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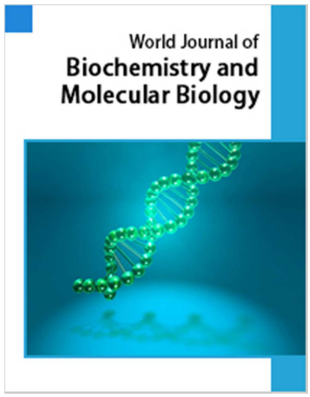

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Iron and Iron Binding Proteins: Clinical Utilities and Diagnostic Approaches

Yoseph Cherinet Megerssa[1, *], Demo Yemane Tesfaye[2]

[1]Department of Biomedical Science, College of Veterinary Medicine and Agriculture, Addis Ababa University, Bishoftu, Ethiopia
[2]Department of Medical Laboratory Sciences, College of Health Sciences, Hawassa University, Hawassa Ethiopia

Email address

yoseph.cherinet@aau.edu.et (Y. C. Megerssa)
[*]Corresponding author

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Abstract

Iron is important for the function of oxygen-binding molecules and for the electron transport and energy metabolism capacity of cells; deficiency of it causes Iron Deficiency Anemia (IDA). However, the redox activity of iron can also cause damage, primarily by the production of reactive oxygen radicals. Hence, iron concentration is tightly regulated at both cellular and systemic level. Estimation of iron status, thus, is very crucial, especially in woman of child bearing age in developing countries, where iron deficiency is highly prevalent. Different approaches and laboratory techniques have been geared to asses iron status from different angle.

1. Introduction

Iron is required for the function of oxygen-binding molecules, mainly hemoglobin and myoglobin, and iron-containing enzymes, including the cytochrome system in mitochondria [1].

Normally, very small quantities if iron are present in most cells of the body, and in other extracellular fluids. Physiologically, the body rigorously conserves its iron supply, so that less than 0.1% of the body iron content is lost daily, mostly in desquamated cells [1].

In a 70kg male, it is estimated that there is about 3.5 g of iron and is distributed into a number of different compartments that include hemoglobin (67%), storage iron (ferritin, hemosiderin) (27%), myoglobin (3.5%), labile pool (2.2%) and other tissue & transport iron (0.28%) [1].

Cellular iron is mostly bound within iron protoporphyrin (heme) and iron–sulfur clusters, which serve as enzyme cofactors, or stored within the core of ferritin multimers. Without adequate iron, cells lose their capacity for electron transport and energy metabolism. However, the redox activity of iron can also cause damage, primarily by the production of reactive oxygen radicals. As such, iron concentrations must be tightly regulated both at the cellular and the systemic level. A crucial element in the maintenance of systemic iron homeostasis is effective communication between cells that absorb iron from the diet (duodenal enterocytes), use iron (mainly erythroid precursors), and store iron (hepatocytes and tissue macrophages). The peptide hormone hepcidin interacts with the cellular iron exporter ferroprotin and thus is now recognized as the key regulator of systemic iron homeostasis [2].

Iron is transported from one organ to another by a plasma iron transport protein, apotransferrin. The apotransferrin-Fe^{3+} complex is called transferrin. Normally, there is a total of approximately 2.5 mg of iron in plasma. When transferrin binds to the transferrin receptor of cells, the transferrin/receptor complex is internalized into an endosome. It then becomes acidified, releasing the iron from transferrin and reducing it to ferrous iron (Fe^{2+}), which is then into the cell through the divalent metal transporter, DMT 1. The apotransferrin is then transported back to the cell surface, ready to transport another transferrin molecule to the interior of the cell. This series of reactions has been designated the transferrin cycle [1].

2. Clinical Conditions Related to Iron Status

Iron deficiency and iron overload (hemosiderosis & hemochromatosis) are the major disorders of iron metabolism. The high prevalence of iron deficiency and iron deficiency anemia, especially in women of childbearing age, and its association with central nervous dysfunction, impaired work performance and response to exercise, impaired immune response, thermogenesisand energy metabolism, and adverse outcome of pregnancy represent a serious health problem. Worldwide, iron deficiency is the most common nutritional disorder. Women are more prone to suffer from the consequences of iron deficiency anemia because of menstrual blood loss and enhanced iron requirements during pregnancy and lactation, especially in developing countries [3]. Hemosiderosis is a term used to imply iron overload without associated tissue injury. It occurs locally in sites of bleeding or inflammation, and may be widespread in persons who have been given large amounts of iron, either as iron medication or as blood transfusions [1].

Hereditary hemochromatosis (HH), a disorder that causes iron overload, is the most common autosomal recessive disorder, affecting 1 in 300 individuals in Northern European populations [4]. Mutations in the hemochromatosis (*HFE*) gene (C282Y gene) that are responsible for HH have been identified. Two point mutations, G-to-A transition at cDNA position 845 produces a substitution of tyrosine for a highly conserved cysteine residue at position 282 (C282Y in the protein) [3] and C-to-T transition at nucleotide 187 produces substitution of Aspartic acid for Histidine residue at position 63 (H63D) are mainly identified with HH. A third mutation, A193T (S65C, Cysteine for Seerine), has been reported to be associated with mild iron overload, although the clinical significance of this mutation is still uncertain [5]. Homozygosity for this mutation has been found in 67–100% of patients with HH, and 3.2–13% of Caucasians have been found to be heterozygous for this gene alteration [3]. Primary causes of hemochromatosis usually stem from inherited abnormalities of proteins implicated in iron transport and regulation that may lead to excessive absorption of iron from the gastrointestinal tract. HH is characterized by an increase in iron absorption inappropriate to body iron stores, which leads to iron deposition in parenchymal organs such as the liver and the pancreas. Initial clinical symptoms of tissue iron overload typically occur at adult age and are often nonspecific and vague. In later stages, disease manifestations may include arthropathy, diabetes mellitus, hypogonadism and other endocrinopathies, liver cirrhosis, cardiomyopathy, skin pigmentation, and in cirrhotic patients, increased susceptibility to liver cancer [6].

3. Methods to Measure Iron Status

The conventional laboratory tests of iron status; serum iron, transferrin/total iron-binding capacity (TIBC), transferrin saturation, and ferritin are widely used in clinical practice, although they are considerably influenced by acute phase response, which complicates the clinical interpretation of the test results. High sensitivity, as well as specificity, is of special importance for a test of iron status, as the further identification of the cause of the depletion of iron stores is bound to result in tedious clinical and laboratory investigations. Because the absence of stainable iron in bone marrow examination is generally regarded as the definitive marker of iron deficiency, marrow examinations are generally requested to confirm iron deficiency. There is an evident clinical need for noninvasive and sensitive means for the detection of iron deficiency, and in recent years, the serum transferrin receptor (TfR) level and hepcidin assays havebeen introduced [2] [7].

3.1. Serum Iron

The principle of serum iron measurement lies on the fact that iron is released from transferrin by decreasing the pH of the serum. The released iron is reduced from Fe^{3+} to Fe^{2+} (by use of ascorbic acid [8]) and then complexed with a chromogen, such as bathophenanthroline, chromazurol B or ferrozine. Such iron-chromogen complexes have an extremely high absorbance at the appropriate wavelength (for e.g. at 660nm for chromazurol B), which is proportional to iron concentration [1] [9].

The serum iron concentration refers to the Fe^{3+} bound to serum transferrin and does not include the iron contained in serum as free hemoglobin. The serum iron concentration is decreased in many, but not all, patients with iron deficiency anemia and in chronic inflammatory disorders, such as acute infection, immunization, and myocardial infarction. Greater than normal concentrations of serum iron occur in iron-loading disorders such as hemochromatosis, in patients with aplastic anemia, in acute iron poisoning in children, after oral ingestion of iron medication, after parenteral iron administration or in acute hepatitis [1]. The reference range for serum iron is 15.7 ± 6.2 umol/L and is higher for male than female [10].

3.2. Iron Binding Capacity and Transferrin Saturation

The serum unsaturated iron-binding capacity and the total iron-binding capacity (TIBC) are determined by addition of sufficient Fe^{3+} to saturate iron binding sites on transferrin. The excess Fe^{3+} is removed and the assay for iron content is then repeated. [1].

Total iron-binding capacity (TIBC) indicates the total amount of iron to saturate plasma or serum transferrin, which is a binding protein for iron. The measurement of serum iron, TIBC, and the percent saturation of transferrin with iron [(serum iron/TIBC) 100] have been used to assess the state of iron deficiency [8].

The measurement of TIBC consists of three steps: saturation of transferrin by addition of an excess amount of iron, removal of unbound iron by absorption with magnesium carbonate ($MgCO_3$) or ion-exchange resin, and finally determination of iron that is dissociated from transferrin at acidic pH. Then the unbound iron is reduced to Fe^{2+} by ascorbic acid and eliminated by formation of a complex with ferrozineused as a chromogenic reagent. Ferrozine–iron complex that was proportional to the TIBC under acidic pH has an increase in absorbance at 570 nm [8].

Typically, about one third of the iron-binding sites of transferrin are occupied by Fe^{3+}. Serum transferiin, therefore, has considerable reserve iron-binding capacity. This is called the serum unsaturated iron-binding capacity. The TIBC is a measurement of the maximum concentration of iron that transferrin binds. The serum TIBC varies in disorders of iron metabolism. It is often increased in iron deficiency and decreased in chronic inflammatory disorders or malignancies, and it is often decreased also in hemochromatosis [1]. The reference range for TIBC is 48.5 ±6.3umol/L and is higher for females than males and a 30% - 50% saturation is considered normal [10].

3.3. Serum Ferritin

It is a kind of storage iron that consists of a protein shell surrounding an iron core. It consists of an apoferritin shell composed of 24 subunits, which are either L (light) or H (heavy) ferritin chains, and an interior ferric oxyhydroxide $(FeOOH)_x$ crystalline core. It provides a reserve of iron readily available for formation of hemoglobin and other heme proteins and is found in nearly all cells of the body. It is stored in a form in which the iron is shielded from body fluids, so that it is unable to produce oxidative damage, as would be the case if it were in ionic form. In men, the amount of storage iron is approximately 800mg, mostly as ferritin; in healthy women, it ranges up to 200mg. Minute quantities of ferritin are also present in serum in concentrations roughly proportional to total body-iron stores. Liver injury and a large number of pathological processes result in release of relatively large amounts of ferritin into plasma [1]

Serum ferritin assay may be performed by any of several methods, including immunoradiometric asay (IRMA), enzyme-linked immunoseorbent assay (ELISA), immunochemiluminescent and immunoflurometric methods [11] [12]. Ferritin is found to have reference range of; 15 to 306 ug/L for men and 5 to 103 ug/L for women according to a study conducted using radioio immuno assay [7] but another study conducted using immunofluorometric assay reported a hospital ferritin reference range of 20-240ug/L for men and 10-100ug/L for woman [13], hence the reference range is method dependent.

Although it is an acute-phase protein, under normal conditions it roughly reflects the body iron content. The circulating protein is largely apoferritin. The plasma ferritin concentration declines very early in the development of iron deficiency, long before changes are observed in blood hemoglobin concentration, RBC size, or serum iron concentration. Thus measurement of serum ferritin concentration is used as a very sensitive indicator of iron deficiency that is uncomplicated by other concurrent disease. Alternatively, a large number of chronic diseases result in increased serum ferritin (SF) concentration e.g. Hepatitis [1] The introduction of serum ferritin for the evaluation of the iron status constituted a marked diagnostic improvement since low levels are only seen in ID (absence of iron stores) and is not associated with any other known condition. On the other hand, infections, inflammatory conditions, and liver diseases usually increase SF, also in ID subjects, and can thus lead to an underestimation of the true prevalence of ID. Also, a temporarily decreased energy intake may increase the SF concentration [14]. All in all, measurement of serum ferritin is currently the accepted laboratory test for diagnosing iron deficiency, and a ferritin value <12 ug/L is a highly specific indicator of iron deficiency [15].

3.4. Serum Transferrin Receptor

The Tfr receptor is a transmembrane protein with two identical polypeptide chains, each weighing 85kD. Each subunit has a 61-amino acid N-terminal cytoplasmic domain, a transmembrane region, and a large extracellular domain. TfR is shed from cells by proteolytic cleavage at Arg100-Leu101, just external to the plasma membrane and just after the two interchain disulfide bonds. The product circulates in the blood as soluble TfR (sTfR), a 74-kDa monomerbound to transferrin. The amount of circulating sTfR is proportional to the total amount of cell-associated TfR [16]. Iron delivery to erythroblasts is mediated by the interaction of plasma transferrin with cell surface transferrin receptors. From the cell membrane the TfR-transferrin-iron complex is internalized via an endocytic vesicle, and in the intracellular compartment iron dissociates from TfR-transferrin complex. The iron remains in the cytosol, while the TfR- transferrin complex is recycled back to the cell surface. Virtually all cells have transferrin receptors on their surface, but in the normal adult, about 80% of them are in the erythroid marrow [7], therefore the concentration of circulating soluble transferrin receptor (sTfR) is primarily determined by erythroid marrow activity [17] Soluble TfR present in human plasma is not an acute-phase reactant unlike serum ferritin,

hence is relatively a sensitive marker for iron deficiencyand stays normal in patients with inflammation [14]. When the iron supply is inadequate, there is an up-regulation of transferrin receptor synthesis to enable the cell to compete more effectively for iron [17]. Though it was assumed that sTfR would be decreased in concentration in Iron overload study findings are inconclusive and are not useful for the diagnosis of iron overload, mainly due to the considerable overlap in the values between subjects with iron overload and those with normal iron stores [18].

Both serum ferritin and serum transferrin receptor (sTfR) are known to undergo a characteristic sequence of changes, as body iron stores decrease from normal iron-replete levels to those found in IDA. During the depletion phase (stage I), in which sTfR concentration remains stable, there is a progressive decrease in serum ferritin. When the storage deficit is sufficient to restrict the synthesis of hemoglobin and other functional iron compounds, iron-deficient erythropoiesis (IDE) ensues (stage II). The only indicator of early IDE is the compensatorily elevated sTfR concentration. Finally, IDA (stage III) develops as the hemoglobin (Hb) concentration falls below the lower limit of normal as a result of progressive depletion of the functional iron compartment [13].

Serum TfR is usually measured using an enzyme-linked immunosorbent assay (ELISA) method by using a polyclonal or monoclonal antibodies [4] [15] [16]. The reference range for sTfR is 1.16 – 2.2 mg/L, and is found in a little higher concentration in females than male [10]. The combination of measurements of iron stores and of functional tissue iron as represented by the sTfR:SF ratio has potential advantages in distinguishing IDA fromACD(Anemia of chronic diseases). Measurements for SFis taken as ug/l. In calculating the sTfR:Fer ratio, the sTfR values are converted to ug/l and the logarithm (base 10) of the ratio is used: $Log^{TfR:Fer}$ (ug/l: ug/l). The TfR-Index is calculated as the ratio of sTfR to logarithm (base 10) of SF:sTfR/Log^{SF} ratio [12].

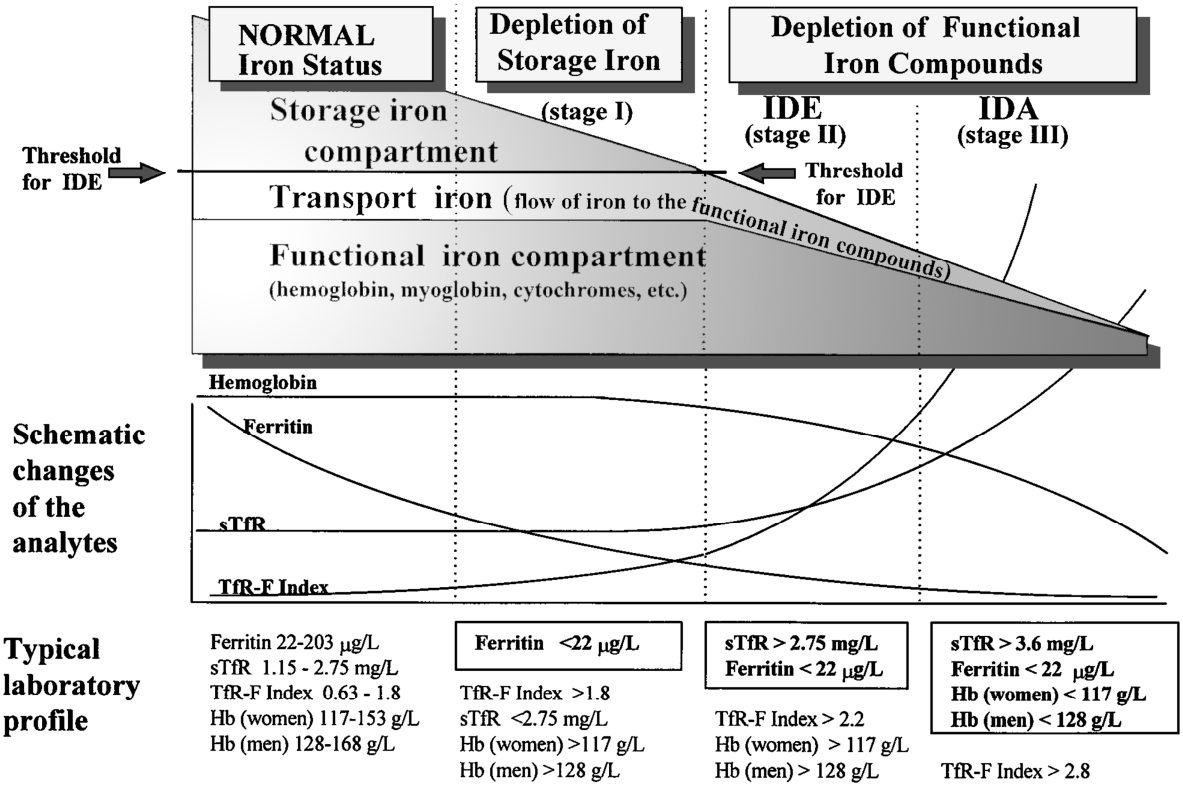

Figure 1. *Phases of advancing ID. From Pauli Suominen, et al. Serum Transferrin Receptor and Transferrin Receptor-Ferritin Index Identify Healthy Subjects With Subclinical Iron Deficits. Blood 1998 [12].*

3.5. Reticulocyte Hemoglobin Content

Because reticulocytes are the earliest erythrocytes released into blood and circulate for only 1 to 2 days, the reticulocyte hemoglobin content (CHr) provides a measure of iron available to red cells recently produced by the bone marrow [19]. CHr has been shown to be an early indicator of iron-restricted erythropoiesis in patients receiving erythropoietin therapyand is a strong predictor of iron deficiency [20].

Automated devices that measure the hemoglobin reticulocyte are commercially available and haveenabled the integration of reticulocyte analysis with the complete blood count (CBC) in high throughput automated hematology analyzers [1]. This measurement has been found to be useful in differentiation the anemia of iron deficiency from that of chronic inflammation [21].

The CHr is determined from measurements of light scatter at two different angles after isovolumetric sphering of oxazine 750 stainedreticulocytes (or by new methylene blue; both stain the RNA content of the reticulocytes [21] [22].

From the amount of light scattered at the two different angles, the hemoglobin concentration and cellular volume of individual reticulocytes are independently determined, suggesting that this light scatter may be used for monitoring functional iron deficiency [19] [23]. CHr reference range is method dependentand may range from 25.9–35.9 pg [21].

CHr has some diagnostic limitations. Independent of iron status, CHr is often reduced in alpha and beta thalassemias and hemoglobinopathies that cause microcytic anemia. It can also be increased in iron-deficient patients with confounding megaloblastic anemia due to the high mean reticulocyte volume associated with megaloblastosis. Therefore, it is important that CHr values be interpreted in the context of the patient's overall erythrocyte physiology, including knowledge of recent blood transfusions, iron therapy, vitamin B12 or folate deficiency, and the results of Hb analysis. But CHr can be combined with sTfR, ferritin ratio to differentiate IDA with ACD [21].

3.6. Hepacidin Assay

Hepcidin is a hepatocyte-produced peptide hormone that regulates systemic iron homeostasis. The mature bioactive form of hepcidin is a 25 amino acid peptide. Subsequent amino-terminal processing of the 25 amino acid form can result in the appearance of two smaller hepcidin forms of 22 and 20 amino acids, which are without apparent biological function. Under physiological conditions hepcidin-20 and hepcidin-22 are present in the urine, but not, or at very low concentrations, in the serum. Interestingly, these smaller hepcidin isoforms occur only in serum of patients with diseases that are associated with increased concentrations of hepcidin-25, such as acute myocardial infarction (AMI), sepsis, anemia of chronic disease (ACD), metabolic syndrome, and chronic kidney disease (CKD). In vivo studies in mice have demonstrated that only full-length 25 amino acid hepcidin induces significant hypoferremia when injected intraperitoneally. These findings are corroborated by in vitro studies that showed the truncated 22 amino acid and 20 amino acid forms have greatly diminished and almost complete loss of ferroportin regulatory activity, respectively, compared with 25 amino acid hepcidin [2].

By modulating hepcidin production, an organism controls intestinal iron absorption, iron uptake, and mobilization from stores to meet the body iron need. Hepcidin concentrations are decreased in conditions that demand increased serum iron concentrations (i.e., increased erythropoietic activity and iron deficiency), whereas concentrations are increased in infection and inflammation [24]. During inflammation, interleukin (IL) 6 induces hepcidin production, which leads to internalization and degradation of ferroportin, a decrease in dietary iron absorption, and an increase in macrophage iron. As a consequence, inflammation causes decreased circulating iron and impaired iron distribution within the body for which the IL-6-hepcidin axis appears to be responsible [20].

Since the discovery of hepcidin (in 2001) and its crucial role in iron homeostasis, there has been substantial interest in developing reliable assays to measure hepcidin concentrations in body fluids. Accurate assessment of hepcidin concentrations in serum would improve our understanding of iron metabolism disorders and allow hepcidin to become a useful tool in the differential diagnosis and clinical management of these diseases [24]. Hepcidin clearance is assumed to occur via cellular codegradation with ferroportin at its sites of action, and via excretion by the kidneys. Because of its low molecular weight and small radius, unbound hepcidin is likely to freely pass into the glomerular filtrate [2].

The quantification of hepcidin has been found to be complicated by its tendency to aggregate and to stick to laboratory plastics, necessitating implementation of robust laboratory procedures. Furthermore, progress in developing a conventional immunochemical hepcidin assay has been hampered by difficulties in generating specific antihepcidin antibodies in hosts such as rabbits. This difficulty is due to the small and compact structure of hepcidin, which leaves scarce antigenic epitopes, and the high degree of conservation of hepcidin among a wide range of species, which diminishes the elicitation of an immune response in host animals [2].

Few investigative tools have been available for measuring hepcidin in biological fluids. Assays based on mass spectrometry (MS) require relatively expensive equipment, but these methods are advantageous because they can be used to distinguish between hepcidin-25, -22, and -20 [24].

First-Generation Hepcidin Assays: In several early studies a commercial serum-based immunoassay was used that measures the hepcidin precursor prohepcidin rather than the bioactive peptide [2]. The relevance of these studies is questionable, because of the fact that prohepcidin concentrations correlate with neither urinary nor serum hepcidin concentrations, nor do they correlate with relevant physiological responses [25]. The first assays to measure bioactive hepcidin-25 were an immunodot assay, surface-enhanced laser desorption ionization - time of flight mass spectrometry (SELDI-TOF MS). However, these assays could be used to measure hepcidin only in a semiquantitative manner [26].

Second-Generation Hepcidin Assays: In recent years, substantial progress has been made by the introduction of an internal standard to quantify hepcidin-25 in serum and urine by an updated TOF-MS assay [24]. Micro-HPLC-tandem mass spectrometry method, which has a limit of quantification up to 0.2 nmol/L, has also been applied to quantitate hepcidin-25 in serum [20]. Acompetitive ELISA (cELISA) for human serum hepcidin has also been developed. Other study groups have also reported reliable hepcidin assays that can be divided in 3 main methodologies: (a) MS; (b) immunochemical assays, comprising cRIA, cELISA, and a 2-site ELISA (100); and (c) a ligand-binding assay [27].

Strengths and Limitations of Hepcidin Assays: MS assays require relatively expensive equipment, but they have the advantage of distinguishing between hepcidin-25, -22, and -20 [24]. ELISA assays will measure total hepcidin

concentrations, with (depending on the specificity of the antibody) different contributions from each of these 3 isoforms. However, this concern might be overcome by exploiting antibodies that are hepcidin-25 specific [28]. At the same time it should be noted that the relevance of measuring hepcidin-25 instead of total hepcidin for clinicaldecision-making has not been systematically investigated. Immunoassays have the potential for more widespread use in clinical laboratories. Furthermore, sample throughput is likely to be higher for ELISA than for MS assays. However, MS methods can also be automated, and would have to be optimized for hepcidin because its amphiphatic character makes it readily stick to laboratory plastics, especially when relatively small sample volumes are used in large tubes [2].

Urine hepcidin measurements may be attractive, especially for use in research and measurements in children and patients in the underdeveloped world, because this matrix allows noninvasive sampling. Nevertheless, although a significant relationship was found between the hepcidin concentrations of urine–serum sample pairs from healthy controls and patients with disorders of iron metabolism, urine hepcidin concentrations may not always accurately reflect serum hepcidin concentrations. Interpretation of urinary hepcidin data is difficult because urine concentrations may also depend on glomerular filtration, tubular reabsorption, local production by tubular epithelial cells, and production by interstitial inflammatory cells. These issues make measurement of hepcidin somewhat disadvantageous in urine compared to serum and imply that for reliable interpretation of urine hepcidin concentrations as an alternative for serum concentrations, information on renal hepcidin production and handling is needed. Another potential drawback of urine hepcidin measurements is its sensitivity to oxidation and the relatively high concentration of the smaller hepcidin isoforms, which cannot be distinguished from hepcidin-25 by most immunoassays. Altogether, these limitations should be kept in mind during the interpretation of urine hepcidin data and the decision to develop dedicated point-of-care devices for urine hepcidin measurements. So far there is no reference method and calibrator for hepcidin measurment [2].

Hapcidin measurment is known to have promising application in the diagnosis of hereditary hemochromatosis, Iron-loading anemia's, Iron deficiency anemia, Chronic kidney diseases (CKD) etc. but efforts must be undertaken to assess the relevance of specifically measuring hepcidin- 25, to harmonize assay outcomes throughout the world, to define clinical decision limits, and to make assays available to clinical laboratories before hepcidin assays can be fully included in clinical practice. [2].

4. Conclusions and Recommendation

Circulating transferrin receptors reflect the body's iron status, and that the inverse relationship between transferrin receptor expression and ferritin expression. Ferritin is an acute Iron Deficiency marker before hematological changes. Together with serum iron, TIBC and transferrin saturation, these tastes provide a panel for iron status determination and help discriminate between IDA and ACD, also they provide information about Iron overload, as Iron excess acts as a free radical catalyst causing toxicity and tissue damage.

Efforts should be taken to include new markers like hepcidin in assessing Iron status. Also research efforts are mandatory to alleviate absence of a reference method, a validated commutable calibrator and clinical decision limit or other material for assay harmonization of hepcidin. Furthermore all these iron status markers should made available in clinical laboratories along with the hematological tests.

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