அறிவியல் பத்திரிக்கைகளில் பதிவாக்கப்படும். அப்பொழுது தங்களின் தனிப்பட்ட அடையாளம் வெளியிட படமாட்டாது என்பதை உறுதி அளிக்கிறோம்.

தங்களின் பங்கேற்பு தங்களது சுயவிருபத்தினால் எடுக்கும் முடிவு. தங்களுக்கு விருப்பம் இல்லை என்றால் தயங்காமல் கூறலாம் மேலும் நீங்கள் ஆய்விற்கு நடுவில் விட்டு போகலாம். இதனால் இந்த மருத்துவமனையில் தாங்கள் மேற்கொள்ளும் சிகிச்சைமுறையில் எவ்வித மாற்றமும் இருக்காது. இந்த ஆய்வின் பங்கேற்பதிற்பதினால் உங்களுக்கு சலுகை அல்லது வேறு எந்த சிறப்பு சிகிச்சையையும் அளிக்க இயலாது. உங்கள் மருத்துவர் செய்யும்படி கேட்கும் உங்கள் வழக்கமான சோதனைகளுக்கு நீங்கள் பணம் செலுத்த வேண்டும். இருப்பினும், இந்த ஆய்வின் ஒரு பகுதியாக

மேற்கொள்ளப்படும் கூடுதல் சோதனைகளுக்கு நீங்கள் பணம் செலுத்த வேண்டியதில்லை.

மேலும் சந்தேகங்களுக்கு கீழ்காணும் நபரை அலைபேசி அல்லது மின்னஞ்சல் மூலம் தொடர்பு கொள்ளவும்.

தொடர்பு நபர்

மரு. ஆர்தர் அமித் சூரியகுமார், பி.ஜி ஆர்ப்பாட்டக்காரர், உயிர் வேதியியல் துறை, கிறித்துவ மருத்துவக்கல்லூரி, வேலூர்-632002, அலைபேசி: 9381388414

செல்வி: நிகிதா ஜான், உயிர் வேதியியல் துறை, கிறித்துவ மருத்துவக்கல்லூரி, வேலூர்-632002 அலைபேசி: 9791152307

மரு. மோலி ஜேக்கப் , உயிர் வேதியியல் துறை, கிறித்துவ மருத்துவக்கல்லூரி, வேலூர்-632002 அலைபேசி: 0416 2284267

மரு. மனிஷா பெக், மகப்பேறியல் துறை, கிறித்துவ மருத்துவக்கல்லூரி, வேலூர் அலைபேசி: 0416 2286185

மரு. ஜோ வர்கீஸ, உயிர் வேதியியல் துறை, கிறித்துவ மருத்துவக்கல்லூரி, வேலூர், அலைபேசி: 0416 2284267

ஆய்வின் தலைப்பு : **கர்ப்பத்தில் ஹெப்சிடின் மற்றும் இரும்பு சம்பந்தப்பட்ட அளவுருக்கள் : இந்த அளவீடுகள் எவ்வாறு தொடர்புடையது?**

ஆய்வில் பங்கேற்பவரின் பெயர்: திருமதி தேதி :

மருத்துவமனை அடையாள எண்:

பிற்ந்த தேதி:

இந்த ஆய்வு பற்றிய தகவல் படிவத்தை படித்தும அதன் விவரங்களை மரு. ஆர்தர் அமித் சூரியகுமார் மூலம் கேட்டும் புரிந்து கொண்டேன். [ ]

இந்த ஆய்வில் கல்ந்து கொள்ள என்னை யாரும் கட்டாயப்படுத்தவில்லை. எனது சுய விருப்பதுடன் இந்த ஆய்வில் பங்கேற்கிறேன். இந்த ஆய்விலிருந்து எந்த நேரமும் வெளியேற எனக்கு அனுமதி உள்ளது. இதனால் எனக்கு அளிக்கப்படும் சிகிச்சை முறைகளிலோ , சட்டரீதியான உரிமைகளிலோ வேறுபாடு இருக்காது என்று உறுதியளிக்கப்பட்டது. [ ]

இந்த ஆய்வில் பங்கேற்க சம்மதித்தால், கர்ப்ப காலத்தில் முன்று மாததிற்கு ஒரு முறை 6 மில்லி இரத்தம் எடுத்துக் கொள்ள நான் முழு மனதுடன் சம்மதிக்கிறேன். [ ]

இந்த ஆராய்ச்சிக்காக பிரசவத்தின் பொழுது 6 மில்லி மில்லி தொப்புள் கொடி இரத்தமும், எடுத்துக் கொள்ள நான் முழு மனதுடன் சம்மதிக்கிறேன். [ ]

இதனால் என் உடல் நலத்திற்கு எந்தவித பாதிப்பும் ஏற்படாது என அறிந்து கொண்டேன். [ ]

இந்த இரத்தம், ஆராய்ச்சிகாக மட்டுமே பயன்படுத்தப்படும் என்று புரிந்து கொண்டேன். மீதமாகும் இரத்தத்தை சேகரித்து எதிர்காலத்தில் இது தொடர்புடைய ஆராய்ச்சிக்கு பயன்படுத்த சம்மதிக்கிறேன்.[ ]

எனது மருத்துவ விவரங்களை ஆய்வில் உபயோகிக்க ஒப்புக்கொள்கிறேன். இருப்பினும் எனது தனிப்பட்ட அடையாளம்/ தகவல், மற்றவரிடமோ/ மருத்துவ இதழிலோ வெளியிடப்பட மாட்டாது என்பதையும் தெரிந்து கொண்டேன். [ ]

இந்த ஆய்வின் புலனாய்வாளர், நெறிமுறைகள் குழு மற்றும் ஒழுங்குமுறை அதிகாரிகள் எனது சுகாதார பதிவுகளை தற்போதைய ஆய்வு மற்றும் அதைப் பற்றி மேலும் எந்தவொரு ஆராய்ச்சியிலும் பார்க்க எனது அனுமதி தேவையில்லை என்பதை நான் புரிந்துகொள்கிறேன். மேலும் நான்

ஆராய்ச்சியிலிருந்து விலகினாலும் இந்த அணுகலை ஒப்புக்கொள்கிறேன். ” [ ]

இந்த ஆய்வில் பங்கேற்க முழு மனதுடன் சம்மதிக்கிறேன். [ ]

ஆய்வில் பங்கேற்பவரின் கையொப்பம் /பெருவிரல் ரேகை (தேதி):

சாட்சியின் பெயர்/கையொப்பம் /பெருவிரல் ரேகை (தேதி) மற்றும் முகவரி:

ஆராய்ச்சியாளர் பெயர்/ கையொப்பம் (தேதி)

बायोकेमिस्ट्री विभाग और स्त्रीरोग विभाग
क्रिश्चियन मेडिकल कॉलेज, वेल्लोर– 632002

गर्भावस्था में हेपीसीडिन और आइरन (लोहे) से संबंधित मापदंडों : ये कैसे सहसंबद्ध होते हैं?

अध्ययन दलों के लिए सूचना पत्र

तारीख:

बायोकेमिस्ट्री विभाग, स्त्री रोग विभाग (यूनिट-4), क्रिश्चियन मेडिकल कॉलेज, वेल्लोर, के सहयोग से, एक अध्ययन किया जा रहा है, जिस में आपको को आमंत्रित किया जा रहा है यह देखने के लिए कि माँ के रक्त से बच्चे द्वारा लिए जा रहे लोहे पर क्या प्रभाव पड़ता है। यह डॉक्टरों को बेहतर तरीके से समझने में मदद करने के लिए किया जा रहा है कि यह कैसे होता है।

अध्ययन में भाग लेने वाले सभी लोगों से, चेक अप के दौरान, 6 मिलीलीटर रक्त का नमूना एकत्र करेंगे, (3 बार- पहले, दूसरे और तीसरे तिमाही में)। रक्त संग्रह उसी समय किया जाएगा जब इसे अन्य नियमित परीक्षणों के लिए लिया जाता है। इससे आपको या आपके बच्चे के स्वास्थ्य को कोई नुकसान नहीं होगा।

हमें प्रसव के तुरंत बाद 6 मिलीलीटर कॉर्ड ब्लड और नाल के नमूने की भी आवश्यकता होती, जिसे प्रसव के बाद फेंक दिया जाता है।

हम जानना चाहते हैं कि क्या आप इस अध्ययन के लिए इन नमूनों को देने के लिए तैयार हैं?

एकत्र किए गए नमूनों का उपयोग केवल अनुसंधान उद्देश्यों के लिए किया जाएगा। यदि इस अध्ययन के पूरा होने के बाद कोई नमूना शेष है, तो हम आपके नमूने को संग्रहीत करने और भविष्य में संबंधित अध्ययन के लिए उपयोग करने के लिए आपकी अनुमति का अनुरोध करते हैं।

आपकी सारी जानकारी गोपनीय रखी जाएगी। अध्ययन में भाग लेने से आपको सीधे लाभ नहीं हो सकता है, हालांकि, यदि आप आवश्यक नमूने प्रदान करने के लिए तैयार हैं, तो आप हमें दिखाएंगे कि माँ के रक्त से बच्चे द्वारा लिए जा रहे लोहे पर क्या प्रभाव पड़ता है। यह डॉक्टरों को बेहतर तरीके से समझने में मदद करने के लिए किया जा रहा है कि यह कैसे होता है।

यदि आप अध्ययन में भाग लेने के इच्छुक नहीं हैं, तो आप ऐसा कहने के लिए स्वतंत्र हैं। यह अस्पताल में आपको मिलने वाले उपचार को प्रभावित नहीं करेगा। अध्ययन में भाग लेने से आप को रियायत या कोई विशेष उपचार नहीं मिलेगा। आपको अपने सभी नियमित परीक्षणों के लिए भुगतान करना होगा, जो आपके डॉक्टर कहते हैं। केवल अध्ययन के हिस्से के रूप में किए गए परीक्षण मुफ्त किए जाएंगे। यदि आपके पास और प्रश्न हैं, तो कृपया नीचे दिए गए नंबरों में से किसी एक का उपयोग करके हमसे संपर्क करें।

धन्यवाद

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डॉ. मौली जैकब, प्रोफेसर और प्रमुख, बायोकेमिस्ट्री विभाग, क्रिश्चियन मेडिकल कॉलेज, वेल्लोर- 632002

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अध्ययननंबर:

प्रतिभागियों के लिए सूचित सहमति प्रपत्र

अध्ययन का शीर्षक**: गर्भावस्था में हेपीसीडिन और आइरन (लोहे) से संबंधित मापदंडों : ये कैसे सहसंबद्ध होते हैं?**

प्रतिभागी का नाम: तारीख:

अस्पताल का आई-डी नंबर:

जन्म की तारीख:

मैं पुष्टि करती हूं कि, मैंने अध्ययन के लिए प्रदान किया गया सूचना पत्र को पढ़ा और समझा है। डॉ.आर्थर अमित सूर्यकुमार से मुझे अध्ययन के संबंध सारे प्रश्न पूछने का अवसर मिला है।[ ]

मैं समझती हूं कि अध्ययन में मेरी भागीदारी स्वैच्छिक है। बिना कोई कारण बताए या मेरी चिकित्सा देखभाल या कानूनी अधिकारों को प्रभावित किए बिना, मैं किसी भी समय अध्ययन से पीछे हटने के लिए स्वतंत्र हूं।[ ]

मैं समझती हूं कि यदि मैं अध्ययन में भाग लेने के लिए सहमत हूं, गर्भावस्था के हर तिमाही के दौरान मुझसे 6 मिलीलीटर रक्त नमूना एकत्र किया जाएगा।[ ]

मैं समझती हूं कि अगर मैं अध्ययन में भाग लेने के लिए सहमत हूं, तो प्रसव के तुरंत बाद 6 मिलीलीटर कॉर्ड ब्लड लिया जाएगा।[ ]

मैं समझती हूं कि इससे मेरे स्वास्थ्य पर कोई हानि नहीं होगी।[ ]

मैं समझती हूं कि एकत्र किए गए रक्त के नमूने का उपयोग केवल अनुसंधान उद्देश्यों के लिए किया जाएगा। यदि इस अध्ययन के पूरा होने के बाद कोई नमूना शेष है, तो मैं नमूने को संग्रहीत करने और संबंधित अध्ययनों के लिए भविष्य में उपयोग करने की अनुमति देती हूं।[ ]

मैं समझती हूं कि मेरी पहचान उजागर नहीं की जाएगी या मेरी जानकारी तीसरे पक्ष को प्रकाशित नहीं की जाएगी और मेरी सभी चिकित्सा जानकारी गोपनीय रखी जाएगी। [ ]

मैं समझती हूं कि इस अध्ययन के अन्वेषक, नैतिकता समिति और नियामक अधिकारियों को वर्तमान अध्ययन के संबंध में मेरे स्वास्थ्य रिकॉर्ड को देखने के लिए मेरी अनुमति की आवश्यकता नहीं होगी और इसके संबंध में किए110 जाने वाले किसी भी अन्य अनुसंधान, भले ही मैं अध्ययन से वापस ले लूं। मैं इस पहुंच से सहमत हूं। " [ ]

मैं अपनी मर्जी से उपरोक्त अध्ययन में भाग लेने के लिए सहमत हूं।[ ]

प्रतिभागी का नाम और हस्ताक्षर/अंगूठे का निशान (दिनांक)

साक्षी का नाम और हस्ताक्षर/ अंगूठे का निशान (दिनांक) और पता:

शोधकर्ता का नाम और हस्ताक्षर (दिनांक):

Appendix III

PROFORMA FOR DETAILS OF PARTICIPANT

Name	Hospital No	DOB	Age	Spouse Name	Phone No

Clinical details at the time of recruitment

LMP	Calculated EDD	EDD by scan	GA at recruitment	Blood group	Height (cm)	Weight (kg)	BP	Obstetrics score

Urine test

Alb:

Results of tests done on maternal blood:

Laboratory	Test	1st trimester (GA)
Transfusion Medicine	Hb	
	MCV (mean corpuscular volume)	
Clinical Biochemistry	Serum iron	
	Serum ferritin	
	TIBC	
	Transferrin saturation	
	hsCRP	
Biochemistry	Hepcidin	

Date and time of admission due to onset of labour:
BP at time of admission:
Labour induced: Yes/No
Gestational age at delivery:
Mode of delivery:
Any pregnancy-related complication that developed:

Outcomes of pregnancy

Sex of baby	Birth weight	Length of baby	APGAR score

Cord blood obtained: Yes/No

Results of tests done on cord blood

Laboratory	Test	Result
Transfusion medicine	Hb	
	MCV (mean corpuscular volume)	
Clinical Biochemistry	Serum iron	
	Serum ferritin	
	TIBC	
	Transferrin saturation	
	hsCRP	