Chapter 5
Discussion
5.1 Iron:

Iron is an essential trace element present in the earth crust. The role of iron in biological system especially in human beings is a well established fact. Being an integral part of both heme and non-heme compounds as a structural component it plays crucial role. There are wide spectrum of functions which include transport of oxygen, oxidation and the electron transfer mechanism through which the high energy molecule ATP is synthesized.

Iron is a known one way element with very few ways to get out of the body. Nutritional deficiency of iron is quite prevalent in India especially in people of low income group, women and vegetarians. Iron overload is not a common finding in Indian population unlike iron deficiency. High incidences of iron deficiency could be correlated with high proportion of vegetarian community in India, as vegetarian food items are poor source of iron as compared to non-vegetarian food stuffs. On the other hand iron overload is greatly restricted by the physiological mechanisms through strict check post at gastrointestinal absorption level, which sense the need and availability of the body iron and increase or decrease the iron absorption accordingly.

From the gastrointestinal tract, absorbed iron is taken up by iron-binding proteins, the most predominantly by transferrin (Tf) wherein apo-transferrin binds with two molecules of ferric iron (Fe$^{3+}$) (Vasudevan, 2011). Once absorbed and bound to apo-transferrin, iron gets internalized in the organs like liver and bone marrow for storage purpose in the form of ferritin or hemosiderin or gets utilized for the hemoglobin synthesis. Presence of Tf receptors on the organs’ cells play essential role in the uptake of iron in mammals. The mechanism of Tf binding on the cell surface was explained by Richardson and Ponka (1997); however there are few mechanisms which explain internalization of iron, independent of Tf in various organs (Prus and Fibach, 2011).

5.1.1 Serum iron and iron binding capacity:

The total content of iron in an adult body is 3-5 g (Satyanarayana and Chakrapani, 2009). From which chief portion is present within RBCs and the rest is in liver, bone marrow and muscle. The major form of iron present in the blood is hemoglobin, which is an intracellular iron. The extracellular iron form in the blood is
transferrin, a glycoprotein synthesized in liver which is the predominant form of blood iron. Normal serum transferrin level is 250 mg/dL (Vasudevan and Shreekumari, 2001). Each transferrin molecule can carry two atoms of ferric (Fe$^{3+}$) iron. Plasma transferrin can bind with total 400 µg/dL iron which is termed as Total iron Binding Capacity (TIBC). One third of the total capacity is actually saturated in normal subjects, which in turn represent the measure of serum iron level which is about 120 µg/dL. The portion of transferrin bearing vacant sites for iron binding is termed as Unbound Iron Binding Capacity (UIBC). In iron over load conditions, the level of serum iron get increased and results in high percentage of Tf saturation, whereas opposite is true in case of iron deficiency, wherein the TIBC also gets elevated.

### 5.1.2 Iron ligands:

The transitional nature of atomic iron, due to its incompletely filled 3d orbital shell, makes it an extremely useful physiological ligand and catalyst. Iron can form ligands with albumin, transferrin, phosphates, citrates, hemoglobin, pyruvate, lactoferrin, acetate, hemosiderin as well as ferritin. However, the predominant forms in normal conditions are hemoglobin, albumin, transferrin and ferritin (Roche et al, 2008; Farnaud & Evans, 2003; Skutches et al, 1979; Knovich et al, 2008). The other low molecular weight ligands are more often associated with the iron mediated oxidative stress.

### 5.2.1 NTBI: Heterogeneous nature:

Non transferrin bound iron (NTBI) is the term used for the free iron present in plasma, including 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.

There is sparse information available about the biochemical nature of NTBI, but one may speculate that it is composed of a heterogeneous mixture of complexes whose composition might vary with the degree and type of iron-overload. The solubility of the ferric ion (Fe$^{3+}$), in physiological salt solutions is extremely low, so unless ligands capable of forming multiple coordination points for stable binding are available, it forms insoluble polynuclear aggregates of chlorides and oxides. The
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Citrate anion is one such ligand, and analysis of NTBI in sera from hemochromatotic patients by HPLC and high-resolution nuclear magnetic resonance indicated the presence of citrate-iron and ternary citrate-acetate-iron complexes (Grootveld et al., 1989). Albumin is another candidate ligand, and has been shown to form complexes with Fe$^{3+}$ and with Fe$^{3+}$-citrate in vitro (Lovstad, 1993). Although the affinity of albumin for trivalent metals is rather low, this might be compensated by its high concentration in serum (approx. 0.5 mM). Evidence for the heterogeneous nature of NTBI is provided by its variable accessibility to chelators. High-resolution NMR analysis showed the chelation of NTBI by the high-affinity chelator desferrioxamine (DFO) to be a very slow process, requiring several hours even at 1 mM chelator concentration (Grootveld et al., 1989). Hemochromatotic sera, with Tf saturation levels in the range 80-85% showed no detectable DFO-chelatable iron, according to Grootvelt et al. (1989). Whereas NTBI was traced in these sera after mobilization with oxalate (Breuer et al., 2001). In contrast, thalassemic sera, with Tf-saturation levels >90%, were found to contain both DFO-chelatable iron and NTBI that was mobilized with oxalate. These observations indicate that there are at least two forms of NTBI, one accessible to DFO and the other not. Hemochromatosis patients tend to have only the DFO-inaccessible form, whereas, β-thalassemic patients have both (Breuer et al., 2000). Presumably these forms differ in their ligand types and degrees of aggregation.

5.2.2 NTBI: Different names in vogue:

Along with the nature, wide spectrum of names is in vogue for NTBI or its subfractions. 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. Paffetti et al. (2006) used the term” free iron” to indicate low molecular weight iron free from high affinity binding plasma proteins. Breuer and Cabantchik (2001) suggested that the NTBI denotes those forms of iron in serum which 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).

NTBI is also named according to its chelatability by various researchers e.g. “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 antibiotic bleomycin (von Bonsdorff et al, 2002). “Desferrioxamine chelatable iron” (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) was used 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 BPS (Nilsson et al, 2002).

5.2.3 NTBI existence: Why and When:

There is 20-35% saturation of Tf at the normal serum iron level (120 µmol/L), the only major form of non-heme iron present in plasma. In normal physiological conditions 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 NTBI is often undetectable by most of the methods, and if appeared the level do not exceed 1 µmol/L in normal healthy individual (Anderson, 1999).
Many researchers reported absence of NTBI or its sub-fraction in normal subjects. Nilsson et al. (2002) reported absence of NTBI in control subjects in small study group with BPS based colorimetric method. Esposito et al. (2003) reported absence of labile plasma iron in healthy individuals. Breuer et al. (2001) reported absence of desferrioxamine (DFO) chelatable forms of NTBI in normal sera with the use of fluorescent based method. However with the advantage of more sensitive methods low levels of NTBI have been reported in normal subjects too by various researchers.

Jakeman et al. (2001) reported ≤ 0.1 µmol/L NTBI in normal subjects with graphite furnace atomic absorption spectrometry. 0.154 ± 0.328 µmol/L and 0.038 ± 0.07 µmol/L NTBI and LPI have been reported respectively by Caroline et al. (2005) with a fluorescent based method. Shetty et al. (2008) reported 0.5 ± 0.2 µmol/L free iron in healthy control with the use of BPS based spectrophotometric method. With HPLC based method, Lee et al. (2006) found 0.04 ± 0.13 µmol/L NTBI in control subjects. With the use of bleomycin based colorimetric method, Lele et al. (2009) reported 0.1± 0.06 µmol/L catalytic iron in control group.

Existence of NTBI is convincingly explainable in iron overload conditions, where Tf saturation is high and free plasma iron may appear as a spill over. But in conditions wherein Tf if not fully saturated attendance of NTBI is difficult to explain (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). Logically in presence of vacant sites on Tf, the iron should not be free as it has a great affinity with Tf. Most of the research in this field explains the detrimental effects of NTBI in different pathological conditions; however the cause of its existence is a key question which is not well explained in literature. No logical explanation is available to elucidate NTBI presence in above conditions. Nevertheless different justifications have been proposed to explain the existence of NTBI in accordance with the clinical conditions.

Precise way for NTBI removal and the shelf life data have not been documented in the, though some studies state its clearance from highly vascular organs like liver and heart (Hider, 2002).
5.2.4 NTBI in iron overload conditions:

NTBI is well documented in case of primary as well as secondary iron overload conditions like hemochromatosis and thalassemia.

5.2.4.1 Hemochromatosis and NTBI:

Hereditary hemochromatosis (HH) is the best described as 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). After the first documentation of NTBI in thalassemia by Hershko et al, 1978, NTBI was also detected in hemochromatosis patients (Gutteridge et al, 1985). Afterwards various researchers reported the existence of NTBI in different iron overload conditions (Porter et al, 1996; Batey et al, 1980; Aruoma et al, 1988; Loreal et al, 2000). Serum NTBI levels were found elevated in both homozygotes and heterozygotes of the HFE C282Y mutation of hereditary hemochromatosis (de Valk et al, 2000). In computer simulation studies ferric bound to citrate form of NTBI is predominantly found in hemochromatosis patients (Grootveld et al, 1989). Le Lan et al (2005) also reported high levels of LPI in genetic hemochromatosis patients with high transferrin saturation (75%) along with elevated levels of transaminase.

5.2.4.2 Thalassemia and NTBI:

Hershko et al (1978) was the first to trace the presence of NTBI in thalassemia patients. Elevated level of DCI (desferrioxamine- chelatable Iron) has been reported by Breuer et al (2001) for β thalassemia major patients. Elevation of redox active highly toxic form i.e. labile plasma iron was reported to be high in β thalassemic patient which was absolutely absent in case of normal subjects (Esposito et al, 2003). High value of NTBI was also demonstrated by Sharma et al (2009) in β thalassemia major patients with the use of modified fluorescent based method.

In thalassemia patients, due to universally practiced blood transfusion therapy iron gets overload gradually. In such condition Tf saturation gets elevated as a result iron may get a chance to exist in a form which is devoid of apo-transferrin. However it is difficult to explain the presence of NTBI in conditions where Tf saturation is not
high. In such cases the Tf inaccessible fraction can be the key cause (Breuer et al, 2000).

5.2.5 NTBI and various pathological conditions without iron overload:

In the absence of iron overload conditions the detection of NTBI in various disease conditions was used to be difficult proposition due to non availability of a reliable method. However with advancement in the technology, a range of new methods have come in to existence, which have detected the presence of NTBI in patients of diabetes mellitus, cardiac disease, hemodialysis, hematological malignancies, chronic alcoholics etc, where the Tf saturation is not high.

5.2.5.1 Diabetes mellitus:

Lee et al (2006) reported frequent presence of NTBI in diabetic patients with HPLC based methodology. Shetty et al (2008) found significant elevation of free iron level in diabetic patients with poor glycemic control, but not in diabetics with good glycemic control. Mingwei et al (1998) suggested that glycated proteins present in the patients of diabetes mellitus, do not only bind to iron and copper with high affinity but also have the tendency to extract the iron from the traditional iron carriers and become the cause of NTBI existence in such patients.

5.2.5.2 Hemolytic anemia:

Elevated NTBI level has been reported by several researchers in case of hemolytic anemia. The suggested cause of such elevation is 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).

5.2.5.3 Hematological malignancies and chemotherapy:

Weijl et al (2004) reported that cisplatin chemotherapy induces oxidative damage which rapidly leads to release of iron from intracellular proteins and the appearance of NPBI however the NPBI is not elevated before chemotherapy. The observed high levels of NPBI may be a consequence of the chemotherapy and become a major causative determinant in chemotherapy-induced toxicity (Weijl et al, 2004; Sahlstedt et al, 2009). The reported reasons for the existence of NTBI are 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).

5.2.5.4 Cardiac complications:

With the use of bleomycin based methodology, Lele et al (2009) reported rapid elevation of catalytic iron in MI patients peculiarly before the characteristic elevation of routinely used parameter troponin I. However Daphne et al (2006) found no excess risk of CHD or MI in population with the higher NTBI value as compare to the low NTBI bearing population.

5.2.5.5 End stage renal disease and hemodialysis:

NTBI is also commonly traced in patients undergoing hemodialysis. In such cases it is important to note that IV iron supplementation is very common, which can be the reason for presence of NTBI in such patients (van Wyck, 2004). One such report has been documented by Schaller et al in 2005, who found high values of NTBI and the redox active forms of NTBI in hemodialysis patients receiving 300 mg intravenous iron sucrose. However Prakash et al (2005) traced the presence of NTBI even in hemodialysis patients who were not receiving the IV iron. In such cases traumatic effect of the HD was said to be the culprit. On the contrary Nilsson et al (2002) reported presence of NTBI in some cases of pre HD samples which got cleared during HD and the post HD samples of the same patients were found to be devoid of NTBI.

5.2.5.6 Others:

In atrasferrinaemia, it is pertinent that all the plasma iron molecules exist as NTBI. In the absence of Tf the NTBI level 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, 1999).

Marwah et al (2002) found the elevated serum NTBI level in blood stored for more than 17-22 days after donation, which may increase the risk of occurrence of NTBI and the related complications in the BT recipients.

5.2.5.7 NTBI and oxidative stress:

Oxidative stress has been documented as a cause as well as consequence of free iron, where the stable iron compound like iron sulphur proteins, ferritin and even
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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 Smith, 1988; Balla et al, 1991). This could be the reason why NTBI or its sub-fractions are tested out in the pathological conditions associated with high oxidative stress by various researchers where no iron overload is noticed.

In the form of NTBI, iron is weakly bound to non specific ligands, like albumin, citrate, acetate, DNA etc (Grootveld et al, 1989; Lovstad, 1993; Nilsson et al, 2002). As these associations are weak, iron may get released from these ligands, and exhibit its redox activity. Fenton reaction and Haber-Weiss reactions show iron mediated generation of reactive oxygen species which are as follows (Halliwell and Gutteridge, 1990)

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^- \text{ (Fenton reaction)} \]

\[ \text{O}_2^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{OH}^- + \text{OH}^- \text{ (Haber-Weiss reaction)} \]

\[ \text{O}_2^- + \text{Fe}^{3+} \rightarrow \text{O}_2 + \text{Fe}^{2+} \]

5.2.6 Methods available for NTBI estimation:

Due to heterogeneous and unclear nature of NTBI, no gold standard method available at present time. 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 group of iron collectively termed as NTBI (Pootrakul et al, 2004). Most of the available techniques can be grossly categorized in two parts; one group of methodology determine the NTBI after its chelation and separation of the chelated iron from the biological fluid, where as the other does not need the separation step and NTBI is measured in biological fluid itself mainly by targeting the redox activity of NTBI. In these methods, overestimation as well as underestimation of NTBI has been reported time to time.

In chelation based methods, NTBI is firstly chelated from its weak non specific ligands, at this point the chelator has to be chosen with a great care as a strong chelator can extract iron from the classical iron carrier like transferrin and results in overestimation of NTBI. On the other hand weaker chelator may not be able to wipe off the complete range of non-specifically bound iron resulting in false low value of NTBI.
In the second step, chelated iron is separated from the other portion of the biological fluid usually with the help of ultracentrifugation or ultra-filtration. This cost, time and material demanding step is operated to minimize the donation of NTBI back to the non specific negatively charged ligands as well as transferrin. It also minimizes non specific absorbance of reaction mixture which is a major drawback of spectrophotometric method. Gosriwatana et al (1999) suggested an extra step of saturation of Tf’s vacant site with a masking agent to minimize the under estimation of NTBI. However it is important to understand that, if NTBI actually has the affinity and accessibility with Tf, it should not be existing at all, and if it is existing means the NTBI fraction is the Tf independent fraction and the use of masking agent is questionable. The concentration of the chelator also was proven a deciding factor, as even the same chelator at higher concentration can rudely extract the iron and also influence the traditional iron carriers (Kolb et al, 2009). A little leakage of iron from Tf is obviously big flow of NTBI in blood. That’s why selection of chelator and its concentration should be done with utmost care. The chelated iron is then estimated by various techniques like HPLC, atomic absorption spectroscopy, spectrophotometry etc. (Paffetti et al, 2006; Zhang et al, 1995; Jittangprasert et al, 2004; Collins et al, 2000). These techniques must be sensitive enough for estimation of micro-quantity of iron.

In the second group of methods, NTBI is measured in the biological fluid itself, by targeting its peculiar characteristic of redox activity. In this group the fluorescent based methods are included. These methods explore iron sensitive fluorescent probes like fluorescent labeled apo-transferrin, desferrioxamine etc, along with a reducing agent which activate iron and initiate the series of events ultimately generating the signal (Breuer et al, 2001; Esposito et al, 2003). Sharma et al (2009) experimented the use of two fluorescent probes; one is sensitive to iron and second is insensitive to it, to retrieve the value of fluorescence generated by iron and iron only. In the same methodology the reducing substance was also included like the other fluorescent based method, along with a blocking agent, which can block the vacant sites on transferrin, to prevent false low values (Sharma et al, 2010; Sasaki et al, 2011). The inherent drawback of fluorescent based methodology is the background fluorescence which was attempted to be minimized by use of a serum blank by
various researchers. The availability and accessibility of instruments for fluorescent based method is also a point of concern in Indian laboratories.

Bleomycin based methods are the oldest concept of NTBI determination in biological fluid, which use a chelator antibiotic and the chelated iron is estimated in the actual reaction mixture without any separation. In this method ferric forms firstly get reduced with a reducing agent and get converted to active ferrous form. These ferrous forms then bind to a chelator antibiotic bleomycin, generating the reactive oxygen species which then degrade the DNA and the degraded DNA products are directly proportional to the level of NTBI. This multi step method is time consuming and highly vulnerable with great dependence on source of the bleomycin and harsh effect of a little variation in pH. However pH is a critical factor in all the methods for NTBI estimation as acidity of the medium cause release of iron form Tf and influence the level of NTBI at a great extent. An essential heating step of this method, which could be a source of non specific reaction and cause of non reliable results, further enhances the criticism in this methodology.

NTBI is said to be heterogeneous in nature, it exists in association with a range of negatively charged non specific ligands. But appearance of unambiguous isoforms in a selective pathological condition is unclear. This is mainly because of lack of comparative data among various methods. One such good attempt was done by Jacobs et al (2005), who compared the values of serum NTBI of hemochromatosis patients estimated in six different laboratories using various methods. He found that the mean NTBI values differed widely i.e. in the range of 0.12-4.32 µmol/L. He and his colleagues reported high level of between sample variation and within sample variation, with CV in range of 4.4-192.3 %. This report reveal lacuna in the literature regarding the measurement of specific sub-fraction of NTBI by explicit method. So it is quite possible that even though collectively said NTBI estimating methods, one method determine one fraction and the other determine the other sub-fraction which may or may not be comparable. Both these points are to be cleared before application of the serum NTBI in actual clinical practice.

Presently NTBI is not a well established parameter and commercially neither well standardized kits nor known value standards are available. Absence of a reliable analytical method is one of the reasons why its existence has been questioned often.
However, in the recent years a number of analytical techniques have been experimented in the various research laboratories to establish its existence in certain pathological conditions. The so called heterogeneous nature of NTBI; lack of reliable information about its probable forms as well as methods for estimation further complicate the condition.

5.2.6.1 BPS based estimation of NTBI:

BPS is a bidentate ligand, coordinating to iron via the two nitrogen atoms in a phenanthroline ring system. Both the forms i.e. ferrous (Fe\(^{2+}\)) and ferric (Fe\(^{3+}\)) bind to BPS but only BPS-ferrous complex is colored, the BPS-ferric complex is colorless and can’t get measured by colorimetry or photometry. This approach was first explored for NTBI estimation in biological fluid by Zhang et al (1995). BPS has been successfully used as chromogen for NTBI estimating methods by several researchers and got the highly reliable results which were even successfully compared with the other analytical approaches like electrothermal atomic absorption spectroscopy (Kolb et al, 2009; Jittangprasert et al, 2004). Zhang et al (1995) suggested the use of BPS as chromogen for spectrophotometric measurement of NTBI; however the method also included application of another chelator and a step of ultracentrifugation. These two steps had increased the processing time and made the method more demanding in terms of instrumental needs.

Nilsson et al (2002) suggested a modification in Zhang’s method (Zhang et al, 1995). He used BPS all alone without the application of any other chelator, as BPS exhibit dual role i.e. as a chelator as well as a chromogen. This definitely has cut off the time of test run and need of ultracentrifuge. BPS liberates iron neither from hemoglobin (Hb) nor from classical iron transporter transferrin (Tf) and thus NTBI doesn’t get over estimated (Nilsson et al, 2002). The major problem of this method is the high and fluctuating background color at around 535 nm, at which the readings are to be taken. To minimize such interfering background noise, multiscreen millipore filters were used by Nilsson et al (2002). But even in the well equipped Indian laboratories use of such filtration system is not feasible in routine practice.
5.2.7 Cream of the crop about present methodology:

The present study is based on the fact that BPS alone can be successfully used as a chromogen as well as chelator for NTBI estimation (Nilsson et al, 2002). The major problem reported in Nilsson’s method and even we experienced was the non specific and fluctuating background noise while reading the absorbance at 535 nm in unfiltered reaction mixture. As stated above Nilsson et al (2002) used the multiscreen filtration system to filter the reaction mixture at the end of the incubation and minimize the non specific absorbance; however this step restricted the acceptance of this methodology due to specialized instrumental need. In present study, we have experimented the various routinely available techniques to find their ability to replace the need of multiscreen filter and generate reliable results by minimizing the fluctuating background absorbance.

Various reducing substances were included in bleomycin based method as well as fluorescent based method to convert inactive ferric form of iron to active ferrous form of iron. One of such reducing substance is ascorbate. We have also included ascorbate in the present method.

The present study is carried out in 2 parts. The first part was concentrated on the development of NTBI estimating methodology which can be used in the routine laboratories. This was done by modifying and optimization of the BPS based spectrophotometric method reported by Nilsson et al (2002).

In second part of the study the optimized protocol is explored to estimate serum NTBI values in controls and in subjects of selected pathological conditions. Along with serum NTBI few other relevant biochemical parameters were also studied. The serum NTBI values were then statistically analyzed to check their probable correlation with the other routinely experimented analytes and the mean serum NTBI values of different pathological conditions were compared to check the significant elevation of serum NTBI value in specific pathological events.

5.2.8 Specifications of first part of the study:

As stated above, the first part of the study mainly focused about the experimental trials to develop the best suitable modification in BPS based spectrophotometric method (Nilsson et al, 2002). In the reference method the test was
run in 96 well plate formats and the endpoint signals were read on the ELISA reader with 535 nm filter. As acknowledged in the reference method, increase in the path length can increase the sensitivity of the method; we have increased the reaction volume 8X, and increased the path length by reading the reaction mixture in spectrophotometric cuvette. This replacement nullifies the need of 535 nm filter in ELISA reader and multiscreen filtration system which are not routinely used in the pathological laboratory.

As the primary work, the standard graph 1 was plotted first, with mixture of various aqueous dilutions of ferrous ammonium sulfate, ascorbate and BPS. The range of the standard graph (0.1 - 25.6 µmol/L) was decided by reviewing the probable clinically significant range of serum NTBI in the subject categories studied. Figure 4.1.1 shows that the linearity of method is in the range of 0.1 - 25.6 µmol/L iron in aqueous medium. Below this level the corresponding absorbance became undetectable. The detection limit of the present method is comparable with the other methods operated by the various researchers (Jakeman et al, 2001; Gosriwatana et al, 1999).

Even though the graph is quite linear the slope exhibited in Figure 4.1.3 has been explored for the estimation of serum NTBI, as all the values observed in this study actually are within this narrow range.

Various reducing substances were included in bleomycin based method as well as fluorescent based method to convert inactive iron to active iron, which in turn can initiate the series of reactions and NTBI can be measured (Halliwell, 1994; Evans et al, 1992; Breuer and Cabantchik, 2001; Kolb et al, 2009). Reducing substance like ascorbate was also suggested by Nilsson et al for estimation of total NTBI (Fe²⁺ and Fe³⁺ forms together). However he had not used it as a regular reagent for NTBI estimation. Ascorbate was not the part of the reaction mixture in the reference method, but we have included it as a regular reagent in our method. We have used 1mM ascorbate as an essential reagent which can convert Fe³⁺ forms to Fe²⁺ form and generate the colored complex with BPS which can be measured. The concentration of ascorbate is critically adjusted at 1mM, which is proven to be of not releasing the iron bound to transferrin (Nilsson et al, 2002). Ascorbate is especially included by considering the fact that within the biological system, in presence of various reducing
substances, Fe\(^{3+}\) ions obtain every chance to get converted in Fe\(^{2+}\), i.e. the active form and start the detrimental changes. Thus in the clinical study, the free iron forms matter and not the ferric or ferrous form. To check the effect of 1mM ascorbate on end point coloration, we have plotted another standard graph i.e. standard graph 2, which had no ascorbate. Figure 4.1.1, 4.1.2 and Table 4.1.1 show that the standard graph 1 and standard graph 2 were almost identical, which ensure no effect of 1 mM ascorbate in final color production.

As documented by Nilsson et al (2002) fluctuating absorbance was observed with the actual plasma specimens while using BPS based spectrophotometric method, which must be minimized to achieve reliable results. To replace the multiscreen filtration system used in reference method we tried syringe filters with same properties (hydrophilic PVDF membrane with 0.45µ porosity). This trial i.e. trial 1 was done to minimize the cost and complexity of the accessories used in the multiscreen filtration system. As the biological sample heparinised plasma was used in the reference method, so we also started with heparinised plasma as our test specimen. We used heparinised vacutainer®s without gel separator for trial 1 and 2.

In trial 1A the heparinized plasma was filtered first and then processed where as in case of trial 1B the reaction mixture was filtered after the incubation period. In both the cases high level of fluctuation in absorbance was observed, with NTBI values dispersed in a wide range. The mean NTBI values were 0.82 ± 1.49 μmol/L and 1.17 ± 1.60 μmol/L for trials 1A and 1B respectively (Figure 4.1.4 and Table 4.1.2).

After unexpected results achieved in Trial 1, we experimented trial 2, in which we used routinely available techniques like centrifugation and vortex mixing in place of filtration. In trial 2A the heparinised plasma was re-centrifuged at low speed and vortex mixed twice as explained in the methodology. In trial 2B the plasma was re-centrifuged at low speed but vortex mixture was not used, whereas in trial 2C the plasma was processed as such without re-centrifugation and vortex mixing. However in all 3 variations of trial 2, the NTBI values were dispersed in a broad range with high level of fluctuations in absorbance. The mean NTBI value we got were 0.03 ± 0.75 μmol/L, 0.20 ± 1.60 μmol/L and 0.17 ± 0.61 μmol/L for trial 2A, 2B and 2C respectively as shown in Figure 4.1.5 and Table 4.1.2.
In trial 3, the heparinized vacutainer®s with gel separator was used to collect the blood. The plasma was separated and processed as per the protocol. Just before the time of spectrophotometric reading the reaction mixture was filtered in trial 3A, in trial 3B the heparinised plasma was processed without filtration. Once again the results were not consistent with great variation in NTBI level with high degree of fluctuation. The mean NTBI values we got for trial 3A and 3B were 1.02 ± 2.16 μmol/L and 0.42 ± 0.79 μmol/L respectively (Figure 4.1.6 and Table 4.1.2).

With the known fact that heparin only delay the clotting and not prevent it, it can be possible that the heparinized plasma used after storage may form the fibrin and results in non specific fluctuation in the absorbance and generate inconsistent results. Esposito et al (2003) in his experiments showed that the level of LPI in serum and plasma is same. By considering this fact and by reviewing the sample specifications in the other methodology for NTBI estimation, we started using serum in place of plasma as the specimen. We used plain vacutainer®s without gel separator to collect the blood sample for trial 4 and 5.

The serum was filtered before the reaction and after the reaction in trial 4A and 4B respectively as specifies in material and method. But in these trials also we got considerable inconsistency in the results. The mean serum NTBI values were -0.20 ± 2.60 μmol/L and 0.73 ± 1.70 μmol/L for trial 4A and 4B respectively (Figure 4.1.7 and Table 4.1.2).

While our experimental trial 1, 2, 3 and 4 we found that the filtration was increasing the level of fluctuation rather decreasing it, so we decided to process the remaining experiments without filtration. This unexpected finding could be because of some non specific reaction taking place when the sample with BPS comes into contact with the syringe filter or its accessories which is not mentioned anywhere in the literature. It is noteworthy to mention that we had not used needle any where while filtration. To find out the probable interaction of BPS with the materials used in filtration, we filtered the 1mM aqueous solution of BPS repeatedly and read the absorbance curve for the period of 20 minutes. However in this case neither change nor fluctuation was observed. Thus the cause of such fluctuation remained undetermined.
At this point we were trying to find out the ways which can minimize the fluctuation in the absorbance. Gregory (2001) reported that the four major endogenous compounds known to interfere in the laboratory results are hemoglobin, bilirubin, lipids and paraproteins. We have used the samples which were not visibly hemolysed and icteric, so interference through hemoglobin and bilirubin should be least in our case. Probability of presence of paraprotein was very low in case of control subjects. So we concentrated on the techniques which can minimize the lipids interference from the sample which we consider a probable culprit in our methodology. Jabbar et al (2005) reported that ultracentrifugation is an effective way to minimize lipid interference in the reaction. However as ultracentrifugation is also not available in the medium scale laboratories, we continued our experiment with high speed centrifugation. Dimeski and Jones (2011) showed that the high speed centrifugation is a good alternative of ultra-centrifugation to minimize the lipidic interference in the reaction.

In trial 5 we used the high speed centrifugation to minimize non specific fluctuation and for consistent results. At the end of high speed centrifugation of serum a visible fatty layer was formed, the clear portion of the sample was carefully transferred to another tube which was processed further. An extra care had been taken to minimize the disturbance of top fatty layer formed at the end of high speed centrifugation. This approach had significantly reduced the non specific fluctuation and generated more consistent results. Another modification in the same approach had been successfully incorporated i.e. in between sample pre incubation at 37°C. Experimentally it had been shown that when sample was processed for electrophoresis, the mobility of the lipoproteins could be enhanced when sample was pre-incubated at 37°C (Carlson and Regnstram, 1984). This was suggested to be due to solubilization of the lipoproteins present in the sample by activating LCAT enzyme. The same approach was tried in the present methodology as solubilization of lipoprotein might also decrease the non specific fluctuation in the absorbance. The mean serum NTBI values for trial 5A, 5B and 5C were 0.10 ± 0.16 μmol/L, -0.01 ± 0.25 μmol/L and 0.07 ± 0.79 μmol/L respectively (Figure 4.1.8 and Table 4.1.2). As shown in the results the serum NTBI values were constricted within a narrow range in case of trial 5A, which explore both the steps, i.e. high speed centrifugation and
sample pre-incubation. The serum NTBI values obtained in trial 5B which included the step of high speed centrifugation without sample pre-incubation were within a small range which is not as narrow as trial 5A. Level of serum NTBI in case of trial 5C covered the broader range which was processed without application of high speed centrifugation as well as sample pre-incubation.

In trial 6, we used plain vacutainer®s with gel separator to derive the sample for further processing. The mean serum NTBI values for trial 6A, 6B and 6C were 0.00 ± 0.16 μmol/L, -0.03 ± 0.21 μmol/L and -0.03 ± 0.65 μmol/L respectively (Figure 4.1.9 and Table 4.1.2). As shown in the results, in this case also we found the high level of consistency in trial 6A where in both the steps i.e. high speed centrifugation and sample pre-incubation was included as compared to the others. Gel separator of the vacutainer®s did not influence the endpoint absorbance of the reaction mixture; however it has definitely increased the serum volume output. The results of the trial 5A and 6A were quite comparable, where in both high speed centrifugation as well as sample pre-incubation were operated.

In the absence of the universally accepted method it is difficult to prove the reliability of the method, especially when different methods are said to determine different sub-fraction of NTBI. However if we see the reported values of serum NTBI in normal subjects, most of the researchers found the levels which were very close to one another. In control group 0.154 ± 0.328 μmol/L of serum NTBI value has been reported by Caroline et al (2005). Lele et al (2009) reported 0.1 ± 0.06 μmol/L catalytic iron in healthy subjects, with the cutoff values of 0.3 μmol/L. Lee et al (2006) found 0.04 ± 0.13 μmol/L NTBI in control subjects. In part one of our study, we have incorporated only the healthy adults and the results of the most optimized trials, i.e. trial 5A and 6A the serum NTBI values were 0.10 ± 0.16 μmol/L and 0.00 ± 0.16 μmol/L respectively. These values corroborate well with the values reported by above researchers.

Negative findings of NTBI values are well documented in the literature by various researchers using different methodology, even after operation of an added step for occupying vacant sites of Tf (Porter et al, 1996; De Valk et al, 2000, van der et al, 2006). We also found negative NTBI values in our trials, which were considered for
calculation as such in part 1 of the study. For part 2 the samples with negative value were considered as 0 like the others (van der et al, 2006).

5.2.9 Limitations of the methodology:

The reference method runs the test in 96 well plate format followed by reading with ELISA reader, which is less demanding in terms of the volumetric need of the sample and easy to operate. To increase the sensitivity and to minimize specialized instrumental need we have used plastic test tubes followed by its reading in spectrophotometer. This change increases the need of sample volume, which is important especially when pathological samples are concerned. Simultaneously the process became more laborious, as at a time a single test only can be read. To decrease the need of sample volume, we tried to read the sample after dilution with distilled water, but it could not be possible as the respective changes in the absorbance made it undetectable even with highly sensitive spectrophotometer.

Although exhibiting excellent linearity in the standard graph, the color generated at the end of the reaction was very pale especially in the clinically significant range, so an extremely sensitive spectrophotometer was required even after the increment in sample volume.

5.2.10 Data-map of the present study:

After optimizing the protocol in first part of the study, the same protocol i.e. protocol of trial 5A was operated for rest of the study. In the final protocol the samples were pre incubated after high speed centrifugation, where the major lipidic portion had been removed by high speed centrifugation and a little part present in the sample were made more soluble which gave the most consistent and reliable results.

In second part of the study, NTBI was estimated in assorted pathological conditions, which include both iron over load condition like β thalassemia major and the non iron overload conditions like diabetes mellitus, cardiac complications, hemodialysis patients and CLL. 223 subjects were enrolled who belong to six different groups. Out of these, the Group I (normal control), Group II (cardiac patients), Group III (known diabetic patients), Group IV (patients undergoing hemodialysis), Group V (β thalassemia major patients) and Group VI (CLL) contributed 24.7 %, 11.2 %, 22.4 %, 28.2 %, 9.0 % and 4.5 % respectively (Table
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4.2.1 and Figure 4.2.1). These groups were selected by reviewing the literature which suggests the probable correlation of serum NTBI in these conditions. In all the cases serum NTBI was estimated. Along with serum NTBI, other relevant parameters were also studied to check its probable correlation with NTBI.

5.2.11 Toxicity of NTBI as a general view:

A good number of non classical negatively charged molecules like albumin, citrate, acetate, DNA etc. have been documented to be associated with iron (Grootveld et al, 1989; Lovstad, 1993; Nilsson et al, 2002). The interaction between these molecules and iron is not strong enough and so that iron get a chance to get dissociated and exhibit its reactivity as of it is free. Being the transitional metal, iron has suitable redox potential to slide between ferrous i.e. Fe (II) and ferric i.e. Fe (III) which enable it to be associated with organic ligands, this is essential for its biological functions.

These essential characteristics of iron make it a dangerous entity if left unattended or unbounded. Serum iron if it is not guarded by the Tf, the unbound iron (NTBI) poses a great challenge to the protective mechanism of the body. The detrimental effect of free iron is due to its capability to generate reactive oxygen radicals which increase oxidative stress. Free Ferrous iron (Fe^{2+}) may catalyze a variety of free radical oxidative reactions which in turn lead to various degenerative changes (Chau, 2000; Meyers, 2000; Gackowski et al, 2001). Fenton reaction and Haber-Weiss reactions show iron mediated generation of reactive oxygen species. 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). Unbound iron is 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 final location of its deposition. 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 to various infections (Pai et al, 2006). de feo et al (2001) reported probable role of NTBI in initiation of the alcohol induced liver damage in alcohol abuse.
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Many workers have reported increased level of serum NTBI in various disease conditions. A range of diseases reported with elevated serum NTBI level are genetic hemochromatosis, β-thalassemia major, diabetes mellitus, cancer, end stage renal disease, acute myocardial infarction and chronic alcoholism. Iron overload is reported to amplify detrimental effect of super oxide overproduction in broad spectrum acute and chronic inflammatory conditions (Emerit et al, 2001). Iron induced detrimental events have been explained by various researchers in different pathological conditions. A few related with the present study have been explained here.

5.2.12 Documentation of NTBI: past and present:

5.2.12.1 NTBI in control group:

Many researchers reported absence of NTBI or its sub-fraction in the healthy controls (Nilsson et al, 2002; Esposito et al, 2003). However with the development of more sensitive analytical techniques, recently few researchers could estimate the micro quantity of the NTBI even in healthy subjects.

In healthy subjects 0.154 ± 0.328 µmol/L of serum NTBI was reported by Caroline et al (2005) with a fluorescent based method. Shetty et al (2008) found 0.5 ± 0.2 µmol/L free iron in healthy control with the use of BPS based spectrophotometric method. With the use of bleomycin based colorimetric method, Lele et al (2009) reported 0.1 ± 0.06 µmol/L catalytic iron in control group. With HPLC based method, Lee et al (2006) found 0.04 ± 0.13 µmol/L NTBI in control subjects.

We have analyzed serum NTBI values in 55 apparently healthy subjects. The mean serum NTBI values for this group were 0.02 ± 0.06 µmol/L, which is comparable with the values reported by others (Figure 4.2.10, Table 4.2.15) (Lee et al, 2006).

In none of the control subject serum NTBI was > 0.3µmol/L (Table 4.2.10), so we determined 0.3µmol/L value as the cutoff, any value higher than this has been considered as significantly positive. The same cutoff has been reported by others too (Lele et al, 2009).
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5.2.12.2 NTBI in diabetes mellitus:

Diabetes is disease due to the metabolic abnormalities in carbohydrates metabolism. There are two types of diabetes mellitus. Type 1 diabetes is a complex, multi-factorial disease involving severe destruction of insulin producing pancreatic β cells and called as insulin dependent diabetes mellitus (IDDM) also called juvenile onset (Lernmark, 1999). The other type of diabetes is known as maturity onset or type 2 diabetes or non-insulin dependent diabetes mellitus (NIDDM) (Lebowitz, 1999).

Diabetes leads to various clinical manifestations that is mostly irreversible and due to microvascular or macrovascular disease. Nephropathy, retinopathy and neuropathy are related to microvascular diseases whereas macrovascular disease associated with 2-4 fold increased risk for atherosclerosis and ischemic heart disease. Iron is also involved in pathogenesis where insulin sensitivity and insulin secretion are impaired in iron overload (Jiang et al, 2004; Fernandez-Real et al, 2005). The exact mechanism of iron induced diabetes is uncertain, but it may be mediated by three key mechanisms: 1) insulin deficiency, 2) insulin resistance and 3) hepatic dysfunction. Divalent metal iron transporters (DMT) have also been found to play significant role. DMT are known to be expressed in pancreatic cells at greater extent which facilitates more internalization of iron within the cells and makes the cell more susceptible for iron overload (Andrews, 1999). Due to iron load pancreatic cells get exposed to oxidative stress as explained in Fenton chemistry. 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). In iron overloaded thalassemic patients insulin resistance was observed (Dandona et al, 1983; Mendler et al, 1999; Singh et al, 2001). Thus iron actually redox active iron could be considered as a culprit cause of diabetes mellitus as it decrease the insulin production from pancreatic cells and induce resistance of insulin at liver. Along with the above causes, 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 A1c 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).

Lee et al (2006) reported presence of serum NTBI in 59 % newly diagnosed and 92 % of advanced diabetic patients. He reported $0.62 \pm 0.43 \mu\text{mol}/\text{L}$ and $0.24 \pm 0.29 \mu\text{mol}/\text{L}$ NTBI in advanced diabetics and newly diagnosed diabetic patients respectively. In the group of diabetic patients, we found $0.31 \pm 0.64 \mu\text{mol}/\text{L}$ serum NTBI, which is comparable with the values reported by Lee et al (2006) (Figure 4.2.10, Table 4.2.15). The mean serum NTBI value obtained in diabetic group was significantly higher than the values obtained for control group ($p=0.002$). The serum NTBI positivity and significant positivity were 42 % and 22 % respectively in cases of diabetes mellitus (Table 4.2.9 and Table 4.2.20).

5.2.12.3 NTBI in cardiac complications:

Myocardial infarction may be due to occlusion of coronary artery following the rupture of vulnerable atherosclerotic plaque due to unstable collection of lipid and macrophages in the walls of the arteries and resulting in the restriction of blood supply and oxygen shortage and if it is untreated for long time it causes damage or death of the cardiac muscles.

Cardiovascular pathology has multi factorial origin. One of the causes is increased supply of free radicals or non radical oxidant which causes number of organ dysfunction. This link can also be observed as higher cardiac complications in thalassemia and other overload conditions where high iron enhances the oxidative stress. Iron mediated heart diseases have been explained by several pathways, mainly by tissue cell loss and atherosclerosis through ROS production. As a well known fact atherosclerosis is one of the major causes of coronary heart disease. Inflammation theory describes atherosclerosis as proliferation of smooth muscle (Ross et al, 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). These 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 increase the 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. In case of reperfusion injury too, through ROS iron causes lipid peroxidation, fragility of intracellular lysozyme and release of hydrolytic enzymes and ultimately causing myocardial and endothelial cell necrosis (Halliwell and Gutteridge, 1990; Horwitz and Rosenthal, 1999; Voogd et al, 1994).

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 could 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).

Lele et al (2009) reported 1.5 ± 2.0 µmol/L, 0.2 ± 0.16 catalytic iron for of acute MI, suspected ACS without MI patients respectively. In present study, in group II patients, i.e. Cardiac patients, 2 samples were collected first was on the day of hospitalization and the second on the second day of admission to evaluate the change in NTBI level. The serum NTBI values we estimated were 0.05 ± 0.08 µmol/L and 0.08 ± 0.14 µmol/L for day 1 and day 2 (Table 4.2.15). In none of the cases, the serum NTBI was significantly higher than that of the control subjects (p=0.104 and 0.083 respectively) (Table 4.2.15). At the same time no significant difference in serum NTBI value was observed in-between day 1 and day 2. The mean serum NTBI we found for day 1 as well as day 2 was considerably lower than the values reported by Lele et al (2009) for catalytic iron in acute MI patients. The difference in the results could be due to measurement of different isoforms detected by different methodology.

5.2.12.4 NTBI in hemodialysis patients:

Kidney is major organ involved in the maintenance of homeostasis. It is involved in the efficient excretion of waste products and reabsorption of essential biomolecules and crucial in the maintenance of water, electrolyte and pH. Renal failure can be defined as failure of kidney to excrete metabolic end products from blood as a result the level of various macromolecules get altered with major influence
on electrolytes, pH, proteins, fluid level (Hussin, 2008). Anemia is usually been associated with end stage renal disease mainly because of loss of RBC through kidney and decreased hematopoiesis due to restricted availability of the erythropoietin.

NTBI or its different forms have been reported as a cause as well as consequence of end stage renal disease. Catalytic iron has been reported in urine of chronic kidney disease patients (Nankivell et al, 1992). Reactive oxygen species produced due to NTBI is suggested as major cause of chronic kidney disease, it is mediated 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). 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 where as metal chelators improves the conditions in progressive kidney disease (Descamps-Latscha et al, 2004). When this disease progresses to end stage renal failure, dialysis becomes essential to save patient’s life. In case of end stage kidney disease, anemia is commonly encountered and so intravenous infusion is also a common practice. Such IV iron can enhance the risk of NTBI existence, which provoke the ROS mediated detrimental consequences and worsen the condition.

In patients of end stage renal disease Breuer et al reported 0.1 - 13.5 µmol/L NTBI (Breuer et al, 2000). Schaller et al in 2005, found high values of NTBI and the redox active forms of NTBI in hemodialysis patients receiving 300 mg intravenous iron sucrose. However Prakash et al (2005) traced the presence of NTBI in hemodialysis patients who were not receiving the IV iron. On the contrary Nilsson et al (2002) reported presence of NTBI in some cases of pre HD samples but not in post HD samples.

In the present study we have included 63 end stage kidney disease patients undergoing hemodialysis. In such group from each individual 2 samples were collected, one before HD and one after HD, except for the cases where sufficient samples were unavailable. Two samples were studied to evaluate the effect of HD on NTBI values, as there are contradictory reports in the literature regarding the serum NTBI levels before and after hemodialysis (Nilsson et al, 2002, Prakash et al, 2005). The serum NTBI values we found were 0.20 ± 0.32 µmol/L, 0.25 ± 0.76 µmol/L for pre HD and post HD samples respectively. In both the cases the serum NTBI values
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were significantly higher than the values of control group (p <0.05) (Table 4.2.15). However no significant difference was observed in serum NTBI values between pre-HD and post-HD samples. Our study does not support the hypothesis which indicates that NTBI appears in post hemodialysis patients due to traumatic effects of hemodialysis procedure. Rather we found higher percentage of significant positivity in pre-HD sample as compared to the post-HD samples i.e. 23.8 % Vs 13.5 % which somewhat support the results presented by Nilsson et al (Nilsson et al, 2002) (Table 4.2.20). The drop in significant positivity could be due to the removal of NTBI during the process of hemodialysis.

5.2.12.5 NTBI in β thalassemia major:

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 is essential for normal growth and development and also reduces hepatosplenomegaly due to extramedullary hematopoiesis as well as bone deformities (Cunningham et al, 2004; Old et al, 2001). To do so frequent and long term blood transfusion therapy is the only available option. Each unit of blood contains 200 - 250 mg of iron and body iron stores are normally only 0.5 - 1.5 g (Prabhu et al, 2009). 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 an average 0.5 mg/ kg body weight/ day (Hoffbrand, 2001). In such case prolonged blood transfusion definitely results in iron overload. In case of iron over load and due to high Tf saturation, the level of NTBI and oxidative stress gets elevated which further switch on oxidative damage. Hydroxyl radicals generated through free iron cause damage to proteins, lipids and DNA (Bacon and Britton, 1990). The initial reaction with each of these molecules is the formation of peroxides (e.g. lipid peroxides) that can interact with
other molecules to form cross links. These cross-linked molecules perform their normal functions either poorly or not at all (Prabhu et al, 2009). Red cells alone do not bear the brunt of the reactive oxygen species. Damage to cells of other organs start to accumulate within a year of commencing transfusion therapy. Hepatocytes are the major storage site for body iron. With iron overload, these cells are relentlessly bombarded by reactive oxygen species and eventually die (Bonkovsky, 1991). They are replaced by fibroblast cells. The collagen laid down by fibroblasts produces liver fibrosis and, eventually, cirrhosis. Likewise, cardiac cells are damaged with iron overload (Buja and Roberts, 1971). Normal cardiac function requires the coordinate activity of all the cells in the heart. Damaged, poorly functioning cells often fail in this regard. The clinical manifestations include congestive heart failure (due to injury to myocytes) and arrhythmias (due to damage to the cells of the cardiac conducting system) (Liu and Olivieri, 1994; Schellhammer et al, 1967).

The initial study of NTBI was undertaken in iron overload conditions like hemochromatosis and thalassemia. Breuer et al (2000) documented 0.9-12.8 µmol/L of NTBI after the use of a weak mobilizer oxalic acid in case of thalassemic sera. Breuer et al (2001) reported 0.4 - 1.1 µmol/L NTBI in case of hereditary hemochromatosis and 1.5-8.6 µmol/L DCI (Desferrioxamine Chelatable Iron) in thalassemic sera with the probe fluorescein-desferrioxamine. Esposito et al (2003) reported 1.0-16.0 µmol/L labile plasma Iron (LPI) which was determined with the fluorogenic dihydrorhodamine123 and reactive radical generation prompted by ascorbate. Le Lan et al (2005) reported 0.761 ± 0.504 µmol/L NTBI and 0.250 ± 0.289 µmol/L LPI in genetic hemochromatosis with iron overload. Sharma et al (2009) found comparatively lower levels of NTBI i.e. 0.375 ± 0.028 µmol/L in β thalassemia major with the modified fluorescent based method.

With BPS based spectrophotometric method, we found 0.61 ± 0.54 µmol/L NTBI in twenty β thalassemia major patients receiving regular blood transfusions. Mean serum NTBI value in this group was significantly higher than the values observed in the control group (p < 0.001), rather it is the highest amongst all the groups studied. Even though being the highest value group in the present study, the mean serum NTBI value we found in β thalassemia major patients was far lower than the DCI value reported by Breuer et al (2001), NTBI value reported by the same
researcher and LPI reported by Esposito et al (2003). However NTBI values we got in our study was considerably higher than the values reported by Sharma et al (2009).

5.2.12.6 NTBI in hematological malignancies:

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. Redox active forms of iron, due to their ability to generate ROS known to cause damage in DNA which has been considered as a cause of hematological cancer (Galaris et al, 2008). NTBI mediated complication in hematological malignancies are poorly explained in the literature but it is has been suggested to be though increased oxidative stress and increased sensitivity of the infection (Halliwell et al, 1988; Harrison et al, 1994). Furthermore in hematological malignacies 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 with chemotherapy (Bonsdorff et al, 2002; Bradley et al, 1997; Halliwell et al, 1988).

Bonsdorff et al (2002) found 0 - 0.97 µmol/L NTBI in non hemolysed serum samples collected from the patients suffering from hematological malignancies. Weijl et al (2004) reported 10.6 ± 6.6 µmol/L NPBI within 1 - 4 days after chemotherapy in cancer patients. He also reported simultaneous elevation of total plasma iron and ferritin with marked decrease in latent iron binding capacity. Amongst the patients of hematological malignancies we included only CLL patients. 10 CLL patients were included for the study, and the mean NTBI values we got was 0.05 ± 0.08 µmol/L, which was lower than the values reported by others (Weijl et al, 2004) (Table 4.2.15). We did not find significant elevation in serum NTBI values in CLL patient group as compared to the control group (p >0.05).

5.2.13 Comparative evaluation of NTBI in various groups studied:

The mean NTBI values were 0.02 ± 0.06 µmol/L, 0.05 ± 0.08 µmol/L, 0.08 ± 0.14 µmol/L, 0.31 ± 0.64 µmol/L, 0.2 ± 0.32 µmol/L, 0.25 ± 0.76 µmol/L, 0.61 ± 0.54 µmol/L, 0.05 ± 0.08 µmol/L for control subjects, cardiac patients day 1, cardiac patients day 2; diabetic patients; hemodialysis patients: pre HD and post HD; β thalassemia major patients and the patients suffering from CLL respectively (Table
4.2.15). Results showed considerable difference in the mean value of control as compared with diabetes patients, hemodialysis patients and β thalassemia major patients (p < 0.05). The mean NTBI of cardiac patients and CLL patients were not significantly higher than the values of control (p > 0.05).

The serum NTBI positivity found were 14.5 %, 40 %, 28 %, 42 %, 39.6 %, 33.3 %, 90 % and 30 % for group I; group II: day 1, day 2; group III; group IV: pre HD and post HD; group V and group VI respectively when the detection limit was 0.1 µmol/L (Table 4.2.9). But more important is the significant positivity i.e. serum NTBI ≥ 0.4 µmol/L. On the basis of this cut off value, the significant positivity of serum NTBI value were around 0 %, 0 %, 6 %, 22 %, 24 %, 13 %, 60 % and 0 % for group I; group II: day 1, day 2; group III; group IV: pre HD and post HD; group V and group VI (Table 4.2.20).

5.2.14. Comparative study of various biochemical parameters in different groups:

Table 4.2.26 shows the mean value of various biochemical parameters studied in different groups. The mean values of all the analytes studied for control group were within the normal range. The overall high value of serum triglyceride in all the groups could be due to random sample collection without overnight fasting except for group III.

In cardiac patients’ group, for day 1 samples, the mean value of RBS, serum creatinine, serum urea and serum ALT were higher than the suggested normal range. As expected the mean value of serum AST, serum CK, CK-MB and serum LDH were also elevated and crossed the normal range for day 1 as well as day 2 samples. Other than these parameters, the mean value of RBS was also little higher than the suggested normal range for day 2 sample.

In the patients suffering from diabetes mellitus, as per the expectation, the mean value of FBS was higher than the suggested normal range and the mean value of control group. All the other parameters were within the suggested normal range.

In case of end stage renal disease patients undergoing hemodialysis, for pre-HD samples the mean value of all the renal parameters i.e. serum urea, uric acid, creatinine were higher than the normal range. For the same samples, the mean serum
AST values were also higher than the normal range. Whereas in case of post-HD samples, only the mean serum creatinine value was higher than the suggested normal value. The value of A/G ratio is almost same for pre- HD and post-HD samples, which is lesser than the suggested normal range.

In case of β thalassemia major patients, in spite of regular blood transfusion, the mean values of serum iron, serum TIBC and % transferrin were within the normal range.

5.2.15 Comparison of serum NTBI with the other parameters:

Various biochemical parameters have been evaluated for their correlation with NTBI in different groups. Neither perfect positive nor perfect negative correlation was observed in any of the group studied. However in diabetic patients’ group, serum total cholesterol showed strong positive; in hemodialysis patients’ group in pre HD samples serum urea showed strong positive; serum albumin and serum globulin showed strong negative; in post HD sample serum uric acid showed strong positive and in β thalassemia major patients’ serum iron showed strong positive correlation with serum NTBI values (Table 4.2.27).