INTRODUCTION

Beta thalassemia major is considered the most severe form of thalassemia. It is the most common chronic haemolytic anaemia in Egypt ***(Khalifa et al., 1985).***

As a result of marrow expansion, distorted bone formation ensues. Also results in iron overload, which is the major cause of beta-thalassemia mortality worldwide ***(Tanno et al., 2010).***

Hepcidin is a protein, synthesized in the liver that reduces iron absorption in the body. In thalassemia, the patient's hepcidin expression is pathologically suppressed ***(Tanno et al., 2010).***

The transforming growthfactor–β (TGF-β) superfamily of proteins comprisesmore than 40 members, broadly divided into 2 branches, definedby sequence homology and the signaling pathways that they activate ***(McPherron and Lee, 2007)***.

The first branch contains TGF-β, activins, and the nodalfamily, and the second encompasses bone morphogenic proteins (BMPs), growth and differentiation factors (GDFs), and Mullerian-inhibiting substance ***(Lakhal et al., 2009).***

Growth Differentiation Factor 15 (GDF15), which has been proven to be over expressed as result of an expanded erythroid compartment, has been identified as a hepcidin

suppressing cytokine that is overexpressed in thalassemic patients ***(Tanno et al., 2010)***.

GDF15 levels in blood plasma have been found to be dramatically elevated in beta-thalassemia patients compared to healthy donors and patients with hereditary hemochromatosis, another form of iron overload disease ***(Porter, 2009).***

AIM OF THE WORK

The aim of this study is to:

1- Assess the expression of growth differentiation factor 15, and its correlation with the complications of iron overload in patients with beta thalassemia major.

2- To outline the relation of the frequency of blood transfusion and compliance to iron chelators with the expression of growth differentiation factor 15.

IRON METABOLISM

1) Human Body Content of Iron

Iron is essential for cellular metabolism and aerobic respiration, however cellular iron overload leads to toxicity and cell death via free radical formation and lipid peroxidation ***(Munoz et al., 2009)***.

For a healthy adult male individual, total body iron is about 3.5 g (50 mg/kg). Most of the iron in the body is distributed within RBC hemoglobin (65%). Approximately 10% is present in muscle fibers (myoglobin) and other tissues (enzymes and cytochromes). The remaining body iron is stored in the liver, macrophages of the reticuloendothelial system, and bone marrow ***(Siah et al., 2006).***

The normal diet contains 15-20 mg of iron, and the body absorbs 1-2 mg/d of dietary iron. This is balanced with losses *via* sloughed intestinal mucosal cells, menstruation and other blood losses. Therefore, internal turnover of iron is essential to meet the bone marrow requirements for erythropoiesis (20-30 mg/d) ***(Conrad et al., 2002; Miret et al., 2003).***

2) Iron Absorption

Nearly all absorption of dietary iron occurs in the duodenum. Several steps are involved, including the reduction of iron to a ferrous state, apical uptake, intracellular storage or

transcellular trafficking, and basolateral release ***(Munoz et al., 2005)*.**

Dietary iron is found in heme (10%) and non-heme (ionic, 90%) forms. Dietary non-heme iron primarily exists in an oxidized (Fe^{3+}) form that is not bioavailable, and must first be reduced to the Fe^{2+} form by a ferri reductase enzyme, before it is transported across the intestinal epithelium by a transporter called divalent metal transporter 1 (DMT-1), which also transports other metal ions such as zinc, copper and cobalt by a proton-coupled mechanism. **(*Knovich et al., 2009*)**

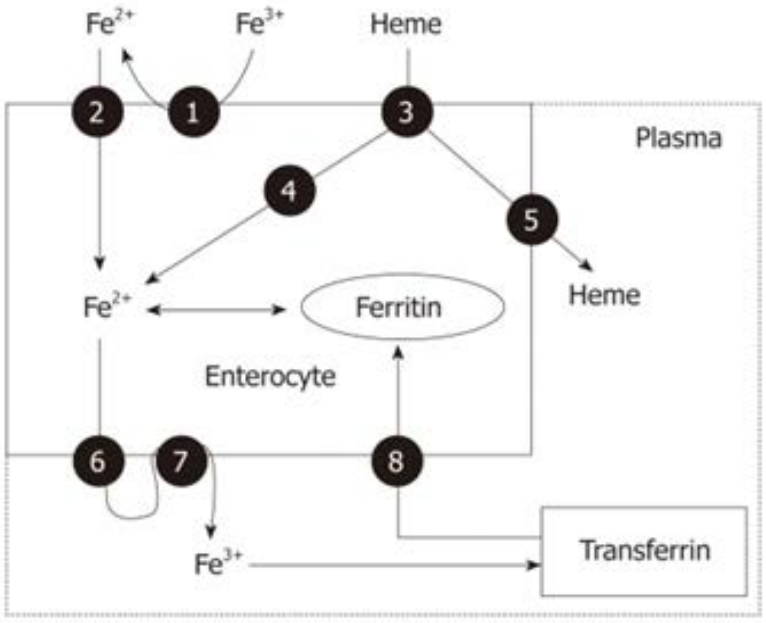

Figure (1): Main pathways of iron absorption by enterocytes in mammals. 1: Ferrireductase; 2: Divalent metal transporter 1 (DMT-1); 3: Heme protein carrier 1 (HPC1); 4: Heme oxygenase; 5: Heme exporter; 6: Ferroportin (Ireg-1); 7: Hephaestin; 8: Transferrin receptor-1 **(*Knovich et al., 2009*)**

Heme iron is absorbed into enterocytes by a putative, heme carrier protein 1, which is a membrane protein found in the proximal intestine ***(Krishnamurthy et al., 2007)***.

Heme carrier protein 1 is also expressed in the kidneys and liver, which suggests that it scavenge free heme or mediate cellular uptake of heme from its circulating carrier protein, hemopexin ***(Andrews, 2005).***

Once internalized in the enterocytes, it is likely that most dietary heme iron is released as ferrous iron by heme oxygenase to enter a common pathway with dietary non-heme iron before it leaves the enterocytes ***(Munoz et al., 2009).***

There is also a siderophore-like iron uptake pathway mediated by lipocalin-2, which is also called neutrophil gelatinase-associated lipocalin, and has been proposed to be a mediator of the transferrin-independent iron delivery pathwaycan transport iron into cells during kidney development ***(Huang et al., 2009).***

Inside the intestinal epithelial cell, iron may either remains in the cell for use or storage (this iron is lost when enterocytes are sloughed into the gut lumen) or exported across the basolateral membrane of the enterocyte into the circulation (absorbed iron). Ferrous iron once exported across the basal membrane by ferroportin 1, is then oxidized by a multi-copper oxidase protein called hephaestin (an enzymatic protein similar

to plasma ceruloplasmin) before being bound by plasma transferrin ***(Mackenzie et al., 2008).***

Regulation of Iron absorption

The absorption of iron is dependent on the body's iron stores, hypoxia and rate of erythropoiesis ***(Siah et al., 2006).***

Ferroportin needs copper-ferroxidases to release iron to plasma transferrin, namely hephaestin in duodenal cells and ceruloplasmin in hepatocytes, and macrophages. When defective, these proteins induce cellular iron retention in specific cell types ***(Mariani et al., 2009).***

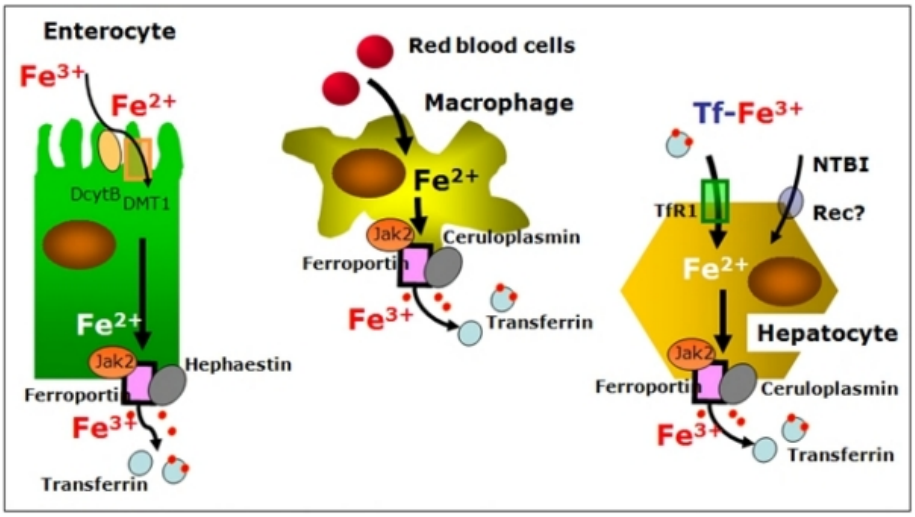

Figure (2): Cells regulating body-iron homeostasis. Enterocytes, macrophages and hepatocytes acquire iron from different sources and deliver it to the rest of the body through the iron exporter ferroportin, which needs copper-ferroxidases to release iron to plasma transferrin. Ferroportin acts under the control of hepcidin and this interaction can explain the systemic regulation of iron metabolism***(Mariani et al., 2009).***

Two models have been proposed to explain how the absorption of iron is regulated. These models have been termed

the crypt programming model and the hepcidin model ***(Siah et al., 2006)***.

A) The crypt programming model:

Proposes that enterocytes in the crypts of the duodenum takes up iron from the plasma. The intracellular iron level of the crypt cells corresponds to the body's iron stores, which in turn determines the amount of iron absorbed from the gut lumen, as these crypt cells migrate upwards to become absorptive cells at the brush border. The crypt cells express both transferrin receptor 1 (TfR1) and TfR2, which mediate the cellular uptake of transferrin-bound iron from plasma ***(Muñoz et al., 2005; Siah et al., 2006).***

The hemochromatosis protein (HFE),an MHC-class 1-like molecule that interacts with β2-microglobulin and forms a complex with TfR1, is highly expressed in crypt cells and seems to enhance transferrin-bound iron uptake from the plasma into crypt cells, and may also inhibit the release of iron from the cell *via* ferroportin 1 ***(Muñoz et al., 2009).***

The intracellular iron concentration controls the interaction of cytosolic iron regulatory proteins (IRPs) 1 and 2 with iron regulatory elements (IREs) which act as iron sensors in mammalian cells and regulate translation or stability of mRNA-encoding proteins***(Wang and Pantopoulos, 2011).***

In the absence of iron, IRP1 binds to IREs of TfR1, DMT-1, and ferroportin 1 mRNA, the transcript is stabilized,

translation proceeds, and the proteins are synthesized. Thus, a high IRP binding activity reflects low body iron stores and results in upregulation of these proteins in the duodenum and increased dietary iron absorption ***(Muñoz et al., 2009).***

B) The hepcidin model:

Hepcidin is a 25-amino-acid (aa) peptide, the principal regulator of iron absorption and its distribution to tissues***(Valore and Ganz, 2008).***

Hepcidin derived from cleavage of an 84 amino acid pro-peptide which is encoded by the hepcidin antimicrobial peptide (HAMP) gene ***(Andreani et al., 2009).***

The main peptide of hepcidin contains eight cysteine residues linked as four disulphide bridges resulting in a molecule with a simple hairpin structure and the bridges in a ladder-like configuration.This structure is characteristic of peptides capable of disrupting bacterial membranes and is similar to other antimicrobial peptides ***(Nemeth and Ganz, 2009).***

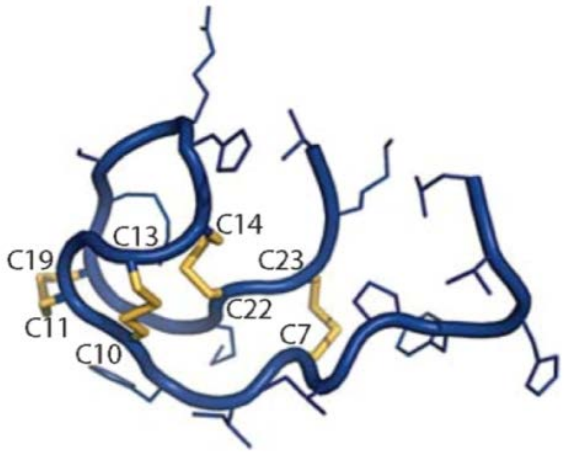

Figure (3): Hepcidin structure***(Nemeth and Ganz, 2009).***

Hepcidin is synthesized predominantly in hepatocytes, but its low levels of expression in other cells and tissues, including (macrophages, adipocytes and brain) may also be important for the autocrine and paracrine control of iron fluxes at the local level ***(Nemeth and Ganz, 2009).***

- **Mechanism of Action of Hepcidin**

The liver peptide hepcidin regulates intestinal iron absorption and iron release from storage cells by binding ferroportin causing its internalization and degradation, thus exerting a general inhibitory effect on iron release in the body ***(Mariani et al., 2009)***.

Ferroportin is both the hepcidin receptor and the only known cellular iron exporter. It is expressed on duodenal enterocytes absorbing dietary iron, macrophages in liver storing iron, in spleen recycling old erythrocytes, and in placental trophoblasts transferring iron to the fetus during pregnancy. Ferroportin is also

expressed in erythroid precursor cells enhancing the sensitivity of precursors to systemic iron levels and helps determine their commitment to expansion and differentiation ***(Zhang et al., 2009).***

The binding likely involves disulfide exchange between one of disulfide bonds of hepcidin and the exofacial ferroportin thiol residue Cys326. Patients with C326S mutations develop early-onset iron overload, and the mutant ferroportin loses its ability to bind hepcidin in vitro ***(Fernandes et al., 2009).***

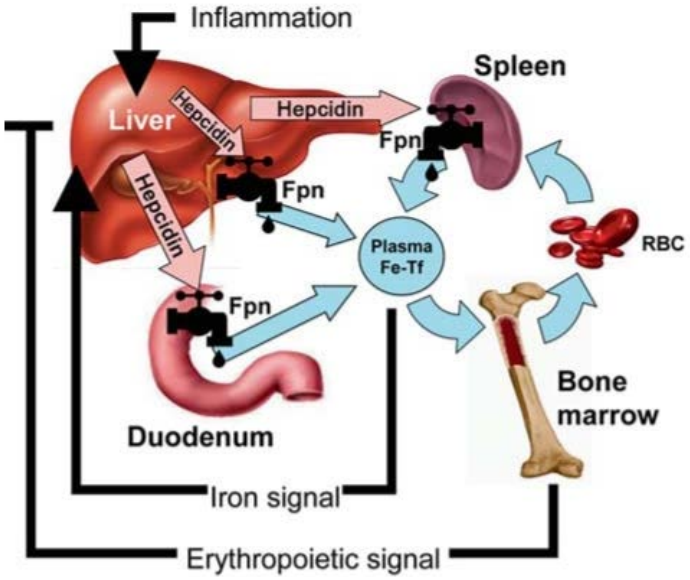

Figure (4): Mechanism of action of hepcidin (hepcidin Fe interaction). Increased iron Tf concentrations stabilize TfR2 protein, HFE is displaced from TfR1 and associates with TfR2. The HFE-TfR2 complex likely interacts with the HJV-BMP receptor (BMPR)***(Nemeth and Ganz, 2009).***

It has been shown that hepcidin-induced internalization of ferroportin requires binding and cooperative interaction with Janus kinase (Jak2). Hepcidin binding to ferroportin results in the phosphorylation of ferroportin, a step necessary for its

internalization, the kinase responsible for the phosphorylation is Jak2***(De Domenico et al., 2008).***

- **Regulation of Hepcidin**

In physiological conditions, hepcidin production is tightly regulated in response to signals released from bone marrow (erythroid regulator) and the iron stores (store regulator) ***(Mariani et al., 2009).***

Iron excess stimulates hepcidin production, and increased concentrations of the hormone in turn block dietary iron absorption thus preventing further iron loading ***(Ramos et al., 2012).***

Conversely, hepcidin is suppressed in iron deficiency, allowing increased absorption of dietary iron and replenishment of iron stores ***(Nemeth, 2010).***

Increased erythropoietic activity also suppresses hepcidin production. Apart from enhancing iron absorption, this enables the rapid release of stored iron from macrophages and hepatocytes and augments the supply of iron for erythropoiesis ***(Nemeth and Ganz, 2009).***

Studies on genetic disorders of iron metabolism and of corresponding animal models have identified the hereditary hemochromatosis proteins (HFE), transferrin factor receptor (TFR2) and hemojuvelin protein (HJV) as the iron-dependent positive regulators of hepcidin expression ***(Piperno et al., 2009).***

The HFE/TfR2 complex signals to the hepcidin appears to involve the BMP pathway because HFE and TfR2 were reported to interact with HJV, it is possible that binding of iron Tf to TfR1 and TfR2 initiates the formation of a supercomplex composed of HFE, TfR2, HJV and BMP receptors ***(Ganz and Nemeth, 2011)***.

Hemojuvelin (HJV) is a BMP co-receptor which augments BMP binding. The consequent activation of intra-cellular SMAD proteins transduces a signal to increase hepcidin transcription.Under low iron conditions matriptase-2 (scissors) cleaves HJV from the cell surface, weakening the BMP6 signal ***(Kroot et al., 2011)***.

Extracellular Tf-Fe^2 mediates a second iron signal.Tf-Fe^2 displaces HFE from TfR1. HFE is then freed to interact with transferrin receptor 2 (TfR2). The HFE-TfR2 complex activates hepcidin transcription via MAPK and/or BMP/SMAD signaling***(Ganz and Nemeth, 2011)***.

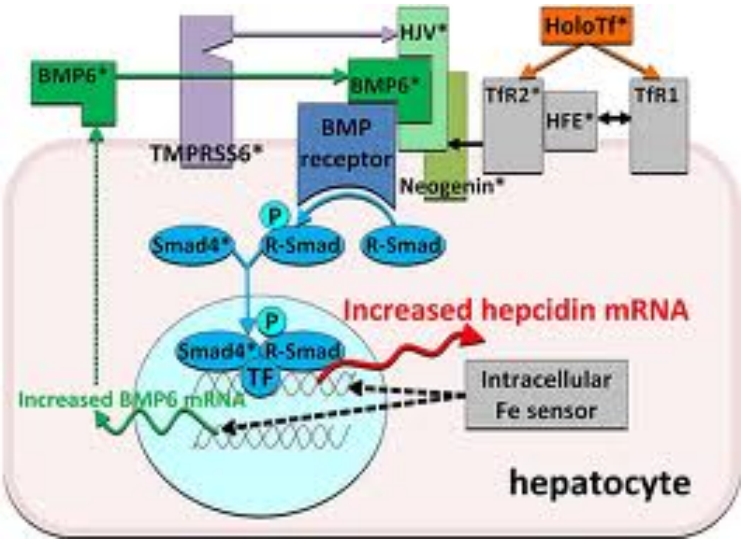

Figure (5): A current model of regulation of hepcidin transcription by iron. Iron as holotransferrin is shown in orange, ironsensors and associated molecule in gray, BMP receptor and its transduction pathway in shades of blue, the ligands and coreceptors of the BMP receptor in shades of green, and the negative regulator protease in purple. *Molecules whose ablationwas shown to cause iron dysregulation ***(Ganz, 2007)***.

Very high erythropoietic activity and increased iron requirement from the bone marrow generates a signal that can over ride hepcidin regulation by iron, leading to increased iron absorption **(*Mariani et al., 2009*)**

Hypoxia induced factor 1 alfa (HIF-1a) that is stabilised in hypoxia/iron deficiency, reduces hepcidin transcription by binding a HIF responsive element of hepcidin promoter***(Smith et al., 2008)*.**

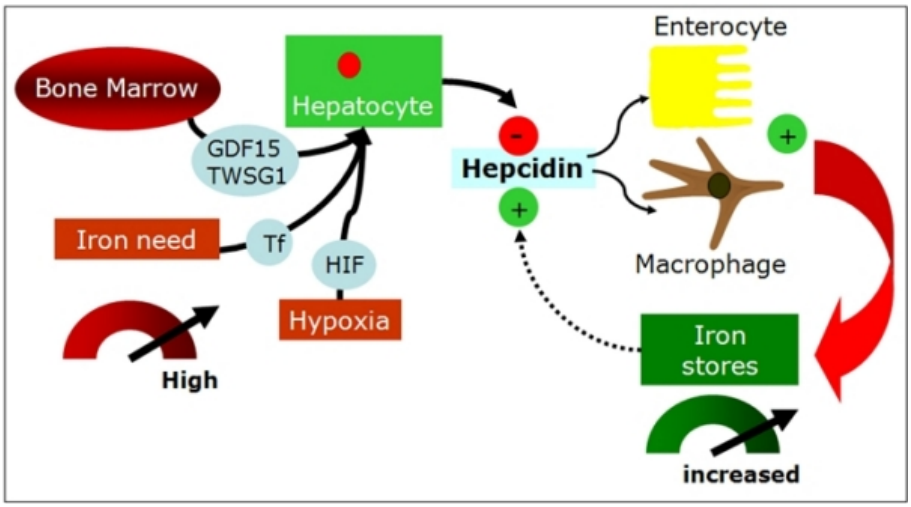

Figure (6): Hepcidin regulation by erythroid- iron- and hypoxia-related signals in iron-loading anemias (GDF= growth differentiation factor; TWSG = twisted gastrulation; TF= holo-transferrin; HIF= hypoxia inducible factor) ***(Mariani et al., 2009).***

The synthesis and release of hepcidin is also rapidly mediated by bacterial lipopolysaccaride and cytokine release, especially interleukin-6. Thus the hepcidin gene is an acute-phase responsive gene which is overexpressed in response to inflammation, and this is now thought to be responsible for the anaemia of chronic disease, where iron is retained by enterocytes, macrophages and hepatocytes ***(Wessling-Resnick, 2010).***

- **Hepcidin Dis-regulation**

i) Decreased Hepcidin

Hepcidin deficiency results in the development of systemic iron overload because of excessive iron absorption. In