REVIEW OF LITERATURE

3.1 Global Blood Situation

- Data from 178 countries indicate that about 81 million units of blood and 20 million liters of plasma were donated annually in the period 2001-2002. Analysis of blood donations versus the Human Development Index (HDI) of countries reveals a significant difference in blood donations between low, medium and high HDI countries; 61% of the global blood supply was donated in developed (high HDI) countries. Only 39% was donated in developing (low and medium HDI) countries where 82% of the world population lives. Differences in blood donation rates largely reflect differences in the level of development of health care systems in these countries.\(^{[44]}\)

3.2 Blood Transfusion Services (BTS):

- Blood Transfusion Service is a vital part of the National Health Service and there is no substitute for Human Blood and its components.

- Transfusion of blood is an important form of therapy in medicine. Without blood donation there cannot be transfusion of blood. A well-functioning blood transfusion service is dependent on forthcoming blood donors who are willing to donate voluntarily without being mandated.\(^{[45]}\)

- Blood is a rich product which can be broken down into many parts. The average amount of blood present in an adult is 4-5 liters or about 8% of the body weight. A person can donate blood 168 times during his/her 18 to 60 years.

- Increasing advancement in the field of Transfusion Technology has necessitated enforcing stricter control over the quality of Blood and its products. In most of the
developed countries, the blood banking system has advanced in all facets of donor management, storage of blood, grouping and cross matching, testing of transmissible diseases, rationale use of blood and distribution.

3.3 Blood Safety and Blood Donors

- People who sell body constituents could act against their self interests and risk being exploited. The international export of products from blood sold by impoverished donors in developing countries prompted the WHO in 1975 to recommend that national blood services should be based on voluntary, non remunerated blood donation to ensure that blood products are as safe as possible.[46]

- The recruitment and retention of safe, voluntary blood donors remains the weakest area, with only a marginal increase in the number of countries achieving 100% voluntary blood donation. To support countries in their campaigns to increase regular voluntary donation, WHO has initiated a collaborative venture with the International Federation of Red Cross and Red Crescent Societies, the International Federation of Blood Donor Organizations and the International Society of Blood Transfusion to promote the global celebration of World Blood Donor Day which is held annually on 14 June every year. It has also developed a set of training materials for blood donor organizers for use in regional and national training activities.[44]

3.4 Iron status in blood donors

- Important questions about the impact of donations on the donor’s iron status are:
  - Who will develop iron deficiency?
  - What is the ideal frequency of donation?
  - What is the effect of iron supplementation to donors?
The increasing percentage of young female donors further underlines the importance of controlling the iron status as the physiological iron loss is high in menstruating females.\[47\]

One whole-blood donation removes about 250 mg of iron (4-10% of the body iron) and leads to a reduction of 0.5 g/dL in the venous Hb immediately after donation.\[48\] The lowest point is reached 2-3 days after donation and Hb regains after 30-40 days.\[49,50\]

Short intervals between donations may increase the risk of iron depletion, while longer intervals and/or iron supplementation after donations may prevent iron deficiency.\[14,25,38,50,51,52\] Ferrous sulphate is the most used formula to compensate for the loss of iron in blood donors.\[53,54\] Unless donors are taking iron supplementation regularly, even two donations per year is associated with a 28% incidence of iron deficiency.\[11\]

Bianco et al found that 13% of donors were rejected and that 41% of all deferrals were explained by low Hb-values.\[55\] The reduction of iron in donors is not sufficiently compensated by a normal diet,\[10\] and it is discussed whether iron supplementation after donations will restore the iron status more quickly.\[54,56,57\] Furthermore, iron supplementation is a controversial issue as a donor may suffer from unknown, hereditary hemochromatosis in the preclinical stage, and it may be considered unethical to offer iron supplementation to these donors.\[55\] Genetic screening for hemochromatosis is not a routine. This is the most frequent disorder of inborn metabolism in the Caucasian population with a prevalence of homozygotic expression of five to seven per thousand.\[53\]

Taken this into consideration there is a certain possibility to give iron supplementation on wrong indications to persons who have contraindications for using iron.\[6,58\] At the opposite, young female blood donors who are vulnerable to iron deficiency, should be...
offered iron supplementation. In general, to avoid a possible harm, it is preferential that the iron status of a donor has been normalized prior to a subsequent donation of blood. Increased frequency of donation may cause stress in the donor. Better knowledge of adjusting the frequency of donations both for donors with low iron status and for donors having iron overload will take better care of the donor on an individual basis.

- In a previous study by Fielding O'Shaughnessy it was shown that there was less storage iron, measured as desferrioxamine chelatable iron, in healthy women than in healthy men. In as many as a third of normal women without anaemia, i.e. with hemoglobin concentration above 120 g per 100 ml, storage iron was in the same range as in severe iron-deficiency anaemia, in which it is known with certainty that storage iron is depleted. As in the well known mild chronic anaemia which afflicts numerous women, the most likely explanation of this finding is the effect of menstrual loss on iron status. It has been shown by direct measurement that about 20% of women lose more than 60 ml menstrual blood per cycle; in some cases the loss exceeds 400 ml per cycle. Many such women seem unaware that their losses are unusual. The net effect of menstrual loss on hematological status depends not only on the amount lost but on dietary iron intake and the efficiency of intestinal absorption. It is uncertain therefore whether storage iron depletion in a high proportion of women is related to menstrual loss which could reasonably be considered pathological, or whether their iron deficiency is related to dietary and intestinal factors.

- Recruiting a sufficient number of new blood donors is a huge challenge in many countries. One of the main reasons for rejection was failing to meet the Hb criteria, predominantly amongst young women. To keep the blood supply sufficient, an
increased pressure is laid upon established donors. The donors with a high frequency of donations are at risk for iron deficiency.

3.5 **IRON:**

Iron was discovered by Known since ancient times. Origin of name: from the Anglo-Saxon word "iron" or "iren" (the origin of the symbol Fe comes from the Latin word "ferrum" meaning "iron"). Possibly the word iron is derived from earlier words meaning "holy metal" because it was used to make the swords used in the Crusades.

3.5.1 **Biological function of iron**

Iron is the most conserved element and its absorption, excretion and metabolism are strictly regulated. It is the fourth most abundant element in the earth’s crust.

- Iron is an essential nutrient for all living organisms as all cells need iron for vital biological processes.[67]
- Erythropoiesis is quantitatively the main consumer of iron. New erythrocytes need about 20-25 mg of iron per day to synthesize hemoglobin molecules.[68]
- The ability of this transition metal to exist in 2 redox (Fe2+ and Fe3+) states makes it useful at the catalytic center of fundamental biochemical reactions
- Iron is an essential nutrient that plays a central role in many metabolic processes. It plays a vital role in many cellular processes, a very important protein ribonucleotide reductase that is involved in DNA synthesis, cell division, proliferation and differentiation requires iron for its enzymatic activity.
- Aerobic metabolism is critically dependent on maintaining normal concentrations of several iron-containing proteins that mediate oxygen transport, storage and utilization, particularly when the tissue demand for oxygen is increased by physical activity.
The very properties that make iron attractive for redox reactions also make it toxic. Free iron has the ability to generate oxidative radicals that damage essential biologic components such as lipids, proteins and DNA. Tight regulation of iron is required to meet the dual challenge of avoiding iron deficiency and iron excess.\[^{69}\]

Cells have acquired specific regulatory controls during evolution which allow them to adapt to changes in the extracellular iron levels and to internal physiological changes like cell proliferation or differentiation. Several protein molecules involved in iron metabolism are known.\[^{70}\] Recently the roles of transferrin receptors (TfR), Divalent Metal ion Transporter 1 (DMT 1), Duodenal cytochrome B (DcyB), Ferroportin, Hephaestin and Hepcidin have been better defined.

### 3.5.2 Distribution of Iron in the body: \[^{71}\]

- Normally, very small quantities of iron are present in most cells of the body, in plasma, and in other extracellular fluids. Physiologically, the body rigorously conserves its iron supply, so that less 0.1% of the body iron content is lost daily, mostly in desquamated cells.

- The total amount of iron in an adult man averages 3-5 grams, and about 70% of this is contained in hemoglobin. Storage iron accounts for about 25% of the total iron.

- The amount of storage iron can undergo more than a tenfold increase or decrease from the normal average without any apparent effect on health. Nevertheless, beyond these extremes, iron excess and iron deficiency will then occur.

Body iron is distributed into a number of different compartments that include [1] hemoglobin, [2] storage iron, [3] tissue iron, [4] myoglobin and [5] a labile pool. The average amount of iron in these compartments is summarized in Table:
Table: Average Iron content of different compartments.

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Iron content (mg)</th>
<th>Total body iron (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemoglobin iron</td>
<td>2000</td>
<td>67</td>
</tr>
<tr>
<td>Storage iron (ferritin, hemosiderin)</td>
<td>1000</td>
<td>27</td>
</tr>
<tr>
<td>Myoglobin iron</td>
<td>130</td>
<td>3.5</td>
</tr>
<tr>
<td>Labile pool</td>
<td>80</td>
<td>2.2</td>
</tr>
<tr>
<td>Other tissue iron</td>
<td>8</td>
<td>0.2</td>
</tr>
<tr>
<td>Transport iron</td>
<td>3</td>
<td>0.08</td>
</tr>
</tbody>
</table>

**[1] Hemoglobin:**

Hemoglobin is made of two pairs of globin chain that bears the haem molecule. Haem molecule has a central ferrous iron to which oxygen is attached loosely. This property of hemoglobin makes it ideal for carrying oxygen. It contains most of the body’s iron. Each molecule of Hb contains 0.34% of iron by weight; hence 1 milliliter of erythrocytes contains 1 mg of iron. In a 70 Kg male, the RBC mass is about 2 L, containing, therefore, about 2 g of hemoglobin iron.

**[2] Storage Iron:**

- **Storage iron** is the pool of iron in the body that is not being used by tissues. Healthy children and adults (apart from infants’ aged 6–11 months and pregnant women) usually have some iron stores to act as a buffer against iron deficiency during periods when dietary iron may be temporarily insufficient.

- Iron is stored in the form of ferritin and hemosiderin. Iron is stored in the epithelial cells of the gastrointestinal tract and in the reticuloendothelial cells of the liver, spleen, and bone marrow.

- Both ferritin and hemosiderin provide a store of iron that is available for protein and heme synthesis. Normally much of the stored iron in the body (about 1 g in men and less in pre-menstrual women and children) is present as ferritin, but during iron overload the proportion present as hemosiderin increases.

**A. Ferritin:**

- It is the second most common iron binding protein. It is stored in a form in which the iron is shielded from body fluids, so that it is unable to produce oxidative damage, as would be the case if it were in ionic form.
- It is an alpha 2 globulin. It consists of a protein, an apoferritin shell with a molecular mass of about 500 kDa composed of 24 subunits assembled in form of a cube which are either L (light) or H (heavy) ferritin chains.

- Each side of the cube is made of 4 monomers with a pore in the centre 10 Armstrong in diameter. Ferrous iron moves in and out of the apoferritin core through these pores present on each side. The protein shell encloses a core of an interior ferric oxyhydroxide (FeOOH) crystalline core which usually stores 2000 molecules of iron and can hold up to 4300 atoms of iron, protecting the cells from the reactive cations.\(^{[67]}\)

- Proteins with a similar overall structure are found throughout the plant and animal kingdom as well as in bacteria.

- In men, the amount of storage iron is approximately 800 mg, mostly as ferritin; in healthy women, it ranges up to 200 mg. Minute quantities of ferritin are also present in serum in concentration roughly proportional to total body iron stores. Stored ferritin in the tissues is normally in equilibrium with a small amount of ferritin leaking into the blood stream.

- In healthy individuals iron stores can indirectly be quantified by measuring serum ferritin (μg/L). Both the macrophages and the hepatocytes have large capacity for iron storage.

- Liver injury and a large number of pathological processes result in release of relatively large amounts of ferritin into plasma.

- Serum ferritin concentrations are normally within the range 15–300 μg/l. There is a good correlation between serum ferritin concentration and storage iron mobilized as a result of phlebotomy. This suggests a close relationship between the total amount of
stored iron and the serum ferritin concentration in normal individuals. Serum ferritin concentration decreases with blood donation. and increases with alcohol intake.

There are two types of monomers of ferritin; L and H

- L monomers (light type) are also called basic ferritin. They contain 174 amino acids. L monomers have 15 hydrophilic sites which are responsible for binding iron very readily and tightly, therefore also responsible for low turnover of iron. It is this property, which makes it suitable for storage. L monomer rich ferritin is therefore found in liver and spleen. Serum also has ferritin rich in L monomers.

- H monomers (Heavy type) are also called acidic ferritin. They contain 182 amino acids. H monomers have 7 hydrophilic sites, which is less than L monomer, due to which it takes up and releases iron readily. It is found in heart, kidney, placenta, monocytes, lymphocytes and erythrocytes.

Ferritin has > 20 isoferritins which have variable number of H and L chains. Ferritin apart from its function in storage is also thought to have a role in metal detoxification as it diverts excess iron.

B. Hemosiderin:

It is formed when ferritin is broken down in secondary lysosomes. Ferritin is a soluble protein but is degraded to insoluble hemosiderin which accumulates in lysosomes. Hemosiderin therefore appears to represent the end point of the intracellular storage iron pathway. It is aggregated, partially deproteinized ferritin. It is composed predominantly of FeOOH crystals, which have been stripped off their apoprotein shell. It contains 25-30% of iron by weight. In contrast to ferritin, hemosiderin is insoluble in aqueous
solutions. Like ferritin, hemosiderin is normally found predominantly in the monocyte macrophage system cells of the liver, spleen and bone marrow. It can be detected easily by Pearls’ reaction as bluish green pigment.\textsuperscript{[77]}

[3] **Myoglobin:**

Myoglobin very closely resembles a single hemoglobin subunit, containing a single heme per molecule. It is present in all muscle cells and acts as an oxygen reservoir.

[4] **The Labile Iron Pool:**

Approximately 80 mg of iron are found in the labile pool. This compartment has no clear anatomical location; rather it is a concept derived from kinetic measurements with radio labeled iron.\textsuperscript{[78]}

[5] **Tissue Iron:**

Numerous cellular enzymes and coenzymes require iron, either as an integral part of the molecule or as a cofactor. Notable are the peroxidases and cytochromes, all of which, like hemoglobin, are heme proteins. Other enzymes, such as aconitase and ferredoxin, contain iron that is coordinated with sulfur in a so called iron sulfur cluster. These enzymes and coenzymes, which appear in all nucleated cells of the body, are referred to collectively as the tissue iron compartment, normally amounting to approximately 8 mg.

With exception of storage iron, which is believed to serve merely as a reserve of iron, the function of iron compounds involves the transport and utilization of oxygen. Hemoglobin binds oxygen as blood circulates through the lungs and then releases oxygen to the tissues as blood traverses the capillary bed. Myoglobin transports Oxygen across the muscle cell and stores it in muscle. The cytochromes, ion-sulfur proteins, and other mitochondrial
iron compounds are required for the oxidative production of cellular energy as ATP. Thus, the iron compounds are essential for an uninterrupted and unimpaired combined chain of oxygen supply and cellular metabolism and therefore decisive for normal muscle function and work performance.\cite{71}

### 3.5.3 Transport of Iron:

- Iron is transported from one organ to another by a plasma iron transport protein, apotransferrin - a beta globulin which is synthesized in liver. Its rate of synthesis is inversely proportional to the iron stores.
- The apotransferrin Fe 3+ complex is called transferrin. It is a 678 amino acid glycoprotein. Each molecule of transferrin carries 2 atoms of iron.
- Transferrin is a carrier protein for iron in blood plasma. Ferric iron (Fe3+) bound to transferrin is carried via the circulatory network to hematopoietic and other tissues.
- Iron is released from transferrin through transferrin receptor pathway in the marrow for erythropoiesis and transferrin is reutilized to carry iron.
- Normally, there is a total of approximately 2.5 mg of iron in plasma. It is the most dynamic compartment of iron and has a turnover of 10 times in 24 hours.
- When transferrin binds to the transferrin receptor of cells, the transferrin/receptor complex is internalized into an endosome. It then becomes acidified, releasing the iron from transferrin and reducing it to ferrous iron (Fe2+), which is then transported into the cell through the divalent metal transporter, DMT1. The apotransferrin is then transported back to the cell surface, ready to transport another transferrin molecule to the interior of the cell. This series of reactions has been designated the **transferrin cycle**.
- Transferrin keeps ferric iron soluble and prevents iron from reacting with other molecules by attenuating its redox activity. It aids in delivery of iron to cells by binding to a specific cell-surface transferrin receptor, TfR 1.

**Transferrin Receptor (TfR):**

- TfR is a transmembrane glycoprotein present on the surface of body cells. Transferrin receptor is present in low concentrations in all cells, but is expressed at high levels by cell types with large needs of iron such as developing erythroid precursors (80% of the receptors) and peak levels are reached in intermediate normoblasts. Thereafter their concentration reduces as maturation is achieved.

- TfR is responsible for internalization of transferrin bound iron into normoblasts, through an endocytic vesicle. Iron dissociates from TfR-Tf complex when pH of the vesicle is 5.0. Iron remains in the cell while the transferrin with receptor is recycled back to cell surface. Apotransferrin reenters the circulation.

- The number of TfRs on the cell surface reflects iron requirement, the higher the iron requirement of the cells, the higher is the density of receptors at the cell membrane. A soluble part of the TfR is measurable in serum with levels increasing in the presence of iron deficiency [a better marker of early iron depletion], as well as in conditions with increased erythropoiesis and low in anemia of chronic diseases.[79]

- Serum transferrin receptor is the truncated form of tissue receptor and exists as transferrin-receptor complex. The serum level of TfR reflects the amount of membranous TfR.

- There are two types of TfRs.
o TfR 1 binds diferric transferrin with high affinity. It mediates iron delivery to erythroblasts by binding with plasma transferrin.

o TfR 2 is similar to TfR 1 (45% structural homology to extracellular domain of TfR 1). It binds to Tf with low affinity. It is highly expressed in hepatocytes and erythroid cells. Mutation in this leads to states of iron overload. Its role in iron regulation by hepcidin has been postulate.

- Membrane transferrin receptors are essential to supply transferrin iron to tissues; the total number of receptors determines the amount of iron uptake with the exception of liver parenchymal and reticuloendothelial cells, which acquire iron from a different route.

- Most tissues modulate their iron content by varying the TfR expression.

- Synthesis of ferritin increases with high iron levels, while synthesis of TfR 1 is reduced.

  The opposite occurs in individuals with a low serum ferritin level.\textsuperscript{[80]}

3.5.4 Regulation of Iron Homeostasis:

The amount iron loss from the body depends only minimally upon the iron burden. At the end of the life of red cells (100-120 days) iron is released from them and enters the plasma pool, from where it is taken to reticuloendothelial cells of the marrow for storage.

With adequate daily absorption and normal losses, iron homeostasis is maintained.

**Normal Iron Balance:**

Regulation of body iron content is achieved almost entirely by modulation the amount of ion absorbed from the upper intestinal tract.
**Daily requirements:**

Diet should contain 10 to 15 mg of elemental iron and with approximately 8 to 10% absorption the net requirement per day is 1 mg in males and 1.5 mg in females in their reproductive period.

Normally, approximately 1 mg of iron is absorbed each day, principally from the duodenum. Heme is absorbed directly as such through a specific receptor.\[81\] To be absorbed, inorganic iron must be in the ferrous state (Fe\(^{2+}\)).

**Iron Cycle:**

Iron is highly conserved in humans with minimal losses occurring through gut, skin and menstruation and in pregnancy in women. A small amount of iron is lost from stools, urine perspiration, exfoliation of skin and milk (during lactation). Daily loss in males is about 1 mg and in females about 2 mg; there is a very delicate iron balance in females.

**Dietary Iron:**

- Bioavailability of iron is dependent on its chemical form in the food and the presence of other food items that enhance or inhibit the absorption. An average diet may contain 10-20 mg of iron per day, but only 1-2 mg is absorbed from the gut, and the rest comes from breakdown of erythrocytes in the macrophages.\[82\]

- Dietary iron consists of two components, heme iron and non-heme (inorganic) iron. To be absorbed from food, iron must either be in the form of heme or converted to soluble ferrous salts and small molecular iron chelates (ascorbate, citrate, and other organic acids and amino acids).\[80\]
There is an inverse relationship between the need for iron and the rate of iron absorption. Thus iron absorption is greater in iron depleted individuals than in those with iron overload.\cite{80,83}

**A] Haem Iron:**\cite{77,80}

- It is present in form of myoglobin and hemoglobin in meat especially liver, kidney and egg yolk.
- From heme iron, 15-35 % is absorbed
- Haem bound to protein is released by acid and proteases in stomach. Ferrous ion in heam is oxidized to ferric iron. Hemin thus formed is absorbed as such.
- This mode of absorption is unaffected by other dietary factors and is a very efficient way of iron absorption.
- Also this mode of absorption is not subject to regulation.

**B] Non-Haem Iron:**\cite{77,84}

It is present in non-meat sources such as legumes, green leafy vegetables, fruits and cereals.

- Non-heme iron accounts for approximately 90 % of dietary iron, but only 2-20 % will be absorbed, depending on the iron status of the individual and the ratio of enhancers and inhibitors in the diet.
- Non heam iron is released from the food as ferric or ferrous ions by action of acid.
- Iron is absorbed in ionic state only. Ferric ions are insoluble in high pH, which is present in duodenum. Ferrous ion on the other hand is soluble at alkaline pH of duodenum. Hence most of the non-haem iron that is absorbed is in ferrous state.
- This form of iron absorption is subject to intestinal regulation, which is influenced by amount of stored iron and the rate of erythropoiesis.
This route of absorption is markedly increased in hereditary haemochromatosis.

3.5.5 **Bioavailability and absorption of iron**[^85]

Most iron absorption takes place in the proximal duodenum and upper jejunum. To be available for various body processes iron needs to pass through the intestinal mucosa into the capillary network to be incorporated into transferrin the transport protein. There seems to be no intestinal lymphatic uptake of iron. Transport of iron across the enterocytes occurs in three stages:

1] **Reduction and uptake of solubilized, luminal iron through the apical membrane:**

- There is high pH in duodenum due to which ferrous ion is soluble, however ferric ions precipitate and hence are chelated with amino acids or sugars to maintain them in soluble form.
- Ferrous ion has a more efficient mechanism of absorption.
- The low pH at the brush border and DcytB, a ferric reductase enables conversion of ferric to ferrous state, which is then absorbed.
- Haem iron and non-haem iron in form of ferrous and ferric iron is absorbed by different mechanisms.
- Haem iron enters enterocytes via haem receptors. This is then degraded to free iron, bilirubin and carbon monoxide by action of haem oxygenase.
- Ferrous ion uptake is mediated through DMT 1[Divalent Metal transporter 1]. Binding if IRE [Iron response elements]+DMT 1 to IRP[Iron regulatory protein] takes place in an iron dependent way and in case of low iron pools increases the stability of mRNA and therefore increases the intestinal iron absorption.
Most of the Fe3+ ions are converted to Fe2+ before absorption. However, some of Fe3+ is directly absorbed by IMP (Integrin & Mobilferrin Pathway). Exact mechanism is however not known.

2) **Intracellular processing of iron:**

Iron that is transported across the cell membrane is either stored in the enterocytes or is transported across the basolateral membrane into the capillary network.

The path taken is governed by the body’s demand for iron.

When there is iron excess, iron is sequestered by apoferritin where it is tightly bound in form of F bodies/ ferritin bodies and less is available for transportation to transferrin.

Reverse happens with low body iron, less of it is sequestered and more is transported to the basolateral membrane.

3) **Transfer of iron through the basolateral membrane into the portal circulation:**

Iron transport across the enterocytes is an active process.

It is limited to duodenal and part of jejunal basolateral membrane and takes place against an electrical gradient.

Basolateral membrane transporter has been cloned [iron regulatory protein]IREG1/ferroportin/ MTP1[membrane transport protein]

An accessory protein, hephaestin (a ceruloplasmin homologue) is required for transfer of iron across the basolateral membrane and for binding of iron to transferrin as it converts Fe2+ to Fe3+.

In IDA, there is up regulation of duodenal DMT 1, Dcyt B and ferroportin 1 mRNA and protein.
Iron status in voluntary blood donors

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[Courtesy from Usha Rusia and Mrinalini Kotru. Molecular mechanisms in Iron Metabolism. By M.B Agarwal Hematology Today- 2005]

Figure 2
Absorption and transport of iron

[Diagram showing absorption and transport of iron]

[Courtesy from Usha Rusia and Mrinalini Kotru. Molecular mechanisms in Iron Metabolism. By M.B Agarwal Hematology Today- 2005]
Other molecules involved in Iron metabolism:

1] **Duodenal cytochromes B (DcytB)**[^86, 87]

It is a diheam protein and a member of cytochromes B561 family of ascorbate dependent reductases.

- It is present in the intestinal brush border and solubalises dietary iron for absorption by reducing ferric iron to ferrous iron.
- Recent data has shown that it along with divalent metal ion transporter 1 (DMT 1) is rapidly down regulated on receiving large doses of oral iron, suggesting a possible molecular mechanism involved in the mucosal block.

2] **Divalent Metal Ion Transporter 1 (DMT 1)**[^87, 88, 89]

It is also called divalent cation transporter 1 (DCT -1) or Nramp2.

- It is a single chain hydrophobic protein which acts as a proton coupled apical transporter of divalent metal cations like Fe2+, Mn2+, Co2+, Cd2+, Cu2+ and Ni 2+.
- Principal function of this transporter is mainly uptake of Fe2+ iron. Fe3+ iron is not transported by DMT 1.
- Evidence suggests that DMT 1 functions in non transferrin bound iron uptake which is necessary for iron assimilation and utilization by transferrin/transferrin receptor pathway.
- Its role in the mucosal block in situations of excessive dietary iron has also been shown.

3] **Iron Regulatory Protein (IRP)**[^89, 90, 91]

It is also called IRE-BP (Iron response element binding protein) and IRF (Iron regulatory factor).

[^86, 87]: Ref 86, 87
[^87, 88, 89]: Ref 87, 88, 89
[^89, 90, 91]: Ref 89, 90, 91
It plays a key role in iron homeostasis by regulating and coordinating the synthesis of TfR, ferritin and erythroid ALA synthase in erythroid precursors by binding to the stem loop structures that constitute the Iron responsive element (IRE) which is present on mRNA of these molecules.

IRP exists as IRP 1 and IRP 2, the former being in larger amounts. IRP 1 acts as a ferrostat and allows cells to respond appropriately to iron concentration in the cell. IRP 2 has no enzymatic activity.

Nitric oxide produced by macrophages and other cells in inflammation, induces the IRE binding activity of IRF, and hence stimulates iron deficient state in the cell.

4] **Mobiliferrin:** [59, 87]

- It is a cytosolic iron transport protein. It is found in apical cytoplasm of enterocytes.
- It is called mobiliferrin, naming it after the city of discovery as well as characterizing it as the probable shuttle protein that safely transport iron through the cytoplasm.
- A large protein complex is identified which contains integrin, mobiliferrin and a flacin monooxygenase.
- It facilitates the reduction of the ferric iron to the ferrous state, so that it can be utilized for the production of iron containing proteins by the cell.
- This IMP (Integrin Mobiliferrin Protein) pathway is involved in ferric ion transport across the apical membrane, but its exact mechanism has yet not been characterized.
- More recent studies have shown that the mobiliferrin–Integrin-paraferritin system is operative in cells in other organs in the body and in these organs is responsible for receiving iron from transferrin.
5) **Ferroporin 1:** \[87]\n
- It is an iron exporter and is present in the basolateral membrane of the enterocytes, in syncytiotrophoblasts, and in phagocytic cells of the reticuloendothelial system of mammals.
- The protein catalyzes the exit of divalent metal ions from the epithelial cell into the tissues. Because ferroportin extrudes Fe²⁺ from the cell which has a membrane potential negative inside, ferroportin is presumed to function by cation(H⁺ or Na⁺) antiport.
- It is associated with excess iron deposits in human macrophages. It plays an essential role in iron recycling from erythrophagocytosed red cells.
- Its expression is regulated by increasing cytoplasmic iron or copper.

6) **Hephaestin:** \[87, 92]\n
- It is a membrane bound homologue of ceruloplasmin that appears to act as a multicopper ferroxidase.
- It is an accessory protein which is required for transfer of iron across the basolateral membrane and for binding to transferrin.
- It oxidizes Fe²⁺ to Fe³⁺ so that it can be carried by transferrin.
- Mutation in hephaestin in animal studies leads to severe microcytic hypochromic anemia as iron in the enterocytes is not able to leave the cell.

7) **Hereditary Hemochromatosis gene product- HFE:** \[87, 92]\n
- HFE binds transferrin receptor with an affinity close to that of transferrin, reducing the affinity of the transferrin receptor for transferrin and competing with transferrin binding.
The close association of HFE with the transferrin mediated iron uptake pathway and its location in endosomes and on the basolateral side of enterocytes precursor cells implicates a role for HFE in sensing body iron stores.

The hereditary hemochromatosis defect has been attributed to a decrease in the amount of functional HFE protein.

Over expression of HFE in cells grown in culture reduces iron uptake and lowers intracellular ferritin levels.

8] **Role of Hepcidin:** [87, 92, 93]

Hepcidin, a 25 amino acid peptide secreted into the plasma from the hepatocytes, is probably a key regulator of intestinal iron absorption and also of iron recycling via macrophages.[94]

It is predominantly expressed in liver but much smaller quantities are also found in brain, heart, blood and urine.

It is observed to limit absorption of intestinal iron.

Its lack of expression has been noted in iron overload and over expression is seen in iron deficiency.

Most importantly, each of the previously mentioned factors regulating intestinal iron absorption, i.e. iron stores, erythropoietic activity, hemoglobin, oxygen content and inflammation also regulate liver hepcidin expression. In each of these situations, intestinal iron absorption varies inversely with liver hepcidin expression.

It appears that hepcidin acts by inhibiting the expression or activity of one or more of the genes involved in intestinal iron transport.
It provides an important link in the pathogenesis of anemia of chronic disease and its role as an erythroid regulator is of prime importance.

Hepcidin production is up-regulated in iron overload leading to reduced absorption of iron, and down-regulated in iron deficiency, causing increased iron absorption. The liver protein, hemochromatosis protein (HFE), regulates the activation of hepcidin synthesis.[80]

Mutations in the HFE gene reduce the synthesis of hepcidin and iron absorption is increased. This is a fundamental mechanism in hereditary hemochromatosis.

Measurements of hepcidin in serum may be a future method to assess fluctuations in the body’s iron balance.

**Mucosal block mechanism:**[95]

The sites of absorption of iron are duodenum and upper jejunum. Absorption of iron is controlled to a large extent by the amount of iron stores. There are 3 components of Fe absorption:

1] It is transported across the cell into circulation.
2] A part of Fe remains in the cell and is lost as the cell is shed off.
3] A part of diet iron is not absorbed and is lost in feces.

Mucosal cells control the amount of iron absorbed and also the amount that is transferred into plasma- known as mucosal block mechanism, e.g.,

1] When iron stores are nil /reduced in IDA- absorption is enhanced and almost the whole iron is put into circulation.
2] When iron stores are increased as in hemosiderosis, major part of iron is not absorbed; the part absorbed remains in the cell as ferritin and is lost as the cell is shed off; very little absorbed part is transported into plasma.

Thus, extent of iron absorption is dependent upon requirement of Fe by the body.

**Proteins that affect Iron Homeostasis:**

A number of proteins play a role in iron homeostasis. Mutations of these proteins in human and/or their targeted disruption or overproduction in mice result either in iron overload or in iron deficiency. Table summarizes the effects of some of these proteins on iron homeostasis. The exact role of each of these proteins, however, is not fully understood.
Iron status in voluntary blood donors

<table>
<thead>
<tr>
<th>Protein</th>
<th>Effect of Deficiency</th>
<th>Putative Function</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFE</td>
<td>Increased Fe</td>
<td>May transmit signal that upregulates hepcidin</td>
<td>Most patients with hereditary hemochromatosis are homozygous for the 845 A→G (C282Y) mutation of this gene.</td>
</tr>
<tr>
<td>β2-microglobulin Transferrin</td>
<td>Increased Fe</td>
<td>Transports HFE to the membrane</td>
<td></td>
</tr>
<tr>
<td>Transferrin receptor-1</td>
<td>Lethal, Increased CNS Fe</td>
<td>Transports iron in the plasma</td>
<td></td>
</tr>
<tr>
<td>Transferrin receptor-2</td>
<td>Increased Fe</td>
<td>Binds and internalizes transferrin at the membrane</td>
<td></td>
</tr>
<tr>
<td>Ferroportin (SLC11A3)</td>
<td>Increased Fe</td>
<td>May transmit signal that upregulates hepcidin</td>
<td></td>
</tr>
<tr>
<td>Hfehaestin</td>
<td>Fe deficiency</td>
<td>Oxidizes ferrous iron to ferric iron at the intestinal abluminal membrane</td>
<td>Dominant inheritance of iron overload</td>
</tr>
<tr>
<td>DMT1</td>
<td>Fe deficiency in rodents; iron overload in humans</td>
<td>Transports ferrous iron across membranes</td>
<td>Encoded by a sex-linked gene. Deletion is cause of isle mouse.</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>Fe increased</td>
<td>Oxidizes ferrous to ferric iron in the plasma</td>
<td>The naturally occurring mutations found in the mjt mouse and the Belgrade rat are the same.</td>
</tr>
<tr>
<td>Hepcidin</td>
<td>Fe increased</td>
<td>Blocks iron transport by ferroportin</td>
<td>Brain accumulation and neurologic disease</td>
</tr>
<tr>
<td>Steap3</td>
<td>Fe deficiency</td>
<td>Reduces iron in endosome</td>
<td></td>
</tr>
</tbody>
</table>

3.6 Iron Deficiency anemia:

- **Iron deficiency** is a state in which there is insufficient iron to maintain the normal physiological function of tissues such as the blood, brain, and muscles. Iron deficiency can exist in the absence of anaemia if it has not lasted long enough or if it has not been severe enough to cause the hemoglobin concentration to fall below the threshold for the specific sex and age group.\[^{96}\]

- It is one of the most prevalent disorders of humans.\[^{97}\] Iron deficiency anemia (IDA) is the most common micronutrient disorder in the world, negatively affecting the health and socioeconomic wellbeing of millions of men, women, and children.\[^{98}\]

- According to the World Health Organization (WHO), IDA constitutes a significant public health problem requiring immediate attention from governments, researchers and healthcare practitioners.\[^{99}\]

- Iron is a requisite element in human nutrition having a special role in red cell, intestinal, liver and placental physiology. Its importance has been fully recognized as iron deficiency anaemia is highly prevalent in many areas of the world, especially the underdeveloped and developing countries where the problem is often exacerbated by limited access to appropriate healthcare and treatment.\[^{100}\]

- Iron deficiency with or without anaemia have important consequences for human health. As long as iron can be mobilized from body iron stores to compensate for insufficient intake, the iron supply to tissues remains normal.\[^{101}\]

- It is characterized by microcytic hypochromic red cells with MCV <80fl and MCH <25pg. Morphological changes of red cells appear as the iron stores get depleted and iron is not
available in adequate amounts for haem synthesis. In IDA, marrow is not very hyper plastic and is one of the hypo proliferative anemias, since reticulocyte index is <2.5.[95]

Iron deficiency anaemia (IDA) in adults occurs typically due to a gradual decline in the iron content of the body due to a loss of hemoglobin and a depletion of iron stores. Early studies of patients with iron deficiency anaemia characterized by microcytic anaemia, low serum iron concentration, high total iron binding capacity (TIBC), and either an absence of stainable iron in the bone marrow or who had a subsequent response to therapeutic iron, showed that serum ferritin concentrations were less than 12–16 μg/l.[102]

Iron deficiency anaemia is probably the most common cause of anaemia and certainly represents a major cause of morbidity and mortality. The seriousness of this world-wide disorder has led to an accumulation of extensive information on the various roles iron fulfills in the body. Because iron is important for the formation of hemoglobin, myoglobin, and other iron containing substances as the cytochromes, peroxidase, catalase, and the dehydrogenases which are involved in the production of energy, it is essential to understand the means by which iron is utilized in the body.

It is particularly a disease of 1] children, 2] young women and 3] older people, but it occurs in people of all ages and all social strata. In children it is frequently due to dietary deficiency because milk has low iron content. In adults it is almost always the result of chronic blood loss or childbearing.[103]

Pioneering research over the last 50 years, much of it stemming from concepts developed and validated experimentally by Dr Clement Finch and his coworkers, led to the recognition that a negative iron balance resulting from an iron intake insufficient to match losses from the body despite compensatory changes in the rate of absorption and, to a more
limited extent, excretion could be divided into three stages based on the severity of the potential effect on physiological functions. The evaluation of functional impairment was related entirely to erythropoiesis for two reasons: the effects of changes in iron status on blood elements are readily evaluated, while the effect on the enzymes in other tissues necessitates obtaining biopsy samples. The red blood cell pool is the largest functional iron compartment in the body. Its requirements therefore have a dominant influence on studies of iron transport and storage.

Iron deficiency is the result of an imbalance between iron assimilation and iron loss. In normal circumstances it is assumed that physiological demands are within the scope of normal iron balance and iron deficiency can be expected to develop only when intake or losses are outside the physiologic range. The most apparent physiological consequences of iron deficiency are those that can be attributed to anaemia. However, it is more difficult to characterize other manifestations that are unrelated to anaemia, and that may be attributable to compromised metabolic functions in which iron serves either as a cofactor or as an integral part of a protein or enzyme molecule.\[104\]

These manifestations include an impairment of work capacity, disorders of cellular immunity and changes in intestinal function. In the experimental animal, some of the effects of iron deficiency on tissue iron compounds are difficult to relate to the severity of anaemia alone.\[105\]

According to severity iron deficiency is divided into three stages.\[106\] In the first stage, iron depletion [prelatent deficiency]. Iron depletion is the state in which storage iron is absent or nearly absent but the tissues that need iron are able to maintain normal physiological functions and hemoglobin synthesis is unaffected. This state can only be detected by
enhanced iron absorption and increased serum transferrin receptor level. In the second stage, iron deficient erythropoiesis (Latent deficiency), where serum ferritin levels are reduced, transport and functional compartments are affected, but complete blood counts are normal. There is a reduced incorporation of iron into Hb, but the Hb and erythrocyte - indices in blood are still within normal range. In the third and most severe stage, iron deficiency anemia, this is manifested by serial changes in the red cell indices and complete blood count, i.e., microcytosis, increased red cell distribution width (RDW) and lowering of hemoglobin level leading to progressively severe anaemia. Hb falls below normal, eventually accompanied by symptoms and clinical signs.

According to Heinrich and Bothwell, iron deficiency anaemia can be divided into four stages. The three stages were same as described above and the fourth stage or severe iron deficiency has not been completely characterized, it is believed to occur when the hemoglobin levels fall below 5 g/dl.
<table>
<thead>
<tr>
<th>Stage</th>
<th>Iron stores</th>
<th>S.Ferritin(µg/l)</th>
<th>TIBC(µg/dl)</th>
<th>%Tf saturation</th>
<th>Hb</th>
<th>Red cell morphology</th>
<th>S-Tf receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Normal Iron balance</td>
<td>Normal</td>
<td>Normal(20-300)</td>
<td>300-360</td>
<td>Normal</td>
<td>N</td>
<td>NN*</td>
</tr>
<tr>
<td>2</td>
<td>Negative iron balance</td>
<td>Decreased</td>
<td>&lt; 20</td>
<td>&gt;360</td>
<td>Normal</td>
<td>N</td>
<td>NN*</td>
</tr>
<tr>
<td>3</td>
<td>Iron deficient erythropoiesis</td>
<td>Nil</td>
<td>&lt;15</td>
<td>&gt;380</td>
<td>&lt;20%</td>
<td>↓</td>
<td>NN*</td>
</tr>
<tr>
<td>4</td>
<td>Iron deficiency anemia</td>
<td>Nil</td>
<td>&lt;12</td>
<td>&gt;400</td>
<td>&lt;16%</td>
<td>↓</td>
<td>Microcytic hypochromic</td>
</tr>
</tbody>
</table>

NN* = normocytic normochromic

As suggested by Heinrich, these extremely low hemoglobin levels would probably be accompanied by a depression in the levels of functional cell iron (i.e. mitochondrial heme protein) due to atrophic gastritis and intestinal malabsorption.\textsuperscript{[107]}

Laboratory assessment of iron status in man is generally restricted to hemoglobin, transferrin-bound iron, serum ferritin and bone marrow storage iron. Very often, these measurements do not necessarily reflect the status of the other iron-containing compounds such as myoglobin, cytochromes, catalase, peroxidase and metalloflavoproteins such as NADH-dehydrogenase, glycerol-3-phosphate dehydrogenase and succinate dehydrogenase.\textsuperscript{[105]}

These iron-containing enzymes, however, are immediately involved in a wide variety of metabolic functions such as the electron transport and the generation of metabolic energy in the mitochondria. It is no surprise that iron is directly involved in a multiplicity of well characterized role in the performance of muscle work. Indeed, poor work performance in man and in experimental animals is frequently observed in iron deficiency anaemia.\textsuperscript{[109, 110]}

Edgerton et al, and Finch et al authors also point out that iron deficiency reduces the production of the iron containing enzymes. It is likely that the fatigue from iron deficiency is the result of decreased mitochondrial oxidative phosphorylation since the presence of both heme and non-heme iron is essential for electron transport.\textsuperscript{[105]}

Blood donors have an increased risk of iron deficiency when blood is let regularly without replacement therapy.\textsuperscript{[12]} Iron absorption seems to be somewhat more efficient than in comparable non-donors;\textsuperscript{[80]} but blood donors generally have lower iron stores and an increased prevalence of depleted iron stores compared to non-donors.\textsuperscript{[111]}
Prevalence of iron deficiency

Iron deficiency has generally been defined as an absence of iron stores while iron deficiency anaemia as the same but with a hemoglobin concentration below a defined threshold.

It is the most common anemia prevalent in India. The prevalence of iron deficiency in children in India varies from 35 to 45% in various studies. Its frequency is higher in females, more so in pregnancy when the prevalence rate of IDA is as high as 45 to 60%. It is prevalent in both in rural and urban population; however, severity of anemia is more in lower socio-economic group. Even in the world, iron deficiency is the most widespread form of malnutrition affecting nearly two billion people.

3.7 Iron overload:

The thresholds suggested for a serum ferritin concentration during iron overload have varied widely. WHO concluded that thresholds of >200 μg/l for men and >150 μg/l for women were appropriate.\(^{[96]}\) Hemosiderosis, hemochromatosis, and some anemias are conditions associated with iron overload and iron storage diseases. Symptoms of iron overload will vary from person to person and tend to worsen over time. They are due to iron accumulation in the blood and tissues. Symptoms may include: Joint pain, Fatigue, weakness, Lack of energy, abdominal pain, Loss of sex drive, Heart problems.

Hemosiderosis:

- It is a term used to imply iron overload without associated tissue injury.
- It occurs locally in sites of bleeding or inflammation and may be widespread in persons who have been given large amounts of iron, either as iron medication or as blood transfusion.
Hemochromatosis:

♦ It is a condition in which the body accumulates excess amounts of iron; it is one of the most common genetic diseases in humans.

♦ The symptoms of hemochromatosis include the “classic triad” of bronzing of the skin, cirrhosis and diabetes. Other manifestations include cardiomyopathies and arrhythmias, endocrine deficiencies and possibly arthropathies.

♦ Secondary hemochromatosis is the consequence of the increased administration and absorption of iron. The administration of iron includes that given with the transfusions that are required in certain anemic patients and the ill advised and unfortunate administration of iron to anemic patients who are not actually iron deficient. The most common causes of secondary hemochromatosis are thalassemia major and acquired myelodysplastic states, but there are many other circumstances in which secondary iron overload occurs, including pyruvate kinase deficiency and congenital dyserythropoietic anemias.

♦ In contrast to secondary hemochromatosis, hereditary hemochromatosis results from inherited abnormalities of proteins that regulated iron hemostasis. Hereditary hemochromatosis is the most common hereditary form of hemochromatosis. It results from hereditary abnormalities of proteins that regulate iron hemostasis. In recent years, the genetic lesions responsible for many forms of the disease have been discovered. It is an adult onset disorder, formerly called primary hemochromatosis or idiopathic hemochromatosis, which is linked to the HLA loci on chromosome 6. The classical iron overload disorder in hemochromatosis is caused by mutations in the HFE gene.
This is an autosomal recessive iron-overload disease. Individuals with iron overload are treated by phlebotomy, and therefore they can be regarded as valuable blood donors.

**Clinical Significance:**

Iron deficiency and iron overload are the major disorders of iron metabolism. There are, in addition, many diseases in which abnormal distribution of iron may play a primary or secondary role. The latter include disorders, such as 1) hyperferritinemia with cataracts, 2) aceruloplasminemia, 3) GRACILE syndrome (growth retardation, aminoaciduria, cholestasis, iron overload, lactacidosis, and early death), 4) neuroferritinopathy, 5) atransferrinemia and possibly 6) neurodegenerative diseases, such as parkinsonism, Hallervorden-Spatz syndrome, and Alzheimer disease.

**Anemias with iron storage:**

- The most common cause of iron overload is thalassemia, particularly in the parts of the world where it is prevalent. Indeed, the cardiac complications of iron overload are among the most common causes of death in beta thalassemia major.

- Sideroblastic anemias are a group of iron loading disorders, many of which are of unknown cause. In a hereditary type of this disorder, there is deficiency of erythroid specific 5-aminolevulinic acid synthetase in RBC precursors because of mutations involving the X linked gene that encodes this enzyme.

- Iron storage is common in patients with congenital dyserythropoietic anemia and may be found in patients with red cell enzyme deficiencies, particularly pyruvate kinase deficiency.
3.8 Etiology of Iron deficiency anemia:

 Common causes of IDA include lack of iron in diet, increased iron demand and increased blood loss.

1] Dietary deficiency of iron:

This is the commonest cause of IDA in developing countries, more so in socio-economically weaker sections of the society. Iron deficiency is amplified by intake of wrong foods and food fads.

2] Impaired absorption of Iron:

Total/ Partial gastrectomy results in loss of gastric acidity, and hence impaired Fe absorption. In total gastrectomy, food rapidly passes through duodenum, and absorption of iron is further impaired. In Gluten sensitive enteropathy; Fe absorption is impaired and IDA may be the initial presentation of malabsorption syndrome.

3] Increased Blood Loss:

A] Gastrointestinal causes: peptic ulcer, hemorrhoids, aspirin ingestion, ulcerative colitis, NSAIDS intake [causes tiny gastric mucosal hemorrhage], Hiatus hernia, Amoebic colitis, Hook worm infestation [blood loss is about 0.2 ml/worm/day], Diverticulosis, Malignancies of stomach, colon.

B] Uterine Causes: Menorrhagia, Repeated pregnancies – increased requirements and excessive blood loss. In each pregnancy there is an average loss of 650 mg iron equivalent to about 1300 ml of blood.
C] Urinary tract causes: Hematuria due to renal, bladder, prostatic lesions, Chronic dialysis, PNH

D) Miscellaneous: pulmonary hemosiderosis

4] Increased physiological demands:

Infancy, Pregnancy, Adolescence, Lactation- loss of iron is about 0.5 to 1 mg/day.

3.9 Pathogenesis of IDA:

Three factors are responsible for pathogenesis of anemia:

1] Impaired Hb synthesis, 2] Impaired cellular proliferation and 3] Diminished iron containing proteins.

1] Impaired Hb synthesis:

As the S. Iron falls and transferrin saturation achieves a critical value of <15%, the iron is so depleted that Hb production is impaired resulting in microcytosis first followed by hypochromia of red cells.

2] Impaired cellular proliferation:

Cell proliferation is impaired in iron deficiency. This is reflected in the marrow erythroid hyperplasia which is not proportionate to the degree of anemia. When anemia is very severe, misshapen red cells are formed from ineffective erythropoiesis.
3] Diminished iron containing proteins:

Other iron proteins are also reduced in iron deficiency. Many of the enzymes like cytochromes C, cytochromes oxidase and myoglobin are reduced. Diminution of tissue enzymes is responsible for epithelial changes in IDA.

3.10 Clinical Features:

Clinical features of IDA are of both anemia and manifestation of the underlying disease. Early iron deficiency usually causes no physical effects at all. If a person is otherwise healthy, symptoms seldom appear before the hemoglobin in the blood drops below a certain level (10 g per deciliter). The most common symptoms of iron deficiency anemia include:

- Chronic fatigue/tiredness
- Weakness
- Dizziness
- Headaches

1] Onset:

Onset is insidious and most of the patients complain of fatigue, palpitations, breathlessness, ringing in the ears (tinnitus), drowsiness and irritability. As iron stores continue to be depleted, the anemia progresses in severity, chest pain, headaches, leg pains, shock, and even heart failure may occur. These symptoms worsen as the severity of anemia increases. Patients become symptomatic as Hb falls below 7 gm/dl.
2] **Growth and development:**

Growth in infancy is impaired. Iron deficient children are irritable and demonstrate lack of interest in surroundings. When iron deficiency occurs in the critical period of neurodevelopment (6-24 months), changes are irreversible and development of child is affected. Children may develop learning (cognitive) disabilities.

3] **Pica:**

Cravings for specific substances, such as licorice, chalk, dirt, or clay. This may be the cause rather than the effect of iron deficiency and is observed in 40% of the cases.

4] **Epithelial changes:**

Fingernails and toe-nails become thin, flattened (platynychia), brittle and finally spoon shaped (koilonychias). There is atrophy of papillae of tongue making the surface smooth, burning sensation in the tongue or a smooth tongue (bald tongue). There are fissures and ulcerations at the angles of mouth- angular stomatitis.

5] **Pharyngeal webs:**

Plummer Vinson syndrome is characterized by IDA with dysphagia in middle aged women. Dysphagia is because of pharyngeal webs formed by squamous epithelium with underlying loose connective tissue with chronic inflammation. These webs are at the junction of pharynx with esophagus. Dysphagia due to webs is mainly to solid foods.
6) **Chronic atrophic gastritis:**

Atrophic gastritis is similar to that observed in pernicious anemia. There is reduced gastric secretion with hypochlorhydria.

7) **Congestive Heart failure:**

Patients with severe anemia develop hyper dynamic circulation and may manifest with congestive heart failure.

8) **Neuropsychiatric manifestations in childhood:**

Anemia affects infants with long term impairment of mental and motor development. Such children have demonstrated lack of concentration, lower IQ scores and deficit cognitive functions. It has been proven that iron is essential for myelin synthesis and its deficiency affects mental development and these neurological changes are probably irreversible. Poor endurance and lack of physical fitness are associated with IDA which impairs overall growth and development in children.

9) **Immune function:**

There is impaired resistance to infections because of reduced cellular immunity. Following iron therapy such abnormalities improve in a few weeks, even before the hemoglobin levels improve.

3.11 **Tests for Assessing Iron Status:**

- Many different measurements have been advocated for the diagnosis of iron deficiency. Originally, emphasis was placed upon the RBC indices. Hypochromic anemia was
generally considered a synonym for iron deficiency in the first half of the twentieth century. Subsequently, the 1] staining of the marrow for iron, and the measurement of 2] serum iron, 3] iron binding capacity, 4] serum ferritin and 5] erythrocyte protoporphyrin became practical, and are used and studied extensively for their ability to diagnose iron deficiency. Circulating transferrin receptor and reticulocyte hemoglobin values also have been found to have diagnostic utility.

⚠️ Of all these tests, the marrow iron is probably the most reliable but the least practical for any but patients with complex diagnostic problems. However, even it can be misleading 1] when the sample size or observer skill is insufficient, 2] in patients who have been treated with iron by the parenteral route, 3] where stainable iron may be present in the face of deficiency and 4] in patients with myeloproliferative disease.

⚠️ Although most methods very readily identify severe, uncomplicated iron deficiency, the large number of tests that have been advocated for the diagnosis of iron deficiency is a reflection of the fact that none by itself is sufficient to detect mild iron deficiency or iron deficiency in a clinically complex setting. Various combinations of iron indicators have been used, but the thresholds for iron deficiency vary. For example: the threshold at which ferritin indicates iron deficiency may be set as high as 30 μg/l in some developing countries because of the presence of infectious diseases.

3.11.1 **Laboratory diagnosis of Iron deficiency:**

A] Screening:

- Hemoglobin
- Mean corpuscular volume[MCV]
- Percentage hypochromic erythrocytes
- Reticulocyte haemoglobin content
- Transferrin saturation
- Zinc protoporphyrin

B] Definitive:

- Storage iron- serum ferritin, bone marrow haemosiderin
- Tissue iron- soluble transferrin receptor.

3.11.2 The need for new tests to assess iron deficiency

- Iron deficiency and consequently iron deficient anemia is detected by measurements of Hb and serum ferritin. Several studies have confirmed that blood donation is associated with a decrease in serum ferritin.\textsuperscript{[14, 52, 82, 123]}

- Serum ferritin is a measure of the amount of iron in body stores if there is no concurrent infection: when the concentration is $\geq 15 \ \mu g/l$ iron stores are present; higher concentrations reflect the size of the iron store; when the concentration is low ($<12–15$ $\mu g/l$) then iron stores are depleted.

- When infection is present the concentration of ferritin may increase even if iron stores are low; this means that it can be difficult to interpret the concentration of ferritin in situations in which infectious diseases are common.

- Measurements of serum ferritin and transferrin receptor provide the best approach to measuring the iron status of populations. In places where infectious diseases are common, serum ferritin is not a useful indicator because inflammation leads to a rise in the concentration of serum ferritin as a result of the acute phase response to disease. If
Infectious diseases are seasonal, then the survey should be done in the season of lowest transmission.

- In general the concentration of transferrin receptor does not rise in response to inflammation so that, when combined with the concentration of serum ferritin, it is possible to distinguish between iron deficiency and inflammation.

- Studies are needed to determine the best procedures to process, transport and store biological samples in which transferrin receptor will be measured, and to establish internationally applicable thresholds to classify the iron status of populations. There is an urgent need for an international reference material with a certified concentration of transferrin receptor to standardize transferrin receptor assays.[96]

- By comparing serum ferritin and sTfR in frequent donors, Punnonen et al.[124] found that while 63% of the women had serum ferritin below the cut-off level for empty iron stores (defined as serum ferritin < 12 μg/L), only 17% had pathologically increased sTfR (defined as sTfR > 4 mg/L), indicating iron deficient erythropoiesis. In men the percentages were six and eight, respectively.

- The use of sTfR (soluble transferrin receptor) has enabled differentiation between iron deficient erythropoiesis and empty iron stores assessed by analysis of serum ferritin.

- The diagnostic sensitivity of detecting iron deficiency in anemic patients was found to be increased by using the Tfr-F index which is the ratio between the sTfR and the log of serum ferritin.[125]

- Increase of sTfR followed by a reduction of Concentration of hemoglobin in reticulocytes are early steps in the development of iron deficient erythropoiesis, while Hb-reduction and increased percent of hypochrome red cells appear later in the process.[126]
The lack of clear cut-off values with the lower reference limit for Hb varying between 11.5 and 12.3 g/dL\[^{103}\] and for serum ferritin between 12 and 27 μg/L,\[^{127}\] has stimulated the search for new markers to improve assessment of the iron status.\[^{128}\] Also, the use of serum ferritin is hampered by its nonspecific response as an inflammatory acute phase reactant.\[^{129}\] Thus, subjects with borderline values of Hb and serum ferritin would profit by tests which more precisely respond to the transition from iron replete to iron deficient erythropoiesis. In this regard, the potential of new tests such as the Hb-content of reticulocytes (CHr), the fraction of hypochrome red cells (percent hypochrome red cells) and the soluble transferrin receptor in serum (sTfR) are of interest.\[^{130}\]

3.11.3 Methodological and biological variability of measures of iron status

- Assays of blood for indicators of iron status vary greatly in both methodological and biological stability.

- Hemoglobin concentrations are stable and a simple and well-standardized method ensures a relatively low day to day variation in individuals. Automated cell counters can analyze at least 10,000 cells and thus reduce errors.

- The more complicated procedures involved in immunoassays lead to a greater variation in ferritin assays, with a coefficient of variation of at least 5%. This variation, coupled with some physiological variation, gives an overall coefficient of variation for serum ferritin for an individual over a period of weeks of the order of 15%. There is however little evidence of any significant diurnal variation in serum ferritin concentration.\[^{131}\] There is no information on seasonal factors influencing most of these analyses, although seasonal change in red cell parameters have been reported.\[^{132}\]
Starvation or even fasting for a short period can cause an increase in the serum ferritin concentration,\textsuperscript{[133]} while a vitamin C deficiency may reduce the ferritin concentration.\textsuperscript{[134]} Moderate exercise has little effect on serum ferritin concentration.\textsuperscript{[135]} although exhaustive exercise leads to an increase in serum ferritin concentration due to muscle damage and inflammatory reactions.\textsuperscript{[136, 137]}

3.11.4 Screening blood donors for iron deficiency

- A number of studies have shown that regular blood donation reduces storage iron levels.\textsuperscript{[14, 30]}
- The conventional screening test for anaemia, the “copper sulphate” test, lacks specificity so that donors may be deferred unnecessarily.
- Despite the availability of the serum ferritin assay for 30 years there has been little attention to the fundamental relationship between storage iron levels and the ability to donate blood. Screening blood donors by routinely assaying serum ferritin may make it possible to predict the development of iron deficiency anaemia \textsuperscript{[14]} and may identify donors with high iron stores who may give blood more frequently than is usually permitted. However the assay has low predictive power to identify donors who are homozygous for HFE gene C282Y.

Analytical Methodology:

Diagnosis of iron deficiency anemia is based on

1] Peripheral blood findings

2] Bone marrow morphology and iron stores and
3] Iron status

1] Peripheral Blood Changes:

Symptomatic IDA usually has Hb below 8 gm/dl.

- The mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) are all reduced in IDA.\(^{138}\) Mean corpuscular volume (MCV) is the average volume of red cells in a specimen. MCV is elevated or decreased in accordance with average red cell size; i.e., low MCV indicates microcytic (small average RBC size), normal MCV indicates normocytic (normal average RBC size), and high MCV indicates macrocytic (large average RBC size). The reference range \(^{139}\) may vary depending on the individual laboratory and patient's age. The reference ranges for mean corpuscular volume, mean corpuscular hemoglobin and mean corpuscular hemoglobin concentration are as follows:

  - MCV is 80-96 fL/red cell

  - MCH: 27-33 picograms (pg)/cell in adults

  - MCHC: 33-36 g/dL in adults

- Anisocytosis, ovalocytosis, frank microcytosis and hypochromia, occurrence of target cells and elongated (so called pencil) cells are commonly seen.

- Absolute eosinophilia is usually seen in hookworm infestation.

- Thrombocytosis is seen in 50-75% of patients.

- Platelet count may go up to twice the normal but return to normal level after therapy.

- The reticulocyte count is usually normal.
Red cell distribution width (RDW) is expressed either as coefficient of variation (CV) or as standard deviation (SD) of which the RDW- CV is the most useful.\cite{140}

- The normal value of RDW is 13.4±1.2% (mean ± 2 SD); and in IDA it is 16.3±1.8%.
- RDW in β or α-thalassemia minor is 13.6 ± 1.6.
- RDW is highly sensitive (90-100%) but low in specificity (50-70%) in detecting iron deficiency anemia.
- In β-thalassemia minor, microcytosis is more severe and is not proportional to the severity of anemia, but presence of basophilic stippling and more prominence of target cells should distinguish it from IDA. Quantification of Hb-A2 is most helpful as it is elevated in β-thalassemia minor.

**Method for Red Cell Volume and Hemoglobin content:**

MCV and MCHC are the red cell indices used to characterize the blood of patients with anemia. Laser light scattering is used to measure the hemoglobin content and concentration of individual red cells.\cite{141} This is an early indicator of iron deficiency. Its usefulness is limited in patients with MCV values of >100fL, but in others it compares favorably with other measurements of iron status.\cite{142, 143, 144, 145}

**2] Bone Marrow Changes:**

- Bone marrow exhibit both increase in cellularity and erythroid hyperplasia of mild to moderate degree.
- The erythroid cells are often smaller than normal (micro-normoblasts), have scanty cytoplasm with irregular ragged borders.
It is important to assess the iron stores directly from the bone marrow. Hemosiderin is seen as golden yellow refractile granules in unstained bone marrow.

But hemosiderin can be readily and reliably evaluated and graded after staining with Prussian blue.

Marrow hemosiderin stores are graded from 0+ and 6+. Normal marrow is graded 1+ to 3+ and in IDA, hemosiderin is absent.

3] Assessment of Iron Stores:

The status of various iron pools can be evaluated by indirect, non-invasive methods which are available in most laboratories.

3.11.5 Iron Tests

Definition

Iron tests are a group of blood tests that are done to evaluate the iron level in blood serum, the body's capacity to absorb iron, and the amount of iron actually stored in the body.

A number of methods are used to measure iron and related analytes. These include methods for 1] serum iron, 2] iron binding capacity, 3] transferrin saturation, and 4] serum ferritin.

1] Serum Iron Level Test:

The iron level test measures the amount of iron in the blood serum that is being carried by a protein (transferrin) in the blood plasma.
Principle:

Iron is released from transferrin by decreasing the pH of the serum; it is reduced from Fe\(^{3+}\) to Fe\(^{2+}\) and then complexed with a chromogen, such as bathophenanthroline or ferrozine. Such iron chromogen complexes have an extremely high absorbance at the appropriate wavelength, which is proportional to iron concentration.

Purpose

Serum or plasma iron tests are used for the following purposes:

- To help in the differential diagnosis of anemia.
- To assess the severity of anemia and monitor the treatment of patients with chronic anemia.
- To diagnose conditions of iron excess, including iron ingestion, thalassemia, hemosiderosis, and hemochromatosis. A serum iron test can be used without the others to evaluate cases of iron poisoning.

Preparation

Iron absorption and metabolism are influenced by several factors. These should be identified prior to testing via a medical history that includes the following:

- Prescription medications, oral contraceptives and multivitamins that affect iron levels, absorption, or storage. Vitamin B\(_{12}\) ingested within 48 hours may increase the results.
- Blood transfusion within the last four days
- Recent extreme stress or sleep deprivation
- Tests and treatments that use radioactive materials
- Recent eating habits
Precautions

- Blood collected for iron level or TIBC tests should be collected following a 12-hour fast. Fasting is not required for serum or plasma ferritin. Blood samples for iron tests should be taken early in the morning because serum iron levels vary during the day, being higher in the morning and lower at night. This precaution is especially important in evaluating the results of iron replacement therapy.

- Hemolysis must be avoided during collection of blood samples to prevent interference with test results from iron in the red blood cells.

2] Total Iron-Binding Capacity (TIBC) Test:

The TIBC test measures the amount of iron that the blood would carry if the transferrin were fully saturated. Since transferrin is produced by the liver, the TIBC can be used to monitor liver function and nutrition. TIBC is the maximum amount of iron that can be bound to transferrin. It is useful in distinguishing anemia (increased value) from chronic inflammatory disorders (normal value or decreased), however if there is marked hypoproteinemia, the TIBC in IDA may also be decreased.\textsuperscript{138}

Transferrin saturation by iron demonstrates a diurnal pattern, with a morning peak and an early evening trough. The unsaturated iron binding capacity (UIBC) can be measured by spectrophotometric/ colorimetric techniques or radioactive iron. TIBC is a mere summation of the UIBC and SI.
Principle:

The serum unsaturated iron-binding capacity and the total iron-binding capacity (TIBC) are determined by addition of sufficient Fe³⁺ to saturate iron binding sites on transferrin. The excess Fe³⁺ is removed, for example, by adsorption with light magnesium carbonate powder, and the assay for iron content is then repeated. From this second measurement, the TIBC is obtained.

Total iron-binding capacity (TIBC) indicates the maximum amount of iron needed to saturate plasma or serum transferrin (TRF), which is the primary iron-transport protein. Theoretically, 1 mol of TRF can bind 2 mol of iron at two high-affinity binding sites for ferric iron. Therefore, TIBC correlates well with TRF concentration, and the theoretical ratio of TIBC (in μmol/L) to TRF (in g/L) is 25.1:

\[
\text{TIBC (μmol/L)} = 25.1 \times \text{transferrin [TRF] (g/L)}
\]

Transferrin test

Transferrin is a beta globulin and glycoprotein with a short (7-day) half-life. Transferrin is a plasma iron-transport protein, also called siderophilin, formed in the liver that has a half-life of 7–10 days, transports dietary iron from the intestinal mucosa to iron-storage sites and hemoglobin-synthesis sites in the body (bone, muscle, erythrocytes, and lymphocytes). Transferrin is capable of binding more than its own weight in iron (that is, 1 g of transferrin can carry 1.43 g of iron). In normal clients, iron saturation of transferrin is between 20% and 45%. Transferrin enables iron storage by binding to transferrin receptors at the iron-storage sites. Because of its short half-life, values will decrease more quickly in protein malnutrition states than albumin will. Thus transferrin is sometimes
used to evaluate nutritional status. Transferrin also has growth-stimulating properties that are separate from its iron-transport properties. The transferrin test is a direct measurement of transferrin. Transferrin is most often measured by rate immunonephelometry. Some laboratories prefer this measurement to the TIBC. Transferrin saturation is calculated as follows: Transferrin saturation(%) = 100 x serum iron/ TIBC.

**Reference range:** for Adults 20% to 45%[ 200–400 mg/dL]

**Increased:**

Transferrin is *increased* in iron deficiency anemia, pregnancy, hormone replacement therapy (HRT).

**Decreased:**

Congenital absence of transferrin (hereditary atransferrinemia), hemolytic states, hepatic disease (acquired), inflammation (chronic), iron overload, low iron states combined with protein malnutrition, neoplasm, proteinuria (severe) and other protein-losing states, severe burns, chronic infections, renal disease and certain genetic disorders.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diurnal variation</td>
<td>Normal values in morning; low values in midafternoon; very low values near midnight</td>
</tr>
<tr>
<td>Menstrual cycle</td>
<td>Premenstrually, elevated values (SI increased by 10%-30%); at menstruation, low values (SI decreased by 10%-30%)</td>
</tr>
<tr>
<td>Pregnancy</td>
<td>May elevate SI owing to increased progesterone; may lower SI owing to iron deficiency</td>
</tr>
<tr>
<td>Ingestion of iron (including iron-fortified vitamins)</td>
<td>High values; may raise SI by +54 µmol/L (+500 µg/dL) and Tsat to 100%</td>
</tr>
<tr>
<td>Oral contraceptives (progestosterone-like)</td>
<td>High values; may raise SI to &gt;38 µmol/L (&gt;200 µg/dL) and Tsat to 75%; also elevates TIBC</td>
</tr>
<tr>
<td>Iron contamination of syringe, Vacutainer tube, or other glassware (phenomenon may be rare, sporadic, very difficult to prove)</td>
<td>High values; e.g., SI &gt;30 µmol/L (&gt;170 µg/dL); Tsat of 75%-100%</td>
</tr>
<tr>
<td>Iron dextran injection</td>
<td>Very high values; SI may be &gt;180 µmol/L (&gt;1000 µg/dL); Tsat 100%; probably from circulating iron dextran; effect may persist for several weeks</td>
</tr>
<tr>
<td>Hepatitis</td>
<td>Very high values; SI may be &gt;180 µmol/L (&gt;1000 µg/dL) owing to hyperferremia from hepatocyte injury</td>
</tr>
<tr>
<td>Acute inflammation (respiratory infection), abscess, immunization, myocardial infarction</td>
<td>Low or normal SI; normal or low Tsat</td>
</tr>
<tr>
<td>Chronic inflammation or malignancy</td>
<td>Low or normal SI; normal or low Tsat.</td>
</tr>
<tr>
<td>Iron deficiency</td>
<td>Low or normal SI; low or normal Tsat; increased TIBC</td>
</tr>
<tr>
<td>Iron overload (hemochromatosis)</td>
<td>High SI; high Tsat; normal or low TIBC</td>
</tr>
</tbody>
</table>

SI, Serum iron concentration; TIBC, total iron-binding capacity; Tsat, transferrin saturation.

3] Methods For The Determination Of Serum Ferritin.[149]

Principle:

Serum ferritin assay may be performed by any of several methods, including 1] immunoradiometric assay, 2] enzyme linked immunosorbent assay (ELISA) and 3] immunochemiluminescent, and 4] immunofluorometric methods. Reagents for this assay are available in kit form and in automated immunoassay instruments from several manufacturers.

※ The ferritin test measures the level of a protein in the blood that stores iron for later use by the body. The factors controlling plasma ferritin concentration are: 1) synthesis, 2) release from cells, 3) clearance from the plasma. There are no instances yet known in which a very high ferritin concentration is due to abnormalities in ferritin clearance, but abnormalities occur in both synthesis and release.

※ Samples may be stored at -20 °C or -80 °C for several years. Several rounds of freezing and thawing do not lead to changes in serum ferritin concentration, nevertheless freezing and thawing should be kept to a minimum. Ferritin is most often measured by double antibody sandwich immunoassay.

Clinical Significance:

☞ Ferritin is present in the blood in very low concentration and is relatively stable in healthy persons. Although it is an acute phase protein, under normal conditions it roughly reflects the body iron content.

☞ The plasma ferritin concentration declines very early in the development of iron deficiency, long before changes are observed in blood hemoglobin concentration, RBC size, or serum iron concentration. Thus measurement of serum ferritin concentration is used as a very sensitive indicator of iron deficiency that is uncomplicated by other concurrent disease. In
patients who have any of these chronic disorders together with iron deficiency, serum ferritin concentration is often normal.

Plasma ferritin concentration is also increased in patients with iron storage disease and is used to gauge the effectiveness of phlebotomy therapy. A ferritin level may also be ordered when iron overload is suspected.

**Normal reference range:**[150]

<table>
<thead>
<tr>
<th></th>
<th>S. Iron</th>
<th>TIBC</th>
<th>Ferritin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult male</td>
<td>50–160 μg/dL</td>
<td>250–400 μg/dL</td>
<td>20-250 μg/L</td>
</tr>
<tr>
<td>Adult female</td>
<td>40–150 μg/dL</td>
<td>250–400 μg/dL</td>
<td>20-200 μg/L</td>
</tr>
</tbody>
</table>
### Conditions affecting results of various Iron tests:

<table>
<thead>
<tr>
<th>Increased in</th>
<th>S. Iron</th>
<th>TIBC</th>
<th>Ferritin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thalassemia,</td>
<td>Iron deficiency anemia,</td>
<td>Infections,</td>
<td></td>
</tr>
<tr>
<td>hemochromatosis,</td>
<td>polycythemia vera,</td>
<td>inflammatory diseases,</td>
<td></td>
</tr>
<tr>
<td>liver disease - Acute hepatitis,</td>
<td>pregnancy,</td>
<td>liver disease,</td>
<td></td>
</tr>
<tr>
<td>aplastic anemia,</td>
<td>blood loss,</td>
<td>late-stage cancers,</td>
<td></td>
</tr>
<tr>
<td>blood transfusion,</td>
<td>severe hepatitis,</td>
<td>acute leukemia,</td>
<td></td>
</tr>
<tr>
<td>hemolytic anemia,</td>
<td>Hemolysis may cause</td>
<td>lymphomas,</td>
<td></td>
</tr>
<tr>
<td>lead poisoning,</td>
<td>falsely elevated iron values</td>
<td>thalassemia,</td>
<td></td>
</tr>
<tr>
<td>Kidney disease -nephritis,</td>
<td></td>
<td>Alcoholics,</td>
<td></td>
</tr>
<tr>
<td>pernicious anemia,</td>
<td></td>
<td>iron overload in certain types</td>
<td></td>
</tr>
<tr>
<td>polycythemia,</td>
<td></td>
<td>of anemia,</td>
<td></td>
</tr>
<tr>
<td>sideroblastic anemia,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>vitamin B₆ deficiency,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>acute leukemia,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>intramuscular iron injections</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drugs include alcohol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(wine, ethanol).</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Decreased in</th>
<th>S. Iron</th>
<th>TIBC</th>
<th>Ferritin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron deficiency anemia,</td>
<td>Cirrhosis,</td>
<td>chronic iron deficiency,</td>
<td></td>
</tr>
<tr>
<td>Acute or chronic blood loss,</td>
<td>dysmenorrhea,</td>
<td>severe protein depletion</td>
<td></td>
</tr>
<tr>
<td>blood donation</td>
<td>hemochromatosis,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>burns,</td>
<td>hemorrhage,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>carcinoma,</td>
<td>hepatitis,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>gastrectomy,</td>
<td>hypothyroidism,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>infection,</td>
<td>kwashiorkor,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>kwashiorkor[malabsorption]</td>
<td>microcytic anemia,</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
postoperative state, pregnancy, rheumatoid arthritis, schizophrenia (chronic), tetralogy of Fallot, uremia, heavy menstrual periods, thyroid deficiency

myocardial infarction, neoplasm, pernicious anemia, thalassemia, severe burns, anemia caused by infections chronic inflammatory diseases, malignancies kidney disease

### Medications and substances that affect the test result

<table>
<thead>
<tr>
<th>Increased level</th>
<th>S. Iron</th>
<th>TIBC</th>
<th>Ferritin</th>
</tr>
</thead>
<tbody>
<tr>
<td>chloramphenicol, estrogen preparations, dietary iron supplements, alcoholic beverages, methyldopa, Birth control pills.</td>
<td>birth control pills, Iron salts and fluorides.</td>
<td>dietary iron supplements oral contraceptives, theophylline, X-ray therapy.</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Decreased level</th>
<th>S. Iron</th>
<th>TIBC</th>
<th>Ferritin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin, cholestryramine, cortisone, ACTH, colchicine, desferoxamine methicillin, testosterone, metformin</td>
<td>ACTH, asparaginase, chloramphenicol, corticotropin, cortisone, dextran, steroids, and testosterone.</td>
<td>antithyroid therapy, High doses of ascorbic acid.</td>
<td></td>
</tr>
</tbody>
</table>
Ferritin levels are often evaluated in conjunction with other iron tests. A summary of the changes in iron tests seen in various diseases of iron status is shown in the table below.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Iron</th>
<th>TIBC/Transferrin</th>
<th>UIBC</th>
<th>% Transferrin Saturation</th>
<th>Ferritin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron Deficiency</td>
<td>Low</td>
<td>High</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
</tr>
<tr>
<td>Hemochromatosis</td>
<td>High</td>
<td>Low</td>
<td>Low</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Chronic Illness</td>
<td>Low</td>
<td>Low</td>
<td>Low/Normal</td>
<td>Low</td>
<td>Normal/High</td>
</tr>
<tr>
<td>Hemolytic Anemia</td>
<td>High</td>
<td>Normal/Low</td>
<td>Low/Normal</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Sideroblastic Anemia</td>
<td>Normal/High</td>
<td>Normal/Low</td>
<td>Low/Normal</td>
<td>High</td>
<td>High</td>
</tr>
<tr>
<td>Iron Poisoning</td>
<td>High</td>
<td>Normal</td>
<td>Low</td>
<td>High</td>
<td>Normal</td>
</tr>
</tbody>
</table>
3.11.6 Method for the Determination of the Reticulocyte Hemoglobin content (CHr).

- Reticulocytes hemoglobin concentration has been shown to be a reliable method for identifying iron deficiency and has been recommended for the evaluation of the adequacy of the iron supply in patients undergoing dialysis for renal failure who are receiving erythropoietin. [144, 152, 153, 154, 155]

- The measurement has been compared to the “gold standard” for iron deficiency, which is stainable bone marrow iron, and found to be reliable. While it promises to be a useful tool for evaluating iron supply even in clinically complex settings, it currently requires access to a specific brand of flow cytometer. It is therefore unlikely to be helpful in developing countries. Moreover reticulocyte hemoglobin concentrations seem to be unsuitable for evaluating iron status in alpha and beta thalassemia, including carrier states. It is decreased in iron deficiency and in alpha and beta thalassemia.

- Automated devices that measure the hemoglobin content of reticulocytes are commercially available, and this measurement has been found to be useful in differentiating the anemia of iron deficiency from that of chronic inflammation[156] although there is considerable overlap between these diagnostic categories. It is an early sensitive indicator of iron deficiency. Similarly, it can show a response to iron therapy approximately 4 days after it is begun which is much earlier than other hematologic measurements.
3.11.7 Free erythrocyte protoporphyrin (FEP)/ Free erythrocyte Zinc protoporphyrin (EZP)

- The conceptual basis for the measurement of protoporphyrin is a lack of iron in the bone marrow for incorporation into newly synthesized globin and the protein porphyrin as the haemoglobin molecule is reaching its final steps in synthesis. The last step in haemoglobin synthesis is the insertion of iron by the enzyme ferrochetalase. Instead of iron, trace amounts of zinc are incorporated into protoporphyrin instead.

- Zinc protoporphyrin can be detected in RBCs by fluorimetry and is a measure of the severity of iron deficiency. Recent development of micro-extraction procedures and hemato fluorometric methods has made this investigation easily available for diagnosis in contrast to the tedious time consuming macro-extraction methods.

- Elevation of FEP, mainly the EZP level is an early and sensitive sign of iron deficiency, even in the latent phase of iron deficiency. FEP levels in normal red cells range from 30-50 µg/dl. In uncomplicated iron deficiency anemia red cell FEP level range from 100- 1000µg/dl.

- EZP is also elevated in ACD, chronic lead poisoning and sideroblastic anemias. It is normal or decreased in primary disorders of globin synthesis like thalassemia minor( therefore useful to differentiate from iron deficiency).

- EZP in combination with serum ferritin levels have proved to be more specific and sensitive in detecting iron deficiency anemia.

- The normal ratio of iron to zinc in protoporphyrin is about 30 000:1, but a lack of iron available to ferrochetalase during the early stages of iron deficient erythropoiesis results in a measurable increase in the concentration of zinc protoporphyrin.
• Free erythrocyte protoporphyrin (FEP) is the compound left over after the zinc moiety has been removed using strong acids during the extraction and chemical measurement process. A variation of the chemical extraction method does not require this step and can provide direct measurements of ZPP and a very small amount of FEP. Typically FEP is less than 5% of the total. Thus FEP measurements, for all intents and purposes, are nearly identical to the ZPP measurements when the chemical extraction protocols are utilized. In turn, both FEP and ZPP should be interchangeable with the term “erythrocyte protoporphyrin” (EP).

• The concentration of EP is expressed either as μg/dl of whole blood or μg/dl of red blood cells. The conversion of values relies on the accurate measurement of the packed cell volume. A rise in the concentration of zinc protoporphyrin is one of the first indicators of insufficient iron in the bone marrow.\textsuperscript{157, 158} The rate at which the concentration of EP rises in blood samples is proportional to the relative deficit in iron and the amount of erythropoiesis that is occurring. In uncomplicated iron deficiency, the concentration of EP is reported to increase within 1–2 weeks of a lack of iron in the bone marrow.\textsuperscript{159, 160} After iron therapy begins, more than a month is required to re-establish a normal concentration of EP, and well after the restoration of normal plasma iron kinetics.
Biochemical indicators of iron status:

Appendix 1. The main biochemical indicators of iron status.\textsuperscript{[96]}

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Sample</th>
<th>Commonly used methods, a</th>
<th>Units</th>
<th>Indicator of</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone marrow iron (haemosiderin)</td>
<td>Bone marrow aspirate</td>
<td>Microscopical examination of stained marrow cells</td>
<td>Semi-quantitative grading</td>
<td>Depleted or absent body iron stores</td>
<td>Indicates body iron stores and correlates well with other indicators</td>
<td>Invasive and traumatic to collect sample</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>Whole blood</td>
<td>Cyanmethaemoglobin using colorimeter or spectrophotometer or azide-methhaemoglobin using e.g. HemoCue®</td>
<td>g/l</td>
<td>Anaemia</td>
<td>Simple to measure; important functional and public health consequences</td>
<td>Anaemia occurs without iron deficiency; adjustment of thresholds needed for age, sex, pregnancy, altitude, smoking and some ethnic groups</td>
</tr>
<tr>
<td>Haematocrit or packed cell volume (PCV)</td>
<td>Whole blood</td>
<td>Centrifugation of whole blood in capillary tube or value derived from automated flow cytometry</td>
<td>Decimal ratio or %</td>
<td>Proportional volume of RBCs in whole blood</td>
<td>Simple to measure</td>
<td>Same as haemoglobin; depends on factors affecting centrifuge e.g. stable power supply</td>
</tr>
<tr>
<td>Test Description</td>
<td>Type of Blood</td>
<td>Methodology</td>
<td>Unit</td>
<td>Result Interpretation</td>
<td>Reliability/Specificity</td>
<td></td>
</tr>
<tr>
<td>-------------------------------------------------------</td>
<td>---------------</td>
<td>-------------------------------------------------</td>
<td>------</td>
<td>--------------------------------------------------------------------------------------</td>
<td>--------------------------</td>
<td></td>
</tr>
<tr>
<td>Mean cell volume (MCV)</td>
<td>Whole</td>
<td>Calculated from haematocrit and RBC count using haemocyto-meter, or value derived from automated flow cytometry</td>
<td>fl</td>
<td>Average RBC size: low is microcytic; high is macrocytic</td>
<td>Requires expensive machine to be reliable; low in thalassemia and inflammation</td>
<td></td>
</tr>
<tr>
<td>Mean cell haemoglobin concentration (MCH)</td>
<td>Whole</td>
<td>Haemoglobin concentration and RBC count using haemocytometer, or value derived from automated flow cytometry</td>
<td>pg</td>
<td>Haemoglobin in an average RBC; if low, hypochromic; if normal, normochromic</td>
<td>As for MCV Requires expensive machine to be reliable; slow to respond to iron deficiency</td>
<td></td>
</tr>
<tr>
<td>Red cell distribution width (RDW)</td>
<td>Whole</td>
<td>Automated flow cytometry calculates RDW = SD of MCV MCV SD= Standard deviation</td>
<td>%</td>
<td>Abnormal range in size of RBCs &lt;11.5% or &gt;14.5%</td>
<td>Size distribution of RBCs can be characteristic of type of anaemia</td>
<td></td>
</tr>
<tr>
<td>Reticulocyte haemoglobin concentration</td>
<td>Whole</td>
<td>Automated flow cytometry</td>
<td>g/l</td>
<td>Concentration of reticulocytes represents new RBCs 18-36 hours old,</td>
<td>Requires expensive machine to be</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum or plasma iron</td>
<td>Colorimetry</td>
<td>μg/dl</td>
<td>Reliable measures of iron supply to the bone marrow and other tissues; varies diurnally and after meals; sample easily contaminated with iron from outside sources; low in chronic disease.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>-------------</td>
<td>-------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| Serum or plasma iron (not using EDTA) | Iron bound to transferrin in blood | μmol/l | Iron status in voluntary blood donors

Serum or plasma iron | Serum or plasma iron (not using EDTA) | Colorimetry | μg/dl | Reliable measures of iron supply to the bone marrow and other tissues; varies diurnally and after meals; sample easily contaminated with iron from outside sources; low in chronic disease. |

### Erythrocyte protoporphyrin

| Whole blood or dried blood spots | Usually estimated from ZPP (below); expressed as ratio to haemoglobin concentration | μg/dl | Restricted supply of iron to developing RBCs | Useful in young children; whole blood or dried spots can be assayed |

Erythrocyte protoporphyrin | Whole blood or dried blood spots | Fluorescence spectrophotometry or portable Aviv® haematofluorimeter | μmol/mol of haemoglobin | Lack of iron to developing RBCs | Useful in young children; whole blood or dried spots can be assayed |

### Zinc protoporphyrin (ZPP)

| Whole blood or dried blood spots | Fluorescence spectrophotometry or portable Aviv® haematofluorimeter | μmol/mol of haemoglobin | Lack of iron to developing RBCs | Useful in young children; whole blood or dried spots can be assayed |

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Increased in iron deficiency, inflammatory disorders, exposure to lead
<table>
<thead>
<tr>
<th>Measurement</th>
<th>Sample</th>
<th>Commonly used methods, a</th>
<th>Units</th>
<th>Indicator of</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ferritin</td>
<td>Serum or plasma</td>
<td>Immunoassay e.g. enzyme-linked immunosorbent assay (ELISA) or immunoturbidometry</td>
<td>μg/l</td>
<td>Size of iron stores</td>
<td>Reflects iron status</td>
<td>ferritin is an acute phase protein so concentration is increased in inflammatory disease and sub-clinical infection</td>
</tr>
<tr>
<td>Total iron binding capacity</td>
<td>Serum or plasma</td>
<td>Colorimetric assay of amount of iron that can be bound to unsaturated transferrin in vitro; determination from transferrin concentration measured immunologically</td>
<td>μg/dl</td>
<td>μmol/l</td>
<td>Total capacity of circulating transferrin bound to iron</td>
<td>Increased in iron deficiency, low in inflammatory disorders</td>
</tr>
<tr>
<td>Transferrin saturation</td>
<td>Serum or plasma</td>
<td>Calculated from: Serum iron TIBC</td>
<td>%</td>
<td>Saturation of &lt;15% with high TIBC indicates iron deficiency</td>
<td>Proportion of transferrin bound to iron</td>
<td>Same as for serum iron</td>
</tr>
<tr>
<td>Transferrin receptor</td>
<td>Serum or plasma</td>
<td>Immunoassay e.g. ELISA or immunoturbidometry</td>
<td>μg/l</td>
<td>Reflects balance between</td>
<td>Semi-quantitative measure of the rate of erythropoiesis</td>
<td>Affected by the rate of erythropoiesis</td>
</tr>
</tbody>
</table>
### Iron status in voluntary blood donors

<table>
<thead>
<tr>
<th>Body iron stores</th>
<th>Serum or plasma</th>
<th>Ratio of transferrin receptor to ferritin – [log (TfR/ferritin ratio) – 2.8229] b 0.1207</th>
<th>mg/kg</th>
<th>Measure of body iron status including iron deficits, status of storage iron and iron overload</th>
<th>Measure of full range of iron status, validated by phlebotomy studies in adult volunteers</th>
<th>Same limitations as component parts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hepcidin</td>
<td>Serum or plasma, urine</td>
<td>Immunoassay for pro-hepcidin e.g. ELISA</td>
<td>ng/ml</td>
<td>Regulator of iron absorption from gut</td>
<td>Production diminished when iron reserves depleted</td>
<td>Assay methods and interpretation of results is under development</td>
</tr>
</tbody>
</table>

RBC: red blood cell.

a The mention of specific companies or of certain manufacturers’ products does not imply that they are endorsed or recommended by the World Health Organization or the Centers for Disease Control and Prevention in preference to others of a similar nature that are not mentioned. b Cook, Flowers, Skikne.\[161\]