Chapter 2

Review of Literature

A part of this chapter has been published as:
2.1 Source of iron:

Various sources of iron are available in nature, some sources are either rich or moderate and others are poor. Naturally iron content is high in non-vegetarian sources where as vegetarian sources are poor in this aspect. Iron content of different food varies and the same food may also show some variation depending upon its origin and preparation (Walter, 1974). Certain types of animal meat, legumes like soya beans, peas, bengal gram etc. are rich sources where as fruits and vegetables are moderate sources of iron. In typical Indian diet the major portion of iron is coming from cereals because of the bulk quantity taken, although they contain the iron only in moderate amounts. Cooking in iron vessels can improve the iron content in diet. Routinely consumed food items in India like jaggery is a good source of iron where as milk is a poor source of iron.

2.1.1 Iron rich food stuffs:

The following Table shows the approximate content of iron in various food stuffs (Gopalan, 1995).

**Table 2.1: Iron content in various food stuffs**

<table>
<thead>
<tr>
<th>Food stuff</th>
<th>Iron content (mg/100 g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cereals/ Grains</strong></td>
<td></td>
</tr>
<tr>
<td>Bajra</td>
<td>8.0</td>
</tr>
<tr>
<td>Jowar</td>
<td>4.1</td>
</tr>
<tr>
<td>Ragi</td>
<td>3.9</td>
</tr>
<tr>
<td>Wheat</td>
<td>4.9</td>
</tr>
<tr>
<td>Maize</td>
<td>2.3</td>
</tr>
<tr>
<td><strong>Pulses and Legumes</strong></td>
<td></td>
</tr>
<tr>
<td>Bengal gram</td>
<td>9.5</td>
</tr>
<tr>
<td>Rajmah</td>
<td>5.1</td>
</tr>
</tbody>
</table>
2.1.2 Requirement:

Iron requirement in human beings varies greatly and depends upon age, gender and physiological state. First two years after birth, the requirement of iron is high due to rapid growth. In the females of reproductive age the iron requirement is increased due to extra iron loss because of menstruation, gestation and lactation (Harper et al, 1977).
Table 2.2: Age and gender wise basal iron requirement and dietary iron requirement (Reproduced from Gopalan et al, 1995)

<table>
<thead>
<tr>
<th>Group</th>
<th>Basal iron requirement (mg/day)</th>
<th>Dietary iron requirement (mg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>0.840</td>
<td>28.0</td>
</tr>
<tr>
<td>Women</td>
<td>1.500</td>
<td>30.0</td>
</tr>
<tr>
<td>Children</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-3 years</td>
<td>0.354</td>
<td>11.5</td>
</tr>
<tr>
<td>4-6 years</td>
<td>0.551</td>
<td>18.4</td>
</tr>
<tr>
<td>7-9 years</td>
<td>0.780</td>
<td>26.0</td>
</tr>
<tr>
<td>Adolescents (10-12 yrs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>boys</td>
<td>1.027</td>
<td>34.2</td>
</tr>
<tr>
<td>girls</td>
<td>0.945</td>
<td>18.9</td>
</tr>
<tr>
<td>(13-15 yrs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>boys</td>
<td>1.243</td>
<td>41.1</td>
</tr>
<tr>
<td>girls</td>
<td>1.401</td>
<td>28.0</td>
</tr>
<tr>
<td>(16-18 yrs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>boys</td>
<td>1.485</td>
<td>49.5</td>
</tr>
<tr>
<td>girls</td>
<td>1.497</td>
<td>29.9</td>
</tr>
</tbody>
</table>

2.1.3 RDA:

The recommended Daily allowance of iron for an Indian adult is 20 mg (ICMR 1990), out of which about 1-2 mg is absorbed. Pregnant women need 40 mg/day (ICMR 1990).
2.2. Metabolism of iron:

![Diagram of iron metabolism in human body]

**Figure 2.1: Overview of iron metabolism in human body**

### 2.2.1 Absorption of iron from GI tract:

Iron is mainly absorbed from the duodenum and jejunum. Iron metabolism is unique because homeostasis is maintained at the level of absorption and not by excretion; no other nutrient is regulated in this manner. Garnick proposed the ‘mucosal block’ theory for iron absorption. According to which when the body iron store is depleted the absorption is enhanced and in the condition of sufficient iron storage absorption is decreased.

There are several factors which influence the absorption of iron. Iron in ferrous form i.e. $\text{Fe}^{2+}$ only can be absorbed and not ferric i.e. $\text{Fe}^{3+}$. Ferric iron present in food firstly get reduced with gastric HCl. Ascorbic acid, cysteine and –SH group of proteins, thus these factors will favor iron absorption. Phytic acid of cereals and oxalic acid of leafy vegetables decrease iron absorption by forming insoluble salts (Vasudevan and Sreekumari, 2001). At high alkaline pH the ingested iron is precipitated which depresses the iron absorption (Kaplan and Pesce, 1989). Calcium, copper, zinc, lead and phosphorus also inhibit iron absorption. Mal-absorption syndrome and achlorhydria etc. affect the iron absorption. In normal people only about 10% of ingested dietary iron is absorbed (Satyanarayana and Chakrapani, 2009; Vasudevan and Sreekumaris, 2001).
Table 2.3: Factors influencing dietary iron absorption

<table>
<thead>
<tr>
<th>Heme iron absorption</th>
<th>Non-heme iron absorption</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Factors determining iron status of subject:</strong></td>
<td><strong>Factors determining iron status of subject:</strong></td>
</tr>
<tr>
<td>Amount of dietary heme iron, especially from meat</td>
<td>Amount of potentially available non-heme iron</td>
</tr>
<tr>
<td>Content of calcium in meal (e.g. from milk, cheese)</td>
<td><strong>Balance between the following enhancing and inhibiting factors:</strong></td>
</tr>
<tr>
<td>Food preparation (i.e. time, temperature)</td>
<td></td>
</tr>
</tbody>
</table>

**Enhancing factors**

- Ascorbic acid (e.g. certain fruit juices, fruits, potatoes, and certain vegetables)
- Meat, fish and other seafood
- Fermented vegetables (e.g. sauerkraut), fermented soy sauces, etc.

**Inhibiting factors**

- Phytate and other lower inositol phosphates (e.g. bran products, bread made from high-extraction flour, breakfast cereals, oats, rice :especially unpolished rice, pasta products, cocoa, nuts, soya beans, and peas)
- Iron-binding phenolic compounds (e.g. tea, coffee, cocoa, certain spices, certain vegetables, and most red wines)
- Calcium (e.g. from milk, cheese)

Ingested iron is classified as non-heme iron and heme iron.
Non-heme iron derived from plants is mainly composed of inorganic Fe$^{3+}$. It gets absorbed into enterocytes through the divalent metal transporter 1 (DMT1) after reduction of Fe$^{3+}$ to Fe$^{2+}$ (McKie et al, 2002; Trinder et al, 2002).

In contrast, heme-iron derived from meat is absorbed through a heme carrier protein into enterocytes, where it is degraded by hemeoxygenase-1 (HO-1). Iron within enterocytes is then transferred from the luminal to the vascular site of the cell and released into the circulation via the metal transporter, ferroportin in the form of Fe$^{2+}$. Excreted Fe$^{2+}$ is thereafter oxidized to Fe$^{3+}$ by hephaestin, a homolog of ceruloplasmin, and the resulting ferric iron is bound to serum Tf (Sargent et al, 2005). Tf is a non heme iron binding glycoprotein with molecular weight of 70,000 D and binds with 2 Fe$^{3+}$ atoms. Synergistically in presence of bicarbonate (HCO$_3^-$) it is a true carrier of iron (Chatergee and Shinde, 2005). The normal level of plasma Tf is 250 mg/dL which can bind with 400 µg/dL iron which is called as total iron binding capacity (TIBC) of plasma (Vasudevan and Sreekumaris, 2001). In normal subjects the level of serum iron is about 120 µg/dL, which saturate about one third of TIBC.

Figure 2.2: Absorption of iron from GI tract
2.2.2 Uptake of iron in liver cells:

Concerning the uptake of Fe2-Tf, there are three pathways involved: two are dependent on and one is independent of transferrin receptor (TfR) recycling. Transferrin receptor 1 (TfR1) is a classical functional receptor, expressed highly in erythroblasts, but less so in hepatocytes. When serum Fe2-Tf binds to TfR1, Fe2-Tf is internalized by endocytosis. Internalized Fe2Tf-TfR1 complexes within the endosome release iron when endosomal pH is acidified. The resulting apotransferrin-TfR1 complex is then recycled back to the cell surface for reutilization. Transferrin receptor 2 (TfR2), a new homolog of TfR1, is ubiquitously expressed on hepatocyte surfaces and possesses a similar mechanism of recycling. In hepatocytes, there is another Fe2-Tf uptake mechanism that is independent of TfR recycling, which is also considered to be important (Ikuta et al, 2004). In iron-overloaded conditions, NTBI appears in the circulation and is taken up through two molecules such as DMT1 and ZIP14 on hepatocytes (Liuzzi et al, 2006).

Figure 2.3: Uptake of iron in hepatocyte

2.2.3 Bone marrow iron metabolism and erythropoiesis:

Bone marrow erythroblasts require large amount of iron for hemoglobin synthesis. TfR1 is strongly expressed in erythroblasts and functions as the uptake system of extracellular Fe2-Tf. Within erythroblasts, iron is transferred to mitochondria and is incorporated into the center of the heme ring.
2.2.4 Iron distribution:

![Diagram of iron distribution in the human body](image)

**Figure 2.4: Iron distribution in human body**

The human body contains approximately 3–5 g of iron (45–55 mg/kg of body weight in adult women and men, respectively), distributed as illustrated in Figure 2.4. The majority of body iron (~60–70%) is utilized within hemoglobin in circulating red blood cells (Andrews, 1999; Ponka, 1997). Other iron-rich organs are the liver and muscles. Approximately 20–30% of body iron is stored in hepatocytes and in reticuloendothelial macrophages, to a large extent within ferritin and its degradation product hemosiderin. The remaining body iron is primarily localized in myoglobin, cytochromes and iron-containing enzymes. A healthy individual absorbs daily 1–2 mg of iron from the diet, which compensates nonspecific iron losses by cell desquamation in the skin and the intestine. In addition, menstruating women physiologically lose iron from the blood. Erythropoiesis requires approximately 30 mg iron/day, which is mainly provided by the recycling of iron via reticuloendothelial macrophages. These ingest senescent red blood cells and release iron to circulating transferrin. The pool of transferrin-bound iron (~3 mg) is very dynamic and undergoes >10 times daily recycling.
2.2.5 Conservation of iron:

Body tries to safeguard iron and to do so after the lysis of RBC, its iron gets conserved. The average life span of circulating RBCs is approximately 120 days, indicating that 20 mg of iron derived from 20 ml of RBCs are processed by RES/macrophages on a daily basis. After hemolysis heme is derived from phagocytized RBCs and free iron is released within macrophages. Intra-cellular iron is released into the circulation via ferroportin a trans-membrane protein and taken up by haptoglobulin. Haptoglobulin is an α2-globulin produced from the liver. Furthermore, when globin part is removed from Hb the released heme gets bind to hemopexin, a β-globulin and prevent its excretion through urine (Vasudevan and Sreekumaris, 2001). After release of porphyrin, iron gets free and binds with Tf. The Tf bound iron can get incorporated for production of various biomolecules or get settled for storage purpose in the form of ferritin which can get reutilized when ever required.

![Figure 2.5: Conservation of iron](image-url)
2.2.6 Storage:
Iron is mainly stored in liver, spleen and bone marrow. The storage forms are ferritin and hemosiderin. The apo-ferritin has the molecular weight of 5,50,000 daltons. An apo-ferritin can take up to 4000 iron atoms and become ferritin (Vasudevan and Sreekumaris, 2001). Ferritin is a vasodepressor and does not normally appear in plasma, but in iron toxicity ferritin does appear in plasma and so being used as a marker of total iron stores in the body (Satyanarayana and Chakrapani, 2009).
Hemosiderin is suggested to be derived from ferritin with partially stripped shell (Chatergee and Shinde, 2005). It is usually seen in states of iron overload or when iron is in excess, when the synthesis of apoferritin and its uptake of iron are maximum.

2.2.7 Excretion:
Green in 1968 suggested that iron loss is obligatory and does not appear to vary between individuals or with environment (Walter, 1974). Iron is a one way element, which is efficiently utilized and reutilized by the body. Iron loss in normal adult human from the body is minimal (< 1 mg/ day), which may occur through bile, urine, feces, sweat and by shedding of cells, hair loss etc (Satyanarayana and Chakrapani, 2009).

2.2.8 Homeostasis:
2.2.8.1 Maintenance of iron homeostasis:
Balancing the iron levels in the body has to be meticulously achieved to provide iron when it is needed without causing toxicity associated excessive accumulation of iron. This is a co-ordinated act regulated by signals from cells and tissues in response to various physiological inputs. Mammals do not possess any physiological pathway for iron excretion. Thus, body iron homeostasis is regulated at the level of iron absorption. Mis-regulated iron absorption leads to iron deficiency or overload. It is believed that three regulatory cues contribute to the maintenance of homeostasis (Andrews, 1999; Finch, 1994). The first one is called “dietary regulator”. It has long been known that after the ingestion of a dietary iron bolus, absorptive enterocytes are resistant in acquiring additional iron for several days (Stewart et al,
1950). This phenomenon, also described as “mucosal block”, probably results from the accumulation of intracellular iron. High intracellular iron may suppress the expression of DMT1 in an IRE–IRP (Iron Responsive Element-Iron Regulatory Protein) mediated manner (Frazer et al, 2003).

A second signal, called “stores regulator”, controls iron uptake in response to body iron stores. It is well established that in iron-deficient conditions, iron absorption is significantly stimulated by two to three fold. When iron stores are replenished, iron absorption returns to basal levels. It has been hypothesized that this type of regulation requires the programming of precursor crypt cells (Roy and Enns, 2000) in the duodenal epithelium after sensing plasma transferrin saturation.

A third signal, called “erythropoietic regulator” modulates iron absorption in response to erythropoiesis. Because most of the body’s iron is utilized by the bone marrow for hemoglobinization of red blood cells, it is not surprising that this signal has a dominant function in the control of iron homeostasis. In other words, the erythropoietic regulator has a greater capacity to increase iron absorption compared to the stores regulator (Andrews, 1999; Finch, 1994). Moreover, it increases iron absorption independently of body iron stores.

A reason for the pathological iron accumulation encountered in disorders with ineffective erythropoiesis (such as thalassemia syndromes, congenital dyserythropoetic anemias, sideroblastic anemias, or a transferrinemia) is the increased iron absorption under conditions where iron stores are replete. The nature of the erythropoietic regulator has remained for long time mysterious. A dual function compatible with both a “stores” and “erythropoietic” regulator has been proposed for hepcidin, a small circulating peptide derived from the liver (Ganz, 2003).

2.2.8.2 Iron regulatory proteins:

It has been postulated for a long time that a soluble factor acts to synchronize body iron metabolism among different organs. Recently, a basic peptide called hepcidin, an antimicrobial purified from urine, was found to have this role (Park et al, 2001). Hepcidin is considered to be a negative regulator that inhibits both intestinal iron absorption and reticulo-endothelial iron release. It is mainly synthesized in the liver and its production is enhanced during iron overload and inflammation (Inamura
In some patients with genetic hemochromatosis, an abnormality of hepcidin gene has been reported. In these patients, hepcidin production was suppressed and iron absorption increased (Ganz, 2004). Furthermore, hepcidin expression is also down-regulated even in patients without a genetic abnormality of hepcidin. These reports strongly suggest that hepcidin plays an important role in tissue iron deposition in many iron-overloaded conditions including HFE hemochromatosis (Bridle et al, 2003). Currently, several additional molecules such as Tfr2 and hemojuvelin (HJV) are also known to be involved in its regulation (Pietrangelo, 2007; Pietrangelo, 2010). Furthermore, it is becoming clear that there is a role for hepcidin even in secondary iron overload. In a mouse model of β-thalassemia, representing ineffective erythropoiesis, there is an up-regulation of hepcidin and a down-regulation of ferroportin, explaining how hepcidin also contributes to the formation of secondary hemochromatosis associated with ineffective erythropoiesis (Gardenghi et al, 2007; Andrews, 2000).

Figure 2.6: Iron regulation by hepcidin
2.2.9 Forms of iron in plasma and tissue:

Iron exists in the body in different forms. Both in the circulation as well as in various organs it is present in functional or active forms and also as resting inactive forms.

2.2.9.1 Iron in plasma: Tf-bound iron and non-Tf-bound iron (NTBI):

It is well known that plasma Tf is capable of binding and transporting ferric iron to cells via TfRs. The binding capacity of Tf to inorganic iron is very strong, and this characteristic behavior prevents iron from existing in its free form under normal physiological conditions. The Tf saturation in normal physiological conditions is up to 35%, which suggests that there is a sufficient capacity to prevent the release of free toxic iron into the circulation (Cazzola et al, 1985). However, when the iron binding capacity of Tf is saturated in the iron overloaded state, an additional iron form NTBI, appears in the circulation. This form is biologically more toxic than Tf-bound iron. Unlike Tf-bound iron, the cellular uptake of NTBI is not dependent on the TfR, and therefore the resulting iron is diffusely distributed throughout the organs, independent of the presence of the TfR (Cabantchik et al, 2005; Breuer et al, 2000). Unlike serum iron, TIBC and percent-Tf-saturation measurements, the inter-institutional difference of NTBI and LPI measurements are too great and these parameters have not yet been standardized in an acceptable form.

2.2.9.2 Iron in tissue: tissue ferritin and labile iron pool (LIP):

Within cells, iron is stored in the proteins ferritin or hemosiderin.

Each molecule of ferritin can store up to 4,500 Fe$^{3+}$ within the protein shell (Koorts and Viljoen, 2007), and release great quantities of iron when the body is deficient of iron. Most of the ferritin is present in liver, spleen and bone marrow, and a trace amount is found in the blood as serum ferritin.

In addition to ferritin iron, LIP is present within cells in order to facilitate biological actions involving iron atoms and can become cytotoxic or carcinogenic when the concentration exceeds the protective capacity of ferritin. LIP is defined as a low-molecular-weight pool of weakly chelated iron that rapidly passes through the cell. It likely consists of both forms of ionic iron (Fe$^{2+}$ and Fe$^{3+}$) associated with a variety of ligands with low affinity for iron ions. LIP represents only a minor fraction
of the total cellular iron (3–5%) which is responsible for oxidation–reduction reactions and the Fenton reaction. Iron toxicity is developed through the production of ROS (Kruszewski 2003). It has been proposed that iron is complexed by diverse low-molecular weight chelators, such as citrate, phosphate, carbohydrates, carboxylates, nucleotides, nucleosides, polypeptides, phospholipids and other organic ions; however, the actual nature of the intracellular ligands participating in LIP formation remains obscure (Kakhlon and Cabantchik, 2002; Kruszewski, 2003; Kruszewski, 2004).

2.3 Functions of iron in biological system:

The functional role of iron can be grossly divided as heme iron and non heme iron.

2.3.1 Heme iron:

A significant fraction of cellular iron is associated with proteins in the form of heme, common prosthetic group composed of protoporphyrin IX and a Fe^{2+} ion. The tetrapyrrol porphyrin ring is synthesized from the universal precursor 5-aminolevulinic acid (ALA) by a series of reactions in the cytosol and in mitochondria. In metazoans, ALA is generated by the condensation of glycine and succinyl-CoA, which is catalyzed by the 5-aminolevulinic acid synthase (ALAS) (Ryter and Tyrrell, 2000). The insertion of Fe^{2+} into protoporphyrin IX, catalyzed by ferrochelatase in the mitochondria, defines the terminal step of the heme biosynthetic pathway. Heme is then exported to the cytosol for incorporation into hemoproteins. The most abundant mammalian hemoproteins, hemoglobin and myoglobin, serve as oxygen carriers in the erythroid tissue and in the muscle, respectively. Oxygen binding is mediated by the heme moieties. Other hemoproteins include various cytochromes and enzymes, such as oxygenases, peroxidases, nitric oxide (NO) synthases. The heme moiety may also function in electron transfer reactions (e.g. in cytochromes a, b, and c), as a substrate activator (e.g. in cytochrome oxidase, cytochrome P450, catalase) or as an NO sensor (in guanylate cyclase).

Heme degradation is catalyzed by the microsomal heme oxygenase 1 (HO-1) and its homologues HO-2 and HO-3 (Ryter and Tyrrell, 2000). The liberated Fe^{2+} is reutilized. The reaction also generates carbon monoxide (CO), which may be involved
in signaling pathways and biliverdin, which is further enzymatically converted to the antioxidant bilirubin.

2.3.2 Non-heme iron:

The most prevalent forms of non-heme iron in metallo proteins are iron–sulfur clusters, such as 2Fe–2S, 3Fe–4S, or 4Fe–4S (Beinert et al, 1997). They play diverse functional roles, ranging from electron transfer (e.g. the Rieske proteins in complex III of the respiratory chain), transcriptional regulation (the bacterial SoxR and FNR transcription factors), structural stabilization (bacterial endonuclease III), catalysis (e.g. aconitase, an enzyme of the citric acid cycle) etc. Other forms of catalytically active, protein-associated iron may include iron-oxo clusters (e.g. in ribonucleotide reductase, an enzyme required for DNA synthesis) or mononuclear iron centers (e.g. in cyclooxygenase and lipoxygenase, enzymes involved in inflammatory responses).

Catalase is a heme containing enzyme with 4 heme groups and is found in blood, bone marrow, mucosal membrane, liver and kidney. It destroys H$_2$O$_2$ formed in tissue and molecular O$_2$ is evolved in the reaction.

It should also be noted that non-heme iron has a central function in a recently discovered mechanism for oxygen sensing, via the hypoxia-inducible factor (HIF). This controls the transcription of a wide array of genes involved in erythropoiesis, angiogenesis, cell proliferation/survival, glycolysis, and iron metabolism in response to oxygen availability (Bruick, 2003). The stability of the HIF-1a subunit is directly regulated by oxygen.

2.4 Diagnostic tests:

Various tests are available to diagnose iron deficiency and iron overload.

2.4.1 Diagnosis of iron deficiency:

The important laboratory investigations to diagnose iron deficiency can be divided into hematological tests and biochemical tests (Ann Chen et al, 2002).

2.4.1.1 Hematological tests:

Hematology laboratory markers like, hemoglobin (Hb), hematocrit (Hct), red blood cell distribution width (RDW), reticulocyte count and the concentration of iron-containing protein in reticulocytes (CHr) have been proven helpful to evaluate iron deficiency.
Measurement of Hb and hematocrit (Hct) are the routinely used parameters for iron deficiency anemia. Both measurements are inexpensive and are readily available tests for anemia and used most commonly to screen for iron deficiency in routine diagnostic laboratories. However, Hb and Hct are late markers of iron deficiency, are not specific for iron deficiency anemia, and are less predictive as the prevalence of iron deficiency anemia decreases. The red blood cell distribution width (RDW) measures variations in the size of RBCs and increases with iron deficiency. The reticulocyte count measures circulating immature RBCs and decreases with iron deficiency. However, the reticulocyte count increases with blood loss. In severe cases of iron deficiency anemia coupled with the blood loss, the reticulocyte count may be slightly elevated. This parameter often is used for assessing the response to iron supplements. CHr, the concentration of iron-containing protein in reticulocytes, can be measured in some hematology laboratories by using the same automated flow cytometer that provides RBC and reticulocyte indices. CHr has been shown to be an early indicator of iron deficiency in healthy subjects receiving recombinant human erythropoietin.

2.4.1.2 Biochemical tests:

To diagnose iron deficiency biochemically, ferritin, serum iron, total iron-binding capacity (TIBC), transferrin saturation are regularly estimated markers along with specialized analytes like serum transferrin receptor (TfR), ZPP (Zink protoporphyrin) erythrocyte protoporphyrin (FEP).

Ferritin is a storage compound for iron, and serum ferritin levels normally correlate well with total iron stores. As iron stores are depleted, serum ferritin levels decline and are the earliest marker of iron deficiency. Serum ferritin has high specificity for iron deficiency, especially when combined with other markers such as Hb. However, the test is expensive and has limited availability in a clinical setting; therefore, it is not used commonly for screening. In addition, serum ferritin is an acute-phase reactant that can become elevated in the conditions like inflammation, chronic infection, or other diseases.

Serum iron concentration can be measured directly and generally decreases as iron stores are depleted. However, serum iron may not reflect iron stores accurately
because it is influenced by several additional factors, including iron absorption from meals, infection, inflammation, and diurnal variation.

Total iron-binding capacity (TIBC) measures the availability of iron-binding sites. Extracellular iron is transported in the body bound to transferrin, a specific carrier protein. Hence, TIBC indirectly measures transferrin levels, which increase as serum iron concentration (and stored iron) decreases. Unfortunately, this test also is affected by factors other than iron status. For example, TIBC is decreased with malnutrition, inflammation, chronic infection, and cancer. Transferrin saturation indicates the proportion of occupied iron-binding sites and reflects iron transport rather than storage. Tf sat is calculated from two measured values: serum iron concentration divided by TIBC, expressed as a percent. Low Tf sat implies low serum iron levels relative to the number of available iron-binding sites, suggesting low iron stores. Tf sat decreases before anemia develop, but not earlier enough to identify iron depletion. Tf sat is influenced by the same factors that affect TIBC and serum iron concentration and is less sensitive to changes in iron stores than that of serum ferritin. Serum transferrin receptor (TfR) also can be detected in some laboratories via immunoassay. This receptor is present on reticulocytes and is shed from the membrane as the reticulocyte matures. With tissue iron deficiency, there is a proportional increase in the number of transferrin receptors. Although not a readily available test, TfR is useful as an early marker of iron deficiency. ZPP (Zink protoporphyrin) is formed when zinc is incorporated into protoporphyrin in place of iron during the final step of heme biosynthesis. Under normal conditions, the reaction with iron predominates, but when iron is in short supply, the production of ZPP increases and the ZPP/heme ratio becomes elevated. ZPP is not the same as free erythrocyte protoporphyrin (FEP) or erythrocyte protoporphyrin, which is created in the laboratory when zinc is stripped from ZPP and also is used as a marker of iron deficiency without anemia. Although ZPP and FEP can be measured by using affordable, clinic-based methods, both are elevated with lead poisoning and chronic disease, making them less useful for the diagnosis of anemia.

2.4.2 Diagnosis of iron overload:

Accumulation of free iron in body characteristically occurs over decades in patients with hereditary disorders of iron metabolism, but it may take place within a
few years in transfusion dependent patients. The diagnosis of iron overload requires sequential steps. Clinical evaluation, biochemical testing, assessment of total body iron, and molecular tests concur to reach the correct diagnosis.

Table 2.4: Merits and demerits of various methods used to diagnose iron overload

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transferrin saturation</td>
<td>Relatively non-invasive; inexpensive; routine laboratory assay</td>
<td>Values confounded by inflammation, liver function, and ascorbate status</td>
</tr>
<tr>
<td>Serum ferritin</td>
<td>Easy to assess; inexpensive; repeat measures are useful for monitoring chelation therapy; positive correlation with morbidity and mortality</td>
<td>Indirect measurement of iron burden; fluctuates in response to inflammation, abnormal liver function, metabolic deficiencies; serial measurements required</td>
</tr>
<tr>
<td>Liver biopsy</td>
<td>Direct measurement of LIC; validated reference standard; quantitative, specific and sensitive; allows for measurement of non-heme storage iron; provides information on liver histology/pathology</td>
<td>Invasive, painful procedure associated with potentially serious complications; risk of sampling error especially in patients with cirrhosis; requires skilled physicians and standardized laboratory techniques</td>
</tr>
<tr>
<td>LIC assessment by SQUID</td>
<td>Linear correlation with LIC assessed by biopsy; may be repeated frequently</td>
<td>Indirect measurement of LIC; limited availability; high cost; requirement of highly specialized equipment dedicated technicians; not validated for LIC assessment and may underestimate levels</td>
</tr>
<tr>
<td>Quantitative iron assessment by MRI</td>
<td>The technique known as R2 (spin echo) MRI is a validated and standardized method for measuring LIC</td>
<td>Indirect measurement of iron concentration in liver and heart; requires MRI imager with dedicated imaging method; methodology remains to be standardized and validated for assessment of cardiac iron</td>
</tr>
</tbody>
</table>

LIC: liver iron concentration; SQUID: superconductive quantum interference device

Several comprehensive diagnostic and therapeutic algorithms have been proposed. Serum ferritin and transferrin saturation are the tests commonly explored
for diagnosis of iron deficiency as well as iron overload. Along with these tests the specialized tests are also performed for determination of iron accumulation in various organs of the body e.g. liver biopsy, LIC by SQUID and quantitative iron estimation by MRI. The advantage and the disadvantages of these tests are enlisted in Table 2.4.

2.4.3 Normal level of iron and related parameters:

As indicated in the distribution of iron, the major portion of iron is present in RBCs, muscle, liver and bone marrow, a very minute portion is present in blood. In healthy individual the blood iron is almost completely bound to transferrin.

Following Table shows the normal value of serum iron and the other related parameters with their SI units and the respective conversion factors.

**Table 2.5: Normal value of various analytes to evaluate iron status of the body with their SI units and the respective conversion factor**

<table>
<thead>
<tr>
<th>Laboratory test</th>
<th>Normal values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Iron status in the body</td>
<td>--</td>
</tr>
<tr>
<td>Serum iron</td>
<td>50-180 µg/dL × 0.179 9-32 µmol/L</td>
</tr>
<tr>
<td>Transferrin</td>
<td>200-360 mg/dL × 0.01 2-3.6 g/L</td>
</tr>
<tr>
<td>Transferrin saturation</td>
<td>20-50 %</td>
</tr>
<tr>
<td>Ferritin (Ft)</td>
<td>30-300 ng/ml ×2.247 65-670 pmol/L</td>
</tr>
<tr>
<td>Soluble transferrin receptors (sTfR)*</td>
<td>0.76-1.76 mg/L × -- 6.4-25.7 nmol/L</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>12-16 g/dL (women); 13-17 g/dL (men) ×0.6206*** 7.5-10 mol/L (women); 8-10.5 mmol/L (men)</td>
</tr>
<tr>
<td>Mean corpuscular volume</td>
<td>80-100 fl</td>
</tr>
<tr>
<td>Red cell distribution width</td>
<td>11-15</td>
</tr>
<tr>
<td>Mean corpuscular hemoglobin</td>
<td>28-35 pg</td>
</tr>
<tr>
<td>Hypochromic red cells</td>
<td>&lt;5%</td>
</tr>
<tr>
<td>-----------------------</td>
<td>-----</td>
</tr>
<tr>
<td>Reticulocyte hemoglobin content</td>
<td>28-35 pg</td>
</tr>
</tbody>
</table>

*Normal values may differ depending on the assay used.

**To convert the concentration values in conventional units into SI units multiply values by the conversion factor.

***In fact, although widely used, this factor allows for the calculation of the molar concentration of hemoglobin subunits.

**2.5 Clinical aspects:**

Like the other biological elements, excess as well as the deficiency of iron is harmful to the body.

**2.5.1 Deficiency of iron:**

Under physiological conditions, there is a balance between iron absorption, iron transport and iron storage in the human body. However, in shortage of iron supplement, its stored portion gets consumed and results in apparent iron deficiency. Iron depletion occurs grossly in 2 phases. In phase 1, there is a depletion of iron storage. At this level the level of ferritin drops, but as this fraction is not directly involved in biological functions the deficiency is not apparent. Thus, this is a hidden phase which does not elicit any medical examination. Many women in child bearing age remain in this phase without being identified. When phase 1 gets prolonged, the stored iron level gets almost exhausted. Biochemically along with the serum ferritin, Tf saturation and Hb concentration decreases, erythrocyte porphyrin increases as erythropoiesis slows down due to non availability of Fe. In phase 2, the deficiency of iron gets expressed in various ways and the most commonly results in iron deficiency anemia (IDA).

**2.5.1.1 Causes of iron deficiency:**

Iron deficiency (ID) and iron deficiency anemia (IDA) may result from the interplay of three distinct risk factors: increased iron requirements, limited external supply and increased blood loss. Some important ones have been listed in Table 2.6 (Hersko et al, 2006).
Table 2.6: Causes of iron deficiency

<table>
<thead>
<tr>
<th>Main causes of iron deficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Increased demand</strong></td>
</tr>
<tr>
<td>* Growth during infancy and childhood</td>
</tr>
<tr>
<td>* Treatment with erythropoiesis-stimulating agents</td>
</tr>
<tr>
<td><strong>Limited external supply</strong></td>
</tr>
<tr>
<td>* Poor intake</td>
</tr>
<tr>
<td>* Inappropriate diet with deficit in bioavailable iron and/or ascorbic acid</td>
</tr>
<tr>
<td>Malabsorption</td>
</tr>
<tr>
<td>* Gastric resection</td>
</tr>
<tr>
<td>* Helicobacter pylori infection (even without significant bleeding)</td>
</tr>
<tr>
<td>* Malabsorption syndromes (Crohn disease and coeliac disease)</td>
</tr>
<tr>
<td>* Drug interference (gastric anti-acid agents and antisecretory drugs)</td>
</tr>
<tr>
<td><strong>Increased loss</strong></td>
</tr>
<tr>
<td>* Phlebotomy</td>
</tr>
<tr>
<td>* Blood donation</td>
</tr>
<tr>
<td>* Dialysis (particularly hemodialysis)</td>
</tr>
<tr>
<td>* Hemorrhage</td>
</tr>
<tr>
<td>* Surgery</td>
</tr>
<tr>
<td>* Trauma</td>
</tr>
<tr>
<td>* Gastrointestinal bleeding</td>
</tr>
<tr>
<td>* Genitourinary bleeding</td>
</tr>
<tr>
<td>* Respiratory tract bleeding</td>
</tr>
</tbody>
</table>

2.5.1.2 Consequences of iron deficiency:

Too little iron can impair body functions, but most physical signs and symptoms do not show up unless iron deficiency anemia occurs. Someone with early stages of iron deficiency may not show signs or symptoms. This is why it is important to screen for too little iron among high risk groups.

Signs of iron deficiency anemia include (CDC report)

- Feeling tired and weak
- Decreased work and school performance
REVIEW OF LITERATURE

- Slow cognitive and social development during childhood
- Difficulty in maintaining body temperature
- Decreased immune function, which increases susceptibility to infection
- Glossitis

2.5.2 Iron overload:

Being one way element, iron has strict check at entry point; still its overload is observed which is mainly due to excessive absorption, parental iron therapy and repeated blood transfusion. Pathological conditions representing body iron overload are designated as iron overload syndromes, and iron deposition causes organ dysfunction including cell death, fibrosis, and carcinogenesis. Iron overload syndromes are classified as genetic or secondary as shown in Table 2.7. Hemochromatosis is the most common genetic disorder in Western countries (Yen et al, 2006). Its clinical manifestations are systemic iron deposition mainly in liver, heart, brain and endocrine organs. This organ damage is considered to be a result of tissue injuries by iron-induced oxidative stresses (Pietrangelo, 2004). In 1996, the causative gene was identified as HFE in the human chromosome 6 (Feder et al, 1996), and approximately 85% of patients with hereditary hemochromatosis in Western counties have a homologous mutation of C282Y in their HFE gene. Thereafter, other genes such as hemojuvelin (HJV), Tfr2, ferroportin, and hepcidin (HAMP) gene were identified (Franchini, 2006). In spite of the lack of genetic background, iron overload is commonly observed as a secondary condition. The most common condition occurs in patients who require long-term blood transfusions is due to severe anemias. This condition includes genetic disorders such as thalassemia and SCD, and anemia refractory to conventional treatments.

In these patients, ineffective erythropoiesis and continuous accumulation of exogenous iron by transfusion are considered to be responsible for the iron overload. Figure 2.7 illustrates the complex chain of events that occurs in various organs of in thalassemia patients with the treatment related complications (Rund and Rachmilewitz, 2005). The resulting organ failures such as liver failure, cardiac failure, and severe diabetes mellitus affect patients’ outcome (Andrews et al, 1999).
## Table 2.7: Causes and types of iron load:

<table>
<thead>
<tr>
<th>Causes of iron overload</th>
<th>Type 1: HFE gene (6p21.3) mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hereditary Hemochromatosis</td>
<td>Type 2: Subtype A: hemojuvelin gene (1q21) mutation</td>
</tr>
<tr>
<td></td>
<td>Subtype B: hepcidin gene (19q13) mutation</td>
</tr>
<tr>
<td></td>
<td>Type 3: Transferrin receptor 2 gene (7q22) mutation</td>
</tr>
<tr>
<td></td>
<td>Type 4: Ferroportin gene (2q32) mutation</td>
</tr>
<tr>
<td>Ferritin gene mutation</td>
<td>H-ferritin gene mutation</td>
</tr>
<tr>
<td></td>
<td>(mRNA iron responsive-element mutation)</td>
</tr>
<tr>
<td>DMT 1 gene mutation</td>
<td>Transferrin gene mutation</td>
</tr>
<tr>
<td>Ceruloplasmin gene mutation</td>
<td></td>
</tr>
<tr>
<td>Atransferrinemia</td>
<td></td>
</tr>
<tr>
<td><strong>Secondary iron overload</strong></td>
<td></td>
</tr>
<tr>
<td>Ineffective erythropoiesis</td>
<td>Thalassemia, sideroblastic anemia, myelodysplastic syndromes</td>
</tr>
<tr>
<td>Administration of iron for long periods</td>
<td>Take orally or intravenous injection</td>
</tr>
<tr>
<td>Transfusion for long periods</td>
<td></td>
</tr>
<tr>
<td>Dietary iron overload</td>
<td></td>
</tr>
<tr>
<td>Liver dysfunction</td>
<td>Alcoholic liver injury, chronic hepatitis (type C), non-alcoholic steatohepatitis</td>
</tr>
<tr>
<td>Others</td>
<td>Porphyria</td>
</tr>
</tbody>
</table>

In addition to these classical conditions, there are many diseases that show mild iron deposition or dys-regulation of body iron distribution. Such conditions include chronic hepatitis C, alcoholic liver disease, non-alcoholic steatohepatitis and insulin resistance, and iron is an important cofactor that modifies these disease conditions. Furthermore, it is becoming clear that excess iron is also hazardous as it promotes atherosclerosis, carcinogenesis, diabetes, and other lifestyle-related disorders (Bonkovsky et al, 2003).
Figure 2.7: Management of Thalassemia and treatment-related complications

2.5.2.1 Iron toxicity:

Iron overload induces damage in organs like liver, heart, pancreas, thyroid and the central nervous system. Iron’s toxicity is largely based on Fenton and Haber–Weiss chemistry where sufficient amount of catalytic iron yield hydroxyl radicals (OH) from superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), collectively known as “reactive oxygen intermediates” (ROIs) (Halliwell and Gutteridge, 1990).

The iron toxicity is confined mainly due to the redox active forms of iron. In normal subjects such toxicity of iron is well suppressed by transferrin, but when iron gets a chance to be present in a form devoid of transferrin the toxicity gets revealed, such forms are called as Non Transferrin Bound Iron (NTBI). Non transferrin bound...
iron (NTBI) is the term used for all the forms of iron present in serum or plasma which are not bound to Tf and the other traditional iron binding proteins like heme, apoferritin, hemosiderin etc. Unbound iron which does not have the shield in the form of protein has capacity to generate highly reactive free radical and known to cause an immense amount of damage to various organs of the body including heart, liver, pancreas, endocrine glands and erythroid cells etc. depending on the final location of its deposition. Another aspect is that iron is almost a one way element and only few physiological avenues for its removal are known to exist in human body. There is a lacuna in the literature regarding the precise way of removal and the shelf life of NTBI, though some studies state its clearance from highly vascular organs like liver and heart (Hider, 2002). It is difficult proposition to find the equivocal definition of NTBI even after the lapse of 34 years of mentioning of NTBI first by Hershko et al (1978).

2.6 Non Transferrin Bound Iron:

2.6.1 Names and nature of NTBI:

A spectrum of names is accorded to the NTBI due to its diverse biological nature. Various research workers have used different terms like “Non transferrin bound iron”, “free iron”, “catalytic iron pool”, “ labile iron” etc. to address the same or overlapping fraction of plasma iron which is devoid of its classical carriers. According to Paffetti et al (2006) the term” free iron” was introduced to indicate low molecular weight iron free from high affinity binding plasma proteins. Breuer and Cabantchik (2001) suggested that the NTBI denotes those form of iron in serum that are bound to ligands other than Tf. The term “free iron” refers to iron loosely bound to variety of biomolecules in such a way that it retains its ability to catalyze the formation of Reactive Oxygen Species (ROS) (Nilsson et al, 2002). According to Breuer et al (2000) “labile serum iron” or “labile plasma iron” represent iron bound to serum albumin, citrate and other undefined negatively charged ligands. The term “labile iron pool was proposed by Greenberg and Wintrobe in 1946 and reintroduced by Jacobs in 1977 as a ‘transient iron pool’ (Kruszewski, 2003). Kruszewski (2003) defined “labile iron pool” as low molecular pool of weakly chelated iron that rapidly passes through the cell. Others coined the term “catalytic iron pool” which consists of
chemical forms of iron that can participate in redox cycling and are associated with oxidative stress (Kruszewski, 2003; Halliwell and Gutteridge 1990; Kakhlon and Cabantchik, 2002).

Numerous researches assigned different terms for NTBI based on its chelating characteristics. For example “Bleomycin detectable iron”, “Desferrioxamine Chelatable Iron” (DCI) later on called as directly chelatable iron by desferrioxamine, “Mobilizer Dependent Chelatable Iron” (MDCI), “Bathophenanthroline detectable iron” etc. (Nilsson et al, 2002; von Bonsdorff et al, 2002). The term “Bleomycin detectable iron” has been applied to address the forms of NTBI which are detected when associated with bleomycin (von Bonsdorff et al, 2002). DCI was the term used by Breuer et al (2001) for the forms of NTBI which are chelatable with the well known iron chelator desferrioxamine. However the abbreviation “DCI” had been used by the same researcher and his colleagues later as “directly chelatable iron” to indicate the NTBI forms which are readily chelated with desferrioxamine without the assistance of mobilizer (Esposito et al, 2003). At the same time another term “Mobilizer Dependent Chelatable Iron” (MDCI) has been in use to denote the NTBI forms which can be detected only after the action of mobilizer like oxalate or Nitrilo Triacetic Acid (NTA) (Esposito et al, 2003). The term “Bathophenanthroline detectable iron” is applicable for NTBI forms which are directly chelated by a chromogenic chelator (Nilsson et al, 2002).

Bearing +3/+2 valence, iron can’t exist in absolutely free form, rather due to its cationic nature it binds with many negatively charged ligands like albumin, citrate, acetate, DNA etc. (Nilsson et al, 2002; Grootveld et al, 1989; Lovstad, 1993).

In the absence of precise data available, presently its nature appears more heterogenic. Binding of NTBI to various molecules have been reported using different methodologies. In computer simulation studies the major form of NTBI is found to be ferric bound to citrate where as nuclear magnetic resonance analysis point out ferric bound to citrate as well as acetate in hemochromatosis patients (Grootveld et al, 1989; May and Williams, 1977). The interaction of iron is also shown with the major plasma protein albumin even when the Tf is not completely saturated (van der Heul et al, 1972).
2.6.2 Sources and status of NTBI:

There is 20-35% saturation of Tf at the normal serum iron level (20 µmol/L), the only major form of non-heme iron present in plasma. In normal physiological condition the level of Tf is sufficient enough for complete scavenging of even the greater dose of free iron and ensuring its absence in internal milieu. This is the reason why levels of NTBI in normal healthy individual do not exceed 1 µmol/L, and often undetectable by most of the methods (Anderson, 1999). In the iron overload scenarios like thalassemia; hemochromatosis and others iron gets a chance to exist in form which is not bond to Tf (Hershko et al, 1978; Graham et al, 1979; Porter et al, 1996; Batey et al, 1980; Aruoma et al, 1988; Loreal et al, 2000).

Table 2.8: Name and concentration of NTBI or its sub-fraction in various clinical conditions

<table>
<thead>
<tr>
<th>Clinical condition</th>
<th>NTBI / name of its sub-fraction</th>
<th>Concentration (µmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic hemochromatosis with iron overload (Le Lan et al, 2005)</td>
<td>NTBI</td>
<td>0.761 ± 0.504</td>
</tr>
<tr>
<td>Genetic hemochromatosis with iron overload (Le Lan et al, 2005)</td>
<td>LPI</td>
<td>0.250 ± 0.289</td>
</tr>
<tr>
<td>Heredity hemochromatosis (Breuer et al, 2000)</td>
<td>NTBI</td>
<td>4.0 - 16.3</td>
</tr>
<tr>
<td>Thalassemia major(Thailand) (Breuer et al, 2001)</td>
<td>DCI</td>
<td>1.7 - 8.6</td>
</tr>
<tr>
<td>β thalassemia major (Sharma et al, 2009)</td>
<td>NTBI</td>
<td>0.375 ± 0.028</td>
</tr>
<tr>
<td>Diabetes mellitus (Lee et al, 2006)</td>
<td>NTBI</td>
<td>0.62 ± 0.43</td>
</tr>
<tr>
<td>Cancer patients undergoing chemotherapy (Weijl et al, 2004)</td>
<td>NPBI</td>
<td>10.6 ± 6.6</td>
</tr>
<tr>
<td>End stage renal disease (Breuer et al, 2000)</td>
<td>NTBI</td>
<td>0.1 - 13.5</td>
</tr>
</tbody>
</table>

As shown in Table 2.8 free iron has been well documented as NTBI or other relevant sub-fractions in various conditions of iron overload like hemochromatosis and thalassemia along with some non iron overload conditions (Breuer et al, 2001;

In transferrinemia, it is pertinent that all the plasma iron molecules exist as NTBI. In the absence of Tf, NTBI levels were reported to be up to 20 µmol/L, whereas in the presence of insufficient Tf concentration these levels were found to be less than 10 µmol/L (Anderson et al, 1999).

Increased levels of NTBI in the above mentioned situations is easily explainable whereas it is difficult to give a convenient explanation for the raised levels of NTBI where Tf saturation is not high (Loreal et al, 2000; Gosriwatana et al, 1999; Breuer et al, 2000; Anderson, 1999). Due to high Tf saturation the surplus iron gets a chance to escape and bind to non specific ligands. It is worthy to note that presence of NTBI was also found in the certain pathological conditions wherein no iron over load was found to exist. There is ample justification that why earlier studies of NTBI were focused in the diseases where the saturation of Tf was expected to high due to iron overload. Appearance of NTBI in patients with normal Tf saturation poses the key question that in spite of the presence of strong affinity between iron and Tf, why a fraction of iron remains to be free though there is availability of free sites in the Tf to incorporate it? Various hypotheses have been suggested in different clinical conditions, but it is simple to state the presence of free iron in the presence of low saturation Tf is due to either greater inflow or inefficient management as efficient management of iron is of high priority because it is almost one way element as mentioned earlier. The fraction of iron which is inaccessible to Tf is the key cause of the NTBI existence at low Tf saturation (Breuer et al, 2000).

The major sources of plasma iron are dietary iron coming from mucosal absorption in gastrointestinal tract and recycling of iron from the breakdown of aged erythrocytes and macrophages. Endogenous source is more important factor as iron absorption is well regulated at the mucosal level. In hemolytic anemia, increased endogenous inflow of iron due to excess hemolysis and /or compensatory blood transfusion may cause higher saturation of Tf leading to existence of NTBI (Hershko et al, 1978; Graham et al, 1979; Porter et al, 1996; al-Refaie et al, 1992). Lee et al reported the existence of NTBI in diabetes mellitus (Lee et al, 2006). In diabetes mellitus glycochelates are found to play vital role in binding of iron. Qian et
al suggested that glycate
d proteins whose concentration is high in diabetics, do not
only bind to iron and copper with higher affinity but also have the tendency to extract
the iron from the traditional iron carriers (Qian et al, 1998). In case of cancer patients
undergoing for chemotherapy the reasons for the existence of NTBI are due to
temporary shutdown of bone marrow and associated reduced demand of iron
(Halliwell et al, 1988; Carmine et al, 1995; Dürken et al, 1997; Bradley et al, 1997).
The other clinical conditions in which the presence of NTBI is indicated are acute
coronary syndrome, liver disease, end stage kidney disease patients who are
undergoing dialysis, in chronic alcoholics, myelodysplastic syndrome etc (Breuer et al
2000; Lele et al, 2009; Harrison et al 2007; Detivaud et al, 2005; De Feo et al, 2001;
Cortelezzi et al, 2000; Mahesh et al, 2008). Oxidative stress has also been
documented as a cause as well as consequence of free iron, where the stable iron
compound like iron sulphur proteins, ferritin and even heme release NTBI
(Brazzolotto et al, 1999; Halliwell, 1994; Halliwell, 1993; Biemond et al, 1984;
Abdalla et al, 1992; Gutteridge, 1986; Puppo and Halliwell, 1988; Gutteridge and

Various forms of NTBI may coexist depending on degree of iron overload,
cause of iron overload and its duration. Clinically it has been proven that the
accessibility of different iron chelators vary in assorted clinical conditions which
indirectly support the heterogeneity of NTBI. For example hemochromatotic sera with
80-85 % Tf saturation showed no detectable DFO- chelatable iron according to
Grootveld et al (1989), where as Breuer et al (2001) found DCI (desferrioxamine-
chelatable iron) in the similar patient group when mobilizer like oxalate was applied.
In case of thealsemic sera with >90 % Tf saturation, both the forms of DCI
(desferrioxamine- chelatable iron) i.e. with and without oxalate mobilization were
found (Breuer et al, 2000). Pootrakul et al estimated the levels of LPI and DCI
separately in iron overloaded patients and found that both are highly correlated
fractions (Pootrakul et al, 2003). To a small extent where they differ is 25% lower
values of LPI which may stand for minor component of DCI which is not redox
active. Careful segregation of various isoforms of NTBI is very essential to establish
the role of individual isoforms in specific pathological condition.
2.6.3 Estimation of NTBI:

Due to ambiguous nature of NTBI, universally accepted gold standard method is not available at present. However, various researchers apply a range of analytical approaches which target one or the other characteristics of NTBI to estimate overlapping subtractions of the large heterogeneous iron pool collectively termed as NTBI (Pootarakul et al, 2004). Basically there are three approaches on which methods have been developed for NTBI estimation.

1. Indirect estimation of NTBI with anti-tumor antibiotic bleomycin
2. Chelation of NTBI with chelator followed by its separation and estimation using various analytical techniques
3. Direct estimation of NTBI with iron sensitive fluorescent probe

2.6.3.1 Indirect estimation of NTBI with anti-tumor antibiotic bleomycin:

NTBI estimating bleomycin based method is the oldest one, which was firstly suggested by Gutteridge et al (1981). Assay was based on ability of bleomycin to degrade DNA in the presence of ferrous iron (Fe^{2+}), which can generate extremely reactive oxygen radicals due to its redox activity (Gutteridge et al, 1981; Gutteridge and Halliwell, 1987; Evans and Halliwell, 1994). Iron only in ferrous form can catalyze such reaction and to facilitate the reaction all the ferric ions were converted to ferrous form by using reducing agent like ascorbate which further advances the reaction and degrades the DNA present in the reaction mixture. Such degraded DNA products were measured in the form of malondialdehyde (MDA) molecules which react with thiobarbituric acid and generate colored compounds which are read at 532 nm.

Bleomycin is the key ingredient of the assay and so the value of NTBI determined by this technique is frequently addressed as ‘Bleomycin detectable iron’ (BDI) (von Bonsdorff et al, 2002). The method is found to be extremely vulnerable to various factors; the important ones are the pH of the medium and the source of bleomycin (Graham et al, 1979). To maintain specific pH, usage of buffer is imperative, however much care should be taken as it can introduce artifactual iron to the reaction mixture and generate misleading results. Another important aspect is that the ascorbate which is used as reducing agent may not convert all the ferric irons to ferrous iron and can lead to false low results. The method also gets influenced by
hemoglobin and applicable only when Tf saturation is >80% (von Bonsdorff et al, 2002; Sahlstedt et al, 2001).

**Figure 2.8: Bleomycin based method for NTBI estimation**

The procedure includes heating step for production of end point colored complex (Thiobarbituric acid 2-Malondialdehyde) which add some more negative views in the methodology (Burkitt et al, 2001). Another drawback of this method is its indirect way of detection. As the degraded DNA products are measured in the form of MDA and not NTBI, any other factors which can either degrade DNA or form MDA contribute to the results. Conclusively this method is lengthy as well as tedious and associated with negative views. Furthermore suggested chelax treatment of all the chemicals to minimize iron artifact leads to increased cost factor and procedural complexity.

Various modifications have been suggested by research workers time to time to improve this method. One of the significant alterations has been suggested by von Bonsdorff et al (2002) who made the use of the microtiter plate for sample processing which significantly decreased the demand of sample volume and made the test...
possible to be operated in mass analysis. Burkitt et al (2001) suggested using ethydium to bind or determine degraded DNA products which are later on determined with fluorescent based technique (Burkitt et al, 2001). This modification is said to decrease the non specific reactions due to non specific MDA produced while heating the reaction mixture. BDI has been detected in various biological fluids like serum from patients of idiopathic hemochromatosis, thalassemia major, hematological malignancies after chemotherapy, patients with end stage renal disease, in overt diabetes and in acute coronary syndrome (von Bonsdorff et al 2002; Lele et al, 2009; Evans and Halliwell, 1994; Han and Okada, 1995; Shah, 2004). It is also detected in plasma of immature as well as full term infants, in synovial fluid from the rheumatoid patients and in CSF of normal subjects (Gutteridge et al, 1981; Gutteridge and Halliwell, 1987; Evans et al, 1992; Gutteridge, 1992).

2.6.3.2 Chelation of NTBI with chelator followed by its separation and estimation using various analytical techniques:

This approach includes two main steps; in the first step iron is chelated with the help of chelator which is separated from the biological fluid. In the second step, chelated iron is subjected to various techniques which estimate it. Different workers used various chelators like Ethylene Diamine Tetra Acetic acid (EDTA), NTA, oxalate etc. (Breuer et al, 2000; Esposito et al, 2002; Singh et al, 1990). Hershko et al were first to utilize this approach with EDTA (Hershko et al, 1978). Subsequently NTA found to be superior to EDTA as it exhibits minimum iron mobilization from Tf (Singh et al, 1990). In addition to the type of chelator used, its concentration is also found to play a critical role. It has been observed that with the increase in the concentration of NTA there is also a parallel increase in the concentration of NTBI detected (Kolb et al, 2009). In the method described by Gosriwatana et al 80 mM NTA was suggested as compared to the other methods where lower concentrations of NTA was used (Gosriwatana et al, 1999; Singh et al, 1990; Jacobs et al, 2005; Zhang et al, 1995). However Kolb et al (2009) suggested that 4 mM NTA is the best suitable concentration for chelation of NTBI. This chelated fraction of iron is separated from plasma protein with ultra filter of cut off of 30,000 dalton or with ultracentrifuge (Jacobs et al, 2005). This step is operated to minimize the donation of chelated iron to the free sites of traditional iron binding protein like apo-transferrin (aTf). The
separated fraction is then subjected to diverse analytical methods like high-pressure liquid chromatography (HPLC), colorimetry, inductive conductiometric plasma spectroscopy (ICP) etc. (Gosriwatana et al, 1999; Dürken et al, 1997; Singh et al, 1990; Zhang et al, 1995).

**Figure 2.9: Two step methods for NTBI estimation i.e. chelation and analysis**

All such techniques have their own advantages and disadvantages. HPLC has many advantages especially with patients treated with desferrioxamine, however need of specialized HPLC instrument which is free of stain less steel may restrict its wide spread applicability (Gosriwatana et al, 1999; Collins et al, 2000). Recently Sasaki et al (2011) suggested that an extra step to reduce iron contamination mainly in NTA and tris carbonatocobaltate may minimize the background color and improve the sensitivity of the test when HPLC is used as detection system. Paffetti et al (2006) have used the HPLC based technique for various biological fluids like plasma, amniotic fluid, broncho alveolar lavage (BAL) and in brain tissue. Zhang et al (1995) suggested the use of bathophenanthroline disulfonate (BPT/ BPS) as a chromogen.
which gives coloration with iron after its initial chelation with NTA. The similar method with some modifications have been experimented and successfully compared with electro thermal atomic absorption spectroscopy (Jittangprasert et al, 2004). Colorimetric methods appear to be attractive with minimum specialized instrumental need without compromising the reliability. However the donation of NTBI from iron-NTA complex to one of the most commonly used chromogen BPS is time consuming and increase the processing time of the assay. An interesting approach had been explored by Nilsson et al who suggested the direct application of BPS in absence of other chelators where it acts as chelator as well as chromogen (Nilsson et al, 2002). In this assay the loosely bound iron gets chelated with BPS and produce coloration. The problem of background color and fluctuation in absorbance had been diminished with filtering the reaction mixture before spectrophotometric measurement. The BPS based method directly measures the concentration of BPS chelatable iron in the sample. BPS is a well-known chromogen to estimate micro-quantity of iron present in various specimens. Several other chromogens have been examined as an alternative to BPS, which may have somewhat higher extinction coefficients and sometimes absorption maxima at more favorable wavelengths, but BPS is easier to use as there is no need to add masking agents to reduce interference from other metals (Cerriotti and Cerriotti, 1980; Eskeleinen et al, 1983; Derman et al, 1989). More importantly, the BPS Fe complex retains its extinction coefficient at physiological pH, whereas other chromogens must be assayed under acidic conditions, with associated risk of liberating physiologically bound iron, e.g. from transferrin. Also, BPS does not liberate iron bound to either hemoglobin or transferrin even during prolonged incubation. Further advantages of the BPS based method include low cost and easy availability of the chemicals and instruments i.e. spectrophotometer in contrast to other methods exploring HPLC instruments, electron spin resonance (ESR)-spectrometers, etc. (Nilsson, 2002). In the method suggested by Zhang et al (1995) an excess of NTA was utilized to bind free iron in a serum sample. Iron-NTA complex was separated from the biological fluid with ultra-filtration. BPS was added to the ultrafiltrate and chromogen was formed. The detour over NTA, however, seems unnecessary in the light of the good recovery of the BPS Fe complex found in our filtration procedure. It also prolongs the time of analysis to 150 min compared with 15
min for the BPS method. BPS forms complexes with both ferric and ferrous ion, but only the ferrous complex is colored. The ferric complex of BPS, however, was readily and quantitatively reduced by ascorbate. Therefore, measurement of absorbance before and after adding ascorbate will give the concentration of ferrous iron and total iron, respectively, in the sample. Subtraction of the former from the latter gives the ferric concentration. Thus, the BPS-method, in contrast e.g. to the bleomycin method, is able to differentiate between “active” (i.e. ferrous) and “inactive” free iron in a biological sample, with the ferric part constituting a latent pool that can be activated through bio-reduction.

The major problem with the second approach is to select an appropriate type and concentration of the chelator which wipe off all the loosely and non specifically bound iron without influencing the iron bound to Tf, as a little portion of Tf bound iron acts as a big dose of NTBI and leads to its overestimation. Another drawback is due to the opposite flow of iron i.e. donation of chelated iron to free sites of Tf in place of getting entered in analytical assay which can lead to underestimation of NTBI, mainly encountered when Tf saturation is normal. Gosriwatana et al (1999) suggested the use of sodium tris carbocobaltate to block the free iron binding sites on Tf for better reliability. This approach is extensively practiced presently but definitely confined by their labor intensive feature and higher sample volume demand.

2.6.3.3 Direct estimation of NTBI with iron sensitive fluorescent probe:

This technique determines NTBI by measuring quenching or de-quenching of fluorescence. It may or may not include the mobilizer or chelator but usually does not require the separation of chelated iron fraction from the other portion of biological fluid. This approach is quite impressive in terms of least technical efforts but demand the fluorimeter as detection system. Fluorescent based technique for NTBI estimation was introduced by Breuer el al in multiwall plate format (Breuer and Cabantchik, 2001). The method is one step, simple to operate, sensitive and available for 96 well plate format make it suitable for large scale analysis. The assay was based on fluorescent labeled apo-transferrin (Fl-aTf) which undergoes for fluorescent quenching when it binds to iron. The assay also included 10 mM oxalate as mobilizer and 0.1 mM gallium (III) as blocking agent. After the use of Fl-aTf, the use of
fluorescent labeled desferrioxamine (Fl-DFO) was successfully experimented by the same researcher to determine the “DFO chelatable iron” or DCI without the assistance of mobilizer (Breuer et al, 2001). In this method fluorescent quenching of Fl-DFO is measured after its binding with iron. The assay can significantly applicable to check the efficacy of the chelator DFO. MDCI was then analyzed with the help of mobilizer and Fl-aTf in which mobilizer like oxalate was used to wipe up all loosely bound iron, which were then analyzed by fluorescent quenching of Fl-aTF (Esposito et al, 2003). To estimate redox active fraction of NTBI, fluorogenic dihydrorhodamine (DHR) was used (Esposito, 2003). The targeted NTBI forms were termed as “labile plasma iron”, which generate various oxidants in the presence of a bioreductant ascorbate but the reaction gets blocked by iron chelators. The signals were determined by increase in fluorescence due to interaction of DHR with LPI.

Figure 2.10: Fluorescent based one step method for NTBI estimation

The inherited drawback of almost all fluorescent based probes is their sensitivity to environmental conditions such as serum color and turbidity, which has regularly been overcome by parallel examination of serum as control without the key ingredient. The difference in the intensity of the signal is the true indicator of the NTBI (Breuer et al, 2001). Another way is to include second fluorescent probe which is insensitive to iron. Sharma et al (2009) suggested a fluorescent based method which has one more step for separation of chelated iron from the biological fluid, along with the different fluorescent probe and chelator like siderophore plus azotobactin. In the
assay use of tri hydrate cobalt solution was suggested as blocking agent of aTf in place of gallium and NTA as NTBI mobilizer in place of oxalate. Quenching of azotobactins’s fluorescence is measured, which supposed to be due to NTBI. In this assay blocking agent and the mobilizer should be selected with utmost caution which should neither interfere with the attachment of Fe$^{3+}$ with azotobactin nor influence on fluorescent quenching (Sharma et al, 2009). A good number of methods experimented by various researchers, nevertheless their comparability is questioned due to lack of reliable inter laboratory analysis. One such good attempt made by Jacobs et al (2005) to measure NTBI value with different methodologies showed unacceptable difference of NTBI value in between the methods with high CV values. The high CV could be due to the measurement of overlapping sub-fractions of NTBI which are not actually identical.

2.6.4 Pathogenesis:

Serum iron if not guarded by the Tf, the unbound iron (NTBI) poses a great challenge to the protective mechanism of the body. The detrimental of free iron is due to its capability to increase the load of oxidative stress. Free Ferrous iron (Fe$^{2+}$) may catalyses a variety of free radical oxidative reactions which in turn lead to various degenerative changes (Chau, 2000; Meyers, 2000; Gackowski et al, 2001; Papanikolaou and Pantopoulos, 2005).

Fenton reaction and Haber-Weiss reactions show iron mediated generation of reactive oxygen species which are as follows:

Fe$^{2+}$ + H$_2$O$_2$ →Fe$^{3+}$ + OH$^-$ + OH$^-$ (Fenton reaction)

O$_2^-$ + H$_2$O$_2$→O$_2$+OH$^+$ + OH$^-$ (Haber-Weiss reaction)

O$_2^-$+ Fe$^{3+}$→O$_2$+ Fe$^{2+}$

The oxygen metabolites including free radical species, superoxide, hydroxyl radicals and other metabolites like hydrogen peroxide and hypohalous acid are often been collectively called as reactive oxygen metabolites (ROM), or reactive oxygen species (ROS) or simply as oxidants. Traditional metal driven generation of oxygen derived free radicals are known to induce oxidation of proteins, lipids, lipoproteins, nucleoproteins, nucleic acids, carbohydrates and other cellular components (Mohan et al, 2011; Galaris et al, 2008). At this point it is essential to note that all the forms of
NTBI may not exhibit the aforesaid activity. So it is most important to find out the specific isoform or isoforms which do such damage.

During the oxidative stress, ROS can also release the iron from stable form like ferritin and heme which worsen the condition and continue the ongoing detrimental cascade (Chau et al, 2000; Meyers, 2000; Horwitz and Rosenthal, 1999). NTBI is also suggested to play supporting role in growth of certain bacteria and fungi. Thus along with the increased load of reactive oxidants it makes host more susceptible against various infections (Pai et al, 2006). Cellular uptake of NTBI is not meticulously regulated like Tf bound iron and hence could easily give rise to cellular iron overload (Cabantchik et al, 2005).

The biological importance of formation of hydroxyl radical through Fenton chemistry has not been given much importance due to compensation of O2^- by NO^- and formation of ONOO^- instead of OH^- (Koppenol, 2001). However, OH^- is still commonly accepted as the main source of oxidative damage to the cell (Fridovich, 1998; Liochev and Fridovich, 1999; Termini, 2000).

2.6.4.1 Role of NTBI in Cardiac disease:

Numerous studies have reported the role of iron in cardiac diseases. The probable role of iron with Cardio vascular disease (CVD) was postulated by Sullivan in 1981 which was supported by high incidences of cardiac disease in iron overload patients (Sullivan, 1981; Schafer et al, 1981). Several studies have successfully demonstrated the correlation between the iron intake, body iron stores and cardiovascular risk in the general population (Ascherio et al, 1994; Lee et al, 2005; van der ADL et al 2005; Ramakrishnan et al, 2002). Several pathways have been indicated to explain iron mediated heart diseases which are mainly by tissue cell loss and atherosclerosis through ROS production as shown in Figure 2.11. Atherosclerosis is one of the major causes of coronary heart disease.

One of the most prevalent hypotheses for its etiology is inflammation theory which describes atherosclerosis as proliferation of smooth muscle (Ross, 1999). ROS known to be produced by NTBI are evident at the site of inflammation and contribute to cell damage (Salvemini et al, 1996; Cuzzocrea et al, 1997). Such reactive species are identified to cause LDL oxidation which induces plaque formation (Meyers, 2000;
Leake and Rankin, 1990; Knight, 1999). Pratico et al (1999) demonstrated that Fe$^{2+}$ could induce platelet aggregation in dose dependent manner. Iron induced oxidative damage has also been linked to increased loss of cardiomyocytes due to apoptosis (Oudit et al, 2004). Altered cellular metabolism and / or iron mediated stimulation of cardiac fibroblast that may contribute to increased myocardial fibrosis. Iron is also suggested culprit for reperfusion injury through its ROS production which causes lipid peroxidation, fragility of intracellular lysozyme and release of hydrolytic enzymes and ultimately causing myocardial and endothelial cell necrosis as shown in Figure 2.11 (Halliwell and Gutteridge, 1990; Horwitz and Rosenthal, 1999; Voogd et al, 1994).

**Figure 2.11: NTBI induced Cardiac damage**

Experimental cell culture studies had shown that addition of NTBI to human endothelial cell culture increase the surface expression of adhesion molecules and also increase the monocyte adherence to endothelium (Kartikasari et al, 2004; Koo et al, 2003). These consequences can be corrected by addition of iron chelators like desferrioxamine and dipirydyl, which decrease expression of adhesion molecules and monocyte adherence (Kartikasari et al, 2004; Koo et al, 2003; Zhang and Frei, 2003).
However several other studies refuse the probable correlation of iron with cardiovascular risk, which could be due to selection of improper indicators or inappropriate techniques to estimate the respective indicator (Danesh and Appleby, 1999; Derstine et al, 2003; Baer et al, 1994; Corti et al, 1997; Knuiman et al, 2003; Sempos et al, 2000).

2.6.4.2 NTBI in Diabetes mellitus:

The evidence that iron overload lead to impaired glucose metabolism firstly derived from the fact that incidences of diabetes are high in hereditary hemochromatosis (HH).

The exact mechanism how iron is involved in pathogenesis of diabetes mellitus is unclear but it is suggested through added oxidative stress to pancreatic cells. Higher expression of divalent metal iron transporters (DMT) additionally facilitates the entry and accumulation of iron in pancreatic cells as compared to the other cells (Andrews, 1999). In iron overloaded thalassemic patients insulin resistance is significantly increased which indicate the possible influence of iron on hepatic iron uptake (Dandona et al, 1983; Mendler et al, 1999; Singh et al, 2001).

![Diagram: Role of NTBI in Diabetes mellitus](44)
In animal models it has been shown that oxidative stress causes the apoptosis of pancreatic islet cells. This obviously decreases the level of insulin production (Jackson et al, 1995). The role of iron in diabetes is depicted in Figure 2.12. Along with the causes of diabetes i.e. impaired insulin production and/or its action, iron is also suggested to play vital role in development of diabetic complications (Sullivan, 1981). This could be because of higher levels of free radicals which decrease antioxidant defense and damage the cell organelles, enzymes and increase lipid peroxidation. Iron chelator like desferrioxamine had been successfully experimented to decrease the level of $A_1c$ in non insulin dependent diabetes mellitus patients as well as in rats, which supports the link of iron in glycemic control (Redmon et al, 1993).

Common presence of NTBI is noticed by Lee et al (2005) in patients with type 2 diabetes mellitus. In uncontrolled diabetic patients, due to persistent high levels of reducing sugar, they get chance to react with the free amino group of proteins by non-enzymatic reactions and generate sugar bound proteins collectively known as advance glycation end products (AGE) (Singh et al, 2001). These glycochelates are known to bind with higher amount of transitional metals like iron and copper and extract more iron from stable iron compound to increase free iron load (Qian et al, 1998). Involvement of more iron molecules without their traditional or classical proteins may exhibit their redox activity and play vital role in development of long term complications.

### 2.6.4.3 NTBI in Renal disease:

In human studies elevated iron is noted in kidney with higher urinary catalytic iron in patients with chronic kidney disease (Nankivell et al, 1992). ROS produced due to NTBI are suggested as major cause of chronic kidney disease, mainly by 3 ways i.e. by damaging glomerular basement membrane (GBM), by decreasing glomerular filtration rate (GFR) and contributing in morphological changes in kidney (Shah, 2004).

Damage to glomerular basement membrane (GBM) is a known cause of proteinuria, which is an early sign of renal disease. Oxidants are suggested to damage GBM by increasing the susceptibility to proteolytic damage by inactivating $\alpha_1$-proteinase inhibitor, which allow the activity of elastase to damage extracellular
matrix. Oxidants also impair the synthesis of glomerular heparin sulfate, a proteoglycan needed to maintain the integrity of glomerular membrane. Oxidants reduce the glomerular and mesangial cell planer surface and increase myosin light chain phosphorylation. These effects could modulate the surface area of mesangial cells and modify ultra-filtration coefficient and decrease GFR. Furthermore oxidants increase the synthesis of prostaglandins and thromboxane which are suggested to cause proteinuria and/or decreased GFR. Oxidants are also suggested to participate in necrotizing crescentic glomerulonephritis which is characterized by necrosis of GBM causing marked infiltration of neutrophils and mononuclear cells (Shah, 2004).

Figure 2.13: Role of NTBI in Renal damage and its progression

In progressive kidney disease, oxidants are well recognized to damage the kidney by inducing proteinuria which facilitates the protein traffic in glomerulus and kidney tubules. These proteins e.g. albumins, Tf etc. are experimentally proven to cause further oxidative damage induce the activity of NF-κβ leading inflammatory response. On the other side protein mediated oxidative stress continues the cascade and leads to end stage renal disease (Shah et al, 2007). The roles of NTBI and ROS have been depicted in Figure 2.13. Furthermore the role of NTBI is evident by the fact
that advanced oxidized protein products AOPP which are generated by the ROS due to NTBI worsen whereas metal chelators improves the conditions in progressive kidney disease (Descamps-Latscha et al, 2004; Lin et al, 2003).

**2.6.4.4 NTBI and Erythroid cells:**

By causing oxidative stress NTBI are known to affect the erythroid series too. Studies suggest that the mature RBC, reticulocytes, and developing erythroid precursors take up iron through Tf independent pathway (Prus and Fibach, 2011). This pathway has been operated in iron overloaded patients in presence of NTBI. Such fraction instead of getting incorporated in heme synthesis induces ROS generation causing cytotoxicity in mature RBC results in intravascular hemolysis.

Increased LIP in RBC may be the result of leftover of unused iron precursors due to increased uptake from iron overloaded plasma, diminished utilization due to reduced hemoglobin production or degradation of unstable hemoglobin. In addition, RBCs may take up iron from their environment especially when the amount of iron in plasma exceeds the Tf binding capacity. Since mature RBCs are devoid of Tf receptors, this uptake is necessary of non Tf iron (Loken et al, 1987).

NTBI is also found in patients of hematological malignancies undergoing high-dose chemotherapy, particularly those treated with myeloablative therapy and stem cell transplantation (Halliwell et al, 1988; Bradley et al, 1997; Sahlstedt et al, 2001).

The existence of free iron has been traced in a number of pathological conditions by various researchers exploring different technique but sparse data are available which can correlate different NTBI isoforms, their respective clinical existence and the method for its estimation. So at present time it is noteworthy to register the identity of the NTBI sub-fraction recognized by the assorted techniques, which may or may not correlate with one another. Another important aspect is to recognize the key sub-fraction of NTBI which has clinical diagnostic relevance. If both of these points are taken for consideration, it will help the clinician to look for the significant iso-form of NTBI in specialized clinical conditions and the laboratory scientist to select a suitable method to estimate the clinically relevant sub-fraction.
2.6.4.5 NTBI in iron overload conditions:

Hemochromatosis and thalassemia treated with blood transfusion are the two most common and obvious causes of iron overload.

Hereditary hemochromatosis (HH) is the best described of the primary iron overload syndromes that have been attributed to genetic variants in genes of iron metabolism. HH is an autosomal recessive disorder resulting from mutations in the HFE gene, usually manifesting in adults beginning in their 40s and 50s (Feder et al., 1996). In HH, duodenal DMT1 and ferroportin are inappropriately elevated, leading to disproportionate iron absorption over daily losses and gradual accumulation of iron (Andrews, 1999; Pietrangelo, 2004; Stuart et al., 2003).

In C282Y/C282Y hemochromatosis, LPI has been suggested to be marker of toxicity due to its potential for catalyzing the generation of reactive oxygen radicals in vivo (Le Lan et al., 2005). Serum NTBI levels were found elevated in both homozygotes and heterozygotes of the HFE C282Y mutation of hereditary hemochromatosis. The reported increased risk of cardiovascular events in heterozygous hemochromatosis may be explained by NTBI-catalyzed LDL peroxidation (de Valk et al., 2000). NTBI present in human sera has been proven to modify the levels of adhesion molecule expression and subsequently monocyte adherence to the endothelial cells which may contribute to cardiopathy. These events were reversed by the application of iron chelator further confirms the role of NTBI in pathogenesis (Kartikasari et al., 2004).

Non-transferrin bound iron (NTBI) is commonly detected in patients with systemic iron overload whose serum iron-binding capacity has been surpassed. It has been perceived as an indicator of iron overload, impending organ damage and a chelation target in poly-transfused thalassemia patients. Thalassemia is a hereditary anemia resulting from defects in hemoglobin production (Higgs et al., 2001). β- Thalassemia, which is caused by a decrease in the production of β-globin chains affects multiple organs and is associated with considerable morbidity and mortality (Cunningham et al., 2004). Such patients require lifelong care to delay the complications and maintain life (Old et al., 2001; Karnon et al., 1999). Hemolysis and ineffective erythropoiesis together cause the anemia that occurs in thalassemia. Regular transfusion therapy to maintain hemoglobin levels of at least 9 to 10 g/dL for
maintenance of growth and development and also reduces hepatosplenomegaly due to extramedullary hematopoiesis as well as bone deformities (Cunningham et al., 2004; Old et al., 2001). Each unit of blood contains 200 - 250 mg of iron and body iron stores are normally only 0.5 - 1.5 g. Since the body has no physiological mechanism for excreting iron, repeated blood transfusions result in iron accumulation in the reticuloendothelial system and parenchymal cells. The rate of iron accumulation is on average 0.5 mg/kg body weight/day (Hoffbrand, 2001). Iron overload causes most of the mortality and morbidity associated with thalassemia. Iron deposition occurs in visceral organs (mainly in the heart, liver, and endocrine glands), causing tissue damage and ultimately organ dysfunction and failure. However cardiac events due to iron overload are still the primary cause of death (Modell et al., 2000; Olivieri and Brittenham, 1997). Chelation therapy must be given to cause excretion of this amount of iron and so prevent early mortality from an iron-induced cardiomyopathy.

The association between iron overload diseases and NTBI is a well-documented phenomenon (Cabantchik et al., 2005; Hider et al., 2002). NTBI appear in plasma when Tf binding capacity for iron is exceeded due to massive iron overload, such as occurs in thalassemia major (Graham et al., 1979) and other congenital anemias (Porter, 2009), which require repeated blood transfusions, or bone marrow failure where iron utilization is inefficient (Bradley et al., 1997). NTBI is well documented in primary iron overload such as thalassemia intermedia or hemochromatosis even in the setting of < 100% Tf saturation (Gosriwatana et al., 1999; Le Lan et al., 2005; Pootrakul et al., 2004), due apparently to formation of insoluble polynuclear ferrirhydrate species (Evans et al., 2008) and complexes with modified albumin (Silva and Hider, 2009) that are inaccessible to Tf. The initial study of NTBI in the serum of thalassemia patients and others that followed led to the recognition that NTBI may be an important indicator of systemic iron overload and source of tissue iron accumulation (Hershko et al., 1978; Breuer et al., 2000). NTBI appears in iron overloaded plasma in multiple forms, some easily filterable via size exclusion leaving others protein bound, some complexed to small organic ligands which, in turn, might be free or adsorbed to proteins (Evans et al., 2008; Silva and Hider, 2009). Moreover, the composition of NTBI might vary with the degree and source of iron overload, treatment of the patient with chelator or phlebotomy.
2.7 Management of iron overload:

Redox active forms of iron predominantly found in iron overloaded conditions have been proven a dangerous chemical entity damaging various internal organs. In iron overloaded patients, the iron level is attempted to bring down to decrease the clinical manifestations and to manage the condition in a better way.

Currently, the mainstay therapy for excessive iron deposition in patients with primary and secondary hemochromatosis is phlebotomy and iron-chelation, respectively, which are designed to facilitate whole-body iron removal. In patients with primary hemochromatosis, a maintenance phlebotomy schedule should be continued following primary iron depletion to prevent re-accumulation of iron. A reasonable goal is to keep the serum ferritin concentration at 50 ng/mL or less (Niederau et al, 1996; Muhlestein, 2000; Niederau et al, 1985). Phlebotomy therapy that is initiated early can be expected to result in a normal lifespan; however, in patients with liver dysfunction, it can be the reverse (Niederau et al, 1996; Muhlestein, 2000; Niederau et al, 1985). Unfortunately, patients with primary hemochromatosis are often diagnosed and treated only after iron-overload becomes advanced (Niederau et al, 1996; Muhlestein, 2000; Niederau et al, 1985). In patients with secondary iron-overload, iron chelation therapy is the main therapy available. It utilizes the parenteral iron chelator, desferrioxamine, or the oral iron chelator, deferiprone. Chelation has been shown to improve ventricular function, prevent ventricular arrhythmias, and reduce mortality in patients with secondary iron-overload (Olivieri et al, 1994; Olivieri, 1999; Brittenham et al, 1994; Modell et al, 2000; Hahalis et al, 2005; Anderson et al, 2002).

In comparison to standard chelation mono therapy with desferrioxamine, combination treatment with additional deferiprone reduces myocardial iron, and improves ejection fraction and endothelial function in thalassemia major patients with mild-to-moderate cardiac iron loading. Though chelation therapy has been consistently shown to reduce the cardio vascular burden from secondary iron-overload, it is cumbersome, associated with toxic side effects, and has only a limited impact on clinical outcome (Olivieri et al, 1997; Hahalis et al, 2005). Patients with heart failure should be managed with the same basic principles as patients with dilated cardiomyopathy and systolic heart failure (Hahalis et al, 2005). Clearly, early use of
angiotensin-converting enzyme (ACE) inhibitors and beta-adrenergic blockers, together with device therapies such as pacemakers and automated internal defibrillators, should be routine therapy for patients with iron-overload systolic heart failure (Hahalis et al, 2005).

However in conditions where the iron is not overloaded but still toxicity exists, the anti oxidants are the probable way to minimize its detrimental effects. Such treatment is especially indicated in patients receiving iron intravenously. A single oral dose of 1200 IU of vitamin E efficiently reduces oxidative stress in patients receiving 100 mg of iron (III) hydroxide sucrose intravenously during a hemodialysis session, when taken before the hemodialysis session. This beneficial effect was demonstrated in patients on hemodialysis with normal vitamin E status before supplementation, indicating a protective effect of high plasma vitamin E concentrations.

To determine the efficacy of chelator, serum ferritin has been used as an indicative parameter for years. Serum NTBI is an emerging parameter for this purpose (Breuer et al, 2000). However this parameter needs to be more standardized before its wide scale application in clinical practice.

2.8 Clinical conditions included in the study:

2.8.1 Cardiac complications:

Myocardial infarction (MI) or acute myocardial infarction (AMI), commonly known as a heart attack, results from the interruption of blood supply to a part of the heart, causing heart cells to die. This is most commonly due to occlusion (blockage) of a coronary artery following the rupture of a vulnerable atherosclerotic plaque, which is an unstable collection of lipids (cholesterol and fatty acids) and white blood cells (especially macrophages) in the wall of an artery. The resulting ischemia and restricted availability of oxygen, if left untreated for a sufficient period of time, can cause damage or death (infarction) of heart muscle tissue (myocardium). Typical symptoms of acute myocardial infarction include sudden chest pain, shortness of breath, nausea, vomiting, palpitations, sweating, and anxiety (Mallinson, 2010). Women may experience fewer typical symptoms than men, most commonly shortness of breath, weakness, a feeling of indigestion and
fatigue (Kosuge et al. 2006). A sizeable proportion of myocardial infarctions (22-
64%) are "silent", that is without chest pain or other symptoms (Valensi et al, 2011).

Routinely done diagnostic tests are an electrocardiogram (ECG),
echocardiography, cardiac MRI and various blood tests. The most often used blood
markers are the creatine kinase-MB (CK-MB) fraction and the troponin levels.
Immediate treatment for suspected acute myocardial infarction
includes oxygen, aspirin, and sublingual nitroglycerin (Erhardt et al, 2002). Ischemic
heart disease was the leading cause of death for both men and women worldwide in
2004 (WHO, 2008). Important risk factors are previous cardiovascular disease, older
age, tobacco smoking, high blood levels of low density lipoprotein (LDL) and low
levels of high density lipoprotein (HDL) cholesterol, diabetes, high blood pressure,
lack of physical activity and obesity, chronic kidney disease, excessive alcohol
consumption, the abuse of illicit drugs (such as cocaine and amphetamines), and
chronic high stress levels (Steptoe and Kivimaki, 2012; Devlin and Henry, 2008;
European guidelines on cardiovascular disease prevention in clinical practice:
executive summary: Fourth Joint Task Force of the European Society of Cardiology
and Other Societies on Cardiovascular Disease Prevention in Clinical Practice).

The role of iron in coronary heart disease (CHD) was proposed by Sullivan in
1981 as an explanation for the sex difference in risk. Now an impressive body of
evidences indicates that free radicals and non-radical oxidants might cause a number
of cardiovascular dysfunctions. Both direct damage to cellular components and/or
oxidation of extracellular biomolecules, e.g. LDL, might be involved in the etiology
of cardiovascular diseases. The key molecules in this process seem to be iron and
copper ions which catalyse formation of the highly reactive hydroxyl radical (Kohgo
et al, 2008). In acute myocardial infarction LPI is suggested to be elevated when large
fluxes in iron occur (Baykan et al, 2001; Griffiths et al, 1985).

2.8.2 Diabetes mellitus:

Diabetes mellitus is a group of metabolic diseases due to impaired insulin
production or its action or both, in which a person has high blood sugar (Greenspan's
basic & clinical endocrinology, 2011). This high blood sugar produces the classical
symptoms of polyuria, polydipsia and polyphagia. If the proper and timely
intervention is not done this disease can cause many metabolic derangements involving CNS, kidney, retina and many others parts of the body.

There are three main types of diabetes mellitus (DM). Type 1 DM results from the body's failure to produce insulin where the patient is dependent on insulin to sustain life. This form was previously referred to as "Insulin-Dependent Diabetes Mellitus" (IDDM) or "juvenile diabetes". Type 2 DM results from insulin resistance, a condition in which cells fail to use insulin properly, sometimes combined with an absolute insulin deficiency. This form was previously referred to as Non Insulin-Dependent Diabetes Mellitus (NIDDM) or "adult-onset diabetes". The third main form, gestational diabetes occurs when pregnant women without a previous diagnosis of diabetes develop a high blood glucose level. It may precede development of type 2 DM.

Table: 2.9 Comparison of Type 1 and Type 2 Diabetes mellitus (Williams, 2011)

<table>
<thead>
<tr>
<th>Feature</th>
<th>Type 1 Diabetes</th>
<th>Type 2 Diabetes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onset</td>
<td>Sudden</td>
<td>Gradual</td>
</tr>
<tr>
<td>Age at onset</td>
<td>Mostly in children</td>
<td>Mostly in adults</td>
</tr>
<tr>
<td>Body</td>
<td>Thin or normal</td>
<td>Often obese</td>
</tr>
<tr>
<td>Ketoacidosis</td>
<td>Common</td>
<td>Rare</td>
</tr>
<tr>
<td>Auto-antibodies</td>
<td>Usually present</td>
<td>Absent</td>
</tr>
<tr>
<td>Endogenous insulin</td>
<td>Low or absent</td>
<td>Normal, decreased or increased</td>
</tr>
<tr>
<td>Concordance in identical twins</td>
<td>50%</td>
<td>90%</td>
</tr>
<tr>
<td>Prevalence</td>
<td>~10%</td>
<td>~90%</td>
</tr>
</tbody>
</table>

Other forms of diabetes mellitus include congenital diabetes, which is due to genetic defects of insulin secretion, cystic fibrosis-related diabetes, steroid diabetes induced by high doses of glucocorticoids, and several forms of monogenic diabetes.

All forms of diabetes have been manageable since insulin became available in 1921, and type 2 diabetes may be controlled with medications. Both types 1 and 2 are chronic conditions that cannot be cured. Pancreas transplants have been tried with
limited success in type 1 DM; gastric bypass surgery has been successful in many with morbid obesity and type 2 DM. Gestational diabetes usually resolves after delivery. Diabetes without proper treatments can cause many complications. Acute complications include hyperglycemia, diabetic ketoacidosis, or nonketotic hyperosmolar coma. Serious long-term complications include cardiovascular disease, chronic renal failure, and diabetic retinopathy (retinal damage). Adequate treatment of diabetes is thus important, as well as blood pressure control and lifestyle factors such as smoking cessation and maintaining a healthy body weight. Globally, as of 2012, an estimated 346 million people have type 2 diabetes (http://www.npr.org/blog/health/2012/06/21/155505445/how-to-spot-a-neglected-tropical-disease). Diabetes is associated with various micro-vascular and macro-vascular complications; oxidative stress is one of the main causes of diabetic complications (West, 2000; Rösen et al, 2001; Turk et al, 2002; Dierk et al, 2004). Iron being a transition metal in free form has been suggested to generate free oxygen radicals mainly by Fenton chemistry. Lee et al (2005) reported higher incidences of NTBI existence in diabetic patients as compare to normal. Sulieman et al (2004) reported higher mortality in cardiac patients with presence of NTBI as compared to its absence.

### 2.8.3 End stage renal disease and hemodialysis:

All individuals with GFR < 60 ml / min / 1.73 m² for 3 months are classified as having CKD, irrespective of the presence or absence of kidney damage.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>GFR (mL/min(1.73)m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Kidney damage with normal GFR</td>
<td>&gt;90</td>
</tr>
<tr>
<td>2</td>
<td>Kidney damage with mild decrease in GFR</td>
<td>60-89</td>
</tr>
<tr>
<td>3</td>
<td>Moderate decrease in GFR 30</td>
<td>30-59</td>
</tr>
<tr>
<td>4</td>
<td>Severe decrease in GFR</td>
<td>15-29</td>
</tr>
<tr>
<td>5</td>
<td>Kidney failure</td>
<td>&lt;15</td>
</tr>
</tbody>
</table>

Table 2.10: Staging of chronic kidney disease (Clellan et al, 1997)
REVIEW OF LITERATURE

The rational for including these individuals is that the reduction in the kidney function to this level or lower represents loss of half or more of the adult level of normal kidney function, which may be associated with a number of complications. In young adults, normal GFR is approximately 120-130 ml/min/1.73m$^2$ and decline with age (Smith, 1951; Davis and Shock, 1950; Lindeman et al, 1985; Rowe et al, 1976). All individuals with kidney damage are classified as having CKD, irrespective of the level of GFR.

Renal failure

Renal failure is a condition in which the kidneys fail to remove metabolic end products from the blood and regulate fluid, electrolyte, and pH balance of extracellular fluids. The underlying causes for the renal failure may be due to impairment in kidney or due to some systemic disease or urinary tract disorder of non renal origin.

Biochemically, renal impairment may be complete or partial is typically detected by an elevated serum creatinine. In physiology renal impairment is described as a decrease in glomerular filtration rate (GFR). When the kidneys malfunction, problems frequently encountered include abnormal fluid levels in the body, deranged acid levels, abnormal levels of potassium, calcium, phosphate, later anemic conditions (Hussain, 2006). When this disease is progressed from initial stages to end stage renal failure the measures of dialysis become necessary to save patient’s life (William, 2004).

Approximately 19 million of the United States adults suffer from chronic kidney disease (CKD) (Nolan and Anderson, 1998; Brady and Singer, 1995), and an estimated 80,000 persons annually are diagnosed to have chronic kidney failure.

Major causes of chronic renal failure are diabetes mellitus and hypertension, which account for approximately 60 percent of new cases.

Chronic renal failure is characterized by a slow, insidious, and irreversible impairment of renal excretory and regulatory function (William, 2004).

It is mainly caused by following reasons:
1. Chronic disease of the kidneys (ex. glomerulonephritis)
2. Chronic infection (ex. tuberculosis)
3. Anomalies exist since the child birth
4. Vascular disease (ex. hypertension)
5. Endocrine disorders (ex. diabetes)
6. Obstructive processes in the kidneys (ex: kidney stones)
7. Nephrotoxins (toxic chemicals that affect the kidneys)
8. Autoimmune diseases

Though several conditions and diseases can cause chronic kidney failure but diabetes and hypertension are the two most common causes (Kutty and Kocher, 2003). Patients of end stage renal disease are routinely treated by hemodialysis.

**Hemodialysis (HD)**

Hemodialysis removes waste and excess fluid from the blood when the kidneys cannot do so sufficiently. The blood is drawn intravenously, sent through a machine called a dialyzer and returned to the body through a blood vessel. Inside the dialyzer, the blood is passed over a membrane that filters waste and fluid into a dialysate solution. The dialysate is then pumped out to a disposal tank and new dialysate is pumped in the process of removing excess fluid is known as ultrafiltration.

The blood is circulated and diffused numerous times during a dialysis session; each circulation through the machine removes more waste and excess fluid. Hemodialysis is usually performed three or more times a week for 4 hours (Daugirdas et al, 2001; Daniel et al, 2007; Robert, 2004). Shah (2004) explained the role of iron in kidney damage through ROS production, and the cyclic regeneration of new NTBI which continue the detrimental cascade till the end stage disease. Nilsson et al suggested the clearance of serum NTBI after hemodialysis session where as controversially Prakash et al suggested hemodialysis as a culprit to generate NTBI due to its upsetting procedure (Nilsson et al, 2002; Prakash et al, 2005).

**2.8.4 β Thalassemia major:**

β thalassemias are a group of inherited blood disorders caused by reduced or absent synthesis of the β chains of hemoglobin resulting in variable phenotypes ranging from severe anemia to clinically asymptomatic individuals. Three main forms have been described: thalassemia major, thalassemia intermedia and thalassemia
minor. Individuals with beta thalassemia major usually present within the first two years of life with severe anemia, poor growth, and skeletal abnormalities during infancy. Affected children will require regular lifelong blood transfusion. Beta thalassemia intermedia is less severe than beta thalassemia major and may require episodic blood transfusions. Transfusion-dependent patients will develop iron overload and require chelation therapy to remove the excess iron. Bone marrow transplant can be curative for some children with beta thalassemia major (Muncie and Campbell et al, 2009). Transmission is autosomal recessive; however, dominant mutations have also been reported. Genetic counseling is recommended and prenatal diagnosis may be offered (Galanello and Origa, 2010).

Globally, there are at least 60,000 individuals born with Thalassemia Major (TM) each year (Weatherall and Clegg, 2001). Regular blood transfusions are mandatory for long-term survival, but over a period of years these cause a secondary state of tissue iron overload. Myocardial iron deposition can result in cardiomyopathy, and heart failure remains the leading cause of death (Olivieri et al, 1994; Borgna-Pignatti et al, 2004; Telfer et al, 2006). NTBI is well documented in thalassemia (Breuer et al, 2000). It is also been considered as a marker to determine the efficacy of chelator in iron overload patients (Hershko et al, 1978; Breuer et al, 2000).

2.8.5 Hematological malignancy:

Hematological malignancies are the types of cancer that affect blood, bone marrow and lymph node. As the three are intimately connected through the immune system, a disease affecting one of the three will often affect the others as well: although lymphoma is a disease of the lymph node, it often spreads to the bone marrow, affecting the blood and occasionally producing a paraprotein.

While uncommon in solid tumors, chromosomal translocation is a common cause of these diseases. This commonly leads to a different approach in diagnosis and treatment of hematological malignancies.

Redox active forms of iron, due to their ability to generate ROS, known to cause damage in DNA which have been considered as the cause of hematological cancer (Galaris et al, 2008). Furthermore in hematological malignancies NTBI is considered to be elevated due to temporary shutdown of bone marrow function and
utilization of iron. Elevated serum NTBI has been reported especially in patients treated chemotherapy (Bonsdorff et al, 2002; Bradley et al, 1997; Halliwell et al, 1988).

**Table 2.11: Relative proportions of hematological malignancies in the United States**
(Horner et al, 2009)

<table>
<thead>
<tr>
<th>Type of hematological malignancy</th>
<th>Percentage</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukemias</td>
<td>--</td>
<td>30.4%</td>
</tr>
<tr>
<td>Acute Lymphoid Leukemia (ALL)</td>
<td>4.0%</td>
<td>--</td>
</tr>
<tr>
<td>Acute Myeloid Leukemia (AML)</td>
<td>8.7%</td>
<td>--</td>
</tr>
<tr>
<td>Chronic Lymphoid Leukemia (CLL)</td>
<td>10.2%</td>
<td>--</td>
</tr>
<tr>
<td>sorted under lymphomas according to current WHO classification; called small lymphocytic lymphoma (SLL) when leukemic cells are absent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic Myelogenous Leukemia (CML)</td>
<td>3.7%</td>
<td>--</td>
</tr>
<tr>
<td>Acute Monocytic Leukemia (AMOL)</td>
<td>0.7%</td>
<td>--</td>
</tr>
<tr>
<td>Other Leukemias</td>
<td>3.1%</td>
<td>--</td>
</tr>
<tr>
<td>Lymphomas</td>
<td>--</td>
<td>55.6%</td>
</tr>
<tr>
<td>Hodgkin’s lymphoma (all four subtypes)</td>
<td>7.0%</td>
<td>--</td>
</tr>
</tbody>
</table>
2.9 Biochemical parameters included in the study:

2.9.1 Serum total iron, TIBC and transferrin saturation:

These parameters determine the level of iron present in the serum. It does not include the heme iron present in the blood. Serum iron is almost completely in association of a β1 globulin protein Transferrin (Tf). Its molecular weight is 70,000 dalton and it can bind with 2 atoms of iron in ferric (Fe³⁺) state synergistically in presence of HCO₃⁻. Normal conditions Tf is 30-33% saturated with iron. Major function of Tf is to transport iron to RE cells and bone marrow for its incorporation into immature RBC (Chattergea and Shinde, 2005). The diurnal variation observed in this parameter, with lower values in the evening and higher values observed in morning hours (Vincent, 2000).

Increased serum iron is observed in hemocromatosis, hemosiderosis, hemolytic anemia, hepatitis, hepatic necrosis, lead toxicity. Decreased serum iron is observed in insufficient dietary intake, chronic blood loss, inadequate intestinal absorption of iron, pregnancy, iron deficiency anemia and neoplasma. Increased TIBC is observed in estrogen therapy, pregnancy, polycythemia vera and iron deficiency anemia. Decreased TIBC has been reported in malnutrition, hypoproteinemia, inflammatory disease, cirrhosis, hemolytic anemia, pernicious anemia and sickle cell anemia. % Tf saturation increased in hemochromatosis, hemolytic anemia and increased iron intake whereas it decreases in iron deficiency anemia and chronic illness.

These parameters have been regularly studied in patients of iron deficiency as well as iron overload. Iron deficiency anemia results in low serum iron level and decreased transferrin saturation. Hemochromatosis is a common genetic disorder causing iron overload due to increased iron absorption from intestine. However in conditions like β thalassemia major where frequent blood transfusion is must to sustain life, iron gets overload in the body. In this case there are chances that the levels of Tf saturation increase with probable risk of NTBI existence.
2.9.2 Serum cholesterol:

Cholesterol is a lipid found in the cell membranes of all tissues, and it is transported in the blood plasma of all animals. Most of the cholesterol is synthesized by the body and some has dietary origin. It is insoluble in blood, but is transported in the circulatory system bounded to one of the varieties of lipoprotein. Major dietary sources of cholesterol include eggs, beef, poultry, and shrimp. Plants have trace amounts of cholesterol. Plant products (e.g. flax seed, peanut) also contain cholesterol like compounds, phytosterols which are suggested to help lower serum cholesterol (Ostlund et al, 2003). Cholesterol is required to build and maintain cell membranes. Some researches indicate that cholesterol may act as an antioxidant (Smith, 1991), it also aids in the manufacture of bile. It is the major precursor for the synthesis of vitamin D and of the various steroid hormones. Recently, it has also been implicated in cell signalling processes, where it forms lipid rafts in the plasma membrane. It also reduces the permeability of the plasma membrane to hydrogen ions (protons) and sodium ions (Baylor, 2006). It is either synthesized in the endoplasmic reticulum or derived from the diet, in which case it is delivered by the blood stream in low density lipoproteins. Cholesterol is primarily synthesized from acetyl CoA through the HMG-CoA reductase pathway in many cells and tissues (Haines, 2001).

The purpose of cholesterol testing is to identify the patients at risk of atherosclerotic heart disease. Cholesterol has been routinely done as a part of lipid profile which also evaluates lipoproteins and triglycerides because by itself cholesterol is not a totally accurate predictor of heart disease. The increased level of cholesterol is observed in familial hypercholesterolemia, hyperlipidemia, uncontrolled diabetes mellitus, nephritic syndrome, hypertension, myocardial infarction, biliary cirrhosis and other hormonal derangement causing enzymatic deficiencies in lipid metabolism (Pagana and Pagana, 2006). The decreased cholesterol has been observed in malnutrition, advanced stages of cancer, hyperthyroidism, pernicious anemia, sepsis, liver disease, hemolytic anemia (Pagana and Pagana, 2006).
2.9.3 Serum triglyceride:

Triglycerides are the chemical form in which most fat exists in food as well as in the body. They are also present in blood plasma and, in association with cholesterol, form the plasma lipids.

Triglycerides in plasma are derived from dietary lipids or made in the body from other energy sources like carbohydrates. Calories ingested in a meal and not used immediately by tissues are converted to triglycerides and transported to fat cells to be stored. Hormones regulate the release of triglycerides from fatty tissues so they meet the body's needs for energy between meals (Terres et al, 1991).

Excess triglycerides in plasma are called hypertriglyceridemia. It is linked to the occurrence of coronary artery disease in some people. Increased TG levels are observed in glycogen storage disease, apoprotein C-II deficiency, hypothyroidism, high carbohydrate diet, diabetes mellitus, nephritic syndrome and chronic renal failure. Increased TG is seen in mal-absorptive syndrome, malnutrition, abetalipoproteinemia and hyperthyroidism (Pagana and Pagana, 2006).

2.9.4 Serum lipoproteins:

Lipoproteins are the proteins in blood which transport cholesterol, triglycerides and insoluble fats. They are used as markers indicating level of lipids within the blood stream. These lipoproteins can be grouped in to Chylomicrons which are primary triglycerides, LDLs (beta LP) which are primary cholesterol, VLDL (pre beta LP) which are mainly TG and HDL (alpha LP) which are predominantly proteins with small amount of cholesterol (Pagana and Pagana, 2006).

Chylomicrones (CM) are packaged by the enterocytes and released in the circulation as nascent chylomicrons that will mature following the acquisition of apolipoproteins Apo E and Apo C from high-density lipoprotein (HDL)-2. Mature chylomicrons undergo hydrolysis of their triglyceride content by lipoprotein lipase (LPL) in the capillaries that supply muscles and adipose tissues. This leads to the release of free fatty acids (FA) and their uptake by the surrounding adipocytes / myocytes and the formation of Chylomicrons remnants, which are subsequently cleared by the liver and other tissues via Low density lipoproteins (LDL) receptor-related protein (LRP) (Takahashi et al, 1992).
Very low-density lipoproteins (VLDL) are produced by hepatocytes and released into the circulation as nascent VLDL, which mature following the acquisition of Apo E and Apo C from HDL-2.

Mature VLDL undergoes lipolysis of its triglyceride (TG) content by LPL in the capillaries supplying muscles and adipose tissues. This leads to the release and uptake of FA by the surrounding adipocytes/myocytes and the formation of VLDL remnants, commonly known as intermediate-density lipoproteins (IDL). Most of the IDL formed in this manner undergo further lipolysis by hepatic lipase to become LDL.

The primary function of HDL is the retrieval and transport of surplus cholesterol from the extra hepatic tissues for disposal in the liver (Genest et al., 1999). This process, which is commonly known as reverse cholesterol transport, is critical for cellular cholesterol homeostasis and protection against atherosclerosis, renal disease, and other complications.

Increased LDL and VLDL has been observed in familial LDL lipoproteinemia, nephritic syndrome, hypothyroidism, high alcohol consumption, chronic liver disease, hepatoma, gammopathies, familial hypercholesterolemia type IIa, cushing syndrome, apo-protein CII deficiency where as decreased has been observed in familial hypolipoproteinemia, hypoproteinemia and hyperthyroidism.

Decreased HDL is seen in metabolic syndrome, familial low HDL, hepatocellular disease and hypoproteinemia.

Dyslipidemia is often observed in patients with chronic renal failure, resulting in abnormal concentrations and composition of plasma lipoproteins. Plasma triglyceride concentration is frequently elevated in patients with CRF. However, plasma cholesterol concentration is usually normal, even reduced and only occasionally elevated in patients with End-Stage Renal Disease (ESRD). Elevation of plasma triglycerides in ESRD patients is accompanied by increased plasma concentration and impaired clearance of VLDL. This is associated with the accumulation of atherogenic VLDL remnants, commonly known as IDL (Majumdar and Wheeler, 2000). Similarly, clearance of Chylomicrons is impaired and plasma concentration of Chylomicrons remnants is elevated in CRF patients. In contrast, plasma concentration of LDL is usually normal and only occasionally elevated in ESRD patients. Plasma HDL concentration is consistently reduced, and maturation of
cholesterol ester-poor HDL-3 to cholesterol ester rich cardio-protective HDL-2 is impaired in CRF (Gutman et al, 1973). In addition to these quantitative abnormalities, the composition of plasma lipoproteins is altered in CRF e.g. the cholesterol content of VLDL is relatively increased and its triglyceride content is relatively reduced in CRF. In contrast, CRF results in a relative reduction in cholesterol and relative increase in the triglyceride content of LDL. Similarly, cholesterol ester and free cholesterol content of HDL are consistently reduced, whereas its triglyceride content is elevated in CRF (Tsaltas and Friedman, 1968). The above compositional abnormalities are present in nearly all patients with mild to severe renal insufficiency (even those with normal plasma total cholesterol and triglyceride levels) and point to redistribution of cholesterol from HDL to VLDL and IDL and defective removal of triglycerides from LDL and HDL particles (Fytili et al, 2002; Bagdade et al, 1976; Chan et al, 1981).

Insulin associated with diabetes mellitus is also an important hormone which and influence the lipid metabolism. It increases the cholesterol synthesis and increases the activity of lipoprotein lipase in white adipose tissue. Insulin increases the number of LDL receptors, so chronic insulin deficiency might be associated with a diminished level of LDL receptors. This causes the increase in LDL particles and result in the increase in LDL cholesterol value in DM (Surayawanshi et al, 2006).

Abnormal lipid metabolism results in cardiac disease like myocardial infarction, which leads to the death of the heart tissues. Comparison of the lipid profile of cardiac patients, diabetic patients and normal persons showed that as compared to normal persons, both the cardiac and diabetic patients have elevated level of total cholesterol, triglyceride and LDL-cholesterol, but lower level in HDL-cholesterol the age dependent manner. Female have higher HDL-cholesterol level than men and therefore they are less prone to cardiac diseases (Irshad et al, 2004).

2.9.5 Plasma glucose:

Glucose is an aldo-hexose acts as energy currency of the biological system which is necessary for maintaining normal body tissue functioning. Although most glucose comes from dietary intake of carbohydrates, liver can convert fats and protein into glucose when not enough glucose is available to the cells. Liver also stores extra
glucose in form of glycogen. With the excess of glucose intake the glucose which
can’t be stored in the glycogen can get converted to fat and gets stored in adipose
tissue. The level of glucose is influenced by hormones like insulin, glucagon,
corticosteroids, epinephrine, and growth hormone; many drugs and conditions like
pregnancy.

Increased level of plasma glucose is seen in diabetes mellitus, cushing
syndrome, pheochromocytoma, chronic renal failure, glucagonoma, acute pancreatitis,
diuretic therapy, corticosteroid therapy and acromegaly. Whereas decreased plasma
sugar is observed in hormonal abnormalities like insulinoma, hypothyroidism, addison’s disease; extensive liver disease and in conditions like insulin over dose and
starvation.

Elevation in serum or plasma sugar level is a common finding in diabetes
mellitus (Blessing et al, 2011). Goyal et al (2006) have made an important
contribution to our knowledge of glucose metabolism during acute myocardial
infarction. Glucose levels at admission as well as after 24 hours have strong
prognostic implications but more importantly, the change in glucose in the first 24
hours was shown to be an independent predictor of adverse outcome. Abnormalities
of glucose metabolism and hyperinsulinemia have been demonstrated in patients with
end-stage renal disease (Leonardo et al, 2002).

2.9.6 Serum albumin and total protein:

Proteins are important constituents of muscles, enzymes, hormones,
transporters and several other key functional and structural entities of the body which
confer the osmotic pressure within the vascular space. Total serum proteins are
combination of pre-albumin, albumin and globulin.

Serum albumin is a most abundant blood plasma protein and is produced in
the liver and forms a large proportion of all plasma proteins. It normally constitutes
about 60% of human plasma protein, being synthesized primarily by hepatic
parenchymal cells except in early fetal life, when it is synthesized largely by the yolk
sac (Johonson et al, 1999). The rate of synthesis is controlled primarily by colloidal
osmotic pressure and secondarily by protein intake and the normal plasma half-life of
albumin is 15 to 19 days (Gltlin and Perricelli, 1970).
Levels of serum proteins are measured routinely to evaluate the nutritional status. Decreased total protein level is observed in malnutrition, pregnancy, liver disease, protein loosing enteropathies, protein loosing nephropathies, increased capillary permeability, inflammatory disease, and familial idiopathic dys-proteinemia due to decreased level of albumin. Increased level of total protein is observed in multiple myeloma, chronic inflammatory disease, malignancies, hyper-immunization, cirrhosis, acute and chronic infection due to increased level of gamma globulin. There are several conditions in which albumin level decreases and globulin level increases in the serum, so total protein can be misleading and serum albumin/globulin must be checked.

Decreased level of serum protein due to its filtration through glomeruli has been well accepted in CRF patients. Dialysis used as a treatment can further worsen the conditions due to loss of amino acids, peptides and protein while dialysis procedure (Victoria and Joel, 2000), which in turn increase the dietary need of the proteins. Serum albumin concentration at the start of chronic dialysis therapy is a good predictor of prognosis (Himmelfarb et al, 1977). In the study of Joki et al (2001), hypo-albuminaemia at the initiation of dialysis has been found as an important predictor of advanced CAD, particularly in male diabetic patients.

2.9.7 Serum creatinine:

Creatinine is a catabolic end product of creatine phosphate, which is used in skeletal muscle contraction. The daily production of creatine and subsequently creatinine depends on muscle mass which is quite stable. It gets excreted by kidney and so any dysfunctioning of kidney cause retention of this material in body and elevation of creatinine in serum.

The serum creatinine test along with BUN is used to diagnose impaired renal function, unlike the BUN, the creatinine level is least affected by hepatic function. Increased serum creatinine is observed in disease affecting renal functions, rhabdomyolitis (skeletal muscle injury causing release of myoglobin in blood stream), acromegaly and gigantism. Whereas decreased level is observed in decreased muscle mass.
In kidney failure patients, creatinine builds in the blood until it is removed by dialysis. A high level of creatinine may cause itching and damage to nerve endings. High creatinine levels may cause numbness and tingling of toes (Washington, 2002).

2.9.8 Serum urea:

Urea is formed in the liver as the end product of protein metabolism and digestion. During ingestion, protein is broken down to amino acid. In liver these amino acids are catabolized to form ammonia, which are combined to form urea. Urea is then deposited in the blood and transported to kidney for excretion. Thus urea is directly related to metabolic functions of liver and excretory functions of kidney. It serves as an index for functions of these organs. Blood urea level gets influenced by protein intake, level of muscle mass, GI bleeding and over hydration.

The increased level of serum urea is observed in pre renal conditions like hypovolemia, shock, burns, dehydration, congestive heart failure, myocardial infarction, GI bleeding, excessive protein ingestion, excessive protein catabolism, starvation; renal conditions like renal disease, renal failure and nephrotoxic drugs and in post renal azotemia due to obstruction. Decreased serum urea level is seen in liver failure, over hydration, negative nitrogen balance, pregnancy and nephritic syndrome. Renal dysfunction predicts increased mortality in cardiovascular patients. High creatinine and urea levels in diabetic patient may indicate a pre-renal problem such as volume depletion. Judykay (2007) suggested that high creatinine levels observed in diabetic patients may be due to impaired function of the nephrons.

2.9.9 Serum uric acid:

Uric acid is a non protein nitrogenous compound that is the final breakdown product of purine catabolism. It is primarily made by liver of which 75% of uric acid is excreted by kidney and 25% by intestinal tract.

Increased level of uric acid is observed in increased ingestion of purines, genetical error in purine metabolism, metastatic cancer, multiple myeloma, leukemias, cancer chemotherapy and hemolysis. Decreased level is seen in chronic renal failure, acidosis and hypothyroidism.

Uric acid is associated with cardiovascular morbidity and mortality (Alderman, 2002). Hyperuricemia is associated with carotid atherosclerosis. Serum uric acid
levels are a strong independent predictor for coronary heart disease (Longo-Mbenza et al, 1999), total mortality in non-diabetic subjects and stroke in non-insulin-dependent diabetics, poor outcomes and further vascular events in all stroke patients, and also higher mortality in hemodialysis patients.

2.9.10 Serum creatine kinase and creatine kinase-MB:

CK is predominantly found in skeletal muscle and brain, as well as in heart muscle. Its molecular weight is 85,000 daltons and exists in several iso-enzymatic forms. It is formed from two dimers, M and B (each with a molecular weight of 40,000 daltons), and thus three different iso-enzymes are possible- MM, BB and MB with their predominant existence in skeletal muscle, brain and heart respectively.

The value of serum CK may gets influenced by strenuous exercise and recent surgery, early pregnancy and muscle mass. The increased level of total CK value is seen in disease or injuries affecting heart muscle, skeletal muscle and brain. Increase in CK-BB sub-fraction is found in disease affecting CNS, electro convulsion therapy, adeno carcinoma and pulmonary infarction. Increase in CK-MB fraction is observed in AMI, cardiac defibrillation, myocarditis, ventricular arrhythmia, cardiac ischemia. Increased CK-MM specifically gets elevated in rhabdomyolysis, muscular dystrophy, myositis, recent surgery, trauma, shock, and hypothyroidism.

Table 2.12: Characteristic features of CK iso-enzymes (Vasudevan, 2011)

<table>
<thead>
<tr>
<th>Iso-enzyme</th>
<th>Electrophoretic mobility</th>
<th>Tissue of origin</th>
<th>Mean % in blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>MM (CK3)</td>
<td>Least</td>
<td>Skeletal muscle</td>
<td>80%</td>
</tr>
<tr>
<td>MB (CK2)</td>
<td>Intermediate</td>
<td>Heart</td>
<td>5%</td>
</tr>
<tr>
<td>BB (CK1)</td>
<td>Maximum</td>
<td>Brain</td>
<td>1%</td>
</tr>
</tbody>
</table>

After MI, CK-MB rise at about 4-6 hours, reaches to peak at 12-24 hours and returns to normal by 12-48 hours if there is no further damage (Pagana and Pagana, 2006). CK-MB iso-enzyme level is helpful in both quantifying the degree of infarction and timing of onset of infarction. It is often used to determine the efficacy of thrombolytic treatment (Pagana and Pagana, 2006).
2.9.1 Serum lactate dehydrogenase:

LDH is a ubiquitous tissue enzyme that catalyses the reaction of pyruvate to lactate using nicotinamide adenine dinucleotide (NAD).

As LDH is widely distributed through the body the level of total LDH is a non specific marker. However analysis of the iso-enzyme can generate more significant information about the specific organ damage. In myocardial infarction, total LDH activity is increased, while H4 iso-enzyme is increased 5-10 times more. The LDH level starts to rise within 6-12 hours of infarction, peaks at 24-48 hours, and return to baseline at 6-8 days.

Table 2.13: Characteristic features of LDH iso-enzymes (Vasudevan, 2011)

<table>
<thead>
<tr>
<th>Iso-enzyme</th>
<th>Subunit</th>
<th>Electrophoretic mobility at pH 8.6</th>
<th>Activity at 60°C for 30 min.</th>
<th>Tissue of origin</th>
<th>Mean % in blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>LDH-1</td>
<td>H4</td>
<td>Fastest</td>
<td>Not destroyed</td>
<td>Heart muscle</td>
<td>30%</td>
</tr>
<tr>
<td>LDH-2</td>
<td>H3M1</td>
<td>Faster</td>
<td>Not destroyed</td>
<td>RBC</td>
<td>35%</td>
</tr>
<tr>
<td>LDH-3</td>
<td>H2M2</td>
<td>Fast</td>
<td>Partially destroyed</td>
<td>Brain</td>
<td>20%</td>
</tr>
<tr>
<td>LDH-4</td>
<td>H1M3</td>
<td>Slow</td>
<td>Destroyed</td>
<td>Liver</td>
<td>10%</td>
</tr>
<tr>
<td>LDH-5</td>
<td>M4</td>
<td>Slowest</td>
<td>Destroyed</td>
<td>Skeletal muscle</td>
<td>5%</td>
</tr>
</tbody>
</table>

Increased level of LDH is seen in hemolytic anemia, hepatocellular damage, muscular dystrophy, carcinoma, leukemia and any condition which causes necrosis of body cells. The study of iso-enzyme of LDH is of more significance (Vasudevan, 2011).

2.9.12 Serum aspartate amino transferase:

AST catalyzes the transfer of an amino group between aspartic acid and pyruvate to form oxaloacetate (alpha-ketoglutarate) and alanine. The level of AST is very high in metabolically active tissues like heart muscle cell, liver, skeletal muscle cells and to lesser degree in kidney, pancreas, RBC. The enzyme exists in two structurally different forms; one is found principally in the cytoplasm and the other in the mitochondria. The cytosol form that is found in serum and its half-life in serum is probably about 20 hours.
Increased level of AST is observed in heart disease like MI, cardiac operation, cardiac catheterization and angiopathy; liver disease like hepatitis, liver cirrhosis, hepatic metastasis, hepatic surgery; skeletal muscle disease like skeletal muscle trauma, multiple traumas, progressive muscular dystrophy, heat stroke, primary muscle disease and other diseases like acute hemolytic anemia, pancreatitis.

AST was used as a marker of myocardial ischemia in olden days. The AST level starts to rise within 24-36 hours of infarction, peak at 4-5 days and return to baseline at 10-12 days. It is a marker of liver injury and shows moderate to drastic increase in parenchymal liver diseases like hepatitis and malignancies of liver (Vasudevan, 2011). Increased AST is reported in iron overload patients suffering from liver dysfunction along with increase in NTBI (Harrison et al, 1996).

2.9.13 Serum alanine amino transferase:

ALT catalyzes the transfer of an amino group between aspartic acid and pyruvate to form oxaloacetate (alpha-ketoglutarate) and alanine. It is predominantly found in liver, lesser quantity is found in kidney, heart and skeletal muscle.

Very high ALT values are seen in acute hepatitis, moderate increases are seen in chronic liver diseases. The increase ALT activities are also found in myocardial infarction, renal infarction, progressive muscular dystrophy, gaucher’s disease, niemann-pick disease, infectious mononucleosis, myelocytic leukemia, diabetic ketoacidosis and hyperthyroidism (Vasudevan, 2011; Kaplan, 1996). Erhardt et al (1974) reported that a late rise in ALT following acute myocardial infarction (AMI) beginning about 4 days after the AST maximum.

2.9.14 Serum potassium:

Potassium is the major cation within the cell, and difference between the intracellular and extracellular potassium level is important in maintaining membrane electrical potential especially in neuromuscular tissues. Potassium is excreted by kidney without getting reabsorbed. So if the potassium is not adequately supplied to the body, serum potassium level drops rapidly. Potassium is important for protein synthesis and maintenance of oncotic pressure and cellular electrical neutrality as well as maintenance of pH by kidney. The level of serum potassium is influenced by aldosterone, sodium reabsorption and pH of the system. It is essential for proper
functioning of muscle including heart. Too much or too little potassium can cause the heart to stop working. There is a long list of diets which have high potassium foods. Increased level of potassium is observed in excessive intake through diet or direct iv infusion, acute or chronic renal failure, hyperaldosteronism, aldosterone inhibiting diuretics, crush injuries to tissue, hemolysis or transfusion of hemolysed blood, infection, acidosis and dehydration. Decreased potassium is observed in low dietary intake, burns, GI disorders, cushing syndrome, alkalosis etc.

In CRF patients the diseased kidney is not normally able to rid the blood of potassium that is eaten in foods and the serum (blood) potassium increases. So in such cases salt substitutes are to be avoided because they are made from potassium (Tietz, 1999). During hemodialysis, diffusion occurs inside the dialyzer, causing potassium ions to leave the blood and enter the dialysate’s. As long as the dialysate’s concentration of potassium is lower than the blood level, serum potassium will continue to drop (the potassium ions in the blood will continue to cross over the dialyzer membrane and then simply go down the drain. This slow removal of potassium usually occurs throughout the entire dialysis treatment.

2.9.15 Serum chloride:

Chloride is the major extracellular anion, works primary to maintain electrical neutrality mostly as a salt with sodium. Chloride is necessary for nerves and muscles to work together. There are two main re-absorption mechanisms for chloride the first is via an antiporter in exchange for secretion of other anions. The seconds occurs in the final two thirds of the proximal tubule. In cortical nephrons, cell membrane permeability to chloride is increased and re-absorption is driven by the concentration gradient, which is accompanied by passive sodium re-absorption (Tietz, 1999).

Increased level of serum chloride is observed in dehydration, excessive infusion of normal saline, metabolic acidosis, renal tubular acidosis, cushing syndrome, kidney dysfunction, hyper parathyroidism. Decreased chloride level is seen in over hydration, syndrome of inappropriate secretion of anti-diuretic hormone, congestive heart failure, vomiting, chronic diarrhea, addison’s disease diuretic therapy etc.
2.9.16 Serum sodium:

Sodium is the major cation of extracellular fluid necessary for maintaining the hydration in body. The higher the level of sodium in the blood, the more water is retained by the body. Too much sodium will make the person thirsty and lead to edema and also cause shortness of breath. Extra sodium and water in body may cause rise of blood pressure.

Increased sodium level is observed in increased dietary sodium intake, excessive sodium iv fluid, cushing syndrome, hyperaldosteronism, GI fluid loss, excessive sweating, diabetes insipidus and osmotic diuresis. Decreased sodium is seen in low intake in diet, increased loss of sodium in addison’s disease, diuretic administration, chronic renal insufficiency etc.

CRF patients retain nitrogenous wastes and have an elevated plasma osmolality. While urea exhibits osmotic activity in serum, no sustained gradient can be stabilized across cell boundaries because it readily diffuses through cell membranes. Thus, sodium remains the major indicator of body tonicity and determines the distribution of water across the intracellular - extracellular boundaries; subsequently increase in cell volume, thirst, and among patients with renal insufficiency, systemic blood pressure. As a result of highly conserved plasma tonicity control systems, uremic subjects demonstrate remarkable stability of their serum sodium. Dialysate is a synthetic interstitial fluid capable of reconstituting extracellular fluid composition through urea extraction and extremely efficient solute and solvent (salt and water) transfer to the patient (Michael, 2000).