SERUM GROWTH DIFFRENTIATION FACTOR 15

IN PATIENTS WITH ULCERATIVE COLITIS

DISSERTATION

Submitted to

THE TAMILNADU DR MGR MEDICAL UNIVERSITY

In partial fulfilment for the degree

DOCTOR OF MEDICINE

IN

BIOCHEMISTRY- BRANCH XIII

APRIL 2015

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DEPARTMENT OF BIOCHEMISTRY

CHRISTIAN MEDICAL COLLEGE

VELLORE- 632002, INDIA

CERTIFICATE

This is to certify that the study entitled **"SERUM GROWTH DIFFRENTIATION FACTOR 15 IN PATIENTS WITH ULCERATIVE COLITIS"** is the bona fide work of Dr. Chinmai Jagadish, who conducted it under the guidance and supervision Dr. Molly Jacob, MD, Ph.D. The work in this dissertation has not been submitted to any other university for the award of a degree.

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DECLARATION

I hereby declare that the investigations, which form the subject matter of this study, were conducted by me under the supervision of Dr. Molly Jacob, Professor of Biochemistry, Christian Medical College, Vellore.

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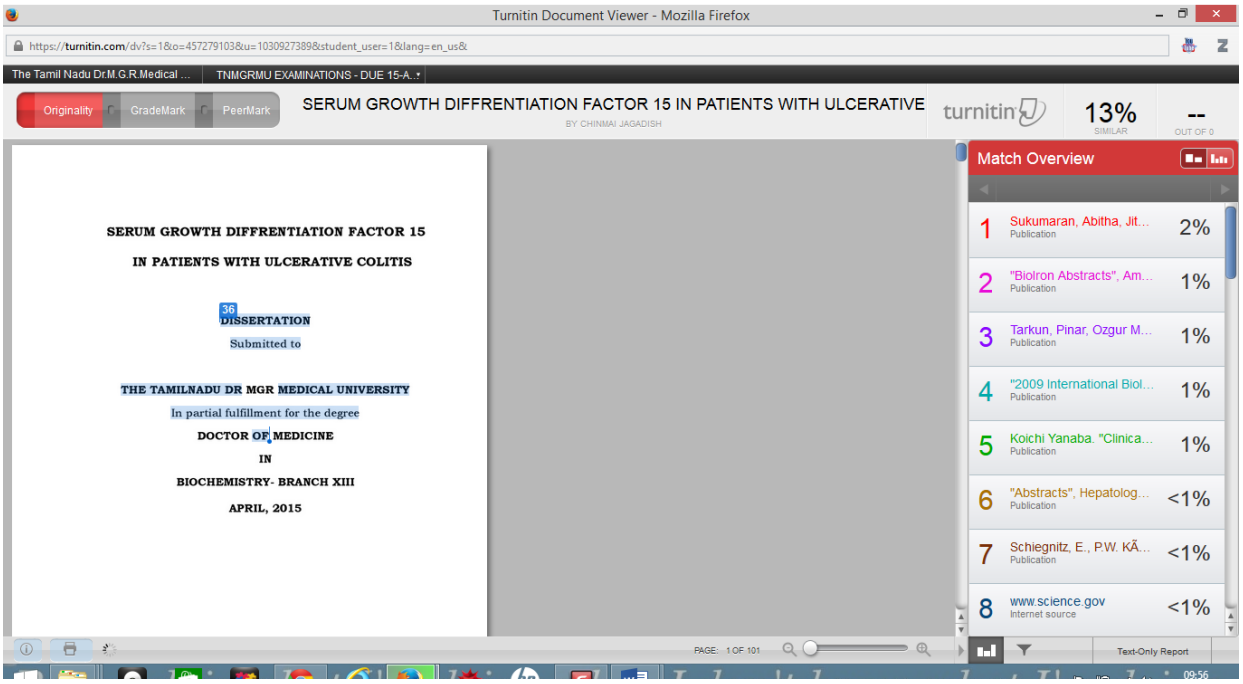
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CONTENTS

ABSTRACT

SERUM GROWTH DIFFERENTIATION FACTOR-15 LEVELS IN PATIENTS WITH ULCERATIVE COLITIS

BACKGROUND

Hepcidin is the central regulator of iron homeostasis. Previous work done in the Department of Biochemistry, CMC, Vellore, has shown that serum hepcidin levels were decreased in patients with ulcerative colitis. Growth differentiation factor-15 (GDF-15) is a known negative regulator of hepcidin.

AIM

The aim of the study was to test the hypothesis that serum GDF-15 levels may be increased in patients with ulcerative colitis (UC), thus accounting for decreased levels of hepcidin in such patients and to determine whether it correlates with hemoglobin and markers of iron status (serum iron and ferritin).

MATERIALS AND METHODS

Twenty patients diagnosed with UC, who were not on iron supplements or erythropoietin therapy, served as cases. Twenty age and gender-matched non-anaemic patients, who underwent upper gastrointestinal endoscopy for evaluation of dyspepsia and who were found to have no endoscopic abnormalities served as controls. A blood sample collected from each subject, after obtaining informed

consent, was used to estimate haematological parameters and serum levels of GDF-15, iron, ferritin, and C-reactive protein (CRP).

RESULTS

Serum GDF-15 levels were significantly higher (912.8 ± 430 pg/ml vs 623.7 ± 285 pg/ml) and hemoglobin levels significantly lower in patients with UC, when compared to control subjects. Levels of GDF-15 and haemoglobin showed a negative correlation with one another.

CONCLUSION

Serum GDF-15 levels were increased in patients with UC. Such raised levels may account for decreased hepcidin levels that have been observed in such patients.

Key words: GDF-15, ulcerative colitis, anaemia, iron, hepcidin

REVIEW OF LITERATURE

INTRODUCTION

Iron is a transitional metal, which is necessary for heme synthesis and a number of other biological functions. It is a redox-active metal and has the ability to accept or donate electrons. Due to this property, it plays an important role in processes like generation of energy by the electron transport chain and helps in metabolism, by serving as a cofactor for various enzymes. However, iron can also be harmful. When present in excess, it can participate in Fenton reactions leading to free radical formation, which leads to oxidative stress in the cell. Therefore, iron levels have to be constantly maintained at a steady state (Wessling-Resnick, 2014).

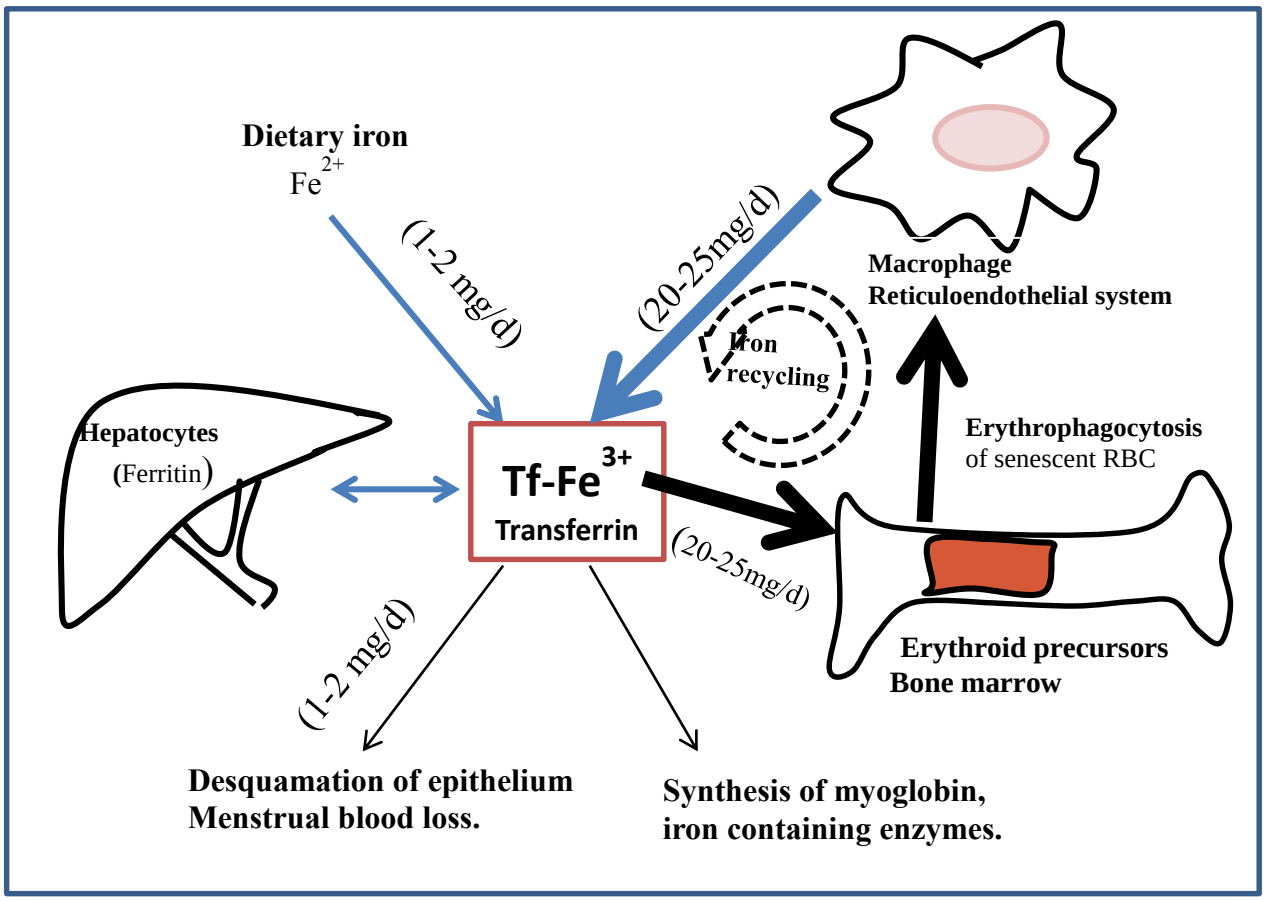
Iron distribution in the body:

An adult male has about 3.8 grams of iron and adult females about 2.3 grams (Wessling-Resnick, 2014). Hemoglobin contains about 67% of the total iron in the body; 27% is present as ferritin and hemosiderrin, 3.7% as myoglobin and only 0.08% of it is in circulation bound to transferrin (Higgins et al, 2012). The major iron consumer in the body is the bone marrow erythroid precursors, where it is utilized for hemoglobin synthesis. About 20-25mg of iron is utilized for this process in a day. There is no regulated excretory route for iron. About 1-2mg is lost through desquamation of

epithelium from mucosal membrane, skin and through menstrual blood loss in females (Adamson, 2011).

Iron needs of the body are met by 2 processes: iron recycling and intestinal absorption. About 20-25mg of iron is recycled through erythrophagocytosis by macrophages of the reticuloendothelial system (Kautz and Nemeth ,2014). Only about 1-2 mg of iron enters the body through intestinal absorption. However, this process is highly regulated.

Figure 1: Depiction of iron distribution in body



INTESTINAL IRON ABSORPTION

The amount of iron absorbed by the intestine is small, when compared to iron recycled by macrophages, in meeting iron needs. However, it is the only means for iron entry into body and is regulated by iron status of body. Intestinal absorption therefore plays a major role in maintaining iron homeostasis (Wessling-Resnick, 2014).

Iron exists as heme and non-heme iron in diet. Heme iron is present mainly in non-vegetarian diet and its bioavailability is high compared to non-heme iron (Wessling-Resnick, 2014). About 10-20mg of iron is present in human diet, of which only 1-2 mg is absorbed, mainly in the proximal duodenum (Adamson, 2011).

Absorption of non-heme iron:

Non-heme iron in the diet exists in the ferric form, which has to be converted to the ferrous form before being absorbed (Wollenberg and Rummel 1987). In the duodenum, ferric iron is converted to ferrous iron by duodenal cytochrome b (Dcyt b) (McKie et al. 2001). Once reduced by Dcytb, ferrous iron is transported across the apical membrane into the enterocyte by divalent metal transporter 1(DMT-

1) (Gunshin et al. 1997). In the enterocyte, ferrous iron enters the labile iron pool from where it is transported out into circulation by ferroportin or stored within the cell as ferritin (Evstatiev and Gasche 2011).

Dcytb is a transmembrane ferric reductase expressed in the brush border of the proximal duodenum (McKie et al. 2001). It is an ascorbate-dependent ferric reductase, where cytosolic ascorbate acts as an electron donor, for luminal reduction of iron (Su and Asard 2006). Its expression is increased in iron-deficient states (McKie et al. 2001). The observation that Dcytb knockout mice did not develop iron deficiency, questioned the necessity of Dcytb in iron absorption (Gunshin et al. 2005). Normal iron status in these mice was attributed to the presence of reducing substances in diet, which may play role in reduction of dietary ferric iron under normal conditions (Choi et al. 2012). However, a reduction in splenic iron was observed in Dcytb knockout mice, when exposed to hypoxia (Choi et al. 2012). The reduction was attributed to increased mobilization of tissue iron to meet increased iron requirement due to hypoxia, as increase of duodenal absorption was not possible (Choi et al. 2012). This observation suggested that Dcytb is necessary in conditions with increased iron requirement, where its expression is up-regulated.

DMT1

Divalent metal transporter 1(DMT1) is also known as DCT1, NRAMP 2, SLC11A2. It belongs to natural resistance associated macrophage protein (NRAMP) family. DMT1 is a membrane-spanning protein located in the apical membrane of enterocytes. Expression of DMT1 is high in the proximal duodenum, where it is mainly involved in Fe^{2+} absorption (Gunshin et al. 1997). Transport of Fe^{2+} by DMT1 is coupled with co-transport of a proton. It is also transports other divalent metal ions like Zn^{2+}, Cu^{2+}, Mn^{2+}, Co^{2+}, Cd^{2+}, Ni^{2+} and Pb^{2+}(Gunshin et al. 1997).

DMT1 is also expressed in erythrocyte membranes and has a role in iron uptake. Autosomal recessive microcytic hypochromic anemia was observed in Belgrade (b) rat and microcytic anemia (mk) mice. Both these animal models were found to have similar missense mutations in DMT1, where glycine is substituted for arginine at 185^{th} position in the polypeptide chain (Fleming et al. 1997; Fleming et al. 1998). This mutation in DMT1 decreased intestinal iron absorption and also erythroid iron uptake, which explains the phenotype observed in these animals (Fleming et al. 1997; Fleming et al. 1998). This finding signifies the important role played by DMT1 in intestinal iron absorption.

Absorption of heme iron:

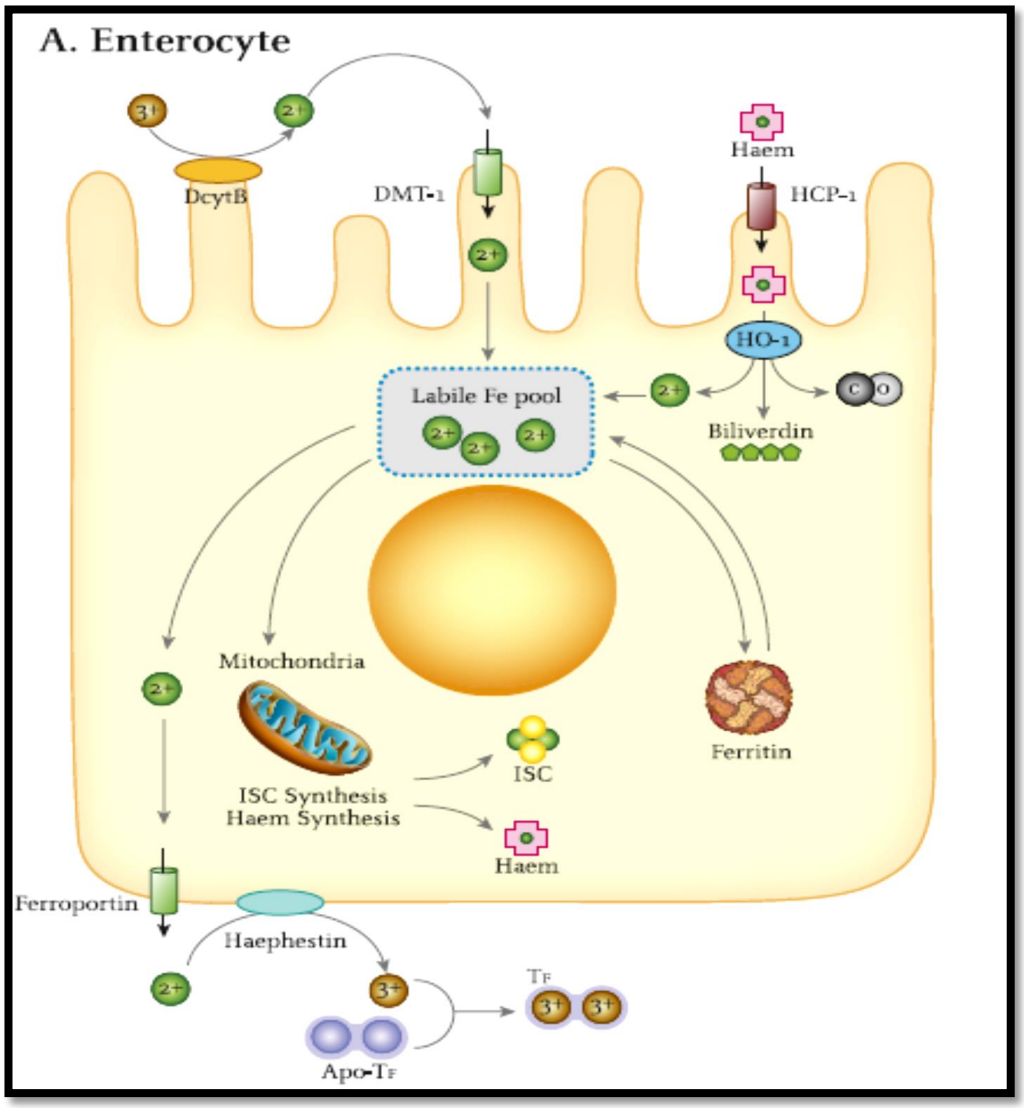
Proteolytic degradation of heme proteins by digestive enzymes in the intestine separates heme from the polypeptide. Heme is then absorbed by putative heme transporters in the apical membrane of the enterocyte (Shayeghi et al. 2005). In the cytosol, Fe^{2+} is released from heme by heme oxygenase-1 (HO1) (Raffin et al. 1974). The fate of Fe^{2+} released from heme is similar to that of non-heme iron from the diet.

Efflux of iron into circulation from enterocytes:

Iron (Fe^{2+}) from enterocytes, enters the circulation with the help of iron export protein, ferroportin. Ferroportin is a transmembrane protein; it is also called SLC 40A1, IREG1 or MTP 1. It is highly expressed in the proximal intestine, macrophages, placenta and to lower extent in hepatocytes (McKie et al. 2000; Abboud and Haile, 2000; Donovan et al. 2000). In the proximal duodenum, it is localized to the basolateral membrane of enterocytes (McKie et al. 2000). It plays a major role in iron absorption, by transporting Fe^{2+} across the basolateral membrane (Donovan et al. 2005). Expression of ferroportin is regulated by iron status and is increased by iron deficiency and hypoxia (Donovan et al. 2005). Fe^{2+} transported across the basolateral membrane by ferroportin, is oxidized by

hephaestin, before it is released into circulation. SLA (sex-linked anemia) mice with a mutation in hephaestin, manifests with moderate to severe microcytic anemia (Vulpe et al. 1999). In SLA mice, luminal uptake of iron into enterocyte was normal, but release of iron into circulation was impaired due to the mutation (Vulpe et al. 1999). This observation signifies the essential role of hephaestin, a multicopper ferroxidase, in release of absorbed iron into circulation (Vulpe et al. 1999).

Figure 2: Proteins involved in intestinal iron absorption

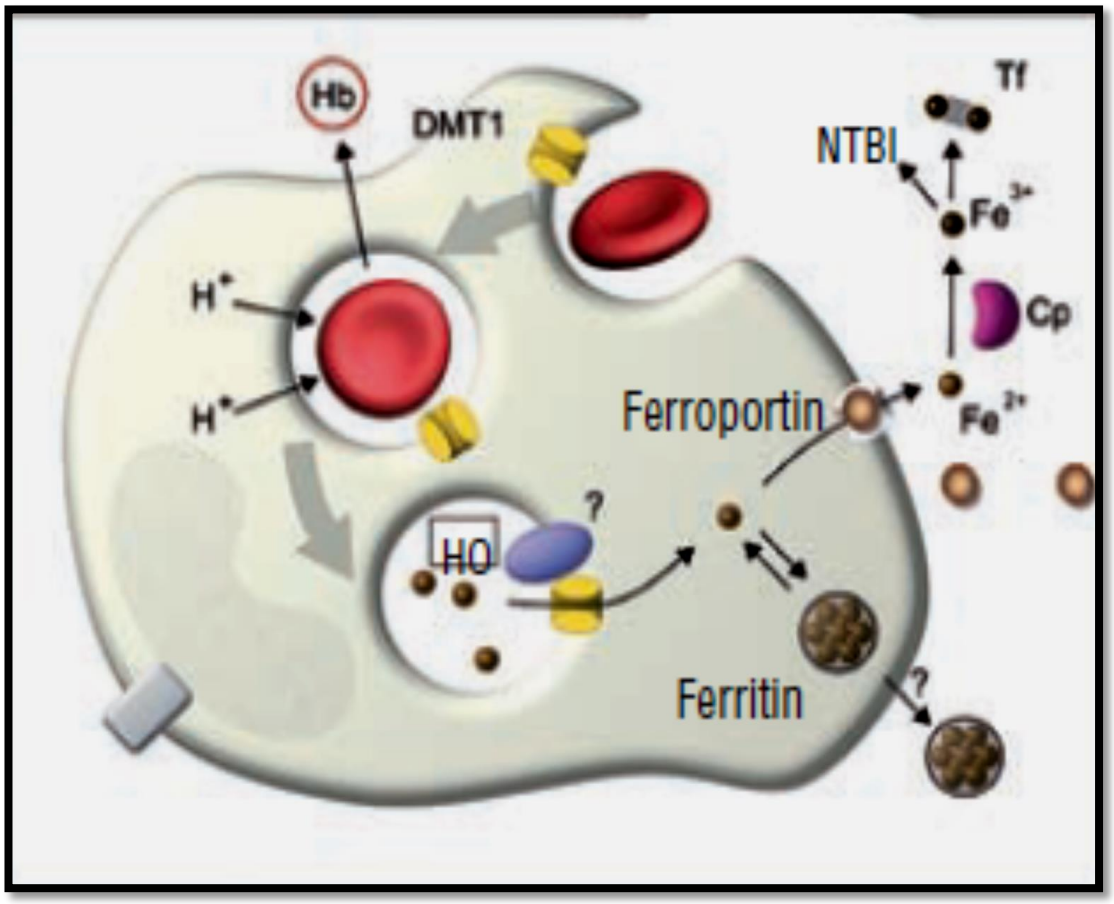


Source: (Evstatiev and Gasche 2011)

IRON RECYCLING

Major iron needs of body are met from iron recycling by macrophages. Senescent RBCs undergo the process of erythrophagocytosis in the reticuloendothelial system. During this process, RBCs are phagocytosed by macrophages. In the phagolysosome, ferrous iron is released from heme by the action of HO-1 (Poss and Tonegawa 1997) and is transported into cytoplasm by NRAMP1 (natural resistance associated macrophages protein 1) (Tabuchi et al, 2000). Ferrous iron either enters the labile iron pool from where it is transported out of the macrophage by ferroportin (Donovan et al. 2005) or is stored as ferritin. Ceruloplasmin in blood oxidizes ferrous iron to the ferric form (Harris et al. 1999), which then binds to transferrin in circulation.

Figure 3: Iron recycling by macrophages



Source :(Kemna et al. 2008)

IRON IN CIRCULATION

Iron absorbed from the intestine or released from macrophage enters the circulation in the ferric form. As ferric iron is insoluble, it is transported in circulation bound to apo-transferrin (Baker 1994). Apo-transferrin is a bi-lobed glycoprotein with 2 iron-binding sites, that can bind ferric iron and the binding is reversible. At a given point of time, about 30-40% of binding sites are occupied by iron (transferrin saturation). Saturation of transferrin is utilized clinically in assessing iron status of the body (Higgins 2012).

In conditions with iron overload, increased iron in circulation exceeds the capacity of transferrin to bind iron (Barisani et al. 1995). The unbound iron that accumulates in circulation is known as non-transferrin bound iron (NTBI). The process of NTBI uptake by the cell is not well understood. L-type voltage dependent calcium channel (LVDCC) is shown to be involved in the NTBI uptake by myocardium, thus accounting for cardiomyopathy observed in iron overload (Oudit et al. 2003). DMT1 and Zip14 mediate NTBI uptake by the hepatocytes.

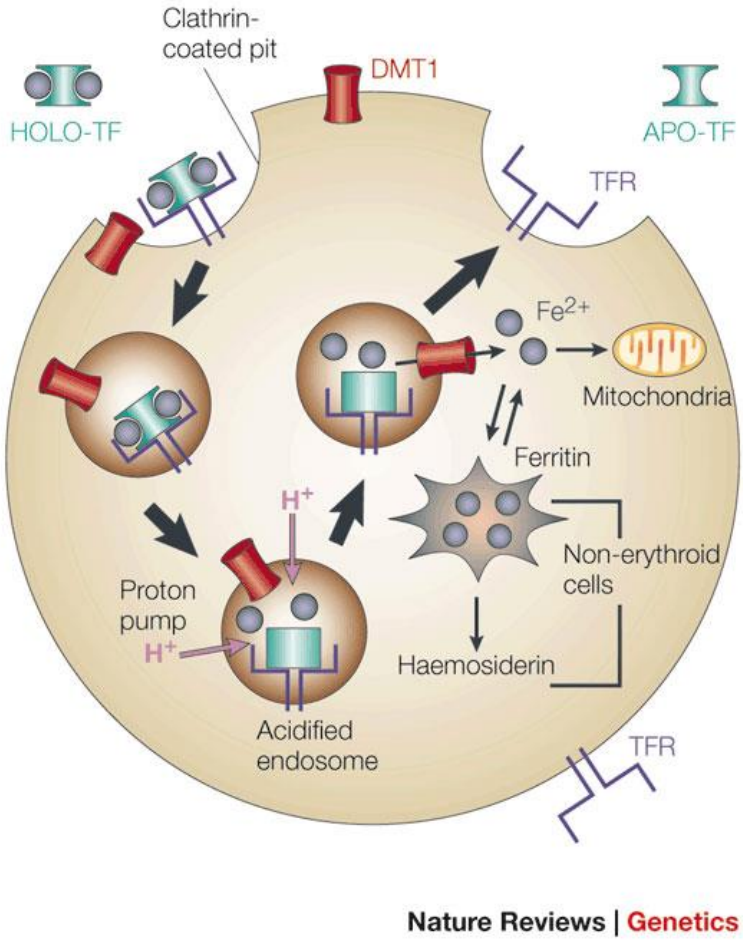
PERIPHERAL UPTAKE OF IRON

Cellular uptake of iron occurs through receptor-mediated endocytosis. Transferrin receptors on the cell surface binds to holo-transferrin and the whole complex is endocytosed. There are 2 types of transferrin receptors (TfR) - TfR1 and TfR2 (Kawabata et al. 1999).TfR1 is expressed in all cells, while TfR2 is expressed in hepatocytes and erythroblasts. TfR1 is the receptor involved in iron uptake into a cell. Targeted disruption of TfR1 in mice was associated with severe anemia (Levy et al. 1999), while mutation in TfR2 is mainly associated with iron overload (Roetto et al. 2002).

The major portion of iron is utilized for erythropoiesis; two-thirds of iron in the body is directed towards this process. Transferrin binds to TfR1 and the complex is internalized by endocytosis. The endosome is acidified by Na^+-H^+ ATPase. The acidic pH causes dissociation of ferric iron from transferrin. The ferric iron is reduced to its ferrous form by the six transmembrane epithelial antigen of prostate 3 (STEAP3) (Ohgami et al 2005). Ferrous iron is carried out of endosome by DMT1 (Fleming et al, 1998). The apo-transferrin and transferrin receptors in the endosome are then transported back to the plasma membrane (Klausner et al, 1983).

The iron thus taken up into the cell is diverted to meet cellular iron needs. In erythroid progenitor cells, most of the iron from labile pool is taken up by mitochondrial mitofferin for synthesis of heme (Ponka,1997). Mitoferrin has been found to be the chief iron transport protein in mitochondria, by Shaw et al, who observed that mutations in mitofferin was associated with severe hypochromic anemia (Shaw et al, 2006).

Figure 4: Transferrin cycle



Source: (Andrews, 2000)

FERRITIN: STORAGE FORM OF IRON

Once the cellular iron needs are met, the excess iron in the labile pool is sequestered into ferritin. Ferritin is a hetero-polymer made up of heavy (H) and light (L) chains and is ubiquitously expressed in all cells of the body. It has 24 protein subunits, which form a shell-like structure around the core containing iron in a ferrihydrate form. Ferrous iron is taken up and oxidized by a catalytic site on the H chain of ferritin (Lawson et al. 1989). Iron in ferritin is easily mobilized as and when the cellular need arises (Higgins, et al, 2011).

Ferritin is also present in serum in small quantities, the source of which is not known. Serum ferritin is mainly made up of L chains, which may be glycated and is poor in iron content (Santambrogio et al. 1987) and is said to reflect iron stores of the body (Lipschitz, Cook, and Finch 1974). Its levels are reduced in iron deficiency anemia. But it is not a reliable marker of iron deficiency, if it is associated with acute or chronic inflammation, in which case ferritin levels are increased (Lipschitz, et al 1974).

INTRACELLULAR IRON HOMEOSTASIS

Cellular iron content is maintained by regulating various proteins involved in iron storage and utilization and transporters of iron across the cell membrane. These proteins are regulated post-transcriptionally, at the level of translation, by altering the stability of mRNA or repressing its translation (Hentze et al. 1987). The mRNA of these proteins contains hair-pin loop-like domains in the 5' or 3'untranslated region (UTR) known as iron-responsive elements (IRE). Ferritin (H and L chain) (Leibold and Munro,1988), ferroportin, erythroid 5-aminolevulinic acid synthase (Cox et al. 1991) and mitochondrial aconitase have IRE in the 5' end (Zheng et al. 1992), while IRE of transferrin receptor1 is in the 3' end. These IREs provides binding sites for iron-responsive proteins (IRPs) (Rouault et al. 1990; Anderson et al. 2012).

IRPs are of two types - IRP1 and IRP2. IRP1 is a cytosolic protein with dual function. It is active in the absence of iron and binds to the IRE of mRNA. It is inactive in the presence of iron and functions as cytosolic aconitase (Zheng et al. 1992). Binding of IRPs to IRE in the 5' end of mRNA inhibits its translation (translational arrest) and binding to IRE in 3' end promotes translation, as it stabilizes mRNA by preventing its degradation. IRP2 is active in the absence of iron.

In the presence of iron, it is rapidly ubiquitinated and proteasomally degraded.

When cells have sufficient iron, iron-sulphur clusters are formed, which bind to IRP1 and inhibit it from binding to the IRE. This promotes translation of ferritin and ferroportin, which have IRE at the 5' UTR and decreases translation of transferrin receptor 1 which has IRE at the 3' UTR, by increasing mRNA degradation (Pantopoulos 2004).

When the iron content of the cell is low, iron-sulphur clusters are not formed. IRP1 is activated and binds to the IREs. Binding of IRP to 5' IRE represses translation. Therefore, translation of ferritin (H and L chain), ferroportin, erythroid 5-aminolevulinic acid synthase and mitochondrial aconitase are reduced. Binding of IRP1 to 3' IRE stabilizes the mRNA and promotes translation of transferrin receptor 1. This promotes iron uptake by the cells and decreases the translation of proteins involved in iron storage and utilization (Anderson et al. 2012).

Figure 5: Regulation of cellular iron content by Iron Responsive proteins

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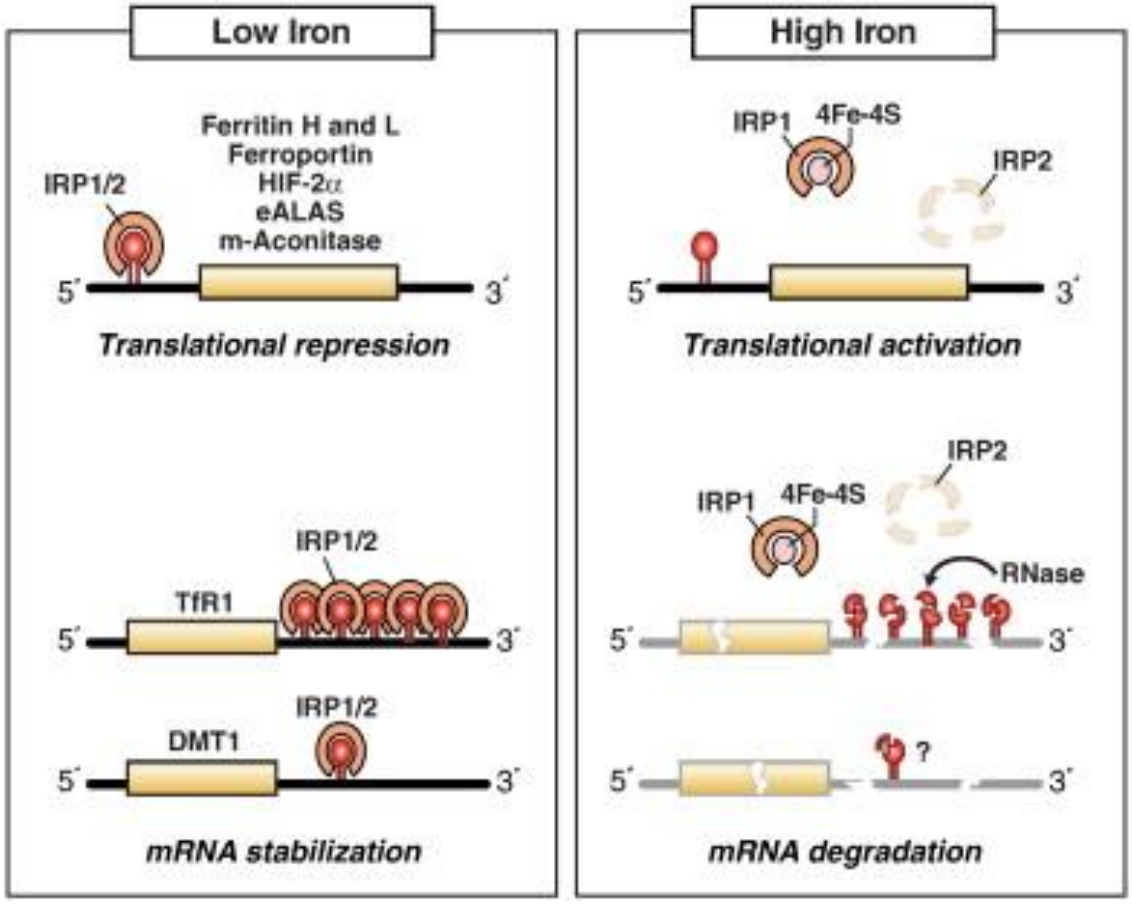


Source: (Anderson et al. 2012)

SYSTEMIC IRON REGULATION

Systemic iron levels are maintained by regulating its entry into the circulation. This is done through regulation of intestinal iron absorption and release of iron from reticuloendothelial macrophages and hepatocytes. As there are no physiological means of iron excretion from the body, iron homeostasis is tightly regulated at the level of intestinal absorption. Hepcidin is the principal iron-regulatory peptide involved in systemic iron regulation.

Hepcidin was discovered in human blood ultra-filtrate, as a result of a search for disulphide-rich antimicrobial peptides. It was found to be highly expressed in the liver and was therefore called LEAP 1 (liver-expressed antimicrobial peptide) (Krause et al. 2000). Subsequently it was named hepcidin by Park et al, who isolated it from urine (Park et al. 2001). Hepcidin is a 25-amino acid-containing peptide, with 8 cysteine (~60%) residues.

The observation that iron overload in mice induced hepcidin expression provided the first link between hepcidin and iron metabolism (Pigeon et al. 2001). Further, it was shown that upstream stimulatory factor 2 (USF-2) knockout mice were hepcidin-deficient as well and developed iron overload in hepatocytes and low iron levels in macrophages (Nicolas et al. 2001). This was similar to

findings in HFE knockout mice and other hemochromatosis mouse models (Nicolas et al. 2001).On the other hand, transgenic mice models with hepcidin over-expression developed severe iron deficiency anemia (Nicolas et al. 2001). Some types of juvenile hemochromatosis patients had mutations in hepcidin (Roetto et al. 2003). Low hepcidin levels were observed in hemochromatosis patients with mutation in HFE and TfR2 (Bridle et al. 2003; Nemeth et al. 2005; Papanikolaou et al. 2004). These findings highlight the role of hepcidin in the maintenance of iron levels in the body.

Hepcidin regulates iron levels by acting on ferroportin, the only known iron export protein expressed mainly in macrophages, enterocytes and syncytiotrophoblast in placenta (McKie et al. 2000; Abboud and Haile, 2000; Donovan et al. 2000). It post-translationally regulates ferroportin levels by internalization and degradation of the protein, thus reducing its numbers in the membrane (Nemeth et al. 2004). Low hepcidin levels were associated with increased expression of iron-related protein like ferroportin (Viatte et al. 2005).

REGULATION OF HEPCIDIN SYNTHESIS

Hepcidin is regulated mainly at the level of transcription by factors such as iron status of the body, hypoxia, erythroid activity and inflammation. Low iron levels, increased erythropoiesis, and hypoxia act as negative regulators, thus decreasing hepcidin, thereby increasing iron absorption and release. Increased iron levels, and inflammation act as a positive regulators, thereby decreasing iron absorption and lowering body iron levels (Hentze et al. 2010). Hepcidin is a type 2 acute phase protein. Inflammation up-regulates its transcription via cytokines like IL-6, which decreases iron absorption and also promotes sequestration of iron in macrophages, whereas tumor necrosis alpha can decrease its expression (Nemeth 2003).

REGULATION BY IRON LEVELS

Under physiological conditions hepcidin levels are chiefly regulated by iron availability. Two main signaling pathways are involved:

1) Hfe-TfR2 (HFE-transferrin receptor 2) pathway
2) HJV-BMP (hemojuvelin–bone morphogenic protein pathway) signaling

1) Hfe-TfR2 signaling:

Most of our understanding of Hfe-TfR2 pathway in regulation of iron levels via hepcidin is based on the observation of mutations associated with iron dysregulation in hemochromatosis**.** The proteins involved in this pathway are Hfe and transferrin receptor 2. Mutations of these proteins were observed in patients with hemochromatosis (Feder et al. 1996; Roetto et al. 2002), who were found to have low levels of hepcidin associated with iron overload. Iron overload in these patients was attributed to a low level of hepcidin, leading to increased iron absorption (Bridle et al. 2003; Nemeth et al. 2005; Papanikolaou et al. 2004). Further, it was shown by Nicolas et al that a mouse model produced by crossing $Hfe^{-/-}$ and hepcidin over-expressing transgenic mice, constitutively expressed

hepcidin and prevented iron overload, in contrast to that seen $Hfe^{-/-}$ mice (Nicolas et al. 2003).

Hfe is a transmembrane protein, which is highly expressed in hepatocytes (Zhang et al. 2004). It is structurally similar to major histocompatibility complex (MHC) class I molecules with α_1,α_2, α_3 and α_4 associated with β2 micro globulin (Feder et al. 1997). Alpha 1 and 2 domains interact with the ecto-domain of transferrin receptor 1 (TfR1) and form a complex (Bennett, Lebrón, and Bjorkman 2000). As binding site of Hfe and Tf-Fe (holo-transferrin) overlap, TfR1 affinity towards Tf-Fe is decreased, due to formation of a complex (Feder et al. 1998).

Hfe can also bind to TfR2, which has ~ 45% homology with TfR1 and is highly expressed in hepatocytes (Kawabata et al. 1999). Hfe displaced from TfR1 by Tf-Fe binds to TfR2. Unlike TfR1, which is involved in cellular iron uptake and whose expression is regulated by cellular status, TfR2 is not necessary for cellular iron uptake, and its membrane stability is increased by ligand (Tf-Fe) binding (Robb and Wessling-Resnick 2004). TfR2 binding differs from TfR1 in that it can bind to both Hfe and holo-transferrin simultaneously (Chen et al. 2007; Gao et al. 2009).

Mouse models with mutation in TfR1, which increase its binding affinity to the Hfe had decreased hepcidin levels and iron overload comparable with that of hemochromatosis. A mouse model, with a TfR1 mutation decreasing its binding affinity to Hfe, had increased hepcidin and iron deficiency. Based on these findings, Schmidt et al suggested that TfR1 sequesters Hfe. Increased concentration of holo-transferrin displaces Hfe from TfR1 and promotes its interaction with TfR2, which leads to hepcidin transcription (Schmidt et al. 2008). Further, this model was supported by the finding that increase in hepcidin levels by holotransferrin depends on interaction of Hfe and TfR2 in cell membranes of hepatocytes (Gao et al. 2010).

2) HJV-BMP (hemojuvelin–bone morphogenic protein pathway) signaling

BMP belongs to the transforming growth factor (TGF) –β super family, which has diverse functions like cell proliferation, differentiation and cell signaling. They are said to have autocrine and paracrine actions. Binding of BMPs to its receptor complex involves dimerization of type I and II BMP (serine/threonine kinase) receptor on the cell membrane, with help of a co-receptor. This binding induces phosphorylation of receptor-regulated SMAD (R-SMAD),

which then complexes with co-SMAD to form a transcription factor that favors transcription (Shi and Massagué 2003).

BMP/HJV/SMAD pathway is involved in hepcidin transcription in hepatocytes. BMP6 is the main ligand (Andriopoulos et al. 2009), which acts in a paracrine fashion i.e., secreted by non-parenchymal cells of liver in response to iron availability (Enns et al. 2013). HJV acts as a co-receptor and helps in BMP-receptor complex formation (Babitt et al. 2005) and induces phosphorylation of R-SMAD and

SMAD 4, leading to transcription of hepcidin in hepatocytes (Wang et al. 2005).

The significance of the pathway in iron metabolism is highlighted by the following observations:

1) BMP-6 knockout mice had low hepcidin levels and severe iron overload (Meynard et al. 2009)

2) Mutation in HJV decreases hepcidin levels and causes iron overload in juvenile hemochromatosis (Babitt et al. 2006)

3) Mice lacking HJV were found to have decreased phosphorylated R-SMAD levels (Babitt et al. 2006).

4) Absence of SMAD in hepatocytes leads to decreased hepcidin levels and iron overload (Wang et al. 2005).

5) Presence of BMP-RE (response element) I and II in the promoter region of the hepcidin gene (Casanovas et al. 2009).

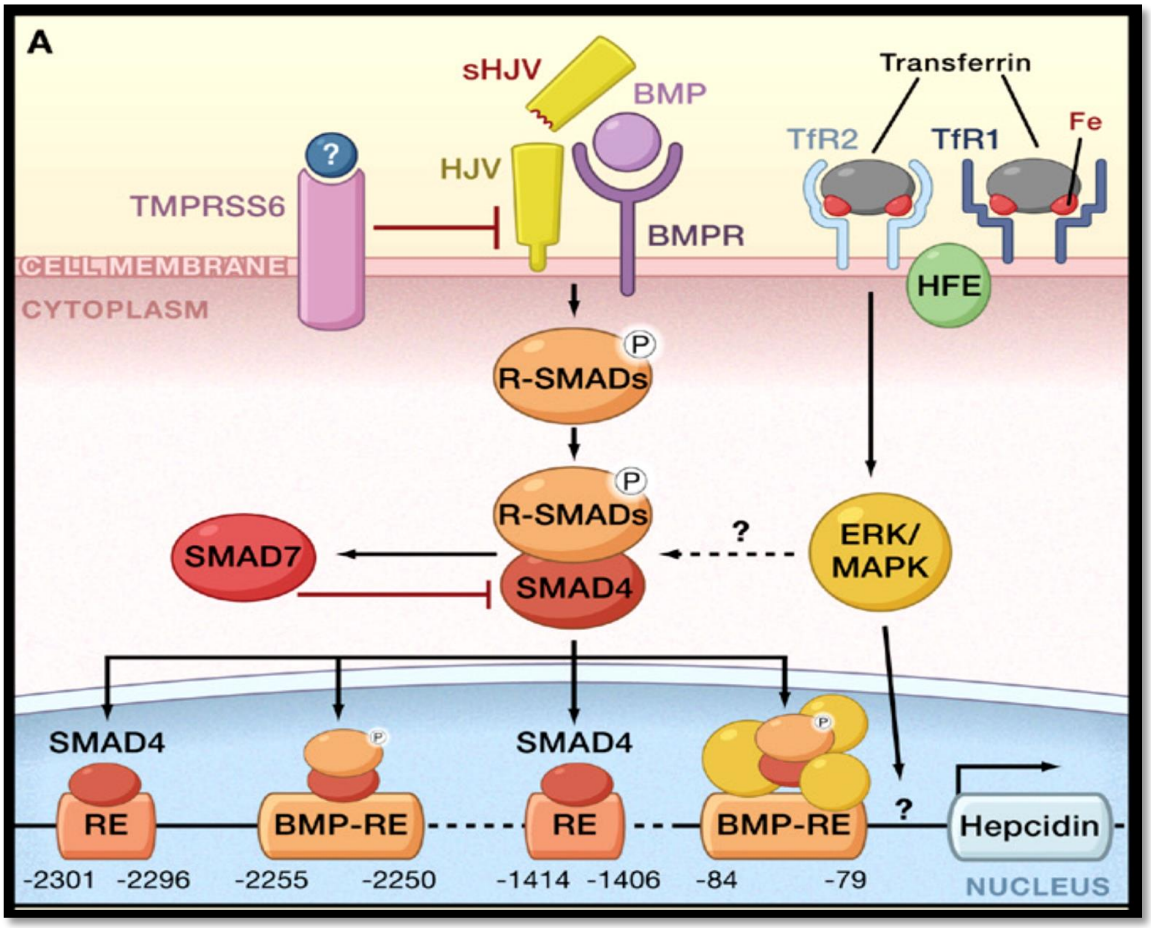
Matriptase 2: a negative regulator of BMP/HJV/SMAD pathway

HJV is a GPI- (glycophophatidlyinositol) anchored protein (Wang et al. 2005) that is highly expressed in liver, heart and skeletal muscles (Niederkofler 2005). Matriptase-2, which is a type 2 transmembrane protein with serine protease activity, is also highly expressed in hepatocyte membrane (Velasco et al. 2002). Its role in iron metabolism was demonstrated by finding mutations in *TMPRSS6* gene in iron-resistant iron deficiency anemia (IRIDA) patients (Finberg et al. 2008). These patients have severe anemia associated with high hepcidin levels. Similar observations were made in mouse

models with mutated matriptase 2 (Folgueras et al. 2008). Recently, it has been shown that its expression is increased by iron deficiency (Du et al. 2008), anemia and hypoxia (Lakhal et al. 2011). The matriptase 2 thus induced is said to decrease hepcidin levels by inhibiting the BMP/HJV/SMAD pathway.

One of the possible mechanisms explained is that matriptase cleaves the membrane-bound HJV, which acts as a co- receptor for BMP receptor dimer, thereby inhibiting hepcidin transcription via BMP/SMAD pathway (Silvestri et al. 2008). But recently, iron and BMP-6, which induce hepcidin, were shown to increase expression matriptase. Based on these findings, it was suggested that matriptase 2 may be involved in fine-tuning of hepcidin synthesis by preventing prolonged up regulation of hepcidin (Meynard et al. 2011).

Figure 6: Regulation of hepcidin by BMP/HJV/SMAD and HFE-TfR 2 signaling



Source: (Hentze et al. 2010)

REGULATION BY ERYTHROID SIGNALS

Erythroid precursors are the major consumers of iron in the body. Erythropoietin (EPO) is the main hormone involved in erythropoiesis. EPO is synthesized mainly in liver and kidney. It is up-regulated by hypoxia associated with anemia.

Increased erythropoiesis in response to anemia increases the iron needs of the precursors. There exists an inverse correlation between hepcidin levels and erythropoietic activity. This suggests that some mediators are involved in decreasing hepcidin synthesis when there is increased erythropoiesis, thereby increasing iron in circulation and its availability to erythroid precursors.

EPO was considered as a possible mediator. EPO administration in healthy volunteers was associated with decreased hepcidin levels (Robach et al. 2009). Similar effect was seen when phlebotomy was done to induce erythropoietic drive in healthy volunteers (Ashby et al. 2010). However, it was found to have no direct effect on hepcidin expression when administered in mice whose bone marrow was ablated (Pak et al. 2006). These findings highlight importance of erythroid mediators in EPO-induced hepcidin suppression.

GDF-15 belongs to the transforming growth factor β (TGF β) super family. Its expression is increased during erythroid maturation(Tanno et al. 2007). In thalassemia, GDF-15 is expressed and secreted in very high quantities. This was found to be responsible for low hepcidin level and increased iron absorption seen in these patients (Tanno et al. 2007). GDF-15 is thought to decrease hepcidin production by inhibiting HJV-BMP pathway.

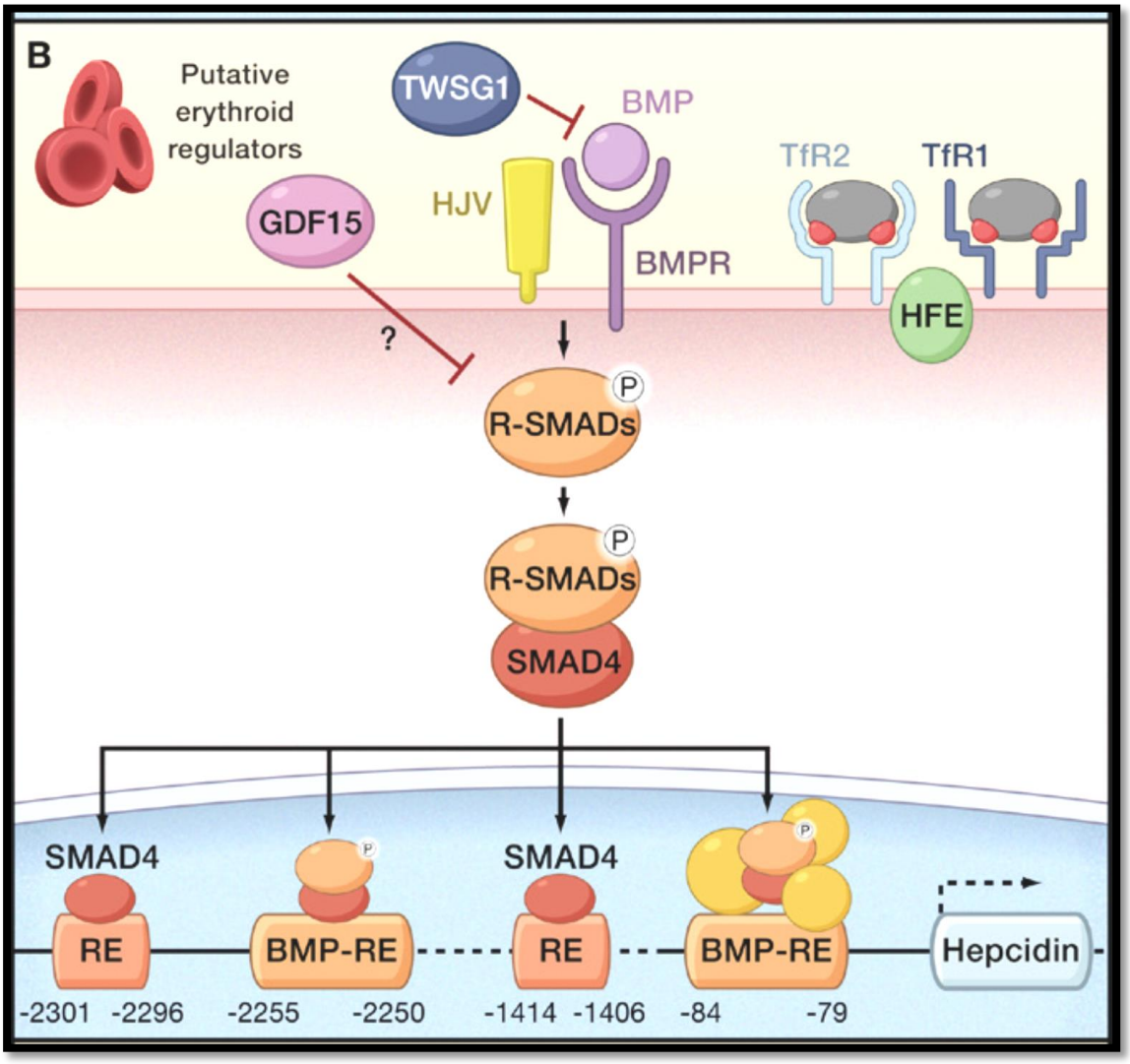
Twisted gastrulation factor (TWGF) is also increased in the thalassaemia mouse model, causing suppression of hepcidin and iron overload in them (Tanno et al. 2009). Its role in humans is not clear.

Erythroferrone (ERFE) is the latest addition to the list of potential erythroid mediators. It is produced by erythroblasts in response to EPO through Janus kinase/STAT 5 pathway (Kautz and Nemeth, 2014). Erythroferrone knockout mice failed to suppress hepcidin expression on administration of EPO and during stress erythropoiesis. Its expression is increased in bone marrow of thalassaemia mouse models. However, the observation that ERFE deficient mice did not have anemia and had normal hematological parameters shows that it is mainly involved in hepcidin suppression in stress erythropoiesis. In vitro studies have shown its expression in

human erythroblast. Its role in vivo is yet to be studied (Kautz and Nemeth, 2014).

All these factors, GDF-15, TWGF and eythroferrone, have been shown to mediate expression of hepcidin mainly when there is stress erythropoiesis. Erythroid mediators, which regulate hepcidin expression under normal steady state erythropoiesis, are still not known.

Figure 7: Regulation of hepcidin synthesis by erythroid regulators via inhibition of BMP/SMAD signaling



Source: (Hentze et al. 2010)

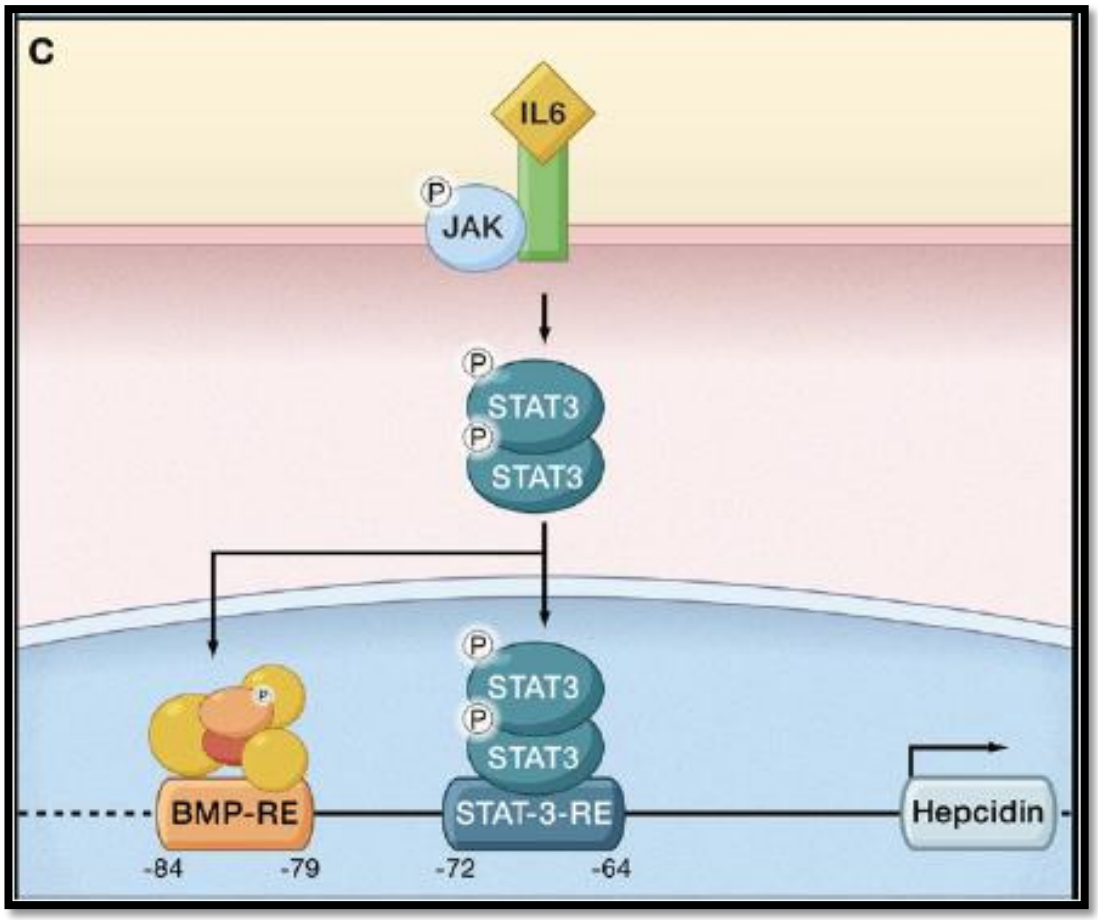
INFLAMMATORY CYTOKINES AND HEPCIDIN:

Anemia is associated with inflammatory conditions like inflammatory bowel disease (IBD), rheumatoid arthritis, chronic kidney disease and also in malignancy. This is referred to as anemia of chronic disease (ACD). These patients have hypoferremia and reduced transferrin saturation in spite of having normal iron stores. This indicates a state of functional iron deficiency during chronic inflammation (Adamson 2011)

Hepcidin is an acute phase reactant. Its levels are found to be increased by cytokines like interleukin 6 (IL-6) (Nemeth 2003), IL-1 (Lee et al. 2005) and decreased by TNFα, which are produced during inflammation (Nemeth 2003). Up-regulation of hepcidin levels, leading to sequestration of iron in macrophages is found to be one of the factors responsible for anemia in ACD. IL-6 was shown to be the main mediator responsible for hepcidin up-regulation during inflammation. IL-6 knockout mice failed to increase hepcidin when inflammation was induced, while wild-type mice increased hepcidin in response to inflammation. Administration of recombinant IL-6 in human volunteers increased hepcidin formation, which further supported IL 6-mediated hepcidin up-regulation. (Nemeth et al. 2004)

IL-6 acts via JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway to increase hepcidin transcription (Wrighting and Andrews 2006). It was observed that IL-6 response was blunted in mice with liver-specific SMAD 4 disruption, which suggest cross-talk between IL-6-JAK/STAT-3 pathway and BMP/HJV/SMAD pathway (Wang et al. 2005).

Figure 8: Cytokine induced up regulation of hepcidin by IL 6 via JAK/STAT signaling



Source: (Hentze et al. 2010)

IRON, INFLAMMATION AND ANEMIA

Anemia associated with chronic immune activation in conditions like chronic infection, inflammation and neoplasm is termed as anemia of chronic disease (ACD) or anemia of inflammation (AI). These patients usually have hypoferremia, low transferrin saturation and a normocytic normochromic blood picture due to functional iron deficiency, as iron is sequestered in its storage form (Weiss and Goodnough 2005).

Increased cytokine levels during inflammation are said to play a major role in the pathogenesis of ACD at various levels. It affects EPO production, erythroid response to EPO, life span of red blood cells and iron availability to the erythroid precursors (Means 1995). Suppressed EPO expression was observed in isolated rat kidney and human hepatoma cell-line due to pro inflammatory cytokines like IL-1, tumor necrosis factor-α (TNF-α) (Jelkmann 1998). Interferon-γ is found to down-regulate EPO receptor in early erythroid progenitors, thus inhibiting erythroid differentiation and proliferation in response to EPO (Taniguchi et al. 1997; C. Wang, et al. 1995)

Increased hepcidin levels in inflammation in response to IL 6 plays a major role in altered iron homeostasis in these patients. Increased hepcidin levels results in sequestration of iron in macrophages and

also decreases intestinal iron absorption. Thus, iron deficient erythropoiesis occurs with normal body iron stores (Spivak 2002).

Diagnosis of ACD, based only on routinely used iron parameters like serum iron, ferritin, transferrin saturation, is difficult. Reduced serum iron and transferrin saturation may be similar in ACD and in iron deficiency and so they are not useful in differentiating the two conditions. Ferritin may be increased or normal as its levels can be increased by inflammation so it does not reflect iron stores in ACD. sTfR (soluble transferrin receptor) measurement and sTfR/log ferritin ratio can be used to differentiate between ACD (ratio < 1), IDA (> 2) and also ACD with IDA (>2) (Weiss and Goodnough 2005).

Treating the underlying disease can restore hemoglobin in ACD. But in certain conditions with relapses and remissions, recurrent anemia is a problem. When ACD is associated with blood loss, there is concomitant iron deficiency. In these conditions, along with treating underlying disease, treating anemia becomes necessary. Treatment usually involves blood transfusions, iron supplementations and erythropoietin therapy (Weiss and Goodnough 2005).

ULCERATIVE COLITIS AND ANEMIA

Ulcerative colitis, together with Crohn' disease, is termed as inflammatory bowel disease (IBD). Ulcerative colitis (UC) is a disease of immune dysregulation of the intestinal mucosa (Longo et al. 2011). It is a chronic inflammatory condition with remissions and relapses. Ulcerative colitis is said to be more common than Crohn's in Asian countries. Prevalence of UC in India is not known due to lack of population-based studies ("Indian Society of Gastroenterology Consensus on Ulcerative Colitis - Springer" 2014). A study from Punjab showed an incidence of 6.02 cases per 100,000 population, which was higher than those reported in other Asian countries ("Indian Society of Gastroenterology Consensus on Ulcerative Colitis - Springer" 2014).

Recurrent anemia is seen in patients with ulcerative colitis. About one-third of ulcerative colitis patients develop anemia during the course of the disease (Gasche et al. 2004). Several factors contribute to anemia in UC. These include iron deficiency due to blood loss associated with the disease, anemia due to ongoing inflammation and vitamin B_{12} and folate deficiency, which occur as a result of

inadequate dietary supply, inadequate absorption from inflamed ileum or due to drug interaction with folate metabolism (Schreiber and Wedel 1997).

Hepcidin levels are known to be increased in anemia of chronic disease. However, hepcidin levels found to be lower in patients with ACD with IDA, which included 4 IBD patients, when compared to patients with ACD (Theurl et al. 2010). Low serum hepcidin with up-regulation of Fe-related proteins was reported in the duodenum of ulcerative colitis patients (Sukumaran et al. 2014). In another study, serum hepcidin was found to be low in IBD patients irrespective of presence or absence of iron deficiency anemia (Arnold et al. 2009). In a mouse model of colitis, hepcidin concentration were found to be low, which was attributed to increase in tumor necrosis alpha, which is known to down-regulate hepatic hepcidin expression (Shanmugam et al. 2012). The reason for reduced levels of hepcidin in UC is currently unknown. One explanation is the fact that hepcidin concentration may differ in different types of IBD, which in turn, may be the due to the predominant cytokine expressed in that particular disease. In any case, determining hepcidin levels and its possible negative regulator may help in identifying patients for iron supplementation (Shanmugam et al. 2012).

Growth Differentiation Factors 15 (GDF15)

Growth differentiation factor 15(GDF-15) is also known as macrophage inhibitory cytokine 1 (MIC-1). It belongs to to the TGF-β super family (Bootcov et al. 1997). It was first discovered in activated macrophages and is said to play role in inhibition of inflammation. Structurally, GDF-15 is a 25kDa dimeric secretory protein, with 2 disulfide bonds It is mainly expressed in the placenta and in maturing erythroblasts (Bootcov et al. 1997).

The role of GDF-15 in erythropoiesis was demonstrated by the erythroblast transcriptome project (Miller et al. 1999), where increased GDF 15 expression was seen in the cultured erythroblasts (Tanno, Noel, and Miller 2010). The cultured erythroblasts failed to enucleate, which normally occurred in course of maturation of erythroblasts. These cells with higher proliferation rate and inability to mature were said to reflect state of ineffective erythropoiesis.

Ineffective erythropoiesis is seen in patients with thalassemia, congenital dyserythropoietic anemia I (CDA I), refractory anemia with ring-sideroblasts (RARS) and pyruvate kinase deficiency (Tamary et al. 2008; Ramirez et al. 2009; Tanno et al. 2007; Finkenstedt et al.

2009). Anemia associated with iron overload was shown to be a common feature in these diseases. Serum GDF-15 levels were found to be higher in these patients when compared to controls (Tamary et al. 2008; Ramirez et al. 2009; Finkenstedt et al. 2009; Tanno et al. 2007).

Thalassemia and CDA I patients have iron overload, which is not only transfusion-related but also due to increased intestinal iron absorption. This increased iron absorption was attributed to decreased levels of the iron-regulatory peptide, hepcidin, observed in these patients (Tanno et al. 2007; Tamary et al. 2008). Increased GDF-15 levels were observed correlate with the decreased serum levels of hepcidin and ferritin. In addition, immunoprecipitation of GDF-15 from the sera of patients with thalassemia resulted in loss of ability to suppress hepcidin expression. Therefore, GDF-15 was considered to be an erythroid marker, which regulates hepcidin in these patients (Tamary et al. 2008; Tanno et al. 2007). Further in vitro studies by Ramirez et al showed the essential role played by GDF-15 in maturation of erythroblasts and its dependence on erythropoietin (EPO) (Ramirez et al. 2009).

Recent evidence has, however, shown conflicting results on the physiological and pathophysiological role of GDF-15 as an erythroid regulator of hepcidin, both in humans and mice. For example, a significant increase in serum GDF-15 was not observed in human volunteers who received erythropoietin or in volunteers who underwent phlebotomy. In both these cases, an erythropoietic drive was present and hepcidin levels were found to be decreased but these did not correlate with serum GDF-15 levels (Ashby et al. 2010). GDF-15 knock-out mice did not show any significant effect on hematological parameters in these mice. Their response to phlebotomy, with respect to iron parameters, was similar to that in wild type mice (Casanovas et al. 2013). This suggested that GDF-15 may not be an important erythroid regulator in mice during normal erythropoiesis.

In a study by Theurl et al (2010), serum GDF-15 levels were found to be elevated in patients with anemia of chronic disease (ACD) and those with ACD and iron deficiency anemia (IDA), when compared with subjects who had neither condition and those with only IDA. However, the levels of GDF-15 did not correlate with serum hepcidin levels observed in these patients. But the study included only four patients with IBD, who were categorized under ACD/IDA group. As correlations between serum GDF-15 and sTfR receptor was observed

in ACD with IDA but not in ACD alone, these authors have suggested a possible link between serum GDF-15 production and iron availability for erythropoiesis, even during inflammation (Theurl et al. 2010).

Even though animal and human studies have questioned the role of GDF-15 in hepcidin expression in normal erythropoiesis and in acute response to erythropoietin (Casanovas et al. 2013 Ashby et al. 2010), the role of GDF-15 in iron homeostasis in chronic inflammatory conditions such as UC has not been adequately studied.

In summary, ACD is a common complication in patients with ulcerative colitis and its prevalence varies between 6-70%. Hepcidin is a major regulator of iron metabolism. Its levels are known to be increased in ACD, thus accounting for iron-restricted erythropoiesis in these patients (Nemeth et al. 2003). Even though ulcerative colitis is a chronic inflammatory condition, low hepcidin levels have also been observed in these patients (Sukumaran et al. 2014, Arnold et al. 2009). The precise reason why hepcidin is suppressed despite the presence of inflammation is not clear. GDF-15 is one of the known negative regulators of hepcidin (Tanno et al, 2007). However, it is not known whether its levels are increased in patients with ulcerative

colitis. We hypothesized that serum GDF-15 may be increased in patients with UC, which may account for low serum hepcidin levels that have been observed in these patients.

THE STUDY

HYPOTHESIS

Hepcidin is the central regulator of iron homeostasis. Work done previously in the Department of Biochemistry, CMC, Vellore, has shown that serum hepcidin levels were decreased in patients with ulcerative colitis. Growth differentiation factor-15 (GDF-15) is a known negative regulator of hepcidin. It was, thus, hypothesized that serum GDF-15 may be increased in patients with UC, which may account for the low serum hepcidin levels that have been observed in patients with UC.

AIM

The aim of the study was to test the hypothesis that serum GDF-15 levels may be increased in patients with ulcerative colitis (UC) and to determine whether it correlates with haemoglobin and markers of iron status (serum iron and ferritin).

MATERIALS

EQUIPMENTS USED

- Elix and Milli-Q ultrapure water systems (Millipore, USA)
- Table-top refrigerated centrifuge (MPW R 350, MPW Poland)
- Microplate reader, model 680 and microplate manager software from Bio-Rad laboratories, Inc. (UK).

CHEMICAL AND REAGENTS USED

- Quantakine ELISA human GDF-15 kit, R&D systems, Inc (MN, US).

Materials in the kit:

1. **GDF-15 microplate:** 96-well polystyrene microplate pre-coated with monoclonal antibody specific for human GDF-15

2. **GDF-15 conjugate:** Polyclonal anti-GDF-15 antibody, conjugated with horse-radish peroxidase

3. **GDF-15 standards:** Recombinant human GDF-15 in buffered protein solution with preservatives (lyophilized)

4. **Assay diluents RD1-9:** Buffered protein solution with preservatives

5. **Calibrator diluent RD5-20:** Buffered protein base with preservatives

6. **Wash buffer concentrate:** 25-fold concentrated solution of buffered surfactant, with preservatives

7. **Color reagent A:** Stabilized hydrogen peroxide

8. **Color reagent B**: Stabilized chromogen (tetramethylbenzidine)

9. **Stop solution:** 2N sulphuric acid

The kit was stored at 2-8^{o}C.

MISCELLANEOUS CONSUMABLES USED

- Glass vacutainer blood collection tubes (BD Biosciences, Plymouth, UK)
- Pipette micro tips (Tarsons Product Pvt Ltd, India).
- Graduated centrifuge tubes (15 ml and 25ml)(Tarsons Product Pvt Ltd, India)

METHODS

The study was approved by the Institutional Review Board (IRB) of Christian Medical College, Vellore, India.

SUBJECTS

Patients diagnosed to have ulcerative colitis (UC) were the subjects of the study. These patients were recruited in the Department of Gastroenterology, CMC, Vellore, in the colonoscopy clinic, where they underwent the procedure as part of their medical management, or in the inflammatory bowel disease (IBD) clinic. Inclusion and exclusion criteria used for recruitment were as follows:

INCLUSION CRITERIA:

1. Adults aged 19-60 years, of both genders, who had been diagnosed to have ulcerative colitis

2. A diagnosis of ulcerative colitis was based on presence of symptoms of diarrhoea associated with blood and mucus, of more than 2 months' duration, where an infective etiology had been excluded and where the disease was confirmed by colonoscopy and mucosal biopsy.

3. Patients were either on or off treatment and had either active or quiescent disease.

EXCLUSION CRITERIA:

1. Patients with severe ulcerative colitis were excluded. This included the presence of toxic mega-colon, more than 6 bloody stools per day or fever with leukocytosis.

2. History of iron supplementation or treatment with erythropoietin

3. Patients not willing to participate in the study

Once patients met the inclusion and exclusion criteria, informed consent was obtained for recruitment into the study and for collection of a venous blood sample. Relevant clinical and baseline socio demographic information was collected using a proforma (Appendix no.3).

Age and gender-matched non-anemic control subjects were recruited from patients in the Department of Gastroenterology, CMC, Vellore, who were diagnosed to have dyspepsia. These patients had no abnormalities detected on endoscopy.

CALCULATION OF SAMPLE SIZE

Sample size was calculated to be 30 cases by using the following formula:

$$n = \frac{Z_{\frac{\propto}{2}}^{2}\ s^2}{d^2}$$

Where,

$Z_{\frac{\propto}{2}}^{2}$= 95% confidence limit

d = absolute precision of 450

S = standard deviation calculated from the mean serum GDF-15 value (SD=1250pg/ml) in patients with ulcerative colitis reported from a previous study (Theurl et al, 2010).

However, one set of reagents for estimation of serum GDF-15 was sufficient only for 40 samples to be done in duplicate. Since the IRB funded the study only to a maximum amount of Rs. 80,000 for 2 years, it was possible to study only 20 patients with UC and 20 control subjects in this study.

INFORMED CONSENT

Information sheets were provided to the patients and their relatives, which explained the purpose of the study. Oral information regarding the study was also given by PI and consent was obtained from the patients. The information sheets and consent forms were prepared in 5 different languages (i.e. English, Hindi, Telugu, Tamil and Bengali). A copy of the English version is provided in Appendix 2. Once consent was obtained from patients, a blood sample was collected from each. The patients' socio-demographic details and relevant patient history regarding disease, medication and co-morbid conditions were obtained. The proforma used for collecting history and data is included in Appendix-3. Results of some hematological parameters such as hemoglobin, mean corpuscular volume (MCV), C-reactive protein (CRP) and reports of colonoscopy and biopsy samples were obtained from hospital records of patients.

COLLECTION OF SAMPLES

Venous blood samples were collected in vacutainer tubes, using aseptic precautions. The sample was allowed to clot and then subjected to centrifugation, using a table-top centrifuge at 1200*g* for 10 minutes. The serum obtained was divided into several aliquots and stored at -70^{o} C. These samples were used for estimation of serum C-reactive protein (CRP), iron, ferritin and GDF-15.

ESTIMATION OF C-REACTIVE PROTEIN

Serum CRP estimation was done in the Department of Microbiology, CMC, Vellore, where it is offered as a routine test.

Analyzer used:

Nephelometer -BN Prospec, Siemens GmbH, Marburg, Germany

Principle of the assay:

This consists of an in-vitro test for quantitative estimation of CRP in human serum by particle-enhanced nephelometry.

The method involved formation of aggregates due to interaction between monoclonal antibodies against human CRP (coated on polystyrene beads) and CRP in a serum sample. The aggregates formed scattered light passed through it. The intensity of scattered light was measured. This was proportional to the concentration of CRP in the serum.

ESTIMATION OF SERUM IRON

Estimation of serum iron was done in the Department of Clinical Biochemistry, CMC, Vellore, where it is offered as a routine test.

Analyzer:

Roche Modular P, clinical chemistry auto analyzer, Germany.

Principle of the assay:

Estimation of iron was done by a colorimetric assay. Iron was released from transferrin in serum, at acidic pH. Free ferric iron was then reduced by ascorbate to its ferrous form. Ferrous iron then reacted with ferrozine to form a coloured complex. The colour that developed was measured photometrically at 560nm. The intensity of colour developed was directly proportional to the concentration of iron in the sample.

ESTIMATION OF SERUM FERRITIN

Estimation of serum ferritin was done in the Department of Clinical Biochemistry, CMC, Vellore, where it is offered as a routine test.

Analyzer:

Siemens, ADVIA Centaur system Xpi, UK

Principle:

Ferritin was measured by a 2-site sandwich immune assay, using a chemiluminometric method. Two antibodies directed against human ferritin were used. The first antibody was an acridinium ester-labeled polyclonal goat anti-ferritin antibody, which was in a liquid phase. The second antibody was a monoclonal mouse anti-ferritin antibody, which was covalently linked to paramagnetic particles and was in a solid phase. Both antibodies were added simultaneously to the sample and incubated for 7.5 minutes at 37^0C. The ferritin in the sample reacted with both the antibodies to form a complex, in which ferritin was sandwiched between the 2 antibodies. The immune complex formed was immobilized in the cuvette with help of a magnetic field, which interacted with paramagnetic particles. Excess antibodies and antigen were removed by washing. Acid and base reagents were added to the cuvette to initiate a chemiluminescent

reaction. Relative light units were measured by the detector; this was directly proportional to concentration of ferritin in the sample.

ESTIMATION OF SERUM GDF-15

Serum GDF-15 levels were estimated by an enzyme-linked immunosorbent assay (ELISA), using commercially available reagents (Quantakine ELISA kit R & D Systems, Inc. Minneapolis, MN).

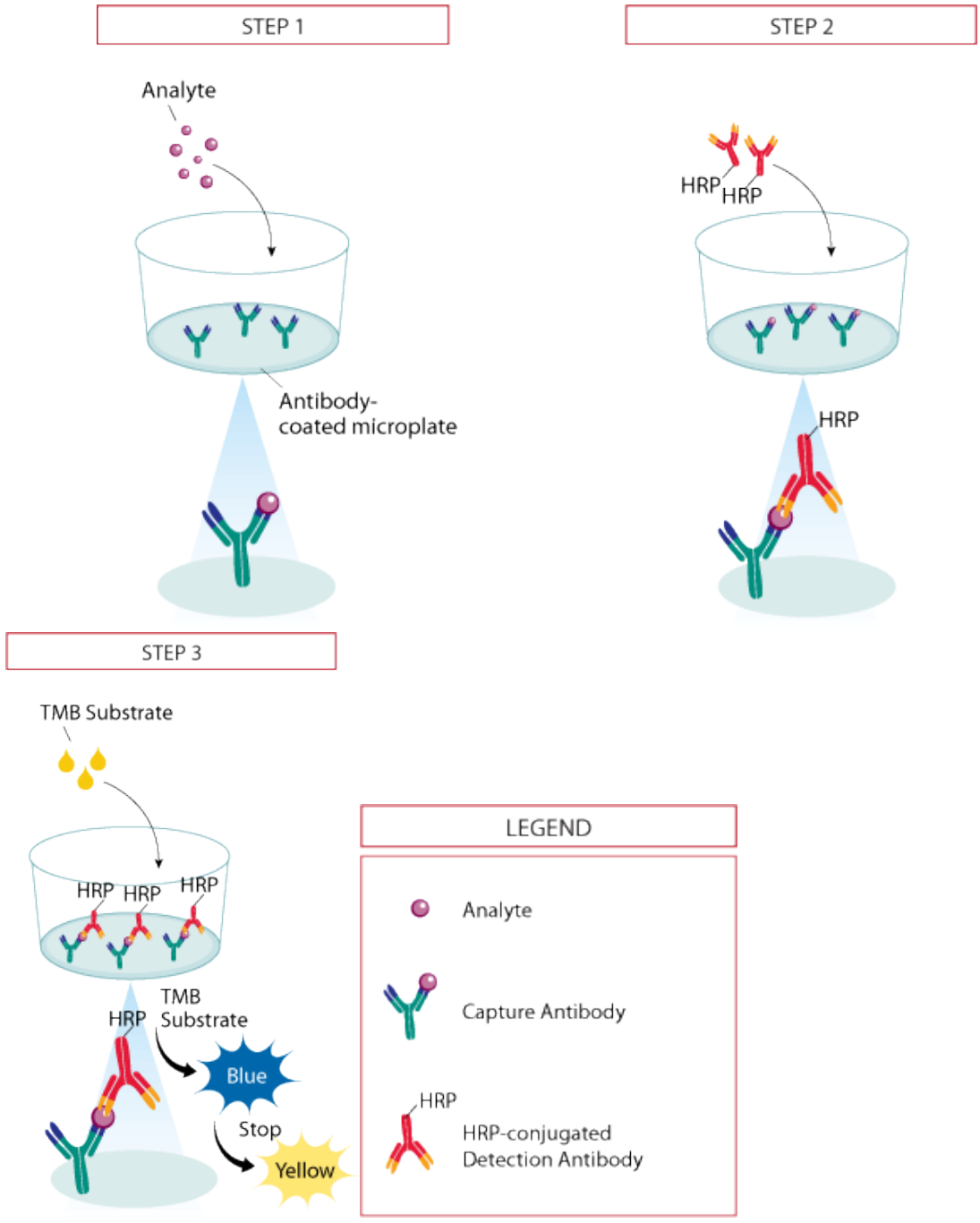
Principle of the assay:

It was a quantitative solid phase sandwich enzyme immunoassay. It contained a 96-well polystyrene microplate, pre-coated with a monoclonal antibody specific for human GDF-15. When a sample/standard was added to a well, GDF-15 in the sample/standard bound to the antibody pre-coated in the well. This formed an antigen-antibody immobilized complex. The unbound GDF-15 in each sample was removed by washing with wash buffer. A polyclonal anti-GDF-15 antibody, conjugated with horseradish peroxidase, was then added to each well. The GDF-15 was sandwiched between the antibody pre-coated on the plate and the

conjugated antibody. The unbound conjugated antibody was removed by washing with wash buffer.

After this, the substrate solution (hydrogen peroxide and tetramethylbenzidine) were added. A colour developed in the well due to the action of the enzyme on the substrate. The intensity of colour developed was directly proportional to the concentration of GDF-15 in the sample/standard. The development of colour was stopped by adding the stop solution. The intensity of the colour that developed subsequently was measured.

Steps involved in estimation of serum GDF-15, using Quantakine ELISA kit, R & D systems.



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rndsystems.com//product_detail_objectname_quantikineelisaassayprinciple.asx

PREPARATION OF REAGENTS:

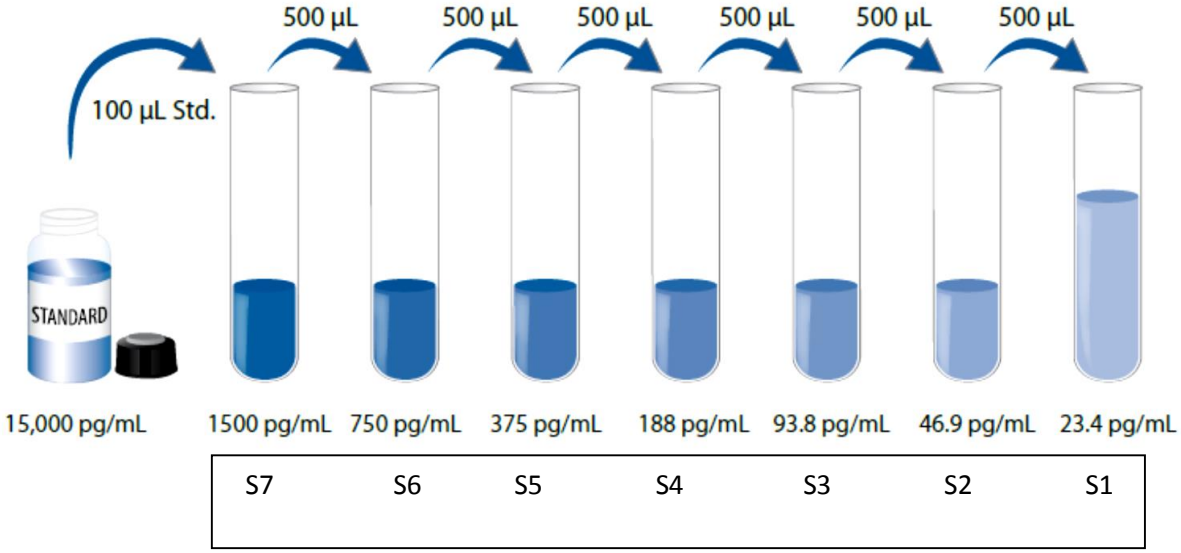
All reagents were brought to room temperature before use.

Wash buffer: 350ml of wash buffer was prepared by diluting 14ml of concentrated wash buffer in 336 ml deionized water.

Substrate solution: Colour reagent A (11ml) and colour reagent B (11ml) were mixed to prepare 22ml of substrate solution. The solution was used within 15 minutes of preparation.

GDF-15 standards:

Stock standard: GDF-15 standard solution was reconstituted with 1ml of calibrator diluent, RD5-20, to prepare a stock solution of 15,000pg/ml. The stock standard was mixed well and allowed to stand for 15 minutes, before preparing serially diluted standards. 900µl of calibrator diluent, RD5-20, was added to a 1500pg/mL (S7) tube. 500µl of calibrator diluent, RD5-20, was pipetted into each tube labeled S1 to S6. Serial dilutions were done, using the stock standard as shown in the figure below (the figure below is from the instruction sheet provided by the manufacturer).



The 1,500pg/ml standard served as the highest standard and the calibrator diluent, RD5-20, as the zero standard or blank.

Sample preparation:

A 5-fold dilution of each sample was done. For this, 50µl of a sample was diluted with 200µl of calibrator diluent, RD5-20.

ASSAY PROCEDURE:

Step 1: 100µl of assay diluent RD1-9 was added to each well.

Step 2: 50µl of standard or diluted sample was added to a well. The micro-plate was covered with adhesive tape provided in the ELISA kit for this purpose, and incubated for 2 hours at room temperature

Step 3: Next, 300µl of wash buffer was added to each well. This was done using a multichannel pipette. The wash buffer was removed. The washing was repeated 4 times. The plate was then inverted and blotted against a clean paper towel to remove all the fluid.

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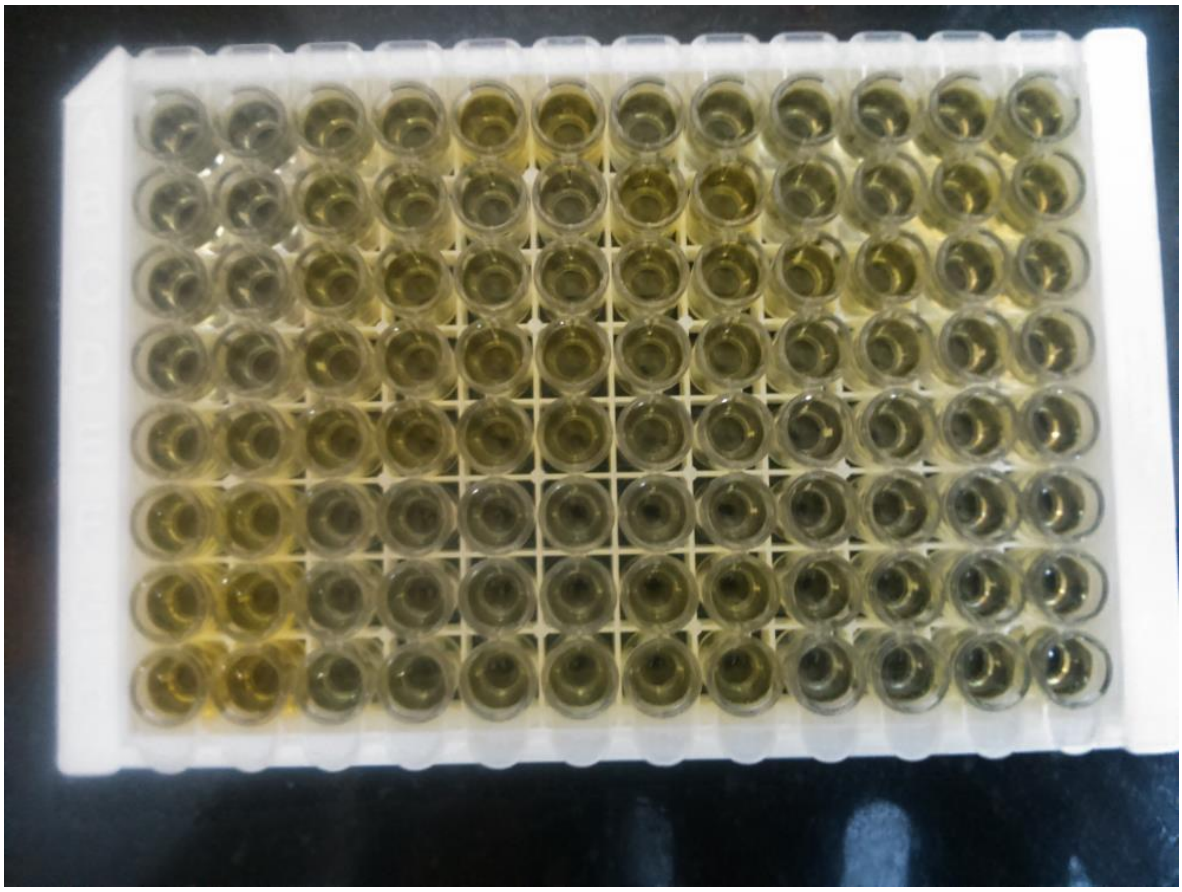
Step 4: 200µl of GDF-15 conjugate was added to each well. The plate was covered with adhesive tape provided in the kit and incubated for 1hour at room temperature.

Step 5: The wells were then washed as described in step 3.

Step 6: 200µl of freshly prepared substrate solution was added to each well. The plate was incubated in the dark for 30 minutes, at room temperature.

Step 7: 50µl of stop solution was added to each well .The colour change from blue to yellow was observed.



Step 8: The optical density (OD) of the solution in each well was determined using a micro plate reader. This was done within 30 minutes after addition of the stop solution. Readings were taken at 450nm. A correction wavelength 540nm was used to correct for optical imperfection of plate. OD obtained at 540nm was subtracted from OD at 450nm automatically by the software, before the final OD was reported.

The standards and all samples were estimated in duplicates. The average of the 2 OD values obtained was used to calculate the concentration of GDF-15 in each sample.

Calculation of results:

A standard curve was generated by 4 parametric logistic curve (4 PL) curve fit, using software of the microplate reader.

The concentration of GDF-15 in each sample was obtained using the microplate reader software.

STATISTICAL ANALYSIS

Statistical analysis of data was done using the Statistical Package for Social Scientists (SPSS), version 17. Distribution of values of parameters studied was checked for normality, using Shapiro-Wilk test. Data were compared between the 2 groups, using unpaired T-test for normally distributed data and Mann- Whitney U test for data that were not normally distributed. Correlation analysis was done using Pearson's correlation coefficient for normally distributed data and Spearman's correlation coefficient for data with skewed distribution.

RESULTS

ANALYSIS OF DISTRIBUTION OF DATA:

Values for age, haemoglobin, mean corpuscular volume (MCV) and serum iron were found to be normally distributed. Values for serum GDF-15, ferritin and C-reactive protein (CRP) were found to have skewed distributions.

CHARACTERISTICS OF SUBJECTS IN THE STUDY

A total of 40 participants were recruited into the study. Twenty patients diagnosed to have ulcerative colitis (UC) served as cases. Twenty age and gender-matched patients, with no evidence of anaemia (hemoglobin equal to or more than l3gm/dl in males and equal to or more than 12gm/dl in females) or inflammation (serum CRP less than 6 mg/L) served as control subjects.

The characteristics of the subjects in the study are shown in Table 1.

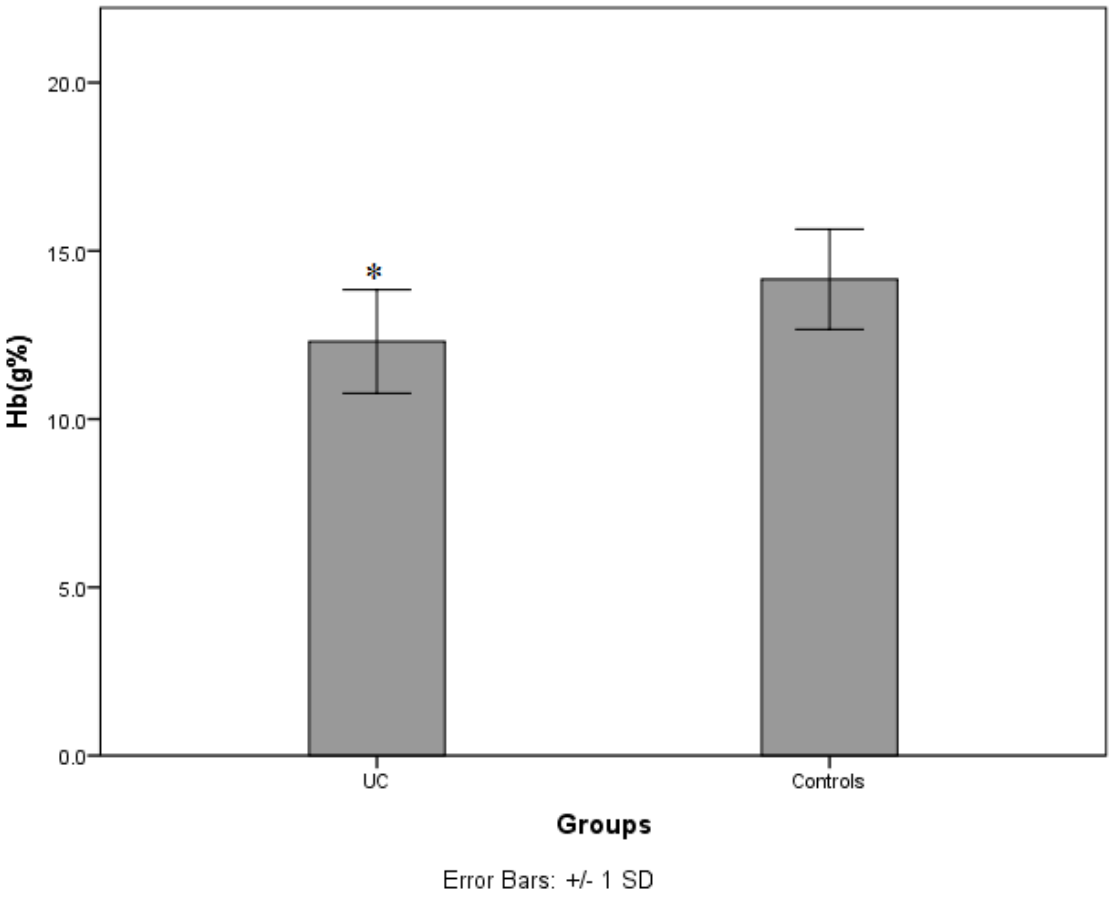
Table 1:

Characteristic	**Patients with ulcerative colitis**	**Control patients**
Number of patients	20	20
Males/females	13/7	13/7
Mean age (years) (± SD)	40.45 (8.17)	39.75 (7.55)

HAEMATOLOGICAL PARAMETERS

The haematological parameters estimated were levels of haemoglobin and mean corpuscular volume. The results for these are shown in Figures 1 and 2.

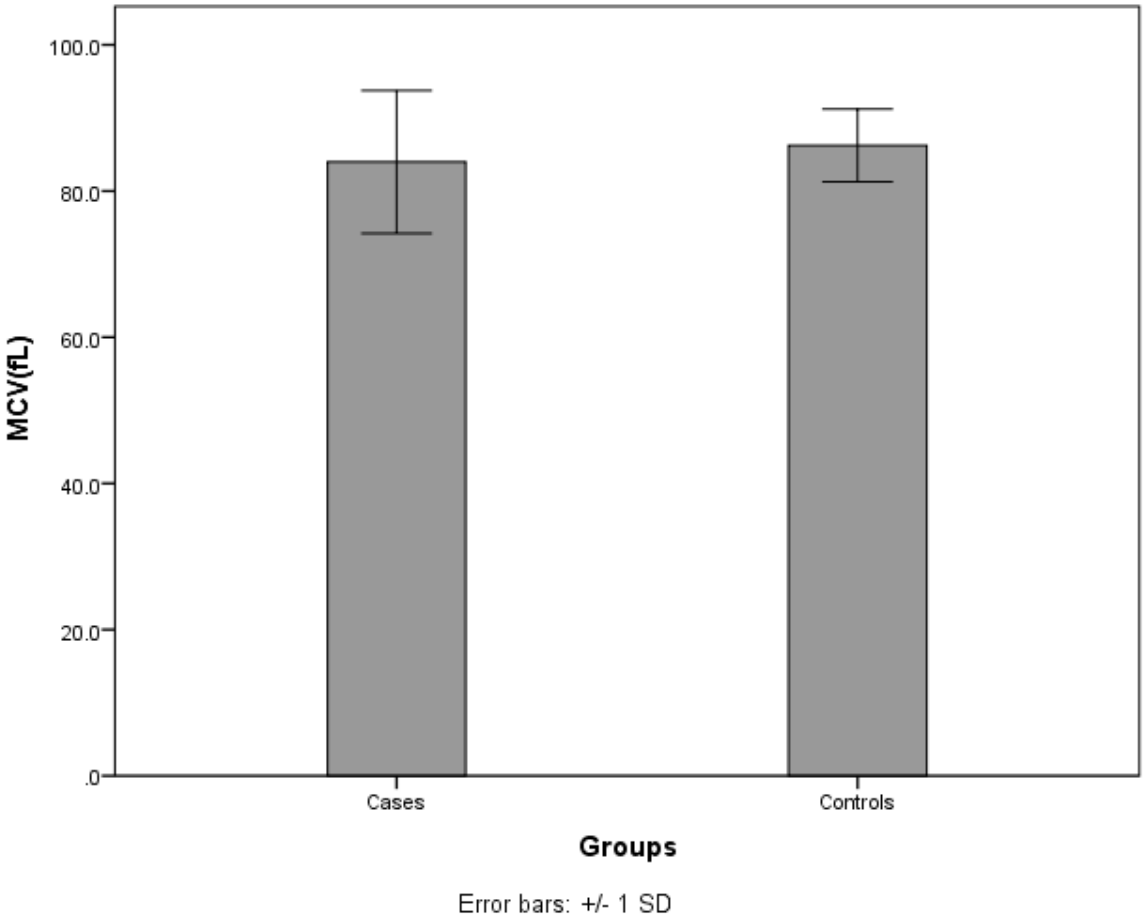
Figure 1: Haemoglobin levels



Data are shown as means (± SD). Data were analysed by unpaired T-test. *p value < 0.05, when compared with control data

Haemoglobin levels were significantly lower in patients with ulcerative colitis than in control patients. Among the 20 patients with ulcerative colitis, 12 were found to be anaemic (hemoglobin levels less than l3gm/dl in males and less than 12gm/dl in females).

Figure 2: Mean corpuscular volume (MCV)



Data are shown as mean (±SD) and were analysed by unpaired T-test.

Mean corpuscular volumes (MCV) in the 2 groups were not significantly different from one another.

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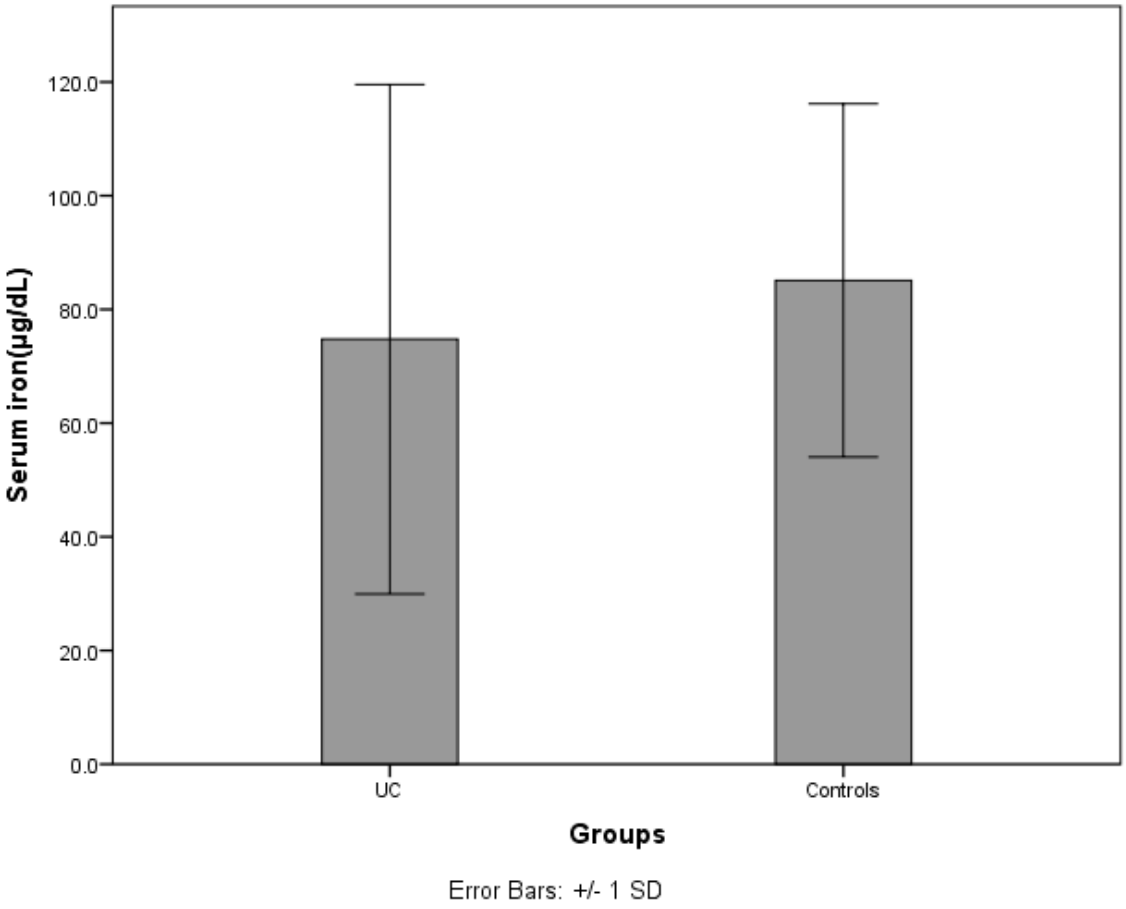
IRON-RELATED PARAMETERS

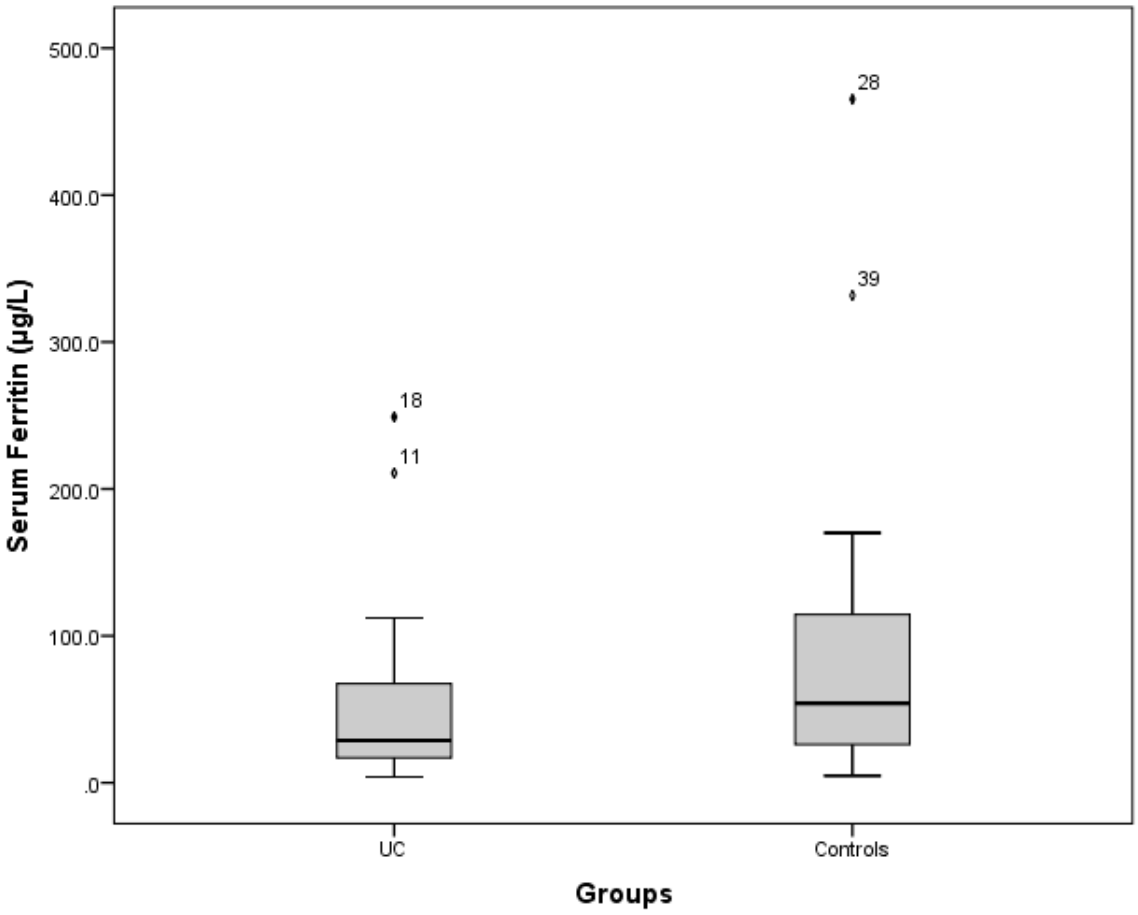
Figure 3: Serum iron levels



Data are shown as mean (±SD) and were analysed by unpaired T-test.

Serum iron levels in the 2 groups were not significantly different from one another.

Figure 4: Serum ferritin levels

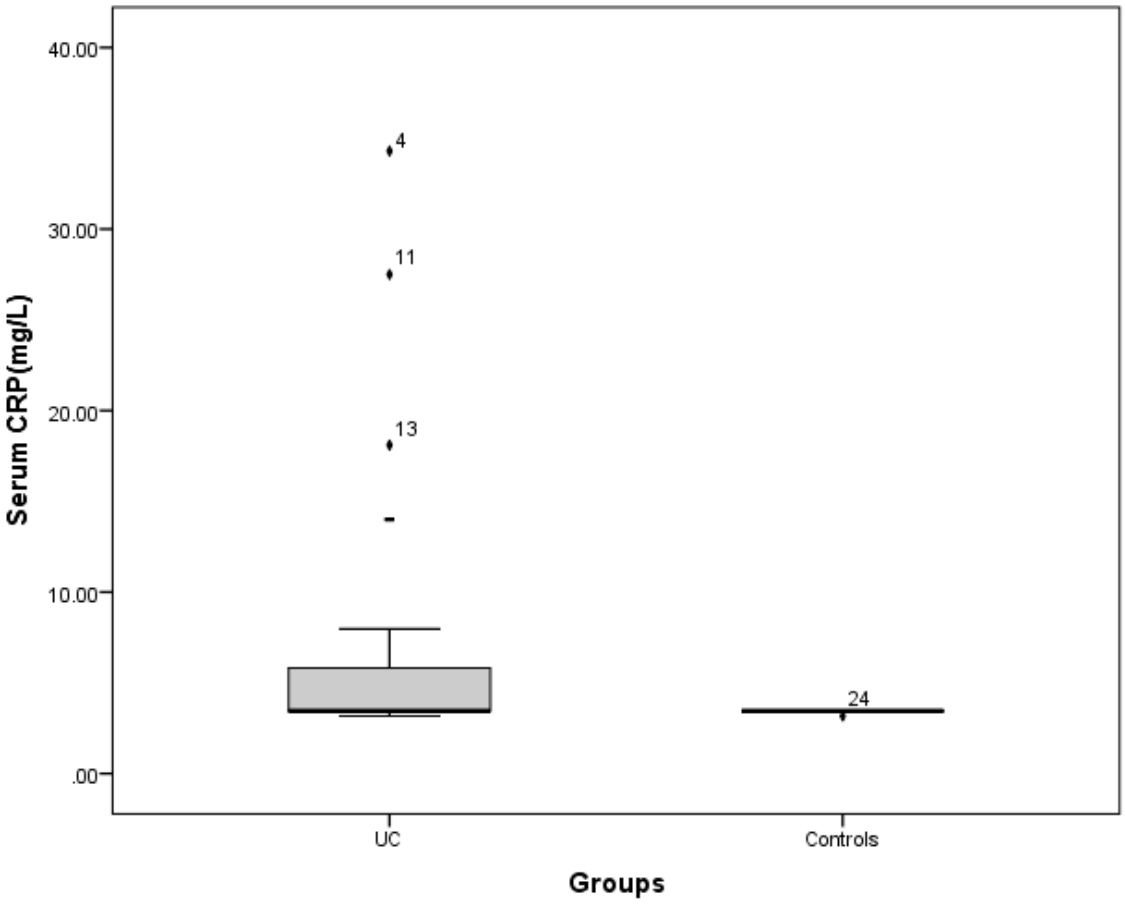


Data are shown as box and whisker plots, with the quartiles and medians shown. The outliers in each group are shown as numbered dots. Data were analysed by Mann – Whitney U test.

Serum ferritin levels tended to be lower in patients with UC, but the decrease was not statistically significant ($p = 0.09$).

MARKER OF INFLAMMATION

Figure 5: Serum C-reactive protein



Data are shown as box and whisker plots, showing quartiles and medians. Outliers in each group are shown as numbered dots. Data were analysed by Mann – Whitney U test.

Serum CRP levels tended to be higher in patients with UC; however, the increase was not statistically significant.

SERUM GDF 15 LEVELS

Serum GDF-15 levels in patients with ulcerative colitis (n= 20) and control patients (n=20) were estimated by ELISA.

A standard curve for GDF-15 was generated using the Bio-Rad Laboratories micro-plate manager software. The concentration of GDF-15 in each sample was obtained from this curve.

Figure 6. Standard curve for GDF-15

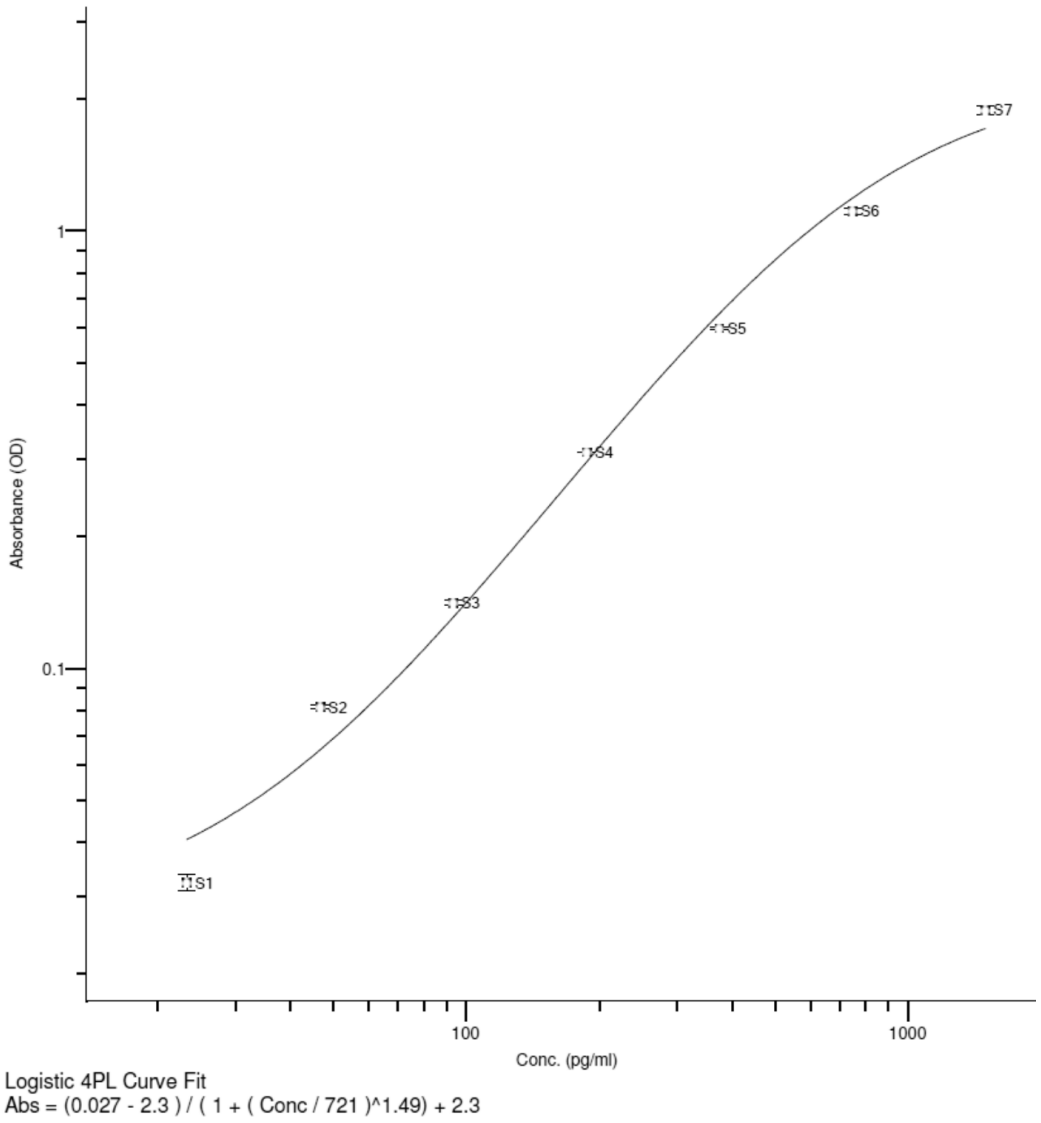
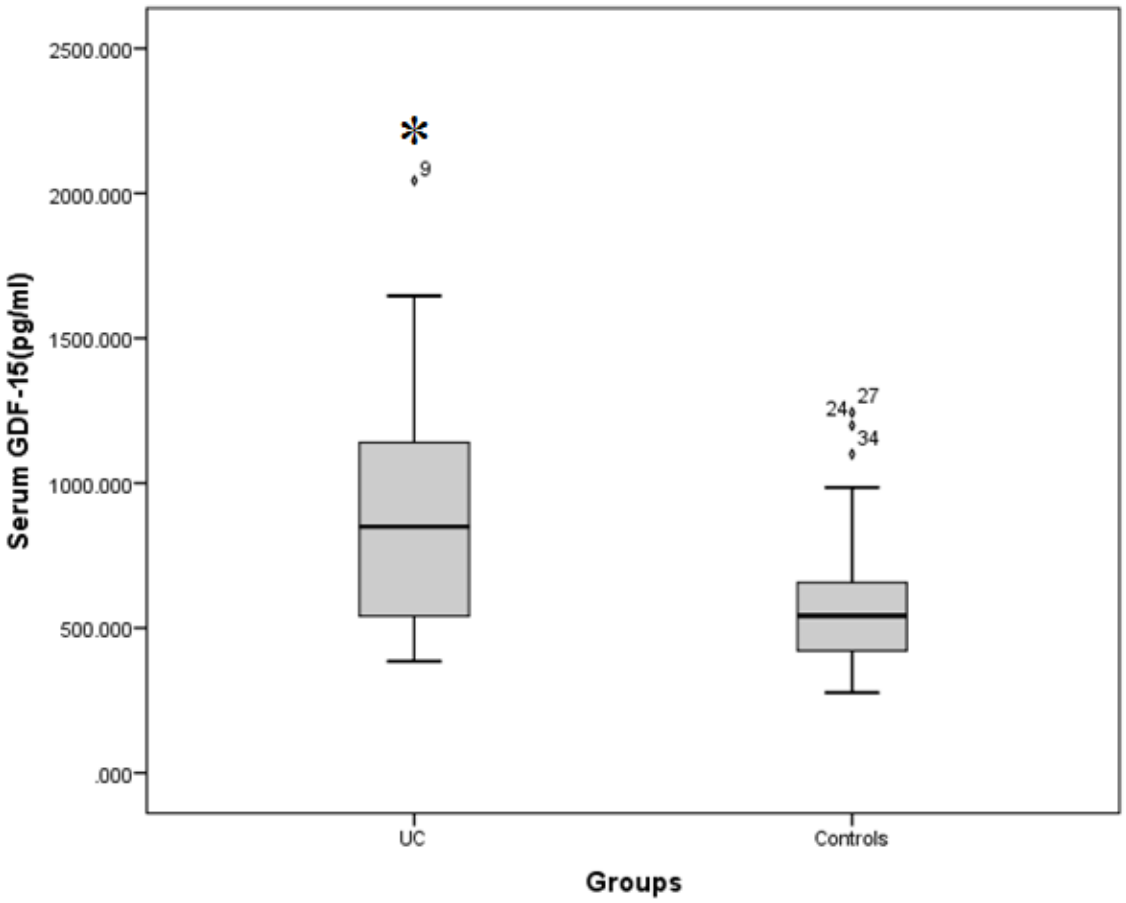


Figure 7: Serum levels of GDF-15 in subjects



Data are shown as box and whisker plots, showing quartiles and medians. Outliers in each group are shown as numbered dots. Data were analysed using Mann-Whitney U test. * indicates p value < 0.05 when compared to controls.

Serum GDF-15 levels were found to be significantly higher in patients with UC.

CORRELATION ANALYSIS

Correlational analysis was done with the total number of 40 patients and also separately for the control subjects and for those with UC.

Table 2: **Results of analysis done with the total number of 40 patients**

	Correlation coefficient	**p value**
Using total data (UC + control) (n=40)		
Serum GDF-15 vs		
Age	0.446*	0.004
Haemoglobin	-0.379*	0.016
Haemoglobin vs		
Serum iron	0.462*	0.003
Serum ferritin	0.490*	0.001
Serum ferritin vs		
Serum iron	0.622*	0.00

Analysis done by Spearman's or Pearson's correlation test, as appropriate.* indicates correlation is significant at the 0.05 level (2-tailed).

Only significant correlations have been shown. There were no other statistically significant correlations among any of the other parameters.

Table 3: Results of correlation analysis done in patients with UC

	Correlation coefficient	p value
Patients with UC (n=20)		
Serum GDF-15 vs		
Age	0.416	0.068
MCV (n=15)	0.55*	0.034
Serum ferritin	0.391	0.088
Haemoglobin	- 0.375	0.104
Serum iron vs		
Haemoglobin	0.587*	0.006
Serum ferritin	0.612*	0.004

Correlation analysis was done by Spearman's or Pearson's correlation test, as appropriate. * indicates correlation is significant at the 0.05 level (2-tailed).

There were no other statistically significant correlations among any of the other parameters.

Table 4: Results of correlation analysis done in control patients.

	Correlation coefficient	p value
Control patients (n=20)		
Serum GDF 15 vs		
Age	0.569*	0.009
MCV(n=14)	-0.616*	0.019
Serum ferritin Vs		
Haemoglobin	0.655*	0.002
Serum iron	0.576*	0.008

Correlation analysis was done by Spearman's or Pearson's correlation test, as appropriate. * indicates correlation is significant at the 0.05 level (2-tailed).

There were no other statistically significant correlations among any of the other parameters.

SUMMARY OF FINDINGS

1. Haemoglobin levels were found to be significantly lower in patients with ulcerative colitis, when compared with control patients.

2. Serum growth differentiation factor 15 (GDF-15) levels were found to be significantly higher in patients with ulcerative colitis, when compared with control patients.

3. When all the subjects in the study were considered together, levels of serum GDF-15 correlated positively with age and negatively with haemoglobin. Haemoglobin and serum levels iron and ferritin bore significant positive correlations with one another.

4. In patients with UC, a significant positive correlation was seen between serum GDF-15 and MCV values. There was also a tendency for GDF-15 to correlate positively with age and serum ferritin and negatively with haemoglobin levels. Haemoglobin and serum levels of iron and ferritin bore significant positive correlations with one another.

5. In control patients, serum GDF-15 levels correlated positively with age and negatively with MCV. Serum ferritin levels correlated positively with haemoglobin and serum iron levels.

DISCUSSION

Anemia is a common complication in patients with ulcerative colitis (UC). Its prevalence has been estimated to vary between 6 and 70% in patients with IBD (Ott et al. 2012). The anemia that develops in patients with UC is of complex etiology; it may be due to inflammation associated with the condition or due to iron deficiency caused by blood loss in stools, or both (Schreiber and Wedel 1997). However, despite the prevalence of anemia in those with IBD, resulting in the quality of life being affected, the condition is often under-treated in clinical practice (Ott et al. 2012).

Hepcidin plays a major role in iron homeostasis (Ganz. 2003). Its levels are increased in inflammatory states (Nemeth et al, 2003; Lee et al. 2005). It was expected that, given the known effect of pro-inflammatory cytokines such as IL-6 in inducing hepcidin, levels of hepcidin would be elevated in those with IBD.

However, an earlier study done in the Department of Biochemistry at CMC, Vellore, found that serum hepcidin levels were significantly decreased in patients with ulcerative colitis (Sukumaran et al. 2014). This was an unexpected finding. In order to account for this, the authors have suggested that that the presence of anemia in these patients was likely to be the reason that caused down-regulation of hepcidin. Thus, it seemed to confirm an earlier postulate that, when anemia and inflammation co-exist, the effect of the former on

hepcidin suppression predominates over that of the latter to induce it (Sukumaran et al. 2014). A similar observation of low serum hepcidin levels has been reported earlier in patients with IBD; this was found to be so, irrespective of the presence or absence of iron deficiency anemia (Arnold et al. 2009). A mouse model of colitis has also been shown to have low hepcidin expression; this was attributed to increased TNF-α levels, which is known to down-regulate hepcidin (Shanmugam et al. 2012).

The finding of low serum hepcidin in patients in patients with UC raised the question of why levels were low in this condition, despite the presence of inflammation. There are several known negative regulators of hepcidin. GDF-15 is one of them. Very high levels of GDF 15 have been reported in patients with ineffective erythropoiesis associated with conditions such as thalassemia and congenital dyserythropoietic anemia I, where it was shown to down-regulate hepcidin expression (Tanno et al 2007, Tamary et al. 2008). Its levels have also been shown to be increased in anemia of inflammation and iron deficiency (Theurl et al. 2010).

Based on the observations of Sukumaran et al (2014), it was hypothesized that the low levels of hepcidin seen in patients with ulcerative colitis may be a result of increased serum GDF-15 levels. It was to test this hypothesis that the present study was carried out.

The results show that GDF-15 levels were, indeed, higher in patients with UC.

Sixty percent of the patients with UC in the present study were found to be anemic. The levels of hemoglobin in patients with UC were significantly lower than in control patients. Serum iron and ferritin levels were measured to assess iron status of the subjects. Serum ferritin levels tended to be lower in patients with UC (with a median value of28.85 μg/L; range: 3.6-249 μg/L) than in controls (median value of 54.1 μg/L; range: 4.7- 465 μg/L) ($p = 0.08$), suggesting a state of iron deficiency. Serum iron levels were, however, not significantly different.

It would be difficult to determine whether the patients in the present study had anemia of inflammation or iron deficiency or a combination of the 2. This is a common dilemma in clinical practice. The lower ferritin levels tend to indicate that a state of iron deficiency may exist. This is consistent with the clinical finding that 14 patients out of 20 patient with UC had blood loss in stools at the time of recruitment. Most of these patients showed signs of inflammation on colonoscopy and in the mucosal biopsy. However, serum CRP levels, a marker of systemic inflammation, were not elevated in these patients. This may be the result of response to treatment. Measurement of additional parameters, such as soluble

transferrin receptor (sTfR) (which is not affected by inflammation) and calculation of the sTfR/log ferritin ratio would have been helpful in differentiating anemia due to inflammation and that due to iron deficiency (Skikne 2008). However, it was not possible to measure sTfR levels in these patients' samples, due to financial constraints.

Correlation analysis showed significant negative correlation between serum GDF-15 and hemoglobin, when all the patients in the study (both control patients and those with UC) were considered together. This relationship did not hold good when the 2 groups were considered separately. These findings are similar to those of observed by Theurl et al (2010). In the present study, MCV values and GDF-15 levels were positively correlated in patients with UC and negatively in control patients. The reasons for this observation are not clear. Studying a larger sample of patients may help better elucidate the relationships involved.

The finding that there was no significant difference in CRP levels between patient with UC and those in the control group differs from those of Sukumaran et al (2014) and Theurl et al (2010). This indicates decreased inflammatory activity in patients with UC in this study, which may be an indication of their improvement due to the treatment they were receiving. However, it has also been shown that CRP responses in patients with UC can be poor, in spite of active

inflammation (Vermeire et al, 2004). Correlation analysis did not show any significant association between serum CRP and GDF-15 levels. It may have been useful to have measured serum interleukin 6 (IL--6) in these patients, as it is known that IL 6 is the main pro-inflammatory cytokine, which is shown to up-regulate hepcidin (Nemeth et al, 2003). However, once again, financial constraints precluded estimation of serum IL-6 in these patients.

In the present study, patients with ulcerative colitis had higher levels of serum GDF 15 when compared to controls. This was consistent with higher serum GDF-15 levels reported by Theurl et al (2010) in patients with ACD and in those with ACD with IDA.

It was not possible to estimate serum hepcidin levels in these patients, once again due to financial constraints. It would have been useful to do this, in order to be able to correlate serum levels of GDF-15 and hepcidin in these patients. It is hoped that it will be possible to do this at a later time point. The median serum GDF-15 levels in patients with UC in this study was 849 pg/ml (range: 385-2043pg/ml); such levels were found to be lower than levels that have been reported in thalassemia mean (66,000 ± 9,600 pg/ml) (Tanno et al, 2007; Tamary et al. 2008). Increase in GDF-15 levels in thalassemia is shown to be due to increased proliferation and apoptosis of erythroid cells, due to an imbalance in the synthesis of

alpha and beta chains of hemoglobin (Tanno et al 2007). In patients with UC, it may be possible that there is an increase in erythroid activity in response to anemia; such an effect may account for raised GDF-15 levels. Theurl et al (2010) found significant correlations between serum levels of GDF-15 and sTfR and have suggested that increases in serum GDF-15 levels may reflect increased erythroid activity, even in presence of inflammation in patients with IDA/ACD. Serum erythropoietin and sTfR levels were not measured in the present study; hence, it was not possible to study possible associations between GDF-15 and erythropoietic drive and erythroid activity.

Interestingly, a positive correlation was found between serum GDF-15 levels and age, in control patients alone and also when all the subjects were considered together. There was a similar trend in patients with UC as well ($r = 0.416$; $p = 0.06$). Such a finding has been reported earlier by Wiklund et al (2010), who have shown that increases in GDF-15 levels occurred with increasing age. Such an increase has been suggested to be due to increase in p53 levels associated with aging, with p53 known to up-regulate GDF-15 expression (Wiklund et al. 2010).

CONCLUSION

In conclusion, serum GDF-15 levels were found to be significantly increased in patients with ulcerative colitis. These patients also had significantly lower levels of hemoglobin. Increases in GDF-15 may reflect increased erythropoiesis in these patients in response to anemia. This would, in turn, down-regulate hepcidin levels, an effect that has been reported in an earlier study from the department. More detailed studies involving estimation of serum hepcidin, sTfR, erythropoietin and other iron-related parameters in these patients would help elucidate the relationships involved further.

LIMITATIONS OF THE STUDY

- The sample size for the study was small.

Due to financial constraints, several related parameters such as serum hepcidin, sTfR, erythropoietin and IL-6 could not be estimated. Measurement of these parameters would have provided information that may have contributed to elucidating the events involved and also in differentiating patients with anemia of inflammation or iron deficiency anemia

FUTURE DIRECTIONS

Estimation of the following parameters need to be carried out for a better understanding of the events involved in the pathogenesis of anemia in patients with ulcerative colitis:

- Serum hepcidin levels
- Serum sTfR and erythropoietin
- Serum IL 6 and serum TNF α levels, which are known to be involved in ulcerative colitis, and also known to alter hepcidin expression.

Measurement of these parameters could help in better understanding of iron metabolism and anemia in patients with ulcerative colitis.

BIBLIOGRAPHY

1. Abboud, Sherry, and David J. Haile. 2000. “A Novel Mammalian Iron-Regulated Protein Involved in Intracellular Iron Metabolism.” *Journal of Biological Chemistry* 275 (26): 19906–12. doi:10.1074/jbc.M000713200.

2. Adamson John W, Iron Deficiency and Other Hypoproliferative Anemias .Harrison’s principles of internal medicine, 18th edition, 2011, (Editors-Longo D, Fauci A Kasper D, Hauser S , Jameson L, Loscalzo). Mc Graw-Hill.

3. Anderson, Cole P., Macy Shen, Richard S. Eisenstein, and Elizabeth A. Leibold. 2012. “Mammalian Iron Metabolism and Its Control by Iron Regulatory Proteins.” *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, Cell Biology of Metals, 1823 (9): 1468–83. doi:10.1016/j.bbamcr.2012.05.010.

4. Andrews, Nancy C. 2000. “Iron Homeostasis: Insights from Genetics and Animal Models.” *Nature Reviews Genetics* 1 (3): 208–17. doi:10.1038/35042073.

5. Andriopoulos Jr, Billy, Elena Corradini, Yin Xia, Sarah A. Faasse, Shanzhuo Chen, Lovorka Grgurevic, Mitchell D. Knutson, et al. 2009. “BMP6 Is a Key Endogenous Regulator of Hepcidin Expression and Iron Metabolism.” *Nature Genetics* 41 (4): 482–87. doi:10.1038/ng.335.

6. Arnold, Jayantha, Arvind Sangwaiya, Bharati Bhatkal, Frank Geoghegan, and Mark Busbridge. 2009. “Hepcidin and Inflammatory Bowel Disease: Dual Role in Host Defence and Iron Homoeostasis.” *European Journal of Gastroenterology & Hepatology* 21 (4): 425–29. doi:10.1097/MEG.0b013e32830e2885.

7. Ashby, Damien R., Daniel P. Gale, Mark Busbridge, Kevin G. Murphy, Neill D. Duncan, Tom D. Cairns, David H. Taube, et al. 2010a. “Erythropoietin Administration in Humans Causes a Marked and Prolonged Reduction in Circulating Hepcidin.”

Haematologica 95 (3): 505–8. doi:10.3324/haematol.2009.013136.

8. Ashby, 2010b. “Erythropoietin Administration in Humans Causes a Marked and Prolonged Reduction in Circulating Hepcidin.” *Haematologica* 95 (3): 505–8. doi:10.3324/haematol.2009.013136.

9. Babitt, Jodie L., Franklin W. Huang, Diedra M. Wrighting, Yin Xia, Yisrael Sidis, Tarek A. Samad, Jason A. Campagna, et al. 2006. “Bone Morphogenetic Protein Signaling by Hemojuvelin Regulates Hepcidin Expression.” *Nature Genetics* 38 (5): 531–39. doi:10.1038/ng1777.

10. Babitt, Jodie L., Ying Zhang, Tarek A. Samad, Yin Xia, Jie Tang, Jason A. Campagna, Alan L. Schneyer, Clifford J. Woolf, and Herbert Y. Lin. 2005. “Repulsive Guidance Molecule (RGMa), a DRAGON Homologue, Is a Bone Morphogenetic Protein Co-Receptor.” *The Journal of Biological Chemistry* 280 (33): 29820–27. doi:10.1074/jbc.M503511200.

11. Baker, E. N. 1994. “Structure and Reactivity of Transferrins.” In *Advances in Inorganic Chemistry*, edited by A.G. Sykes, Volume 41:389–463. Academic Press. http://www.sciencedirect.com/science/article/pii/S0898883808601762.

12. Barisani, D., C. L. Berg, M. Wessling-Resnick, and J. L. Gollan. 1995. “Evidence for a Low Km Transporter for Non-Transferrin-Bound Iron in Isolated Rat Hepatocytes.” *American Journal of Physiology - Gastrointestinal and Liver Physiology* 269 (4): G570–76.

13. Bennett, M. J., J. A. Lebrón, and P. J. Bjorkman. 2000. “Crystal Structure of the Hereditary Haemochromatosis Protein HFE Complexed with Transferrin Receptor.” *Nature* 403 (6765): 46–53. doi:10.1038/47417.

14. Bootcov, M. R., A. R. Bauskin, S. M. Valenzuela, A. G. Moore, M. Bansal, X. Y. He, H. P. Zhang, et al. 1997. “MIC-1, a Novel Macrophage Inhibitory Cytokine, Is a Divergent Member of the TGF-Beta Superfamily.” *Proceedings of the National Academy of Sciences of the United States of America* 94 (21): 11514–19.

15. Bridle, Kim R, David M Frazer, Sarah J Wilkins, Jeanette L Dixon, David M Purdie, Darrell HG Crawford, V Nathan Subramaniam, Lawrie W Powell, Gregory J Anderson, and Grant A Ramm. 2003. “Disrupted Hepcidin Regulation in HFE-Associated Haemochromatosis and the Liver as a Regulator of Body Iron Homoeostasis.” *The Lancet* 361 (9358): 669–73. doi:10.1016/S0140-6736(03)12602-5.

16. Casanovas, Guillem, Katarzyna Mleczko-Sanecka, Sandro Altamura, Matthias W. Hentze, and Martina U. Muckenthaler. 2009. “Bone Morphogenetic Protein (BMP)-Responsive Elements Located in the Proximal and Distal Hepcidin Promoter Are Critical for Its Response to HJV/BMP/SMAD.” *Journal of Molecular Medicine* 87 (5): 471–80. doi:10.1007/s00109-009-0447-2.

17. Casanovas, Guillem, Maja Vujic Spasic, Carla Casu, Stefano Rivella, Jens Strelau, Klaus Unsicker, and Martina U. Muckenthaler. 2013. “The Murine Growth Differentiation Factor 15 Is Not Essential for Systemic Iron Homeostasis in Phlebotomized Mice.” *Haematologica* 98 (3): 444–47. doi:10.3324/haematol.2012.069807.

18. Chen, Juxing, Maja Chloupková, Junwei Gao, Tara L. Chapman-Arvedson, and Caroline A. Enns. 2007. “HFE Modulates Transferrin Receptor 2 Levels in Hepatoma Cells via Interactions That Differ from Transferrin Receptor 1-HFE Interactions.” *The Journal of Biological Chemistry* 282 (51): 36862–70. doi:10.1074/jbc.M706720200.

19. Choi, Jeehyea, Patarabutr Masaratana, Gladys O. Latunde-Dada, Matthew Arno, Robert J. Simpson, and Andrew T. McKie. 2012. “Duodenal Reductase Activity and Spleen Iron Stores Are Reduced and Erythropoiesis Is Abnormal in Dcytb Knockout Mice

Exposed to Hypoxic Conditions." *The Journal of Nutrition* 142 (11): 1929–34. doi:10.3945/jn.112.160358.

20. Cox, T. C., M. J. Bawden, A. Martin, and B. K. May. 1991. "Human Erythroid 5-Aminolevulinate Synthase: Promoter Analysis and Identification of an Iron-Responsive Element in the mRNA." *The EMBO Journal* 10 (7): 1891–1902.

21. Domenico, Ivana De, Diane McVey Ward, Charles Langelier, Michael B. Vaughn, Elizabeta Nemeth, Wesley I. Sundquist, Tomas Ganz, Giovanni Musci, and Jerry Kaplan. 2007. "The Molecular Mechanism of Hepcidin-Mediated Ferroportin Down-Regulation." *Molecular Biology of the Cell* 18 (7): 2569–78. doi:10.1091/mbc.E07-01-0060.

22. Donovan, A., A. Brownlie, Y. Zhou, J. Shepard, S. J. Pratt, J. Moynihan, B. H. Paw, et al. 2000. "Positional Cloning of Zebrafish ferroportin1 Identifies a Conserved Vertebrate Iron Exporter." *Nature* 403 (6771): 776–81. doi:10.1038/35001596.

23. Donovan, Adriana, Christine A. Lima, Jack L. Pinkus, Geraldine S. Pinkus, Leonard I. Zon, Sylvie Robine, and Nancy C. Andrews. 2005. "The Iron Exporter ferroportin/Slc40a1 Is Essential for Iron Homeostasis." *Cell Metabolism* 1 (3): 191–200. doi:10.1016/j.cmet.2005.01.003.

24. Du, Xin, Ellen She, Terri Gelbart, Jaroslav Truksa, Pauline Lee, Yu Xia, Kevin Khovananth, et al. 2008. "The Serine Protease TMPRSS6 Is Required to Sense Iron Deficiency." *Science (New York, N.Y.)* 320 (5879): 1088–92. doi:10.1126/science.1157121

25. Enns, Caroline A., Riffat Ahmed, Jiaohong Wang, Akiko Ueno, Christal Worthen, Hidekazu Tsukamoto, and An-Sheng Zhang. 2013. "Increased Iron Loading Induces Bmp6 Expression in the Non-Parenchymal Cells of the Liver Independent of the BMP-Signaling Pathway." *PLoS ONE* 8 (4): e60534. doi:10.1371/journal.pone.0060534.

26. Feder, J. N., A. Gnirke, W. Thomas, Z. Tsuchihashi, D. A. Ruddy, A. Basava, F. Dormishian, et al. 1996. “A Novel MHC Class I-like Gene Is Mutated in Patients with Hereditary Haemochromatosis.” *Nature Genetics* 13 (4): 399–408. doi:10.1038/ng0896-399

27. Feder, J. N., Z. Tsuchihashi, A. Irrinki, V. K. Lee, F. A. Mapa, E. Morikang, C. E. Prass, et al. 1997. “The Hemochromatosis Founder Mutation in HLA-H Disrupts beta2-Microglobulin Interaction and Cell Surface Expression.” *The Journal of Biological Chemistry* 272 (22): 14025–28.

28. Feder, John N., David M. Penny, Alivelu Irrinki, Vince K. Lee, Jose A. Lebron, Nicole Watson, Zenta Tsuchihashi, Elliott Sigal, Pamela J. Bjorkman, and Randall C. Schatzman. 1998. “The Hemochromatosis Gene Product Complexes with the Transferrin Receptor and Lowers Its Affinity for Ligand Binding.” *Proceedings of the National Academy of Sciences of the United States of America* 95 (4): 1472–77.

29. Finberg, Karin E, Matthew M Heeney, Dean R Campagna, Yesim Ayd?nok, Howard A Pearson, Kip R Hartman, Mary M Mayo, et al. 2008. “Mutations in TMPRSS6 Cause Iron-Refractory Iron Deficiency Anemia (IRIDA).” *Nature Genetics* 40 (5): 569–71. doi:10.1038/ng.130.

30. Finkenstedt, Armin, Paola Bianchi, Igor Theurl, Wolfgang Vogel, Derrick R. Witcher, Victor J. Wroblewski, Anthony T. Murphy, Alberto Zanella, and Heinz Zoller. 2009. “Regulation of Iron Metabolism through GDF15 and Hepcidin in Pyruvate Kinase Deficiency.” *British Journal of Haematology* 144 (5): 789–93. doi:10.1111/j.1365-2141.2008.07535.x.

31. Fleming, Mark D., Michelle A. Romano, Maureen A. Su, Laura M. Garrick, Michael D. Garrick, and Nancy C. Andrews. 1998. “Nramp2 Is Mutated in the Anemic Belgrade (b) Rat: Evidence of a Role for Nramp2 in Endosomal Iron Transport.” *Proceedings of the National Academy of Sciences of the United States of America* 95 (3): 1148–53.

32. Fleming, M. D., C. C. Trenor, M. A. Su, D. Foernzler, D. R. Beier, W. F. Dietrich, and N. C. Andrews. 1997. "Microcytic Anaemia Mice Have a Mutation in Nramp2, a Candidate Iron Transporter Gene." *Nature Genetics* 16 (4): 383–86. doi:10.1038/ng0897-383.

33. Folgueras, Alicia R., Fernando Martín de Lara, Alberto M. Pendás, Cecilia Garabaya, Francisco Rodríguez, Aurora Astudillo, Teresa Bernal, Rubén Cabanillas, Carlos López-Otín, and Gloria Velasco. 2008. "Membrane-Bound Serine Protease Matriptase-2 (Tmprss6) Is an Essential Regulator of Iron Homeostasis." *Blood* 112 (6): 2539–45. doi:10.1182/blood-2008-04-149773.

34. Friedman S and Blumberg RS (2011).Chapter 295: Inflammatory Bowel Disease. Harrison's principles of internal medicine, 18th edition (Editors-Longo D, Fauci A Kasper D, Hauser S , Jameson L, Loscalzo). Mc Graw-Hill. Page no: 2477-2495

35. Gao, Junwei, Juxing Chen, Ivana De Domenico, David M. Koeller, Cary O. Harding, Robert E. Fleming, Dwight D. Koeberl, and Caroline A. Enns. 2010. "Hepatocyte-Targeted HFE and TFR2 Control Hepcidin Expression in Mice." *Blood* 115 (16): 3374–81. doi:10.1182/blood-2009-09-245209.

36. Gao, Junwei, Juxing Chen, Maxwell Kramer, Hidekazu Tsukamoto, An-Sheng Zhang, and Caroline A. Enns. 2009. "Interaction of the Hereditary Hemochromatosis Protein, HFE, with Transferrin Receptor 2 Is Required for Transferrin-Induced Hepcidin Expression." *Cell Metabolism* 9 (3): 217–27. doi:10.1016/j.cmet.2009.01.010.

37. Ganz T. Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. Blood. 2003 Aug 1;102(3):783–8

38. Gasche, C., M. C. E. Lomer, I. Cavill, and G. Weiss. 2004. "Iron, Anaemia, and Inflammatory Bowel Diseases." *Gut* 53 (8): 1190–97. doi:10.1136/gut.2003.035758.

39. Gunshin, Hiromi, Bryan Mackenzie, Urs V. Berger, Yoshimi Gunshin, Michael F. Romero, Walter F. Boron, Stephan Nussberger, John L. Gollan, and Matthias A. Hediger. 1997. "Cloning and Characterization of a Mammalian Proton-Coupled Metal-Ion Transporter." *Nature* 388 (6641): 482–88.

40. Gunshin, Hiromi, Carolyn N. Starr, Cristina DiRenzo, Mark D. Fleming, Jie Jin, Eric L. Greer, Vera M. Sellers, Stephanie M. Galica, and Nancy C. Andrews. 2005. "Cybrd1 (duodenal Cytochrome B) Is Not Necessary for Dietary Iron Absorption in Mice." *Blood* 106 (8): 2879–83. doi:10.1182/blood-2005-02-0716.

41. Harris, Z. Leah, Alison P. Durley, Tsz Kwong Man, and Jonathan D. Gitlin. 1999. "Targeted Gene Disruption Reveals an Essential Role for Ceruloplasmin in Cellular Iron Efflux." *Proceedings of the National Academy of Sciences* 96 (19): 10812–17. doi:10.1073/pnas.96.19.10812.

42. Hentze, Matthias W., Martina U. Muckenthaler, Bruno Galy, and Clara Camaschella. 2010. "Two to Tango: Regulation of Mammalian Iron Metabolism." *Cell* 142 (1): 24–38. doi:10.1016/j.cell.2010.06.028.

43. Hentze, M. W., S. W. Caughman, T. A. Rouault, J. G. Barriocanal, A. Dancis, J. B. Harford, and R. D. Klausner. 1987. "Identification of the Iron-Responsive Element for the Translational Regulation of Human Ferritin mRNA." *Science (New York, N.Y.)* 238 (4833): 1570–73.

44. "Indian Society of Gastroenterology Consensus on Ulcerative Colitis - Springer." 2014. Accessed September 8. doi:10.1007/s12664-012-0259-0.

45. Higgins T ,Eckfeldt J ,Barton J ,Doumas B. Hemoglobin,Iron and Bilirubin . Tietz Textbook of Clinical Chemistry and Molecular Diagnosis. Lopez J. Carl A. Burtis, Edward R. Ashwood and David E. Bruns (eds), (5th edition). 2013 Jan;28(1):104–5.

46. Jelkmann, W. 1998. “Proinflammatory Cytokines Lowering Erythropoietin Production.” *Journal of Interferon & Cytokine Research: The Official Journal of the International Society for Interferon and Cytokine Research* 18 (8): 555–59.

47. Kautz, Leon, and Elizabeta Nemeth. 2014. “Molecular Liaisons between Erythropoiesis and Iron Metabolism.” *Blood*, January, blood – 2014–05 – 516252. doi:10.1182/blood-2014-05-516252.

48. Kawabata, Hiroshi, Rong Yang, Toshiyasu Hirama, Peter T. Vuong, Seiji Kawano, Adrian F. Gombart, and H. Phillip Koeffler. 1999. “Molecular Cloning of Transferrin Receptor 2 A NEW MEMBER OF THE TRANSFERRIN RECEPTOR-LIKE FAMILY.” *Journal of Biological Chemistry* 274 (30): 20826–32. doi:10.1074/jbc.274.30.20826.

49. Kawabata, H., R. Yang, T. Hirama, P. T. Vuong, S. Kawano, A. F. Gombart, and H. P. Koeffler. 1999. “Molecular Cloning of Transferrin Receptor 2. A New Member of the Transferrin Receptor-like Family.” *The Journal of Biological Chemistry* 274 (30): 20826–32.

50. Kemna, Erwin H. J. M., Harold Tjalsma, Hans L. Willems, and Dorine W. Swinkels. 2008. “Hepcidin: From Discovery to Differential Diagnosis.” *Haematologica* 93 (1): 90–97. doi:10.3324/haematol.11705.

51. Krause, Alexander, Susanne Neitz, Hans-Jürgen Mägert, Axel Schulz, Wolf-Georg Forssmann, Peter Schulz-Knappe, and Knut Adermann. 2000. “LEAP-1, a Novel Highly Disulfide-Bonded Human Peptide, Exhibits Antimicrobial Activity.” *FEBS Letters* 480 (2–3): 147–50. doi:10.1016/S0014-5793(00)01920-7.

52. Lakhal, Samira, Johannes Schodel, Alain R. M. Townsend, Christopher W. Pugh, Peter J. Ratcliffe, and David R. Mole. 2011. “Regulation of Type II Transmembrane Serine Proteinase TMPRSS6 by Hypoxia-Inducible Factors.” *The Journal of Biological Chemistry* 286 (6): 4090–97. doi:10.1074/jbc.M110.173096.

53. Lawson, David M., Amyra Treffry, Peter J. Artymiuk, Pauline M. Harrison, Stephen J. Yewdall, Alessandra Luzzago, Gianne Cesareni, Sonia Levi, and Paolo Arosio. 1989. “Identification of the Ferroxidase Centre in Ferritin.” *FEBS Letters* 254 (1–2): 207–10. doi:10.1016/0014-5793(89)81040-3.

54. Lee, Pauline, Hongfan Peng, Terri Gelbart, Lei Wang, and Ernest Beutler. 2005. “Regulation of Hepcidin Transcription by Interleukin-1 and Interleukin-6.” *Proceedings of the National Academy of Sciences of the United States of America* 102 (6): 1906–10. doi:10.1073/pnas.0409808102.

55. Leibold, E. A., and H. N. Munro. 1988. “Cytoplasmic Protein Binds in Vitro to a Highly Conserved Sequence in the 5’ Untranslated Region of Ferritin Heavy- and Light-Subunit mRNAs.” *Proceedings of the National Academy of Sciences of the United States of America* 85 (7): 2171–75.

56. Levy, J. E., O. Jin, Y. Fujiwara, F. Kuo, and N. C. Andrews. 1999. “Transferrin Receptor Is Necessary for Development of Erythrocytes and the Nervous System.” *Nature Genetics* 21 (4): 396–99. doi:10.1038/7727.

57. Lipschitz, David A., James D. Cook, and Clement A. Finch. 1974. “A Clinical Evaluation of Serum Ferritin as an Index of Iron Stores.” *New England Journal of Medicine* 290 (22): 1213–16. doi:10.1056/NEJM197405302902201.

58. Longo, Dan, Anthony Fauci, Dennis Kasper, Stephen Hauser, J. Jameson, and Joseph Loscalzo. 2011. *Harrison’s Principles of Internal Medicine, 18th Edition*. McGraw Hill Professional.

59. McKie, Andrew T, Paola Marciani, Andreas Rolfs, Karen Brennan, Kristina Wehr, Dalna Barrow, Silvia Miret, et al. 2000. “A Novel Duodenal Iron-Regulated Transporter, IREG1, Implicated in the Basolateral Transfer of Iron to the Circulation.” *Molecular Cell* 5 (2): 299–309. doi:10.1016/S1097-2765(00)80425-6.

60. McKie, A. T., D. Barrow, G. O. Latunde-Dada, A. Rolfs, G. Sager, E. Mudaly, M. Mudaly, et al. 2001. “An Iron-Regulated Ferric Reductase Associated with the Absorption of Dietary Iron.” *Science (New York, N.Y.)* 291 (5509): 1755–59. doi:10.1126/science.1057206.

61. Means, R. T. 1995. “Pathogenesis of the Anemia of Chronic Disease: A Cytokine-Mediated Anemia.” *Stem Cells (Dayton, Ohio)* 13 (1): 32–37. doi:10.1002/stem.5530130105.

62. Meynard, Delphine, Léon Kautz, Valérie Darnaud, François Canonne-Hergaux, Hélène Coppin, and Marie-Paule Roth. 2009. “Lack of the Bone Morphogenetic Protein BMP6 Induces Massive Iron Overload.” *Nature Genetics* 41 (4): 478–81. doi:10.1038/ng.320.

63. Meynard, Delphine, Valentina Vaja, Chia Chi Sun, Elena Corradini, Shanzhuo Chen, Carlos Lopez-Otin, Lovorka Grgurevic, et al. 2011. “Regulation of TMPRSS6 by BMP6 and Iron in Human Cells and Mice.” *Blood* 118 (3): 747–56. doi:10.1182/blood-2011-04-348698.

64. Miller, J. L., J. M. Njoroge, A. N. Gubin, and G. P. Rodgers. 1999. “Prospective Identification of Erythroid Elements in Cultured Peripheral Blood.” *Experimental Hematology* 27 (4): 624–29.

65. Nemeth, E. 2003. “Hepcidin, a Putative Mediator of Anemia of Inflammation, Is a Type II Acute-Phase Protein.” *Blood* 101: 2461–63. doi:10.1182/blood-2002-10-3235.

66. Nemeth, Elizabeta, Seth Rivera, Victoria Gabayan, Charlotte Keller, Sarah Taudorf, Bente K. Pedersen, and Tomas Ganz. 2004. “IL-6 Mediates Hypoferremia of Inflammation by Inducing the Synthesis of the Iron Regulatory Hormone Hepcidin.” *Journal of Clinical Investigation* 113 (9): 1271–76. doi:10.1172/JCI200420945.

67. Nemeth, Elizabeta, Antonella Roetto, Giovanni Garozzo, Tomas Ganz, and Clara Camaschella. 2005. “Hepcidin Is Decreased in TFR2 Hemochromatosis.” *Blood* 105 (4): 1803–6. doi:10.1182/blood-2004-08-3042.

68. Nemeth, Elizabeta, Marie S. Tuttle, Julie Powelson, Michael B. Vaughn, Adriana Donovan, Diane McVey Ward, Tomas Ganz, and Jerry Kaplan. 2004. “Hepcidin Regulates Cellular Iron Efflux by Binding to Ferroportin and Inducing Its Internalization.” *Science (New York, N.Y.)* 306 (5704): 2090–93. doi:10.1126/science.1104742.

69. Nicolas, Gael, Myriam Bennoun, Isabelle Devaux, Carole Beaumont, Bernard Grandchamp, Axel Kahn, and Sophie Vaulont. 2001. “Lack of Hepcidin Gene Expression and Severe Tissue Iron Overload in Upstream Stimulatory Factor 2 (USF2) Knockout Mice.” *Proceedings of the National Academy of Sciences of the United States of America* 98 (15): 8780–85. doi:10.1073/pnas.151179498.

70. Nicolas, Gael, Myriam Bennoun, Arlette Porteu, Sandrine Mativet, Carole Beaumont, Bernard Grandchamp, Mario Sirito, Michele Sawadogo, Axel Kahn, and Sophie Vaulont. 2002. “Severe Iron Deficiency Anemia in Transgenic Mice Expressing Liver Hepcidin.” *Proceedings of the National Academy of Sciences of the United States of America* 99 (7): 4596–4601. doi:10.1073/pnas.072632499.

71. Nicolas, Gaël, Lydie Viatte, Dan-Qing Lou, Myriam Bennoun, Carole Beaumont, Axel Kahn, Nancy C. Andrews, and Sophie Vaulont. 2003. “Constitutive Hepcidin Expression Prevents Iron Overload in a Mouse Model of Hemochromatosis.” *Nature Genetics* 34 (1): 97–101. doi:10.1038/ng1150.

72. Niederkofler, V. 2005. “Hemojuvelin Is Essential for Dietary Iron Sensing, and Its Mutation Leads to Severe Iron Overload.” *Journal of Clinical Investigation* 115 (8): 2180–86. doi:10.1172/JCI25683.

73. Ohgami, Robert S., Dean R. Campagna, Eric L. Greer, Brendan Antiochos, Alice McDonald, Jing Chen, John J. Sharp, Yuko Fujiwara, Jane E. Barker, and Mark D. Fleming. 2005. "Identification of a Ferrireductase Required for Efficient Transferrin-Dependent Iron Uptake in Erythroid Cells." *Nature Genetics* 37 (11): 1264–69. doi:10.1038/ng1658.

74. Ott, Claudia, Anne Liebold, Angela Takses, Ulrike G. Strauch, and Florian Obermeier. 2012. "High Prevalence but Insufficient Treatment of Iron-Deficiency Anemia in Patients with Inflammatory Bowel Disease: Results of a Population-Based Cohort." *Gastroenterology Research and Practice* 2012 (July): e595970. doi:10.1155/2012/595970.

75. Oudit, Gavin Y., Hui Sun, Maria G. Trivieri, Sheryl E. Koch, Fayez Dawood, Cameron Ackerley, Mehrdad Yazdanpanah, et al. 2003. "L-Type Ca2+ Channels Provide a Major Pathway for Iron Entry into Cardiomyocytes in Iron-Overload Cardiomyopathy." *Nature Medicine* 9 (9): 1187–94. doi:10.1038/nm920.

76. Pak, Mihwa, Miguel A. Lopez, Victroia Gabayan, Tomas Ganz, and Seth Rivera. 2006. "Suppression of Hepcidin during Anemia Requires Erythropoietic Activity." *Blood* 108 (12): 3730–35. doi:10.1182/blood-2006-06-028787.

77. Pantopoulos, Kostas. 2004. "Iron Metabolism and the IRE/IRP Regulatory System: An Update." *Annals of the New York Academy of Sciences* 1012 (March): 1–13.

78. Papanikolaou, George, Mark E. Samuels, Erwin H. Ludwig, Marcia L. E. MacDonald, Patrick L. Franchini, Marie-Pierre Dubé, Lisa Andres, et al. 2004. "Mutations in HFE2 Cause Iron Overload in Chromosome 1q-Linked Juvenile Hemochromatosis." *Nature Genetics* 36 (1): 77–82. doi:10.1038/ng1274.

79. Park, Christina H., Erika V. Valore, Alan J. Waring, and Tomas Ganz. 2001. "Hepcidin, a Urinary Antimicrobial Peptide Synthesized in the Liver." *Journal of Biological Chemistry* 276 (11): 7806–10. doi:10.1074/jbc.M008922200.

80. Pigeon, Christelle, Gennady Ilyin, Brice Courselaud, Patricia Leroyer, Bruno Turlin, Pierre Brissot, and Olivier Loréal. 2001. “A New Mouse Liver-Specific Gene, Encoding a Protein Homologous to Human Antimicrobial Peptide Hepcidin, Is Overexpressed during Iron Overload.” *Journal of Biological Chemistry* 276 (11): 7811–19. doi:10.1074/jbc.M008923200.

81. Ponka, Prem. 1997. “Tissue-Specific Regulation of Iron Metabolism and Heme Synthesis: Distinct Control Mechanisms in Erythroid Cells.” *Blood* 89 (1): 1–25.

82. Poss, Kenneth D., and Susumu Tonegawa. 1997. “Heme Oxygenase 1 Is Required for Mammalian Iron Reutilization.” *Proceedings of the National Academy of Sciences of the United States of America* 94 (20): 10919–24.

83. Raffin, Steven B., Choong H. Woo, Kenneth T. Roost, David C. Price, and Rudi Schmid. 1974. “Intestinal Absorption of Hemoglobin Iron-Heme Cleavage by Mucosal Heme Oxygenase.” *Journal of Clinical Investigation* 54 (6): 1344–52. doi:10.1172/JCI107881.

84. Ramirez, Jean-Marie, Olivier Schaad, Stephane Durual, Dominique Cossali, Mylène Docquier, Photis Beris, Patrick Descombes, and Thomas Matthes. 2009. “Growth Differentiation Factor 15 Production Is Necessary for Normal Erythroid Differentiation and Is Increased in Refractory Anaemia with Ring-Sideroblasts.” *British Journal of Haematology* 144 (2): 251–62. doi:10.1111/j.1365-2141.2008.07441.x.

85. Robach, Paul, Stefania Recalcati, Domenico Girelli, Cecilia Gelfi, Niels J. Aachmann-Andersen, Jonas J. Thomsen, Anne M. Norgaard, et al. 2009. “Alterations of Systemic and Muscle Iron Metabolism in Human Subjects Treated with Low-Dose Recombinant Erythropoietin.” *Blood* 113 (26): 6707–15. doi:10.1182/blood-2008-09-178095.

86. Robb, Aeisha, and Marianne Wessling-Resnick. 2004. “Regulation of Transferrin Receptor 2 Protein Levels by Transferrin.” *Blood* 104 (13): 4294–99. doi:10.1182/blood-2004-06-2481.

87. Roetto, Antonella, Filomena Daraio, Federica Alberti, Paolo Porporato, Angelita Calì, Marco De Gobbi, and Clara Camaschella. 2002. “Hemochromatosis due to Mutations in Transferrin Receptor 2.” *Blood Cells, Molecules & Diseases* 29 (3): 465–70.

88. Roetto, Antonella, George Papanikolaou, Marianna Politou, Federica Alberti, Domenico Girelli, John Christakis, Dimitris Loukopoulos, and Clara Camaschella. 2003. “Mutant Antimicrobial Peptide Hepcidin Is Associated with Severe Juvenile Hemochromatosis.” *Nature Genetics* 33 (1): 21–22. doi:10.1038/ng1053.

89. Rouault, T. A., C. K. Tang, S. Kaptain, W. H. Burgess, D. J. Haile, F. Samaniego, O. W. McBride, J. B. Harford, and R. D. Klausner. 1990. “Cloning of the cDNA Encoding an RNA Regulatory Protein--the Human Iron-Responsive Element-Binding Protein.” *Proceedings of the National Academy of Sciences of the United States of America* 87 (20): 7958–62.

90. Samad, Tarek A., Ashok Srinivasan, Laurie A. Karchewski, Sung-Jin Jeong, Jason A. Campagna, Ru-Rong Ji, David A. Fabrizio, et al. 2004. “DRAGON: A Member of the Repulsive Guidance Molecule-Related Family of Neuronal- and Muscle-Expressed Membrane Proteins Is Regulated by DRG11 and Has Neuronal Adhesive Properties.” *The Journal of Neuroscience* 24 (8): 2027–36. doi:10.1523/JNEUROSCI.4115-03.2004.

91. Santambrogio, P., A. Cozzi, S. Levi, and P. Arosio. 1987. “Human Serum Ferritin G-Peptide Is Recognized by Anti-L Ferritin Subunit Antibodies and Concanavalin-A.” *British Journal of Haematology* 65 (2): 235–37.

92. Schmidt, Paul J., Paul T. Toran, Anthony M. Giannetti, Pamela J. Bjorkman, and Nancy C. Andrews. 2008. "Transferrin Receptor Modulates Hfe-Dependent Regulation of Hepcidin Expression." *Cell Metabolism* 7 (3): 205–14. doi:10.1016/j.cmet.2007.11.016.

93. Schreiber, S., and S. Wedel. 1997. "Diagnosis and Treatment of Anemia in Inflammatory Bowel Disease." *Inflammatory Bowel Diseases* 3 (3): 204–16.

94. Shanmugam, Nanda Kumar N., Shiri Ellenbogen, Estela Trebicka, Lijian Wang, Subhankar Mukhopadhyay, Adam Lacy-Hulbert, Carey Ann Gallini, Wendy S. Garrett, and Bobby J. Cherayil. 2012. "Tumor Necrosis Factor A Inhibits Expression of the Iron Regulating Hormone Hepcidin in Murine Models of Innate Colitis." *PLoS ONE* 7 (5): e38136. doi:10.1371/journal.pone.0038136.

95. Shaw, George C., John J. Cope, Liangtao Li, Kenneth Corson, Candace Hersey, Gabriele E. Ackermann, Babette Gwynn, et al. 2006. "Mitoferrin Is Essential for Erythroid Iron Assimilation." *Nature* 440 (7080): 96–100. doi:10.1038/nature04512.

96. Shayeghi, Majid, Gladys O. Latunde-Dada, Jonathan S. Oakhill, Abas H. Laftah, Ken Takeuchi, Neil Halliday, Yasmin Khan, et al. 2005. "Identification of an Intestinal Heme Transporter." *Cell* 122 (5): 789–801. doi:10.1016/j.cell.2005.06.025.

97. Shi, Yigong, and Joan Massagué. 2003. "Mechanisms of TGF-B Signaling from Cell Membrane to the Nucleus." *Cell* 113 (6): 685–700. doi:10.1016/S0092-8674(03)00432-X.

98. Silvestri, Laura, Alessia Pagani, Antonella Nai, Ivana De Domenico, Jerry Kaplan, and Clara Camaschella. 2008. "The Serine Protease Matriptase-2 (TMPRSS6) Inhibits Hepcidin Activation by Cleaving Membrane Hemojuvelin." *Cell Metabolism* 8 (6): 502–11. doi:10.1016/j.cmet.2008.09.012.

99. Skikne, Barry S. 2008. “Serum Transferrin Receptor.” *American Journal of Hematology* 83 (11): 872–75. doi:10.1002/ajh.21279.

100. Spivak, Jerry L. 2002. “Iron and the Anemia of Chronic Disease.” *Oncology (Williston Park, N.Y.)* 16 (9 Suppl 10): 25–33.

101. Su, Dan, and Han Asard. 2006. “Three Mammalian Cytochromes b561 Are Ascorbate-Dependent Ferrireductases.” *FEBS Journal* 273 (16): 3722–34. doi:10.1111/j.1742-4658.2006.05381.x.

102. Sukumaran, Abitha, Jithu James, Harish Palleti Janardhan, Anita Amaladas, Lekshmy Madathilazhikathu Suresh, Debashish Danda, Visalakshi Jeyeseelan, Balakrishnan Siddharth Ramakrishna, and Molly Jacob. 2014. “Expression of Iron-Related Proteins in the Duodenum Is up-Regulated in Patients with Chronic Inflammatory Disorders.” *The British Journal of Nutrition* 111 (6): 1059–68. doi:10.1017/S0007114513003334.

103. Sullivan, A. L., J. A. Grasso, and L. R. Weintraub. 1976. “Micropinocytosis of Transferrin by Developing Red Cells: An Electron- Microscopic Study Utilizing Ferritin-Conjugated Transferrin and Ferritin-Conjugated Antibodies to Transferrin.” *Blood* 47 (1): 133–43.

104. Tabuchi, Mitsuaki, Tamotsu Yoshimori, Kazuhito Yamaguchi, Tsutomu Yoshida, and Fumio Kishi. 2000. “Human NRAMP2/DMT1, Which Mediates Iron Transport across Endosomal Membranes, Is Localized to Late Endosomes and Lysosomes in HEp-2 Cells.” *Journal of Biological Chemistry* 275 (29): 22220–28. doi:10.1074/jbc.M001478200.

105. Tamary, Hannah, Hanna Shalev, Galit Perez-Avraham, Meira Zoldan, Itai Levi, Dorine W. Swinkels, Toshihiko Tanno, and Jeffery L. Miller. 2008. “Elevated Growth Differentiation Factor 15 Expression in Patients with Congenital Dyserythropoietic Anemia

Type I." *Blood* 112 (13): 5241–44. doi:10.1182/blood-2008-06-165738.

106. Taniguchi, S., C. H. Dai, J. O. Price, and S. B. Krantz. 1997. "Interferon Gamma Downregulates Stem Cell Factor and Erythropoietin Receptors but Not Insulin-like Growth Factor-I Receptors in Human Erythroid Colony-Forming Cells." *Blood* 90 (6): 2244–52.

107. Tanno, Toshihiko, Natarajan V. Bhanu, Patricia A. Oneal, Sung-Ho Goh, Pamela Staker, Y. Terry Lee, John W. Moroney, et al. 2007. "High Levels of GDF15 in Thalassemia Suppress Expression of the Iron Regulatory Protein Hepcidin." *Nature Medicine* 13 (9): 1096–1101. doi:10.1038/nm1629.

108. Tanno, Toshihiko, Pierre Noel, and Jeffery L. Miller. 2010. "Growth Differentiation Factor 15 in Erythroid Health and Disease." *Current Opinion in Hematology* 17 (3): 184–90. doi:10.1097/MOH.0b013e328337b52f.

109. Tanno, Toshihiko, Prashanth Porayette, Orapan Sripichai, Seung-Jae Noh, Colleen Byrnes, Ajoy Bhupatiraju, Y. Terry Lee, et al. 2009. "Identification of TWSG1 as a Second Novel Erythroid Regulator of Hepcidin Expression in Murine and Human Cells." *Blood* 114 (1): 181–86. doi:10.1182/blood-2008-12-195503.

110. Theurl, Igor, Armin Finkenstedt, Andrea Schroll, Manfred Nairz, Thomas Sonnweber, Rosa Bellmann-Weiler, Milan Theurl, et al. 2010. "Growth Differentiation Factor 15 in Anaemia of Chronic Disease, Iron Deficiency Anaemia and Mixed Type Anaemia." *British Journal of Haematology* 148 (3): 449–55. doi:10.1111/j.1365-2141.2009.07961.x.

111. Velasco, Gloria, Santiago Cal, Victor Quesada, Luis M. Sánchez, and Carlos López-Otín. 2002. "Matriptase-2, a Membrane-Bound Mosaic Serine Proteinase Predominantly Expressed in Human Liver and Showing Degrading Activity

against Extracellular Matrix Proteins." *Journal of Biological Chemistry* 277 (40): 37637–46.

112. Vermeire, Séverine, Gert Van Assche, and Paul Rutgeerts. 2004. "C-Reactive Protein as a Marker for Inflammatory Bowel Disease." *Inflammatory Bowel Diseases* 10 (5): 661–65. doi:10.1097/00054725-200409000-00026.

113. Viatte, Lydie, Jeanne-Claire Lesbordes-Brion, Dan-Qing Lou, Myriam Bennoun, Gaël Nicolas, Axel Kahn, François Canonne-Hergaux, and Sophie Vaulont. 2005. "Deregulation of Proteins Involved in Iron Metabolism in Hepcidin-Deficient Mice." *Blood* 105 (12): 4861–64. doi:10.1182/blood-2004-12-4608.

114. Vulpe, C. D., Y. M. Kuo, T. L. Murphy, L. Cowley, C. Askwith, N. Libina, J. Gitschier, and G. J. Anderson. 1999. "Hephaestin, a Ceruloplasmin Homologue Implicated in Intestinal Iron Transport, Is Defective in the Sla Mouse." *Nature Genetics* 21 (2): 195–99. doi:10.1038/5979.

115. Wang, Cq, Kb Udupa, and Da Lipschitz. 1995. "Interferon-Gamma Exerts Its Negative Regulatory Effect Primarily on the Earliest Stages of Murine Erythroid Progenitor-Cell Development." *Journal of Cellular Physiology* 162 (1): 134–38. doi:10.1002/jcp.1041620116.

116. Wang, Rui-Hong, Cuiling Li, Xiaoling Xu, Yin Zheng, Cuiying Xiao, Patricia Zerfas, Sharon Cooperman, et al. 2005. "A Role of SMAD4 in Iron Metabolism through the Positive Regulation of Hepcidin Expression." *Cell Metabolism* 2 (6): 399–409. doi:10.1016/j.cmet.2005.10.010.

117. Weiss, Guenter, and Lawrence T. Goodnough. 2005. "Anemia of Chronic Disease." *New England Journal of Medicine* 352 (10): 1011–23. doi:10.1056/NEJMra041809.

118. Wilkinson, Nicole, and Kostas Pantopoulos. 2014. “The IRP/IRE System in Vivo: Insights from Mouse Models.” *Frontiers in Pharmacology* 5 (July). doi:10.3389/fphar.2014.00176.

119. Wiklund, Fredrik E, Anna M Bennet, Patrik KE Magnusson, Ulrika K Eriksson, Fredrik Lindmark, Liyun Wu, Nasreen Yaghoutyfam, et al. 2010. “Macrophage Inhibitory Cytokine-1 (MIC-1/GDF15): A New Marker of All-Cause Mortality.” *Aging Cell* 9 (6): 1057–64. doi:10.1111/j.1474-9726.2010.00629.x

120. Wollenberg, P., and W. Rummel. 1987. “Dependence of Intestinal Iron Absorption on the Valency State of Iron.” *Naunyn-Schmiedeberg’s Archives of Pharmacology* 336 (5): 578–82. doi:10.1007/BF00169317.

121. Wrighting, Diedra M., and Nancy C. Andrews. 2006. “Interleukin-6 Induces Hepcidin Expression through STAT3.” *Blood* 108 (9): 3204–9. doi:10.1182/blood-2006-06-027631.

122. Wessling-Resnick Marianne, chapter 10, Iron . Modern Nutrition in Health and Disease Ross AC. Wolters Kluwer Health/Lippincott Williams & Wilkins; 2014. 1616 p.

123. Zhang, An-Sheng, Shigang Xiong, Hidekazu Tsukamoto, and Caroline A. Enns. 2004. “Localization of Iron Metabolism–related mRNAs in Rat Liver Indicate That HFE Is Expressed Predominantly in Hepatocytes.” *Blood* 103 (4): 1509–14. doi:10.1182/blood-2003-07-2378.

124. Zheng, L., M. C. Kennedy, G. A. Blondin, H. Beinert, and H. Zalkin. 1992. “Binding of Cytosolic Aconitase to the Iron Responsive Element of Porcine Mitochondrial Aconitase mRNA.” *Archives of Biochemistry and Biophysics* 299 (2): 356–60.

APPENDIX

APPENDIX 1

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CHRISTIAN MEDICAL COLLEGE
VELLORE 632 002

Ref: IRB/8150/01/2013 18th February, 2013

OFFICE OF THE TREASURER
19 FEB 2013
52882
CMC, VELLORE - 632 004

The Treasurer
Christian Medical College,
Vellore.

Dear Mr. Denzil,

Sub: **FLUID Research grant project NEW PROPOSAL:**
Serum GDF-15 (growth differentiation factor-15) levels in patients with ulcerative colitis.
Dr. Chinmai Jagadish, Post-graduate Demonstrator, Biochemistry, Dr. Molly Jacob, Dr. Joe Varghese, Biochemistry, Dr A. J. Joseph, Gastroenterology, Dr Visalakshi Jeyaseelan, Biostatistics.

Ref: IRB Min. No. 8150 dated 09.01.2013

The Institutional Review Board at its meeting held on 09th January 2013 vide IRB Min. No. ***8150*** accepted the project for *2 years* at a total sanction *Rs. 80,000/- (Rupees EghtyThousand only)*. A sum of Rs 40,000/- will be sanctioned for 12 months after receipt of the revised proposal, subsequent installment of 40,000/- each will be released at the end of the first year following the receipt of the progress report. Kindly arrange to transfer the sanctioned amount to a separate account to be operated by Drs. Chinmai Jagadish, Molly Jacob.

Thank you.

Yours sincerely,

Dr. Nihal Thomas
Secretary (Ethics Committee)
Institutional Review Board

Dr Nihal Thomas
MBBS MD MNAMS DNB (Endo) FRACP(Endo) FRCP(Edin)
Secretary (Ethics Committee)
Institutional Review Board

CC: Dr. Chinmai Jagadish, Department of Biochemistry, CMC
Dr. Molly Jacob, Department of Biochemistry, CMC
File

22-4028. Rf. Dr. Chinmai Jagadish - Biochemistry (8150)

APPENDIX 2

CONSENT FORM IN ENGLISH

SERUM GDF-15(GROWTH DIFFERENTIATION FACTOR 15) LEVELS IN PATIENTS WITH ULCERATIVE COLITIS

INFORMATION SHEET FOR PATIENTS

The Department of Biochemistry at Christian Medical College, Vellore, in association with the Department of Gastroenterology is carrying out a study to understand changes that occur in the way iron is handled in the body in patients with ulcerative colitis. Because of these changes, patients with ulcerative colitis often develop anemia. This affects the quality of life in these patients. How and why anemia develops is not completely understood. We would like to study this problem to gain a better understanding of it. This knowledge may help doctors deal more effectively with the problem. In order to do this study, we will need 10 ml of blood. We would like to ask you if you are willing to provide 10 ml of blood for this purpose.

You may not directly benefit from the study. However, if you are willing to participate in the study and provide a blood sample, it will help us to better understand how anemia develops in ulcerative colitis and may help, in the future, to improve treatment for the condition.

The blood sample collected will be used only for research purposes. If there is any sample remaining after this study is completed, we would like to request you for permission to store the blood and used for future similar studies. Collection of this sample of blood will not cause harm to your health in any foreseeable manner. The medical information you give us will be kept confidential.

If you do not wish to give the blood sample requested, you are free to say so. It will not affect the treatment you will receive in the hospital.

If you have any further queries, please contact me on the number provided below.

Dr Chinmai Jagadish

Post graduate Demonstrator

Department of Biochemistry

Christian Medical College, Vellore -632002

Contact number: 95566736587

Dr Molly Jacob

Professor

Department of Biochemistry

Christian Medical College, Vellore -632002

Dr A. J. Joseph

Professor

Department of Gastroenterology

Christian Medical College, Vellore -63200

INFORMED CONSENT DOCUMENT FOR SAMPLE OF BLOOD

Dr. Chinmai Jagadish has explained to me the details of the study proposed. I have understood what has been said including the following

1. If I agree to participate in this study, a blood sample (10 ml) will be collected from me.

2. This will not affect my health in any foreseeable way.

3. The blood sample will be used only for research purposes. If there is any sample remaining after this study is completed, the sample will be stored and may be used for future studies on ulcerative colitis and iron.

I am willing to donate 10ml of blood voluntarily without any coercion from investigators of this project.

Signature of donor Signature of investigator

Name of the donor:

Signature of witness:

Date:

APPENDIX – 3

PROFORMA USED TO OBTAIN INFORMATION ON

CLINICAL PROFILE OF THE PATIENT

SERUM GROWTH DIFFRENTIATION FACTOR 15 IN PATIENTS WITH ULCERATIVE COLITIS

Proforma

Name:

Hosp no:

Age:

Gender:

Address:

Presenting complaint:

History

Duration of the disease:

Date when diagnosis of UC made:

Number of stools/day currently:

Presence of blood in the stool currently:

Fever:

Any other relevant complaints:

Past history:

Similar complaints in past:

Other diseases:

Surgical history (eg, appendectomy):

Personal history:

Alcohol intake:

Smoking:

Menstrual history:

Drug history:

Drugs for anemia:

Iron supplements

EPO:

Any other drugs used:

Investigations:

Hb:

Serum iron:

Serum ferritin:

CRP:

Serum protein and albumin:

Stool examination:

Parasites:

Colonoscopy findings:

Upper GI endoscopy findings:

Biopsy reports: