SERUM HEPCIDIN LEVELS IN PATIENTS WITH ULCERATIVE COLITIS - DO THEY CORRELATE WITH ANEMIA AND/OR INFLAMMATION?

DISSERTATION

Submitted to

THE TAMILNADU DR.MGR MEDICAL UNIVERSITY

In partial fulfillment for the degree

DEGREE OF MEDICINE

IN

BIOCHEMISTRY - BRANCH XIII

APRIL 2016

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DEPARTMENT OF BIOCHEMISTRY

CHRISTIAN MEDICAL COLLEGE

VELLORE-632002, INDIA

CERTIFICATE

This is to certify that the study titled **"SERUM HEPCIDIN LEVELS IN PATIENTS WITH ULCERATIVE COLITIS - DO THEY CORRELATE WITH ANEMIA AND/OR INFLAMMATION?"** is the bona fide work of Dr. Jagadish R, who conducted it under the guidance and supervision of Dr. Molly Jacob, Professor of Biochemistry, Christian Medical College, Vellore. The work in this dissertation has not been submitted to any other university for the award of a degree.

Dr. Molly Jacob,

Professor and Head of the Department

Department of Biochemistry

Christian Medical College

Vellore

Dr. Alfred Job Daniel,

Principal,

Christian Medical College,

Vellore

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.

Dr. Jagadish R,

PG Registrar,

Department of Biochemistry,

Christian Medical College.

Vellore.

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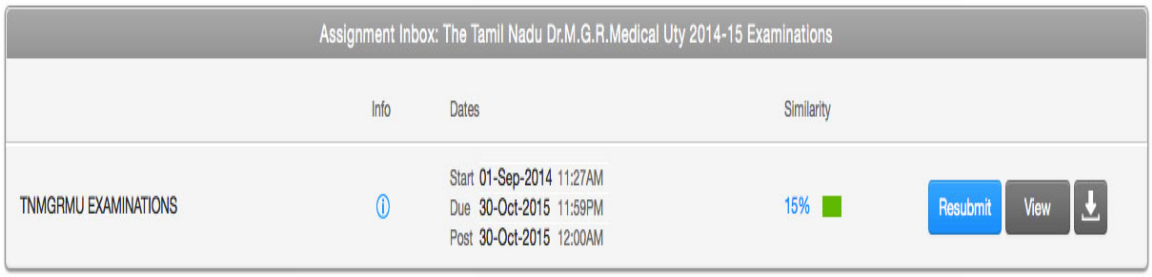

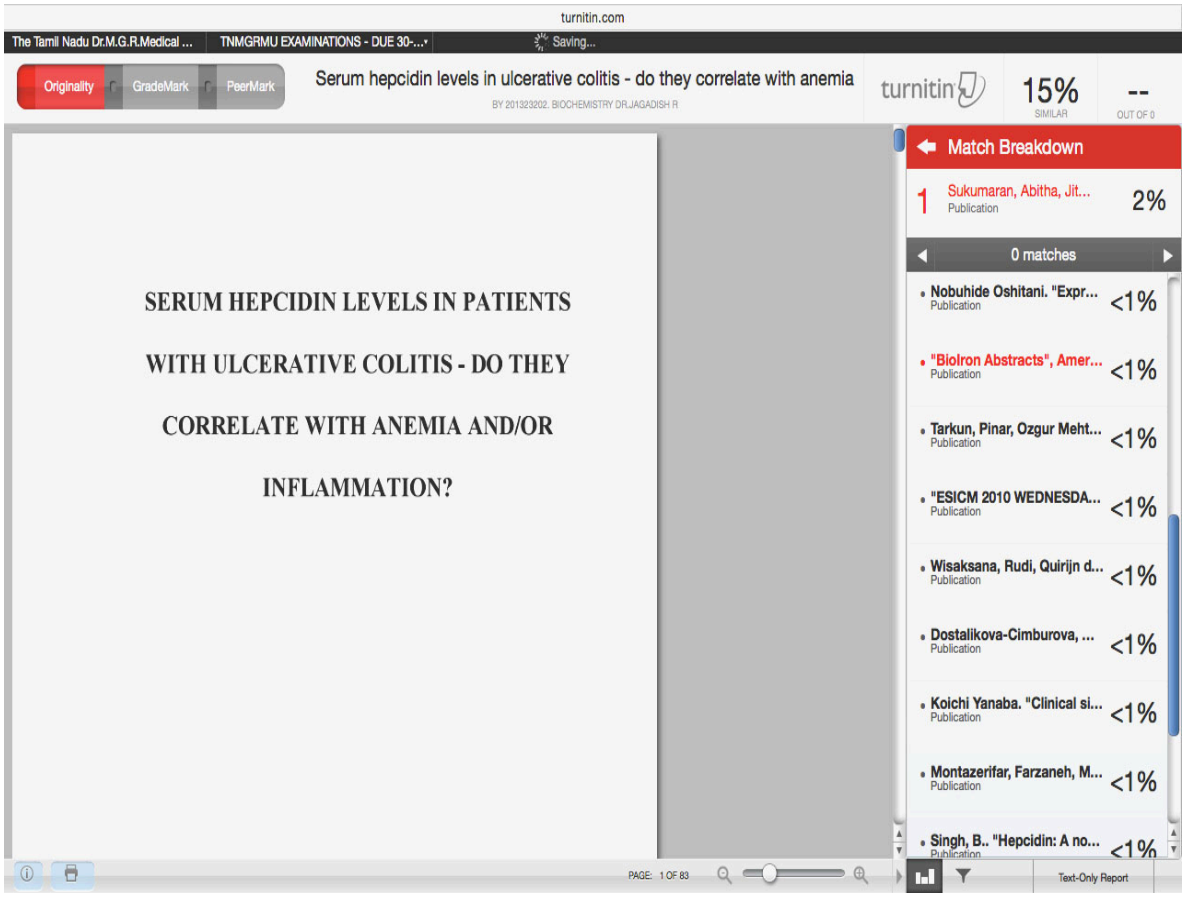

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ABSTRACT

ABSTRACT

Serum hepcidin levels in ulcerative colitis - do they correlate with anemia and/or inflammation?

Background to the study

Anemia is a common complication of inflammatory bowel disease (IBD), which comprises Crohn's disease (CD) and ulcerative colitis (UC). Hepcidin is known to be the central regulator of iron homeostasis in the body. It is up-regulated by inflammation and down-regulated by anemia. Previous work has shown that serum hepcidin levels were decreased in patients with ulcerative colitis, who had co-existent anemia. This was surprising as these patients had a chronic inflammatory state.

Aim:

The aim of the present study was to test the hypothesis that when anemia and inflammation, which are opposing factors involved in regulation of hepcidin, co-exist in patients with UC, the effect of anemia on hepcidin predominates over that of inflammation.

Materials and methods:

A total of 59 patients were recruited into the study. Forty patients who were diagnosed to have ulcerative colitis by standard consensus criteria served as cases. Nineteen patients who attended the Gastroenterology clinic for investigation of dyspepsia and who were found to have no detectable abnormalities, who were non-anemic (Hb $\geq$ 12 g/dL for females and $\geq$13 g/dL for males) and who had no evidence of inflammation (serum CRP

levels < 6 mg/dL) served as control subjects. Informed consent was obtained for collecting blood from the study participants at the time of recruitment. Levels of serum ferritin, iron, hepcidin, C-reactive protein and hemoglobin were estimated in each sample. Data were analyzed using SPSS version 16, using appropriate tests. A p-value of <0.05 was considered to be statistical significant.

Results:

Levels of hemoglobin and serum iron and hepcidin were significantly lower and that of serum CRP were significantly higher in patients with ulcerative colitis, than in control patients. Levels of hemoglobin, serum ferritin, iron and hepcidin were significantly lower in patients with UC who were anemic than in those who were not. Levels of serum hepcidin correlated positively with hemoglobin, iron and ferritin.

Conclusion:

Serum hepcidin levels were significantly lower in patients with UC than in control subjects; they were also lower in UC patients who were anemic than in UC patients who were not. Serum hepcidin levels correlated significantly with markers of iron status, but not with a marker of inflammation (CRP). Hence, it appears that when anemia and inflammation coexist, the influence of anemia on hepcidin predominated over that of inflammation.

Keywords: Serum hepcidin, ulcerative colitis, anemia, iron

REVIEW OF LITERATURE

INTRODUCTION

Iron, a transition metal, is an essential micronutrient. It is required for the synthesis of heme-containing proteins such as hemoglobin, myoglobin, catalase and cytochromes(Murray et al., 2012). In case of iron deficiency, hemoglobin synthesis is impaired and results in anemia. Iron deficiency can occur either due to increased loss of iron or decreased absorption of iron from the diet (Murray et al., 2012). On the other hand, iron in excess can cause detrimental effects to the cell by generating free radicals and inducing oxidative damage(Winterbourn, 1995). Thus, iron levels in the body have to be tightly controlled and regulated.

Iron in circulation in the body is derived from two sources - dietary iron and iron recycled from senescent or worn-out erythrocytes. Hepcidin is a hormone that is known to be the central regulator of iron homeostasis in the body. It inhibits absorption of iron from the diet into the circulation and recycling of iron from macrophages in the reticulo-endothelial system (Murray et al., 2012). It is synthesized predominantly in hepatocytes and is excreted by the kidneys. It blocks iron efflux from cells by binding to, internalizing and degrading ferroportin, which is the iron-exporting protein found in mammalian cells (Nemeth et al., 2004b). Hepcidin is, in turn, regulated by iron availability, inflammation, infection and hypoxia (Nicolas et al., 2002). Inflammation is known to up-regulate hepcidin transcription and increase serum hepcidin levels (Nemeth et al., 2004a). Hence, hepcidin levels are subjected to alterations in patients suffering from inflammatory disorders, like inflammatory bowel disease and rheumatoid arthritis.

INFLAMMATORY BOWEL DISEASE

Inflammatory bowel disease (IBD) is a chronic disease of the digestive tract. It comprises Crohn's disease (CD) and ulcerative colitis (UC). Various etiologies have been proposed for these conditions; these include infections, allergies and immune mechanisms (Danese and Fiocchi, 2011). The most widely accepted view is that these are autoimmune diseases caused by dysregulated T-cell-mediated immune responses against endogenous commensal microbiota (Powrie et al., 1995). Normally, the mucosal immune system in the gastrointestinal tract does not react to endogenous commensals. Typical manifestations of the conditions include bloody diarrhea and abdominal pain, with periods of exacerbations and remissions. The peak age of onset follows a bimodal distribution, occurring between 15- 30 years and 60-80 years (Freidman and Blummberg, 2011). UC affects males and females equally, whereas CD affects males and females in the ratio of 1.1-1.8 respectively (Freidman and Blummberg, 2011). The prevalence of UC is higher than that of CD.

Ulcerative colitis

The etiology, clinical features and diagnosis of ulcerative colitis are briefly reviewed below. The site of involvement in UC is most commonly the rectum. It extends proximally in a continuous fashion to involve the recto-sigmoid, the sigmoid and, in some cases, even the whole colon. A diagnosis of UC is based on the clinical picture, endoscopic appearance and histopathology results, which constitute consensus criteria (Freidman and Blummberg, 2011). It is mostly a superficial disease limited to the mucosa and sub-mucosa, with ulceration being a predominant finding. The clinical features of UC include diarrhea, abdominal pain, intermittent rectal bleeding, tenesmus, passage of mucous in stools and

fever (Freidman and Blummberg, 2011). Intermittent rectal bleeding can result in either passage of frank blood or blood mixed with mucus.

Anemia in ulcerative colitis

Anemia is a chronic complication of inflammatory bowel disease. The prevalence of anemia in IBD is highly variable, ranging between 6 and 74% (Kulnigg and Gasche, 2006; Wilson et al., 2004). Anemia in these patients affects their quality of life (Pizzi et al., 2006; Wells et al., 2006). It is also the most common co-morbid condition associated with mortality in these patients (Cucino and Sonnenberg, 2001). Anemia is also the reason for frequent admissions and delayed discharge in those with UC (Liu and Kaffes, 2012). The causes of anemia include anemia of chronic disease, iron deficiency, vitamin B_{12} deficiency, folate deficiency and/or immune- mediated hemolytic anemia. Of these, the most common causes are iron deficiency and anemia of chronic disease, with iron deficiency reported to be prevalent in 90% of cases (Kulnigg and Gasche, 2006). The pathophysiology of these two types of anemia is different. The cause of anemia in iron deficiency is due to intestinal blood loss from ulcerations in the gastrointestinal tract and impaired intestinal iron absorption (Gasche et al., 2004). The cause of anemia in ACD is due to reduced red cell survival, increased hepcidin levels and/or impaired response to erythropoietin (Freidman and Blummberg, 2011). The differentiation between these two types of anemia is critical for instituting the appropriate treatment in such patients.

IRON AND ITS IMPORTANCE

Iron, a transition metal, is an essential micronutrient. It is required for the synthesis of heme-containing proteins, such as hemoglobin, myoglobin, catalase and cytochromes (Murray et al., 2012). When iron deficiency occurs, heme synthesis is impaired and results in anemia. Iron deficiency can occur either due to increased loss of iron from the body or decreased absorption of iron from the diet(Murray et al., 2012). On the other hand, excess of cellular iron can cause detrimental effects to the cell by participating in the Fenton reaction and generating free radicals (Winterbourn, 1995). Thus, iron levels in the body have to be tightly controlled and regulated.

Iron in circulation in the body is derived from two sources - dietary iron and iron recycled from senescent red blood cells. The amount of iron absorbed from the diet is usually small compared to the amount of recycled iron from macrophages. Intestinal absorption of iron is the only route of entry of iron into the body. In humans, the excretion of iron is not regulated. Therefore, intestinal iron absorption is of major importance in iron homeostasis (Wessling-Resnick, 2014).

Dietary iron

Iron in the diet occurs mainly in two forms - heme and non-heme iron. Heme iron is derived from animal sources. Non-heme iron is derived from plant sources. The bioavailability of heme iron is higher than that of non-heme iron (Miret et al., 2003). Iron in the diet, either as heme or non-heme, enters the enterocyte and into the circulation. The degree of absorption of iron from the diet depends upon various factors such as iron levels in the circulation, the redox state of dietary iron and presence of compounds that can aid in or interfere with its absorption.

Absorption of non-heme iron

Non-heme iron in the diet exists in ferric form; this needs to be converted to its ferrous form, as only the ferrous form of iron is absorbed (Wollenberg and Rummel, 1987). In the duodenum, a protein on the apical surface of duodenal enterocytes, duodenal cytochrome b reductase (dcyt b), converts ferric iron to its ferrous form (McKie et al., 2001). Once reduced by dcyt b, ferrous iron is transported into the enterocyte by a transporter protein called divalent metal transporter-1 (DMT-1)(Gunshin et al., 1997). Inside the enterocyte, iron enters the labile iron pool, from where it is transported into the circulation by ferroportin, a protein found on the basolateral aspect of the enterocyte, or it can be stored inside the enterocyte as ferritin (Evstatiev and Gasche, 2012).

Dcyt b

This is found on the apical surface of enterocytes of the proximal duodenum (McKie et al., 2001). It is a ferri-reductase enzyme; it needs ascorbate as electron donor to reduce luminal ferric iron to its ferrous form (Su and Asard, 2006). Expression of this enzyme is up-regulated in iron deficiency to enhance absorption of iron (McKie et al., 2001). Its role in

physiological iron absorption is, however, uncertain, as dcytb knock-out mice have been shown to maintain normal iron levels (Gunshin et al., 2005). However, under conditions of stress, such as hypoxia, dcytb knock-out mice showed significantly lower splenic iron levels and decreased hemoglobin synthesis, suggesting that dcyt b is essential during conditions of enhanced erythropoiesis (Choi et al., 2012). Hypoxia-inducible factor-2 alpha (HIF-2α), in particular, is known to up regulate dcytb (Luo et al., 2014).

DMT-1 (divalent metal transporter 1)

DMT1 is a transmembrane protein responsible for iron uptake into intestinal cells. It is made up of 12 transmembrane domains and belongs to the Nramp (natural resistance-associated macrophage protein) family of proteins. It is also known as Nramp-2 or SLC11A2. It is found on the apical surface of enterocytes. It is also present in endosomes involved in the transferrin cycle, where it transfers iron out of the endosome, allowing it to be reutilized. A proton is co-transported, along with Fe^{2+}, by DMT-1. It is highly expressed in the proximal duodenum and is mainly involved in iron absorption (Gunshin et al., 1997). Apart from iron (Fe^{2+}), it also transports other divalent cations like Zn^{2+}, Cu^{2+}, Co^{2+}, Pb^{2+}, Cd^{2+}, Mn^{2+} and Ni^{2+} (Gunshin et al., 1997). DMT-1 has a significant role in iron uptake, unlike dcytb, as suggested by development of autosomal microcytic hypochromic anemia in Belgrade (b) rat and microcytic anemia (mk) mice, which are animal models with a missense mutation in the DMT-1 gene (Fleming et al., 1998 and 1997). This mutation resulted in decreased intestinal iron absorption and erythroid iron uptake, explaining the phenotype in these animals. DMT-1 expression is modulated by iron availability, thus making it as a target for pharmacological intervention in iron overload disorders (Andrews, 1999). It has also been postulated to play a role in the uptake of non-transferrin-bound iron (NTBI) (Garrick et al., 1999).

Absorption of heme iron

Heme iron is derived from proteolytic degradation of heme-containing proteins, by digestive enzymes in the intestine. Heme iron is absorbed into the enterocyte by heme transporters in the apical membrane of these cells (Shayeghi et al., 2005). Heme oxygenase-1 (HO-1), a cytosolic enzyme in the enterocyte, releases ferrous iron (Fe^{2+}) from heme (Raffin et al., 1974). The fate of ferrous iron released is the same as that of non-heme dietary iron.

Iron efflux from enterocytes

Iron inside the enterocyte is exported into the circulation by ferroportin. This is a multiple-transmembrane domain protein and is the only known exporter of iron (Donovan et al., 2000). It is also known as IREG1, MTP-1 or SLC 40A1(Donovan et al., 2005; McKie et al., 2000). It is expressed in proximal duodenal enterocytes, macrophages, placenta and, to a lower extent, in hepatocytes (Abboud and Haile, 2000; Donovan et al., 2000; McKie et al., 2000). It is located on the basolateral membrane of cells and plays a major role in iron absorption. Its expression is regulated by hepcidin, the central regulator of iron homeostasis and also by iron availability and hypoxia (Donovan et al., 2005; Nemeth et al., 2004c).

Fe^{2+}, transported across the basolateral membrane by ferroportin, is oxidized to ferric form Fe^{3+} by hephaestin and released into the circulation. Hephaestin is a multicopper ferroxidase, homologous to ceruloplasmin (Vulpe et al., 1999). It plays a significant role in iron absorption. This is based on the development of sex-linked anemia (SLA) in mice with a mutation in the hephaestin gene. These SLA mice showed normal iron absorption but impaired release of iron into circulation (Vulpe et al., 1999).

Recycling of iron

As mentioned earlier, the contribution of recycled iron to circulating iron is much greater than that of dietary iron. Senescent erythrocytes are engulfed by macrophages, resulting in the formation of phagolysosomes. Inside the phagolysosome, heme oxygenase-1 (HO-1) acts on the heme and releases ferrous iron, which is transported into the cytoplasm by DMT-1 (Poss and Tonegawa, 1997; Tabuchi et al., 2000). Ferrous iron in the cytoplasm can either enter the labile iron pool, be stored as ferritin or transported out of the macrophage by ferroportin (Donovan et al., 2005). In the circulation, the ferrous form is converted to ferric form by ceruloplasmin, which acts as a ferroxidase (Harris et al., 1999). The ferric form of iron binds to transferrin (Tf).

Fate of iron in the circulation

Iron is transported in the circulation bound to a protein, apo-transferrrin (Baker N, 1994). In terms of structure, apo- transferrin is a bi-lobed glycoprotein and is able to reversibly bind two ferric ions. In normal conditions, 30-40% of the binding sites on transferrin are occupied by iron. This is measured in terms of transferrin saturation, which is used as an index of iron status (Freidman and Blummberg, 2011).

In conditions of iron overload, the capacity of transferrin to bind iron is exceeded (Barisani et al., 1995). Unbound iron that accumulates in circulation is called non-transferrin-bound iron (NTBI). The mechanism by which NTBI is taken up into cells is not well understood. NTBI uptake by the various cells in the body can lead to various disorders; accumulation in cardiac myocytes, beta cells of the pancreas and neurons leads to cardiomyopathy (Oudit et al., 2003), diabetes mellitus type 2 (Kunutsor et al., 2013) and ataxia and other demyelinating disorders, respectively (Martelli and Puccio, 2014).

Cellular iron uptake

Cellular iron uptake from the circulation is accomplished through receptor-mediated endocytosis. Transferrin receptors on the cell surface bind the transferrin molecule that contains iron (Tf-Fe) and the complex is endocytosed. Tf receptors are of two types – transferrin receptors 1 and 1 (TfR1 and TfR2) (Kawabata et al., 1999). TfR1 is the receptor involved in cellular iron uptake. It is seen present in all cells, unlike TfR2, which is seen only in hepatocytes and erythroblasts.

After endocytosis, the Tf-Fe complex in the endosome dissociates into Fe^{3+} and transferrin. This dissociation happens at an acidic pH, generated by Na-H-ATPase in the endosome. The ferric iron released is converted to ferrous iron by a ferrireductase, STEAP3 (six transmembrane epithelial antigen of prostate-3 (Ohgami et al., 2005). Ferrous iron is transported out of the endosome into cytosol by DMT-1 (Fleming et al., 1998). The transferrin receptors are recycled back to the plasma membrane (Klausner et al., 1983). This chain of events constitutes the transferrin cycle.

Iron taken up by cells is used to meet cellular needs such as synthesis of hemoglobin, Fe-S clusters, etc. Once the cell's needs for iron are met, excess cellular iron is stored in the form of ferritin.

Ferritin

Ferritin is a hetropolymer made up of 24 protein subunits made up of H (heavy) and L (light chains). It is expressed in all cells. It forms a shell-like structure around the bound iron. The H chain of ferritin has a catalytic property and converts ferrous form of iron to its

ferric form (Lawson et al., 1989). Iron inside the ferritin molecule can be easily mobilized whenever there is a demand for iron (Burtis et al., 2012). Ferritin is also found in serum; this consists of L chains and it exists in glycated form (Santambrogio et al., 1987). Serum ferritin is used as an index of iron stores (Cook et al., 1974). Its levels are decreased in iron deficiency. However, it is also an acute phase reactant and its levels are increased in acute or chronic inflammation (Lipschitz et al., 1974). This makes it an unreliable indicator of iron deficiency in the presence of inflammation.

CELLULAR IRON HOMEOSTASIS

Cellular iron homeostasis is achieved by regulating the levels of proteins involved in storage and transport of iron across cell plasma membranes. These include ferritin and transferrin. These proteins are subjected to post-transcriptional regulation (Hentze et al., 1987). Such regulation occurs by alterations in mRNA stability and by repression of translation. Iron responsive elements (IRE) in the mRNA of these proteins play a vital role in such regulation. IREs are hairpin loop-like domains in the 5' or 3' UTR (untranslated region) of the genes concerned. The location of the IRE for each gene differs, with the ones for ferritin (Leibold and Munro, 1988), ferroportin, mitochondrial aconitase (Zheng et al., 1992), erythroid 5-aminolevulinic acid synthase(Cox et al., 1991) and hypoxia-inducible factor alpha (HIF2-alpha (Sanchez et al., 2007) found in the 5' end, while the one for transferrin receptor 1 (TfR1) is found in the 3' end. IREs provide binding sites for iron-response proteins (IRPs)(Anderson et al., 2012), which are cytosolic proteins that are sensitive to variations in cellular iron.

IRPs are of two types- IRP1 and IRP2 (Murray et al., 2012). When a cell is iron-replete, iron forms Fe-S clusters and prevents the binding of IRPs to the IREs on the mRNAs of iron-related proteins described above. This facilitates translation of ferritin mRNA, but not that for TfR1. When a cell is iron-depleted, IRPs bind to IREs at the 5' and 3' ends of UTRs. If it binds to the 5' end, the translation of ferritin is repressed (translational arrest); if it binds at the 3' end, it promotes translation of TfR1. IRP1 has dual functions; it functions as cytosolic aconitase when iron levels are high and binds IRE when iron levels are low (Zheng et al., 1992).

SYSTEMIC IRON HOMEOSTASIS

As mentioned earlier, mammals have no physiological means of excreting iron. Intestinal iron absorption is tightly regulated to maintain homeostasis. Hepcidin is the master regulator of systemic iron homeostasis (Ganz, 2006).

Role of hepcidin in systemic iron homeostasis

Hepcidin, a 25-amino acid peptide, is the master regulator of systemic iron homeostasis. It has 8 cysteine residues, approximately 30% amino acid residues. It is synthesized mainly in the liver, as an 84-amino acid precursor, pro-hepcidin. This undergoes proteolysis to form hepcidin, which is the active form. Hepcidin was first isolated from blood ultrafiltrates, as a result of search of peptides with antimicrobial properties. It was called liver-expressed anti-microbial peptide (LEAP), as it was found to be highly expressed in the liver (Krause et al., 2000). Later, it was isolated in urine and was named hepcidin (Park et al., 2001).

Numerous studies have highlighted the importance of hepcidin in iron homeostasis. The first link between hepcidin and iron metabolism was suggested by showing that iron overload in mice stimulated hepcidin production (Pigeon et al., 2001). Further, it was shown that iron overload occurred in hepatocytes and low iron levels were found in macrophages in mice in which upstream stimulatory factor-2 (USF-2) was knocked out. Those knock out mice were found to be hepcidin-deficient (Nicolas et al., 2001). It was also shown that mice that overexpressed hepcidin had severe iron deficiency anemia (Nicolas et al., 2001). Studies on juvenile hemochromatosis revealed mutations in the hepcidin gene (Roetto et al., 2004).

Hepcidin regulates systemic iron homeostasis by acting on the iron exporter protein, ferroportin. As mentioned above, ferroportin is the only known iron export protein in mammalian cells and is found in enterocytes, macrophages and the placenta. Hepcidin binds to ferroportin causing it to be internalized; it then undergoes ubiquitin-mediated proteasomal degradation. Thus, high levels of hepcidin result in low ferroportin levels and vice versa.

REGULATION OF HEPCIDIN SYNTHESIS

Hepcidin synthesis is regulated by various factors such as iron status, inflammation, hypoxia, and erythroid activity. All these factors act at the transcriptional level. Some of them are positive regulators, while others are negative regulators. Low iron status, increased erythropoiesis and hypoxia are negative regulators of hepcidin (Nicolas et al., 2002; Sasaki et al., 2012). Increased iron levels and inflammation up-regulate hepcidin

levels. Inflammation up-regulates hepcidin synthesis and hence, it is a positive acute phase reactant (Nemeth et al., 2003).

Iron and hepcidin

Availability of iron regulates hepcidin transcription; increased serum iron levels up-regulate hepcidin transcription and vice versa. This is mediated through two main pathways.

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

Hfe-TfR2 pathway

This pathway involves two main proteins - Hfe and TfR2. Hfe is a transmembrane protein, predominantly expressed in hepatocytes (Zhang et al., 2004). It is structurally similar to major histocompatibility complex (MHC) class I molecule and consists of α_1, α_2, α_3 and α_4 domains and β2 microglobulin (Feder et al., 1997). The α_1 and α_2 domains of Hfe interact with the ectodomain of TfR1 to form a complex (Bennett et al., 2000). This affinity of this binding weakens when TfR1 binds holo-transferrin (Tf-Fe), because binding sites of Hfe and Tf-Fe overlap (Feder et al., 1998). This causes displacement of Hfe from TfR1, and promotes its binding with TfR2.

TfR2 shares 45 % homology with TfR1 and is predominantly expressed in hepatocytes. Unlike TfR1, it has the capacity to bind both Tf-Fe and Hfe simultaneously (Chen et al., 2007; Gao et al., 2009). Upon Hfe binding to TfR2, it causes upregulation of hepcidin transcription (Schmidt et al., 2008). TfR2 is not considered important in cellular iron uptake. Instead, it appears to act as a sensor for circulating iron levels (Johnson et al.,

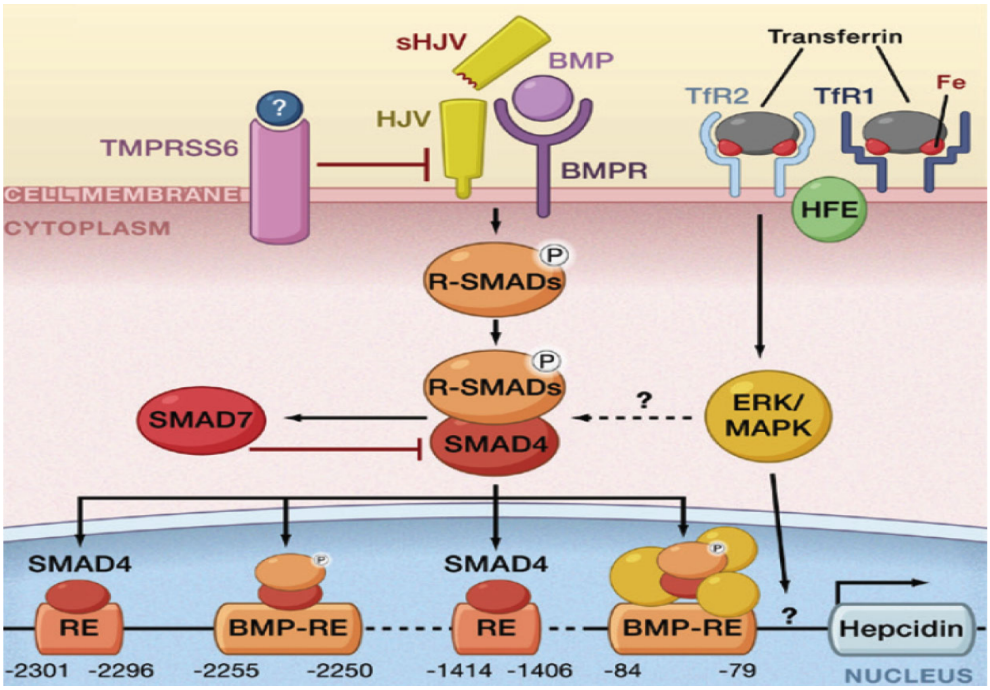
2007; Johnson and Enns, 2004; Robb and Wessling-Resnick, 2004). Thus, when circulating iron levels are high, the binding of Hfe to TfR2 increases, resulting in increased hepcidin transcription (Schmidt et al., 2008).

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

Bone morphogenic proteins, known for their roles in growth, differentiation and cell signaling, belong to the transforming growth factor–β (TGF- β) superfamily. They are recognized by bone morphogenic protein receptors (BMPR), which are serine-threonine kinases. Two types of BMPRs are known – BMPR I and II. These undergo hetero-dimerization, along with co-receptor haemojuvelin, upon binding with its ligand BMP. This leads to receptor-activated SMAD (R-SMAD) protein phosphorylation, which complexes with co-SMAD proteins to form a transcription factor (Shi and Massagué, 2003) (Figure 1).

BMP-6 is involved in iron-mediated induction of hepcidin transcription (Andriopoulos et al., 2009). It is secreted from non-parenchymal cells of the liver, in response to increased cellular iron levels and it acts in a paracrine fashion (Enns et al., 2013). Its co-receptor is haemojuvelin (HJV), which helps in formation of a complex with BMP6 and BMP-6 receptor (Babitt et al., 2005). This complex induces phosphorylation of R-SMAD proteins, especially SMAD-4. Phosphorylated SMAD-4 increases hepcidin transcription (Wang et al., 2005). SMAD-7 is a negative regulator of hepcidin. It inhibits SMAD-4 mediated upregulation (Mleczko-Sanecka et al., 2010)

Figure 1. Regulation of hepcidin by iron bioavailability



(Hentze et al., 2010)

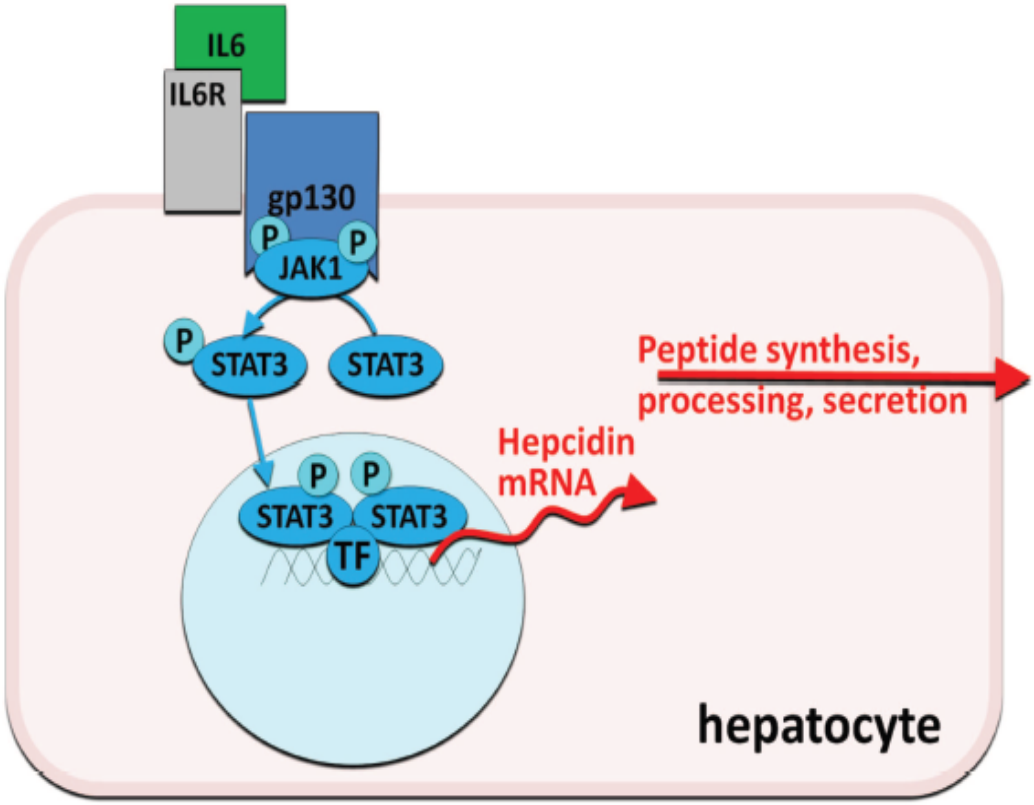
Inflammation and hepcidin

Inflammation is mediated by pro-inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha. IL-6 is known to increase hepcidin levels (Nemeth et al., 2003; Nemeth et al., 2004a). This results in sequestration of iron in macrophages involved in recycling of iron from senescent RBCs, leading to anemia of chronic disease.

IL-6 acts via JAK/STAT (Janus kinase/signal transducer and activator of transcription) signaling to increase hepcidin transcription (Wrighting and Andrews, 2006). This pathway also cross-talks with the SMAD pathway, which mediates BMP/HJV signaling (Wang et al., 2005).

In addition, inflammation also dysregulates erythropoiesis. IL-6 has been shown to decrease the responsiveness of erythroid precursors to erythropoietin (Adamson, 2009). This leads to impaired production of mature RBCs and results in anemia

Figure 2. Regulation of hepcidin by IL-6 during inflammation



(Ganz et al., 2011)

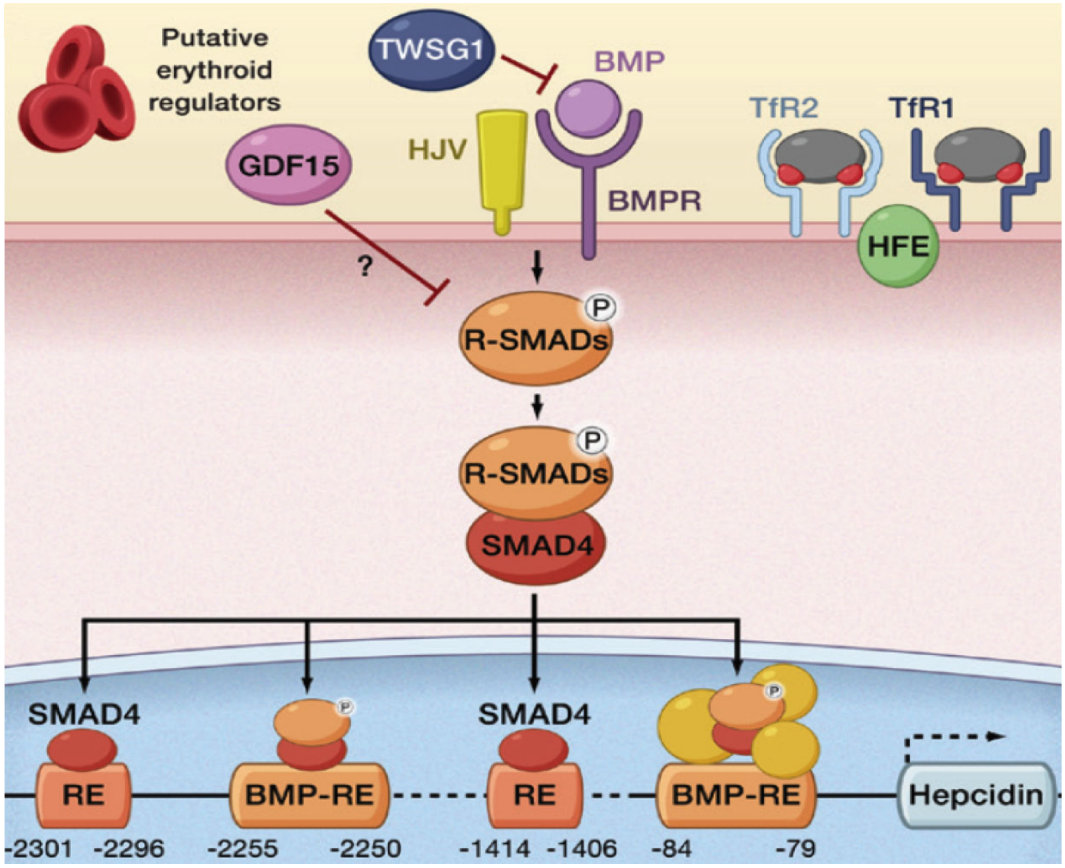
Hepcidin and erythroid precursors

Erythropoiesis is the process of production of mature red blood corpuscles from erythroid precursors. This process occurs as a normal physiological phenomenon. Apart from normal physiology, erythropoiesis can also occur in response to stress conditons such as hypoxia; this is referred to as stress erythropoiesis. In such cases, erythroid precursors have a high demand for iron. The main hormone involved in this process is erythropoietin (EPO), secreted by the kidney and liver (Pak et al., 2006). Increased levels of EPO down-regulate hepcidin transcription (Robach et al., 2009). A similar effect was seen in healthy volunteers in response to phlebotomy (Ashby et al., 2010).

In other conditions of stress erythropoiesis such as thalassemia, other regulators of hepcidin, such as growth differentiation factor-15 (GDF-15) (Tanno et al., 2007a) and twisted gastrulation factor (TWGF) (Tanno et al., 2009), have also been reported; these down-regulate hepcidin transcription. More recently, erythroferrone (ERFE), another factor secreted by erythroblasts in response to EPO, has been shown to down-regulate hepcidin transcription through the JAK-STAT5 pathway (Kautz et al., 2014).

These erythroid regulators are thought to maintain hepcidin levels in situations of stress erythropoiesis. The role of erythroid regulators in steady state erythropoiesis is not clearly known.

Figure 3. Regulation of hepcidin by erythroid regulators



(Hentze et al., 2010)

THE AXIS OF INFLAMMATION, HEPCIDIN, IRON AND ANEMIA

Anemia of chronic disease (ACD) or anemia of inflammation (AI) is commonly seen in chronic inflammatory disorders, such as inflammatory bowel disease, rheumatoid arthritis and chronic kidney disease. This condition is characterized by hypoferremia, low transferrin saturation and a normocytic normochromic anemia, and represents a condition of functional iron deficiency (Weiss and Goodnough, 2005).

Increased levels of inflammatory cytokines are said to play a major role in the pathogenesis of ACD. As mentioned earlier, inflammation affects erythropoietin production, responsiveness of erythroblasts to EPO, red blood cell survival and availability of iron for erythroid precursors (Means, 1995). Pro-inflammatory cytokines, such as interleukin-1(IL-1) and tumor necrosis factor-α (TNF-α), suppressed EPO expression in hepatoma cell lines and isolated rat kidney cells (Jelkmann, 1998). Interferon-γ (IFN-γ), another pro-inflammatory cytokine, was found to down-regulate EPO receptors in erythroid precursor cells (Taniguchi et al., 1997; Wang et al., 1995).

HEPCIDIN AND ULCERATIVE COLITIS

Ulcerative colitis (UC) is a chronic disease of the digestive tract. It is a disease of remissions and exacerbations. It warrants life-long treatment. Anemia is a significant co-morbidity in this condition and it affects the quality of life in such patients (Pizzi et al., 2006; Wells et al., 2006). Iron deficiency and anemia of inflammation are two major causes of anemia in patients with UC. Differentiation of these two types of anemias is essential to institute appropriate treatment. In case of ACD, treating the underlying cause will restore hemoglobin levels; however, but in a disease condition that has frequent remissions and exacerbations, the anemia is likely to recur. The major presenting symptom of ulcerative colitis patients is intermittent rectal bleeding, which predisposes the patient to develop iron deficiency anemia (IDA). In this situation, it is necessary to treat the anemia.

Hepcidin levels have been reported to be increased in patients with ACD (Guagnozzi and Lucendo, 2014; Theurl et al., 2009). However, other studies have reported that hepcidin levels are lower in patients with ACD, who have IDA (Theurl et al., 2010). Similarly, Sukumaran et al (2014) have demonstrated lowered serum hepcidin levels in patients with ulcerative colitis, who had co-existent anemia. Another study has reported lowered serum hepcidin, irrespective of the presence or absence of iron deficiency (Arnold et al., 2009).

It is possible that serum hepcidin levels may help differentiate anemia of chronic disease from anemia due to iron deficiency. The potential utility of this differentiation may be in identifying patients in whom oral iron supplementation may be useful.

THE STUDY

BACKGROUND OF THE STUDY

Anemia is a common complication of inflammatory bowel disease (IBD), which comprises Crohn's disease (CD) and ulcerative colitis (UC). Hepcidin is known to be the central regulator of iron homeostasis in the body. Previous work has shown that serum hepcidin levels were decreased in patients with ulcerative colitis, who had co-existent anemia. This observation is in contrast to other studies that have reported elevations in serum hepcidin levels in patients with inflammatory bowel disease.

AIM

The aim of the present study was to test the hypothesis that when anemia and inflammation co-exist in patients with UC, the effect of anemia on hepcidin predominates over that of inflammation.

OBJECTIVES

The objectives of the present study were to

1. estimate serum hepcidin levels in patients with ulcerative colitis
2. determine whether serum hepcidin levels in these patients correlated with markers of anemia and/or inflammation

The study was approved by the Institutional Review Board (IRB) at Christian Medical College (CMC), Vellore, India (IRB Min No. 8823 dated 07.04.2014).

MATERIALS

EQUIPMENT USED:

1. Elix and Milli-Q ultrapure water systems (Millipore, USA)
2. Table top centrifuge (Thermo Scientific Multifuge 3S+ Centrifuge)
3. -70^{o}C degree freezer (Thermo Scientific)
4. Micro plate reader (Model 680, Bio-Rad Laboratories Inc., UK)

CHEMICALS AND REAGENTS USED FOR ESTIMATION OF HEPCIDIN:

Chemicals and reagents for estimation of hepcidin were obtained from Peninsula Laboratories (Bachem Group, San Carlos, USA). They consisted of the following:

1. Standard diluent (peptide-free human serum)
2. Lyophilized standard, anti-serum against hepcidin, biotinylated peptide
3. Enzyme immunoassay buffer (EIA), streptavidin horseradish peroxidase (HRP), substrate solution (TMB - 3, 3', 5, 5' - tetramethylbenzidine solution) and stop solution (2N HCl)

MISCELLANEOUS CONSUMABLES USED

1. Vacutainer blood collection tubes (BD Biosciences, Plymouth, UK)
2. Micro tips (Tarson Products Private Limited, Kolkata, India)
3. Eppendorf tubes (Tarson Products Private Limited, Kolkata, India)

METHODOLOGY

SUBJECTS

A total of 59 patients were recruited into the study. Forty patients who met inclusion and exclusion criteria for the study and who were diagnosed to have ulcerative colitis (UC), by standard consensus criteria, served as cases.

Inclusion criteria:

1. Adults aged between 19-60 years diagnosed to have UC
2. The diagnosis of UC was based on consensus criteria (clinical picture, endoscopic appearance and histopathology), where an infective etiology has been excluded.
3. Patients were on/off treatment and had active/quiescent disease.

Exclusion criteria:

1. Patients with severe manifestations of UC
2. Patients on erythropoietin and iron supplements
3. Patients not willing to participate in the study

Nineteen patients who attended the Gastroenterology OPD for investigation of dyspepsia and who were found to have no detectable abnormalities, were non-anemic (Hb values equal to or more than 12 g/dL for females and equal to or more than 13 g/dL for males) and who had no evidence of inflammation (serum CRP values less than 6 mg/dL) served as control subjects.

Informed consent

Patients, who were identified on the basis of inclusion and exclusion criteria as listed above, were invited to participate in the study. They were each provided with an information sheet in either English or a vernacular language of their preference. Written consent was obtained from patients who expressed their willingness to participate (enclosed in Appendix 3). Clinical and socio-demographic data for each patient were obtained in the proforma used (enclosed in Appendix 2).

CALCULATION OF SAMPLE SIZE

The sample size that was required to show a relationship between serum hepcidin levels and other parameters of interest was 62 subjects, with 80% power and 5% significance level with an expected correlation of 0.7. This figure was based on data from the publication by Basseri et.al (2013).

The formula that was used for sample size calculation was as follows:

$$n = (Z_{1-\alpha/2} + Z_{1-\beta})^2 / [FZ(\rho_1) - FZ(\rho_0)]^2 + 3$$

(Ref. for the above formula: Machin D, Campbell MJ, Fayers MP and Pinal APY. Sample size tables for clinical studies. Blackwell Science Ltd., 2nd Edition, 1997)

where,

n = sample size

ρ_1 = sample correlation coefficient, i.e. correlation between two variables in the sample (denoted by 'r' in current practice)

ρ_0 = population correlation coefficient, i.e. correlation between two variables in the population (denoted by 'R' or 'ρ' in current practice)

1-α/2 = desired confidence level

1-β = power

Z = standard deviation scores, taken from Z score table.

FZ = cumulative distribution function

Substituting the formula with power of 80 (β = 0.2), significance level of 5% (α = 0.05), and an expected correlation of 0.7

$$n = (Z_{1-0.025} + Z_{1-.2})^2 / [FZ(0.723) - FZ(0.5)]^2 + 3$$

Substituting for the Z deviates of the corresponding values in the formula, the calculated number of subjects was 62. However, the amount of funds sanctioned by CMC, Vellore for MD theses is a maximum of Rs. 100,000. The amount of reagents and chemicals that could be purchased for this amount sufficed only for estimation of serum hepcidin, ferritin, iron, total iron binding capacity (TIBC) and C-reactive protein from 40 patients. Hence, 40 subjects with ulcerative colitis were studied.

SAMPLE COLLECTION

After obtaining informed consent, samples were collected from patients by venipuncture; blood was collected in BD vacutainer tubes. Approximately 6 ml of blood was collected from each patient.

PROCESSING OF SAMPLES

To obtain serum, the clotted blood was centrifuged at 2500 rpm, within 2 hours of blood collection. Serum was separated and divided into multiple aliquots. Parameters such as hemoglobin (Hb), mean corpuscular volume (MCV), total protein, albumin and erythrocyte sedimentation rate (ESR) were obtained from the hospital records of the patients, wherever available.

STORAGE OF SAMPLES

Aliquots of serum samples obtained were stored at -70^{o}C. They were used for estimation of serum hepcidin, C- reactive protein (CRP) , serum iron and serum ferriitn.

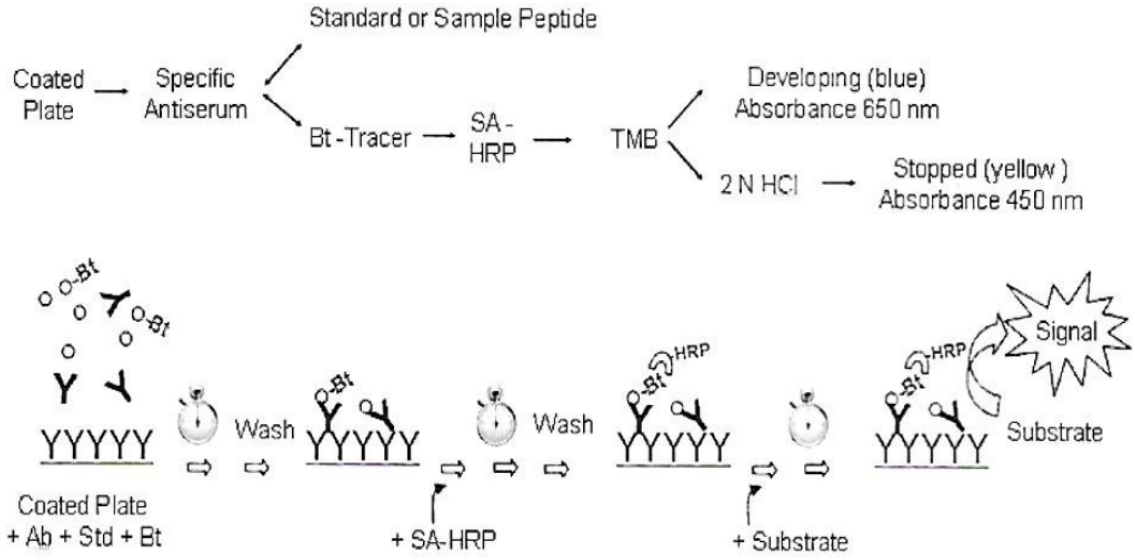
ESTIMATION OF SERUM HEPCIDIN

Reagents for estimation of serum hepcidin were purchased from Peninsula Laboratories (Bachem group, San Carlos, USA)

Principle of the method

The method used was based on a competitive immunoassay. Anti-rabbit antibody against hepcidin-25 was coated on a 96-well plate. A fixed concentration of biotinylated tracer (Bt-tracer) and varying concentrations of the standard or the peptide in the diluted serum samples competed for binding to the anti-serum. The captured Bt-tracer was subsequently bound by streptavidin-conjugated horseradish peroxidase (SA-HRP), which produced a colored product when substrate was added.

Figure 4. Competitive enzyme immunoassay for serum hepcidin



(Enzyme immunoassay protocols, Kit insert)

Components in the kit

1. 96-well plate
2. Enzyme immunoassay buffer (EIA) (50 ml)
3. Standard lyophilized hepcidin peptide (1 μg)
4. Standard diluents (8 ml) (peptide-free human serum)
5. Anti-serum against hepcidin-25 (lyophilized powder)
6. Lyophilized powder of biotinylated peptide
7. Streptavidin-horseradish peroxidase (SA-HRP - 100 μl)
8. Substrate solution (TMB- 3, 3', 5, 5'-tetramethylbenzidine) (11 ml of TMB and hydrogen peroxide[H_2O_2])
9. Stop solution (2N hydrochloric acid, 15 ml)

As per the manufacturer's instructions, standard diluent, lyophilized anti-serum, standard and biotinylated peptide were stored at -20°C. The EIA buffer, SA-HRP, substrate solution and stop solution were stored in a refrigerator at 2-4°C. These reagents were stable for one year under these conditions.

Preparation of the reagents

The unopened kit and reagents were equilibrated to room temperature before preparation of samples and working reagents.

1. **Stock standard:** To 1 μg of lyophilized standard, 1 ml of diluent was added and mixed, using a vortex mixer.

Standard	ng / ml	Range: 0.02-25 ng/ml
Stock	1000	
S1	25.00	Added 5 µl stock + 195 µl diluent
S2	6.25	Added 40 µl S1 + 120 µl diluent
S3	1.56	Added 40 µl S2 + 120 µl diluent
S4	0.39	Added 40 µl S3 + 120 µl diluent
S5	0.10	Added 40 µl S4 + 120 µl diluent
S6	0.02	Added 40 µl S5 + 120 µl diluent
S0	0.00	120 µl diluent

2. **Samples:** The 40 samples in the study were diluted 1 in 10 (12 µl of sample + 108 µl of standard diluent)
3. **Enzyme immunoassay buffer (EIA buffer):** EIA buffer (50 ml) was diluted to 1000 ml with sterile deionized water (18 MOhm) and mixed well.
4. **Anti-serum:** 5 ml of EIA buffer was added to the lyophilized anti-serum and mixed, using a vortex mixer.
5. **Biotinylated tracer (Bt-tracer):** 5 ml of EIA buffer was added to the lyophilized powder of the Bt-tracer.
6. **Streptavidin-HRP:** The tube was vortexed before dilution. It was diluted 1 in 200 with EIA buffer (60 µl of SA-HRP with 12 ml of EIA buffer) and mixed, using a vortex mixer.

Lay out of 96-well plate microtitre plate used in the study

	1	2	3	4	5	6	7	8	9	10	11	12
A	B	B	T1	T1	T9	T9	T17	T17	T25	T25	T33	T33
B	S1	S1	T2	T2	T10	T10	T18	T18	T26	T26	T34	T34
C	S2	S2	T3	T3	T11	T11	T19	T19	T27	T27	T35	T35
D	S3	S3	T4	T4	T12	T12	T20	T20	T28	T28	T36	T36
E	S4	S4	T5	T5	T13	T13	T21	T21	T29	T29	T37	T37
F	S5	S5	T6	T6	T14	T14	T22	T22	T30	T30	T38	T38
G	S6	S6	T7	T7	T15	T15	T23	T23	T31	T31	T39	T39
H	S0	S0	T8	T8	T16	T16	T24	T24	T32	T32	T40	T40

B- Blank, S - Standards, T- Test samples

Procedure

Step 1: Added 25 µl of anti-serum to each well and 25 µl of EIA buffer to the blank well. Incubated at room temperature for 1 hour.

Step 2: Added 50 µl of diluted standard/ sample to each well and 50 µl of diluent in the blank well. Incubated at room temperature for 2 hours.

Step 3: Rehydrated the biotinylated tracer (Bt-tracer) with EIA buffer and added 25 µl of Bt-tracer per well.

Step 4: Sealed the micro-titer plate cling film and incubated the plate at 4°C in a refrigerator overnight.

Step 5: Re-equilibrated the microtiter plate to room temperature the next day.

Step 6: Washed each well in the plate 5 times, with 300 µl of EIA buffer per well each time.

Step 7: Added 100 µl per well of SA-HRP (substrate solution) to each well. Incubated at room temperature for 1 hour.

Step 8: Washed each well in the plate 5 times, with 300 µl of EIA buffer per well each time.

Step 9: Added 100 µl of TMB (substrate solution) per well. Incubated at room temperature for 30-60 minutes.

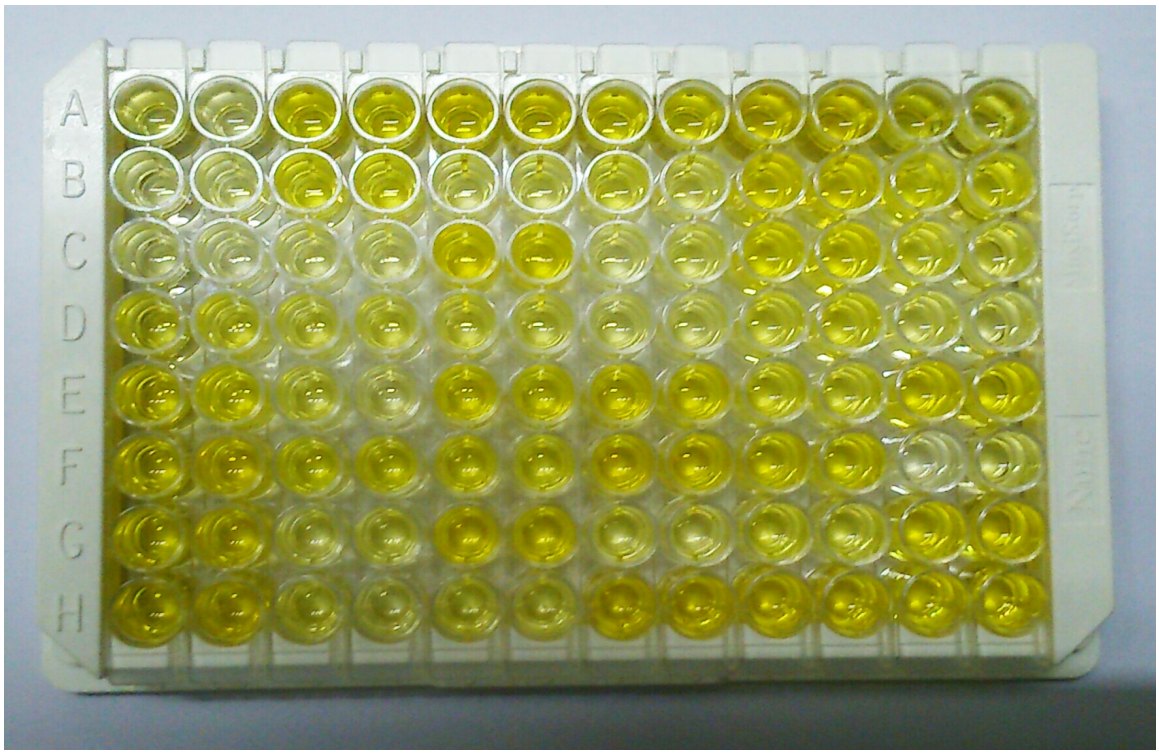
Step 10: Readings were taken at 650 nm, during development of blue color.

Step 11: Terminated the reaction with 100 µl of 2N HCl per well

Step 12: Readings were taken at 450 nm, within 10 minutes of termination of reaction.

Readings of optical density (OD) were obtained, using the software microplate manger of the ELISA plate reader.

Image of micro titer plate after termination of reaction



A standard curve was plotted on a semi-log scale, using Microsoft Office, Excel 2011. The mean of the OD readings for each standard was used for the y-axis and the concentration of the standards (ng/ml) for the x-axis. Serum hepcidin levels in the samples were calculated, by four parameters logistic regression analysis, using the following formula.

$$y = \{a\text{-}d/1+ (x/c)^{b}\} + d$$

The equation was used to calculate the value of fit and plot "smooth line of fit". The four parameters a (maximum), b (slope), c (IC50, point of inflection) and d (minimum) were adjusted, till the value of fit was satisfied and the fit was good.

$$x = c\,(y\text{-}a/d\text{-}y)^{1/b}$$

The equation was used to calculate the concentration of the samples, expressed in ng/mL.

ESTIMATION OF C-REACTIVE PROTEIN (CRP)

CRP levels in the serum samples were estimated in the Department of Microbiology, CMC, Vellore.

Equipment used:

The reagents and equipment used for estimation of CRP were manufactured by Siemens GmbH, Germany. The nephelometer used was BN Prospec, Siemens GmbH, Marburg, Germany.

Principle of the method (particle-enhanced nephelometry)

Polystyrene particles, coated with monoclonal antibodies specific to human CRP, bound with CRP in the test sample and formed aggregates. A beam of light was passed through the solution containing these aggregates. Light was scattered by the aggregates. The

intensity of the scattered light was directly proportional to the concentration of CRP in the sample. The result was calculated by comparison with a standard of known concentration.

Reference interval: < 6 mg/L

ESTIMATION OF SERUM FERRITIN

Estimation of serum ferritin was done in the Department of Clinical Biochemistry, CMC, Vellore.

Analyzer used: Siemens, ADVIA Centaur Immunoassay system Xpi, UK

Principle of the method (two-site sandwich immunoassay using direct chemiluminescence technology)

Two anti-ferritin antibodies were used in this method. The first antibody was polyclonal goat anti-ferritin antibody, labelled with acridinium ester. The second antibody was monoclonal mouse anti-ferritin antibody, which was covalently coupled to paramagnetic particles. These antibodies were sequentially added to the reaction chamber. These antibodies bound the ferritin molecule present in the serum sample. On adding substrate (0.1 N nitric acid, 0.5% hydrogen peroxide and alkaline medium), acridinium ester was excited and released a photon, which was measured in terms of relative light units (RLU). The amount of ferritin present in the sample was directly proportional to the amount of RLUs detected by the system.

Reference interval:

Men and women > 50 years - 20-320 ng/mL

Women < 50 years - 10-290 ng/mL

ESTIMATION OF SERUM IRON

Estimation of serum iron was carried out in the Department of Clinical Biochemistry, CMC, Vellore.

Analyzer used: Roche Cobas c 702 modular analyzer

Principle of the method: guanidine/ ferrozine spectrophotometric method

Transferrin-bound ferric ions in the sample were released by guanidine, and reduced to ferrous form by means of hydroxylamine. Ferrous ions reacted with ferrozine to form a purple colored complex. The absorbance of the sample was measured at 560 nm, using spectrophotometry. The intensity of the color obtained was directly proportional to the concentration of iron in the sample.

Reference interval:

Male - 60- 160 μg/dL

Female - 40-145 μg/dL

ESTIMATION OF TOTAL IRON BINDING CAPACITY (TIBC)

Estimation of serum iron was carried out in the Department of Clinical Biochemistry, CMC, Vellore.

Analyzer used: Roche Cobas 8000c 702 modular analyzer

Principle of the method:

A known amount of ferrous iron was added to the sample at an alkaline pH. The ferrous ions bound to transferrin at unsaturated iron binding sites. The unbound ferrous ions were measured using the ferrozine method (described above under the estimation of serum iron). The difference between the amount of ferrous ions added and the unbound ions measured was taken to be the unsaturated iron binding capacity (UIBC) of the sample.

TIBC was calculated as the sum of serum iron concentration and the UIBC.

UIBC = [Amount of ferrous ion added] - [Amount of unbound ferrous ion]

TIBC = Serum iron + UIBC

Reference interval:

Male - 300-400 µg/dL

Female - 250-350 µg/dL

CALCULATION OF TRANSFERRIN SATURATION (TSAT)

It was calculated as the ratio of serum iron and total iron binding capacity, multiplied by 100.

TSAT = (Serum Iron / TIBC) * 100

Reference interval:

Adults - 25 - 50 %

STATISTICAL ANALYSIS

Statistical analysis of data was done using the Statistical Package for Social Sciences (SPSS), version 16. The data were checked for normality of distribution, using Kolmogorov-Smirnov test. Data were analyzed by parametric and non-parametric tests, as appropriate. Data of the two groups were compared using unpaired t-test, in case of 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 that were not normally distributed. A p- value of less than 0.05 was considered to be statistically significant in all cases.

RESULTS

PART- I

Distribution of data

Data on age and hemoglobin were found to be normally distributed. Data for C-reactive protein, ferritin, iron, and hepcidin were found to have skewed distributions.

Characteristics of subjects of the study

A total of 59 patients were recruited into the study. Forty patients who met inclusion and exclusion criteria for the study and who were diagnosed to have ulcerative colitis (UC), by standard consensus criteria, served as cases. Nineteen patients who attended the Gastroenterology OPD for investigation of dyspepsia and who were found to have no detectable abnormalities, were non-anemic (Hb values equal to or more than 12 g/dL for females and equal to or more than 13 g/dL for males) and who had no evidence of inflammation (serum CRP values less than 6 mg/dL) served as control subjects.

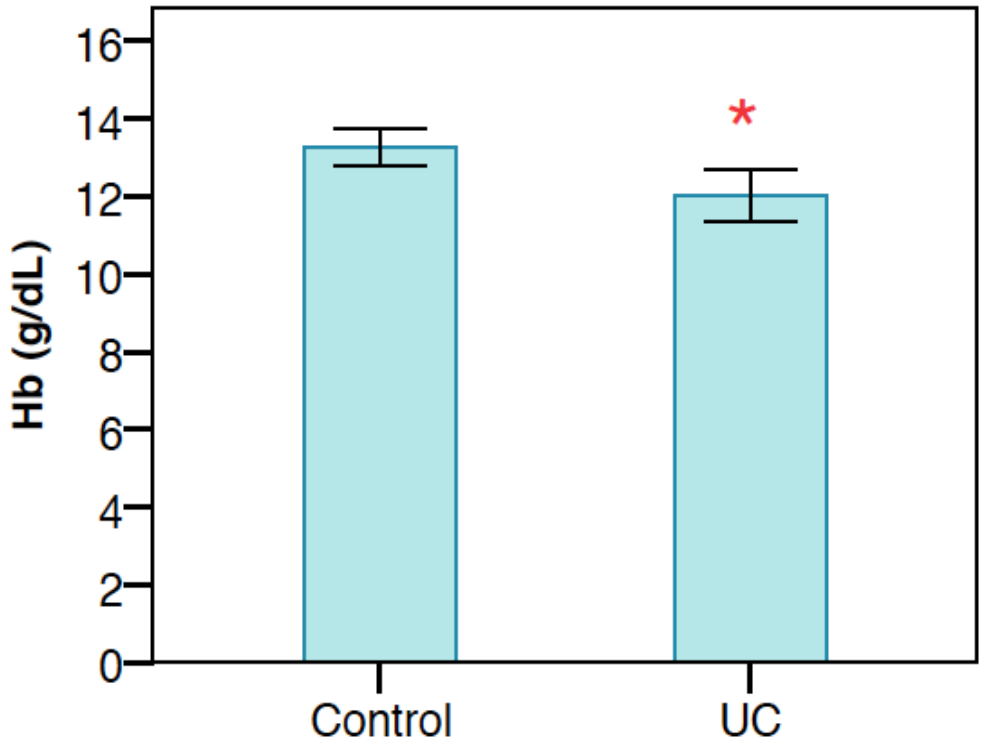
The characteristics of subjects in the study are shown in Table 1.

Table 1

Characteristic	Control patients	Patients with ulcerative colitis
Number of patients	19	40
Male/Female	10/9	22/18
Age in years (mean ± SD)	39.47 (12.77)	41.7 (9.12)

Patients with ulcerative colitis comprised 22 males (55%) and 18 females (45%), while control subjects comprised 10 males (53%) and 9 females (47%). The ages of those with UC were similar to those of control subjects.

Figure 5. Hemoglobin levels in control subjects and patients with UC

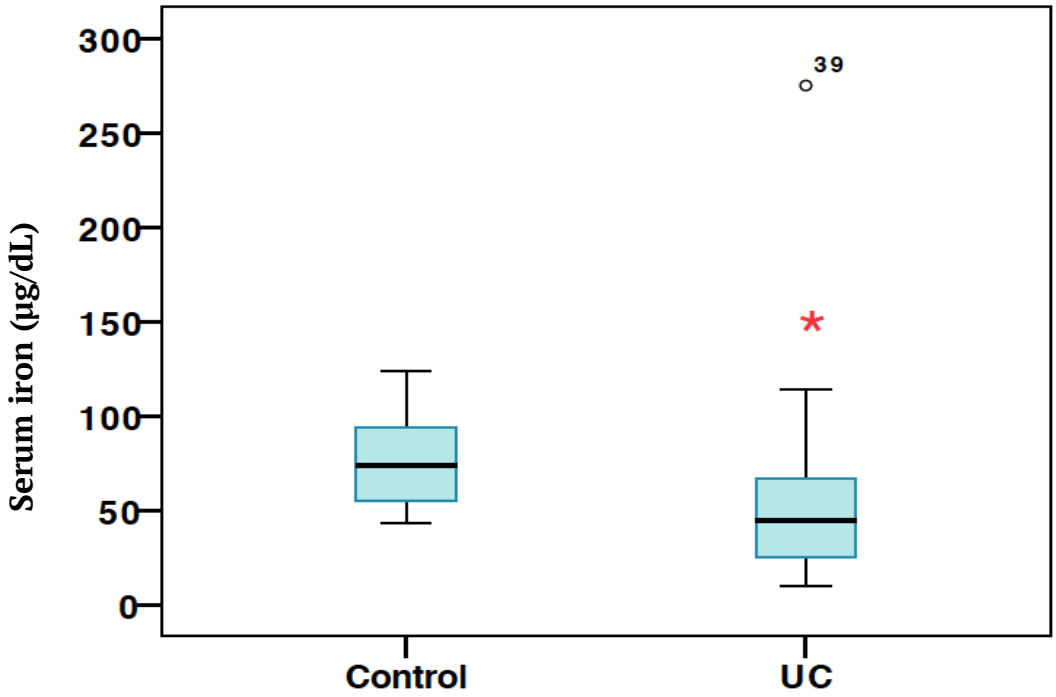


Data are shown as mean (±SD).

*p <0.05 when compared with control data

Hemoglobin levels were significantly lower in patients with ulcerative colitis than in control subjects. Among the 40 patients with ulcerative colitis, 21 (53%) were found to be anemic (Hb < 12 g/dL in females and <13 g/dL in males).

Figure 6. Serum iron levels in control subjects and patients with UC

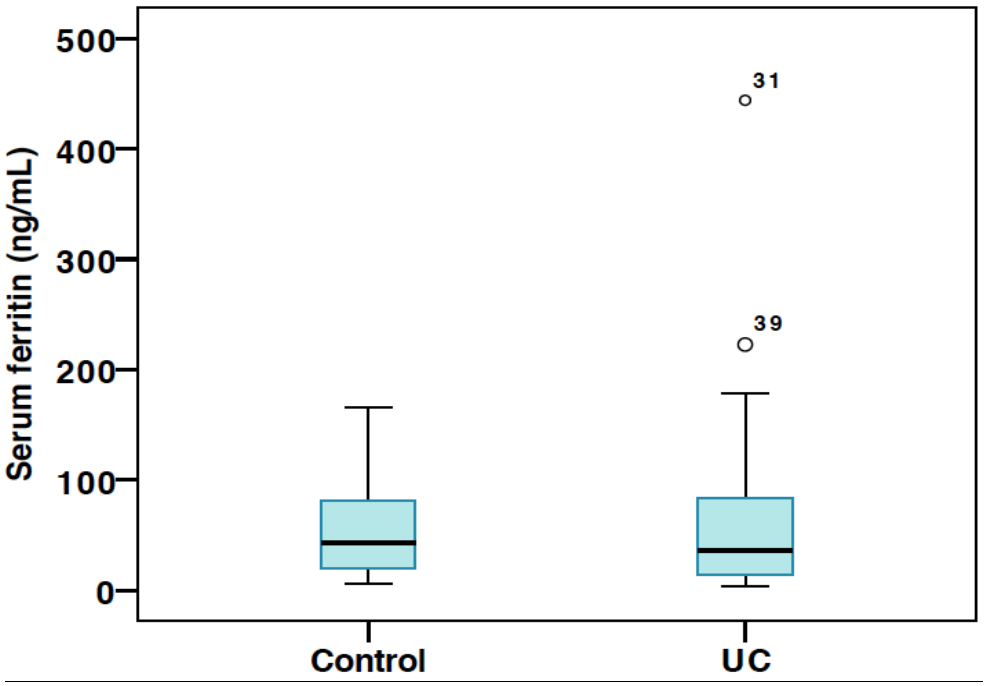


Data are shown in the form of box and whisker plots, with quartiles and medians shown. An outlier is shown as a numbered dot.

*p <0.05 when compared with control data

Serum iron levels were significantly lower in patients with ulcerative colitis than in control subjects.

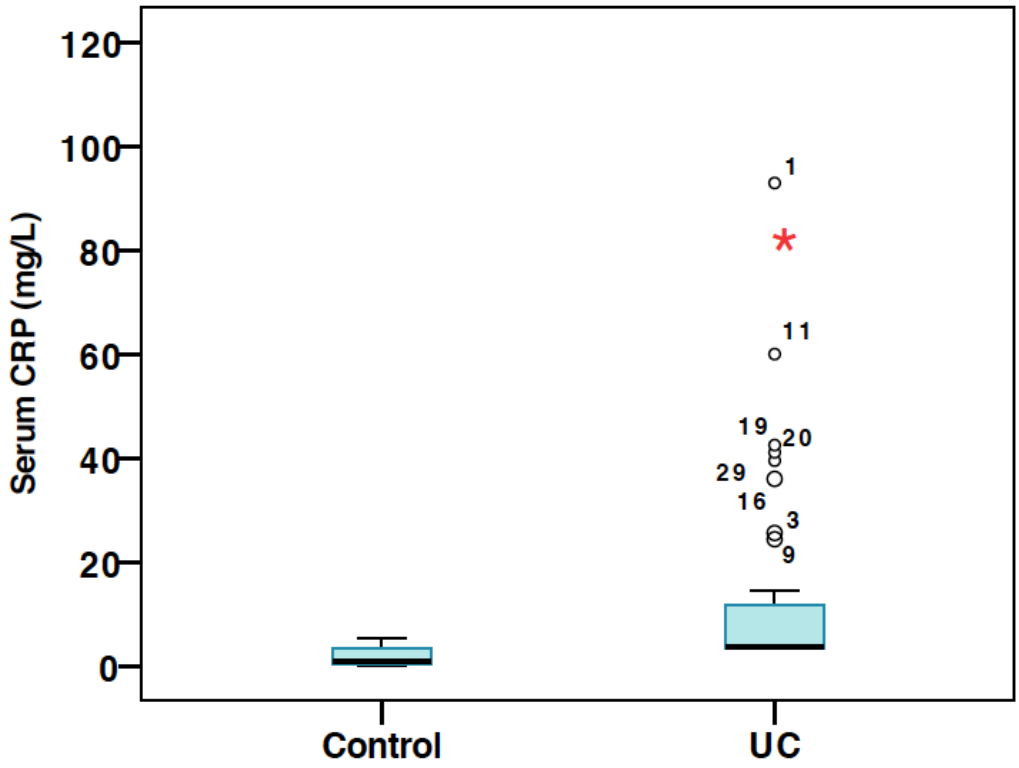
Figure 7. Serum ferritin levels in control subjects and patients with UC



Data are shown in the form of box and whisker plots, with quartiles and medians shown. Outliers are shown as numbered dots.

Serum ferritin levels were similar in patients with ulcerative colitis and control subjects.

Figure 8. Serum CRP in control subjects and patients with UC



Data are shown as box and whisker plots, with medians and interquartile ranges, as shown. Outliers are shown as numbered dots.

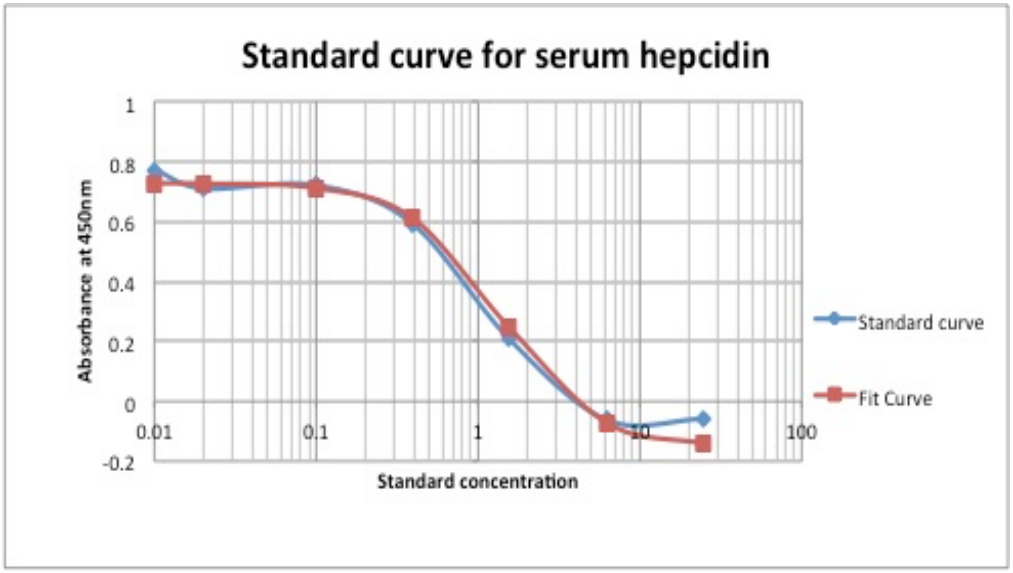
*p <0.05 when compared with control data

Serum CRP levels were significantly higher in patients with ulcerative colitis than in control subjects.

Serum hepcidin levels

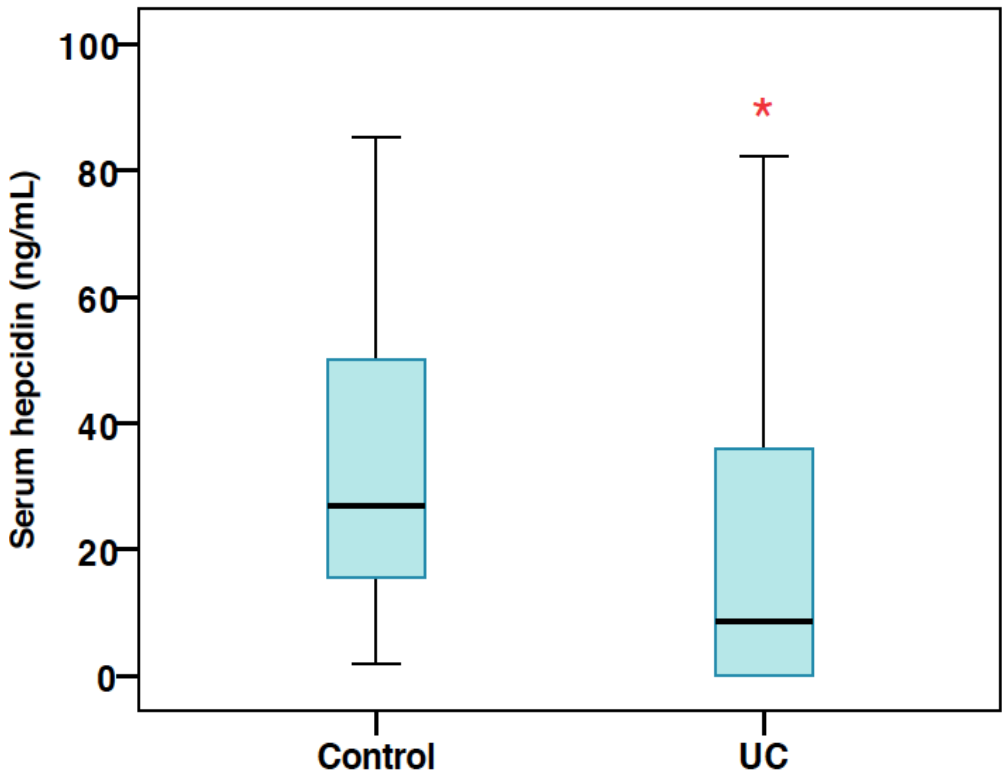
A standard curve for serum hepcidin levels was generated, using the microplate manager software from Bio-Rad laboratories. The concentration of each sample was obtained from this standard curve.

Figure 9. Standard curve for serum hepcidin



The fit curve was generated as per the manufacturer's instructions. The concentration of hepcidin in each sample was calculated from the optical density readings, after drawing a line for best fit.

Figure 10. Serum hepcidin levels in control subjects and patients with UC



Data are shown as box and whisker plots, with medians and quartiles shown.

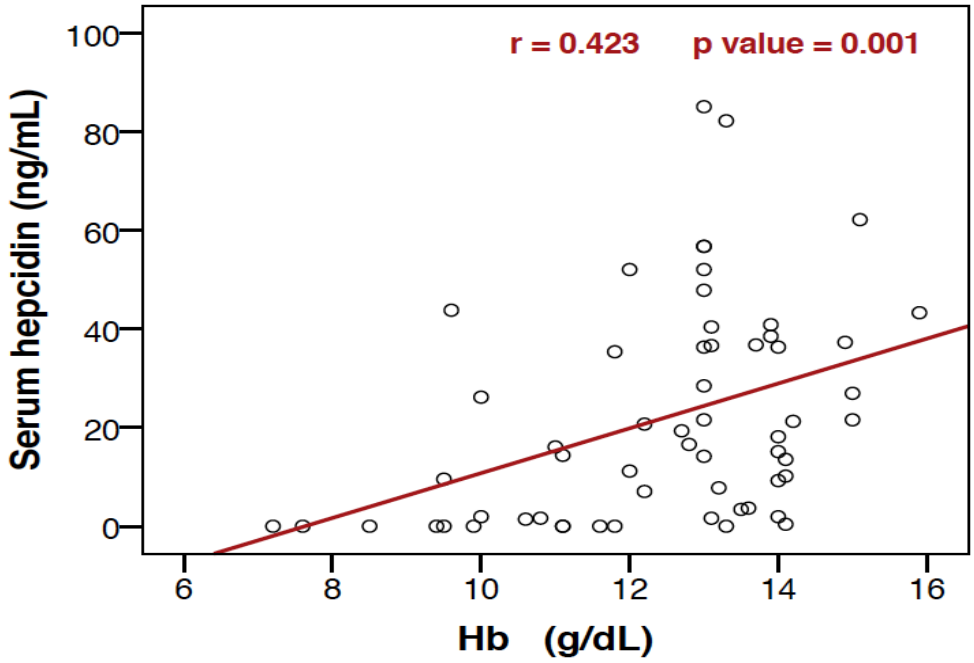
*p <0.05 when compared with control data

Serum hepcidin levels were found to be significantly lower in patients with UC than in control subjects.

Correlation analysis

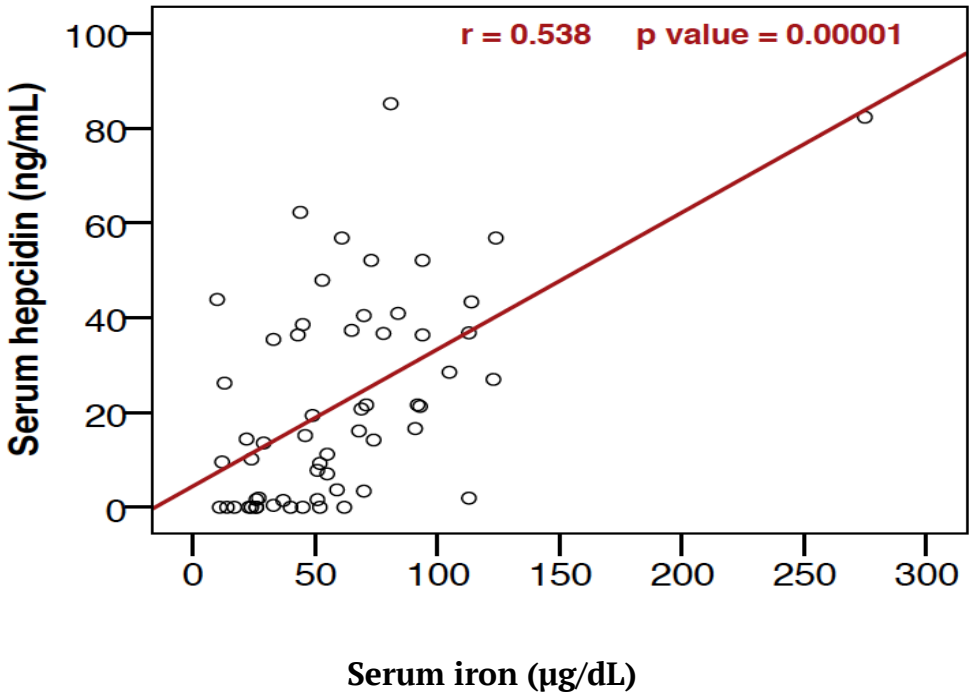
Correlation analysis was done on the data obtained. The data are represented as scatterplots with correlation coefficient (r) and p values shown.

Figure 11. Scatterplot of serum hepcidin versus hemoglobin



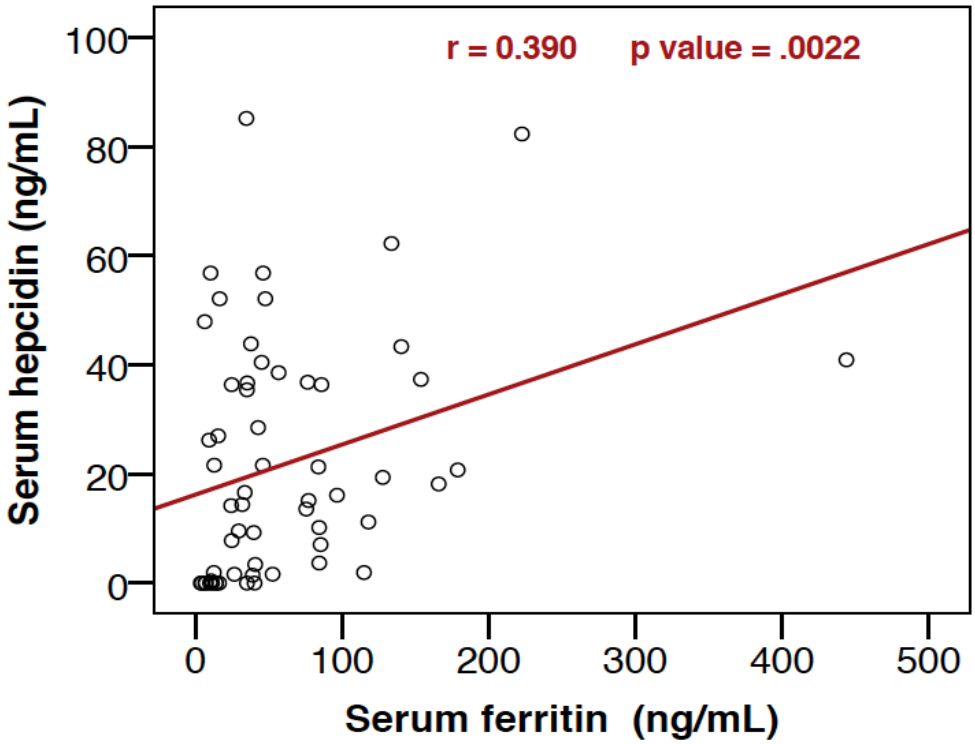
Serum hepcidin levels and hemoglobin values were found to have a significant positive correlation.

Figure 12. Scatterplot of serum hepcidin versus serum iron



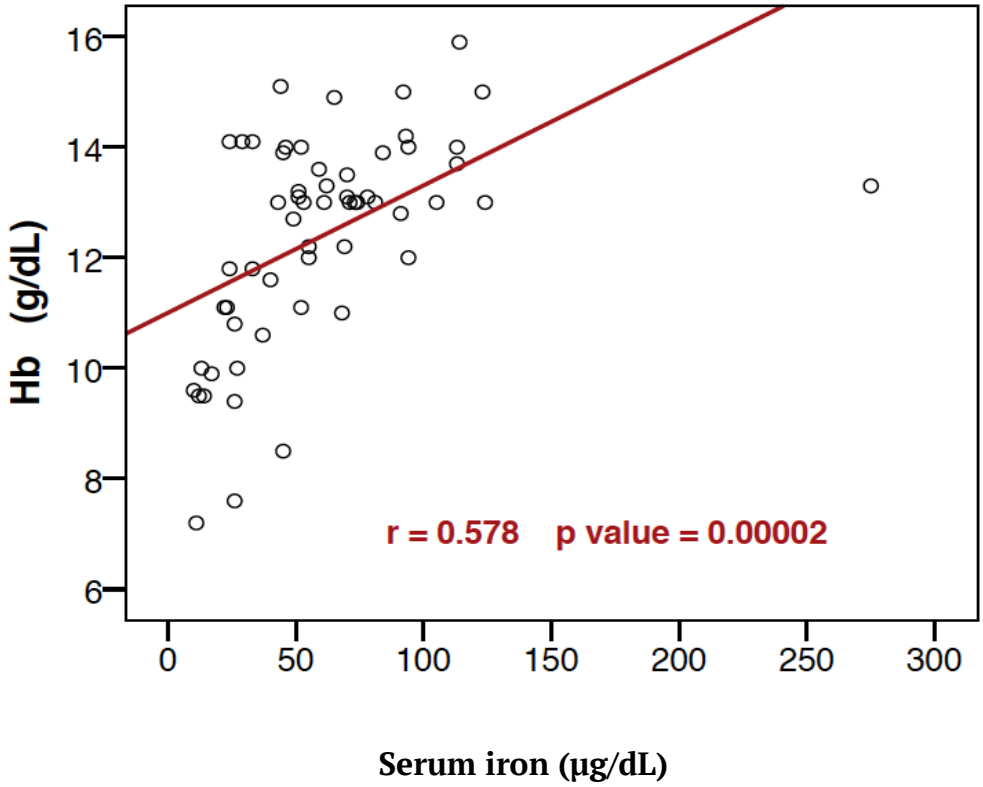
Serum hepcidin levels and serum iron values were found to have a significant positive correlation.

Figure 13. Scatterplot of serum hepcidin versus serum ferritin



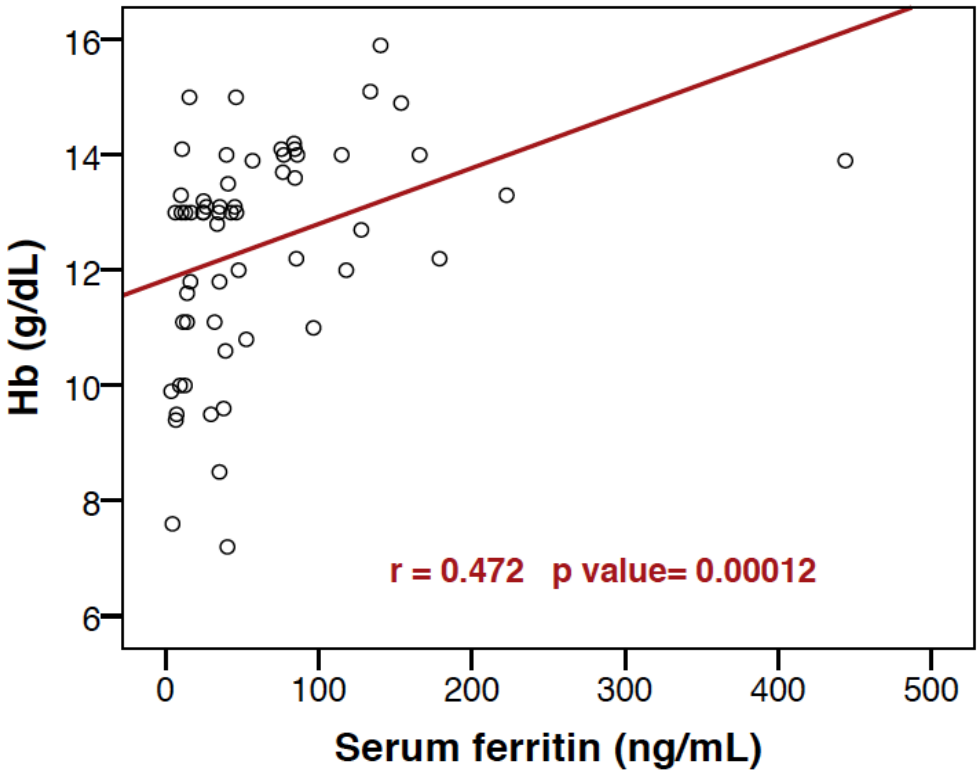
Serum hepcidin and serum ferritin values were found to have a significant positive correlation.

Figure 14. Scatterplot of hemoglobin versus serum iron



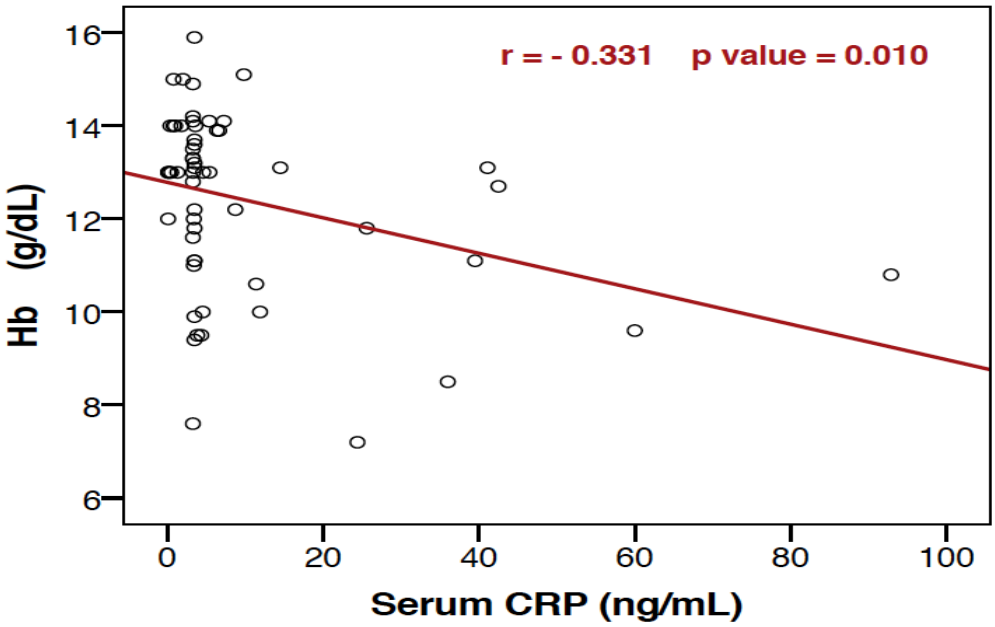
Hemoglobin and serum iron values were found to have a significant positive correlation.

Figure 15. Scatterplot of hemoglobin versus serum ferritin



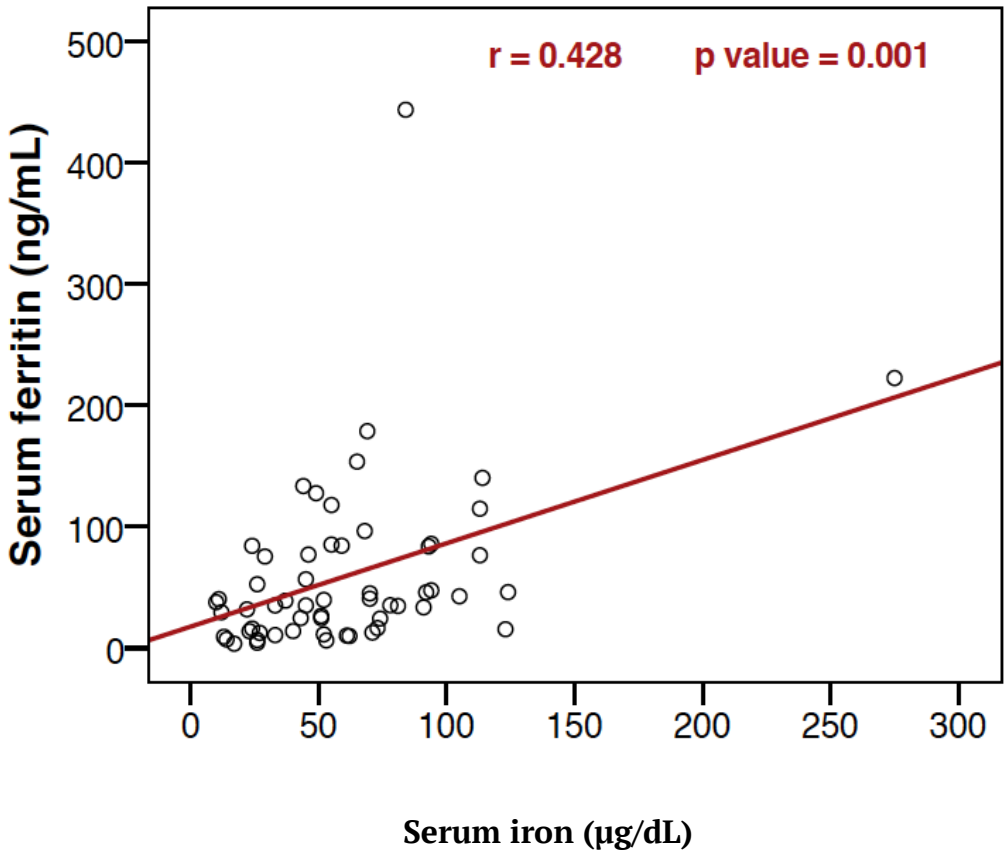
Hemoglobin and serum ferritin values were found to have a significant positive correlation.

Figure 16. Scatterplot of hemoglobin versus serum CRP



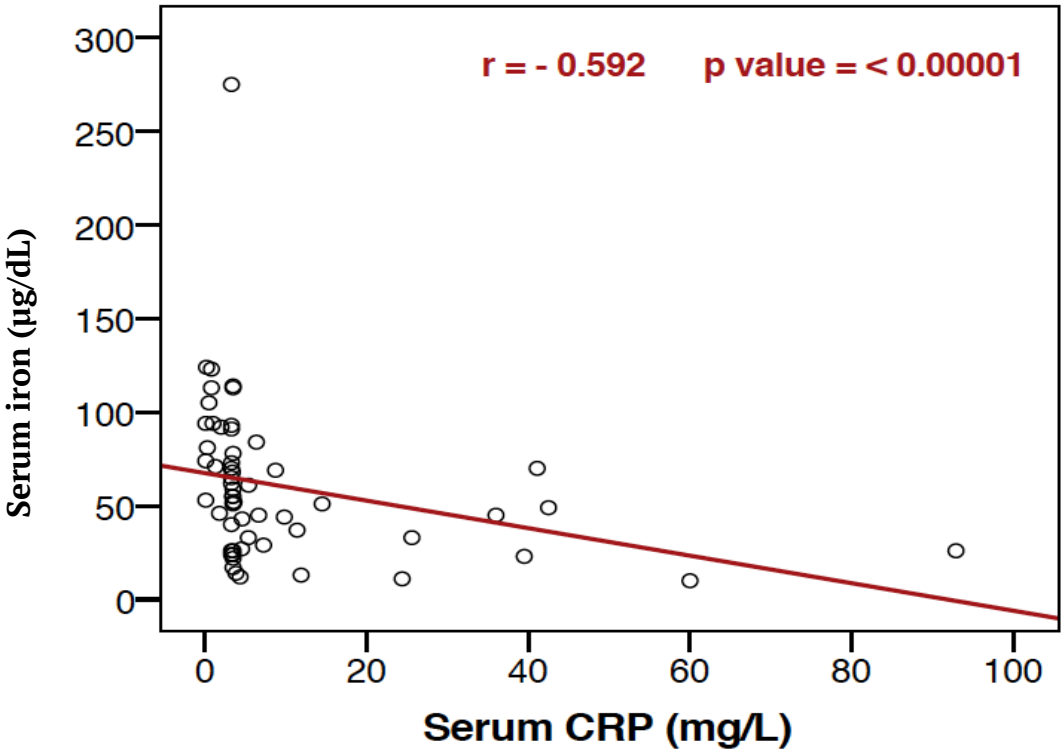
Hemoglobin and serum CRP values were found to have a significant negative correlation.

Figure 17. Scatterplot of serum ferritin versus serum iron



Serum ferritin and serum iron values were found to have a significant positive correlation.

Figure 18. Scatterplot of serum iron versus serum CRP



Serum iron and CRP values were found to have a significant negative correlation.

Summary of results in Part-I

1. Hemoglobin and serum iron and hepcidin levels were significantly lower in patients with ulcerative colitis, compared to control patients.

2. Levels of serum CRP were significantly higher in patients with ulcerative colitis than in control patients.

3. Correlational analyses showed the following:
    i. Levels of serum hepcidin correlated positively with hemoglobin, iron and ferritin.
    ii. Levels of hemoglobin correlated negatively with those of serum C-reactive protein and positively with levels of serum iron and ferritin.
    iii. Serum ferritin and iron levels showed significant positive correlations with one another.
    iv. Serum iron levels correlated negatively with levels of C-reactive protein.

PART-II

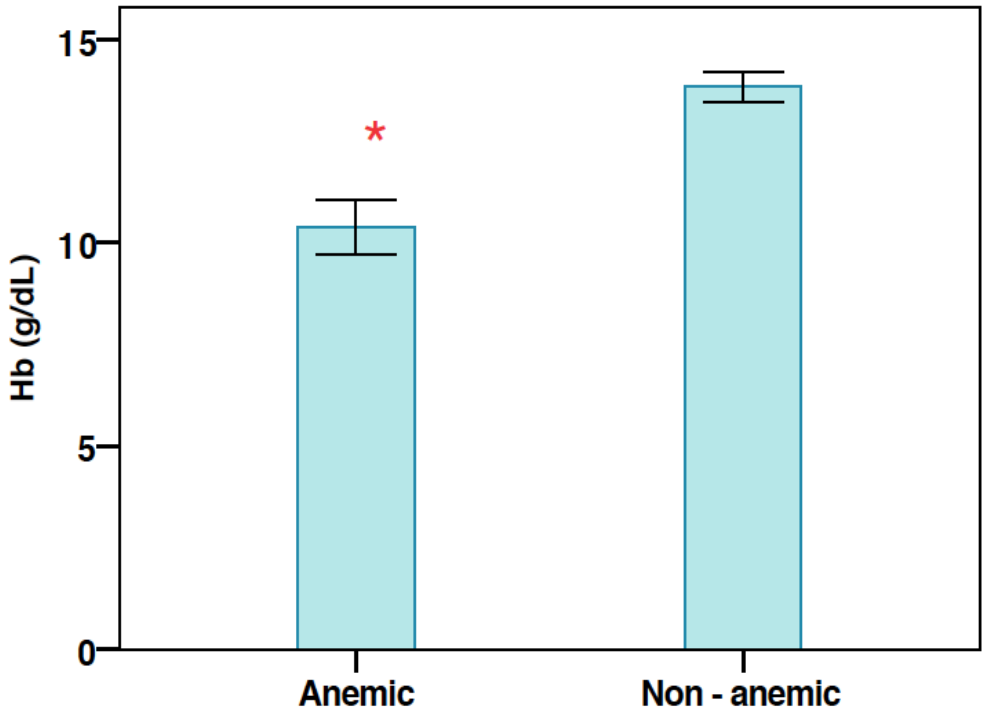
For additional analysis, subjects in the ulcerative colitis group were stratified based on anemic status. Patients were diagnosed to be anemic if their hemoglobin level was less than 13 g/dL for men and less than 12 g/dL for women.

Table 2. Patients with UC with and without anemia

	Anemic	**Non-anemic**
Number of subjects with UC	21	19
Male/Female	9/12	13/6
Age in years (mean±SD)	40.47 (8.727)	42 (9.527)

In patients with ulcerative colitis, 21 (53%) were found to be anemic and 19 (47%) were found non-anemic. Anemic group comprised of 9 males and 12 females, non-anemic group comprised of 13 males and 6 females. The age is similar in between anemic and non-anemic group

Figure 19. Hemoglobin levels in anemic and non-anemic patients with ulcerative colitis

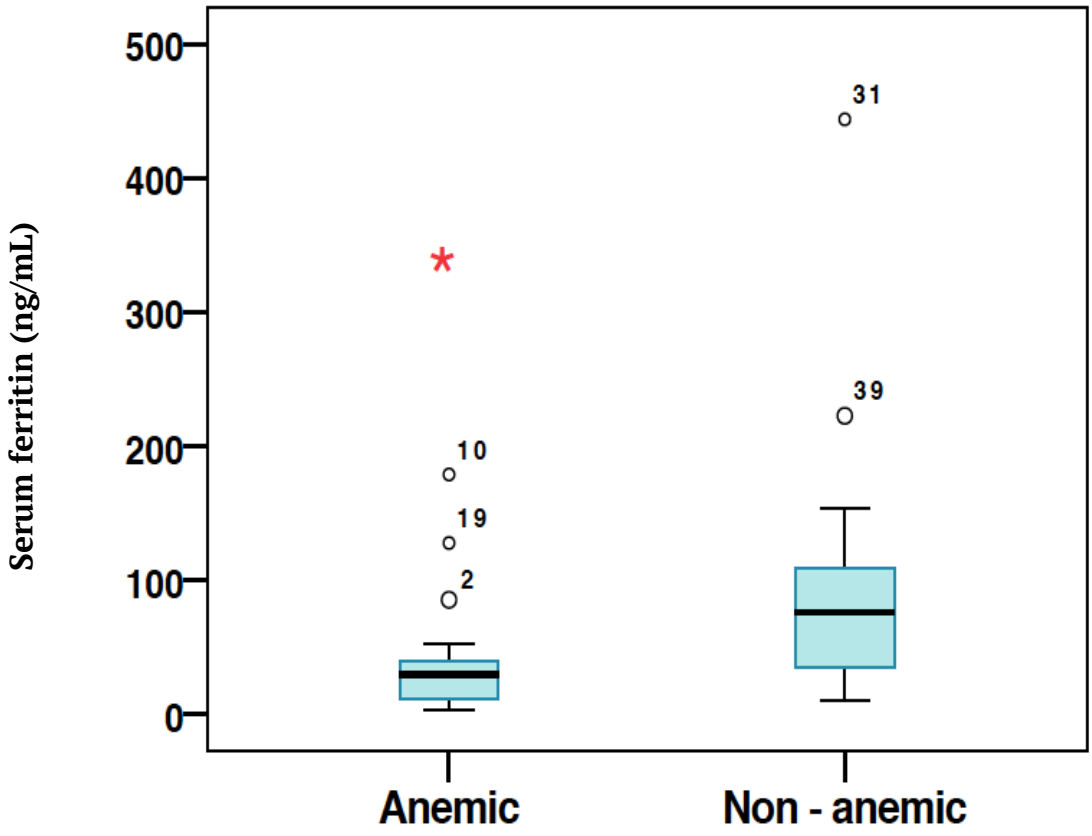


Data are shown as mean ± SD.

*p <0.05 when compared with non-anemic group

Haemoglobin levels in patients with UC, who were anemic, were significantly lower than in those without anemia.

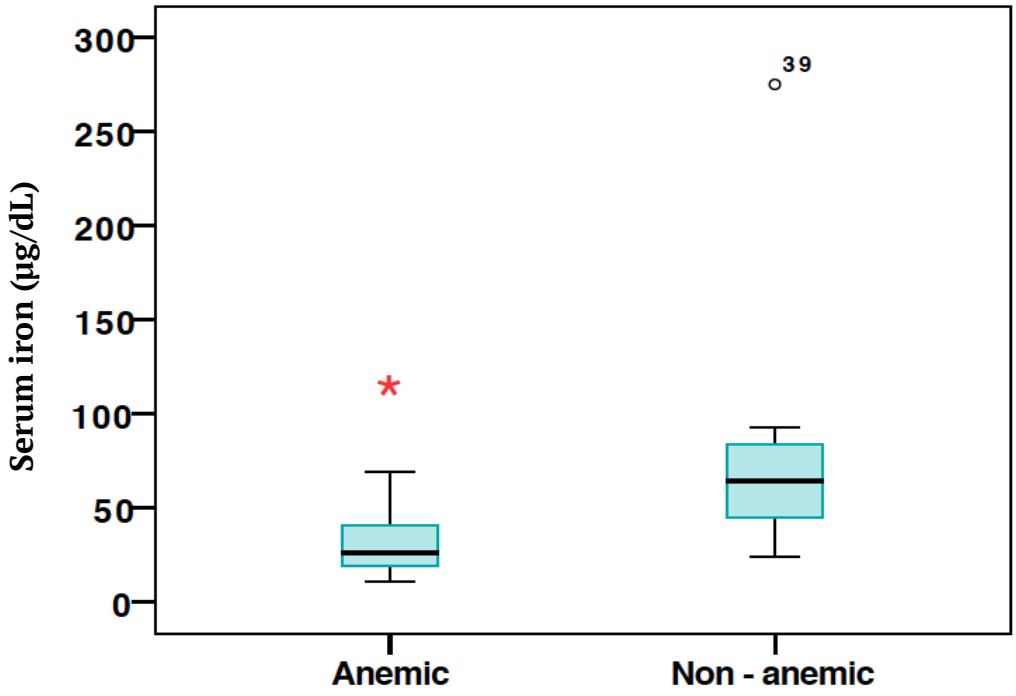
Figure 20. Serum ferritin levels in anemic and non-anemic patients with ulcerative colitis



*p <0.05 when compared with non-anemic group

Serum ferritin levels in patients with UC, who were anemic, were significantly lower than in those without anemia.

Figure 21. Serum iron levels in anemic and non-anemic patients with ulcerative colitis

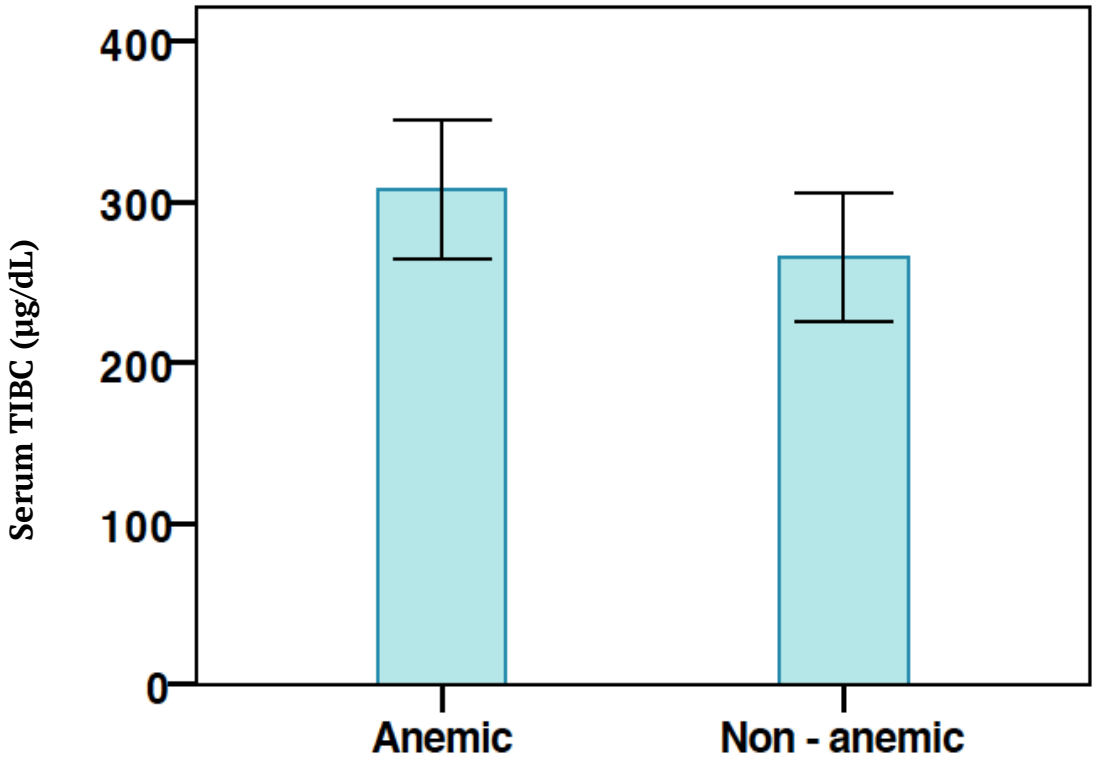


Data are represented as box and whisker plot with medians and quartiles shown.

*p <0.05 when compared with non-anemic group

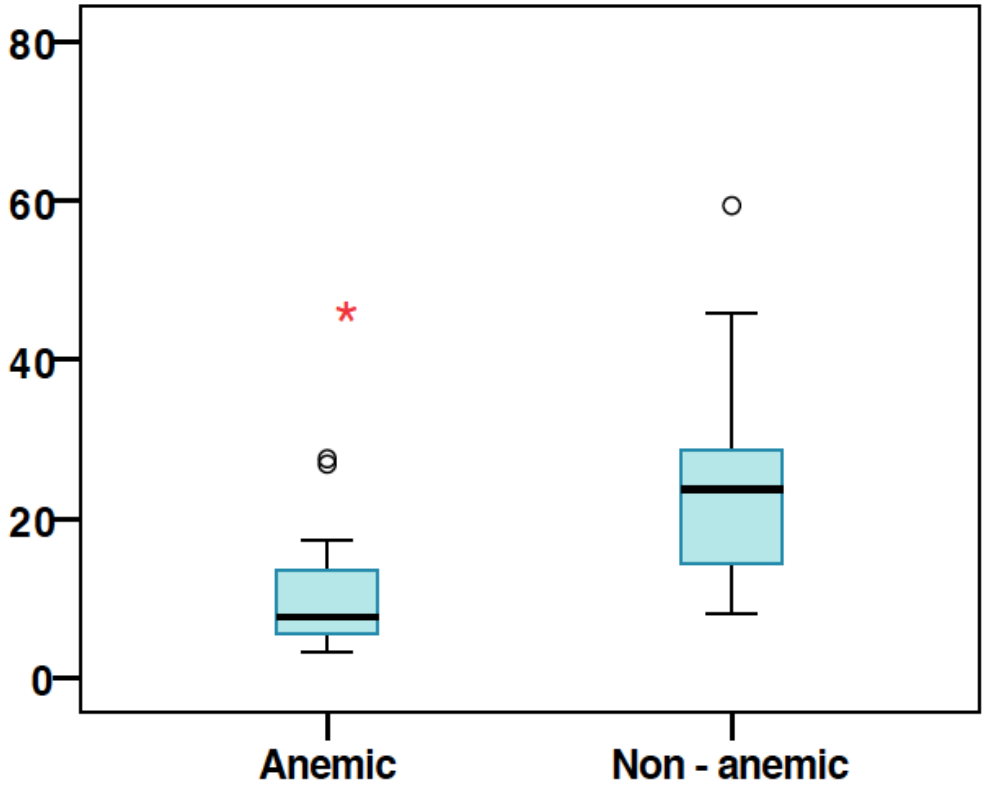
Serum iron levels in patients with UC, who were anemic, were significantly lower than in those without anemia.

Figure 22. Serum TIBC levels in anemic and non-anemic patients with ulcerative colitis



Serum TIBC levels in patients with UC were similar in anemic and non-anemic group.

Figure 23. Transferrin saturation in anemic and non-anemic patients with ulcerative colitis

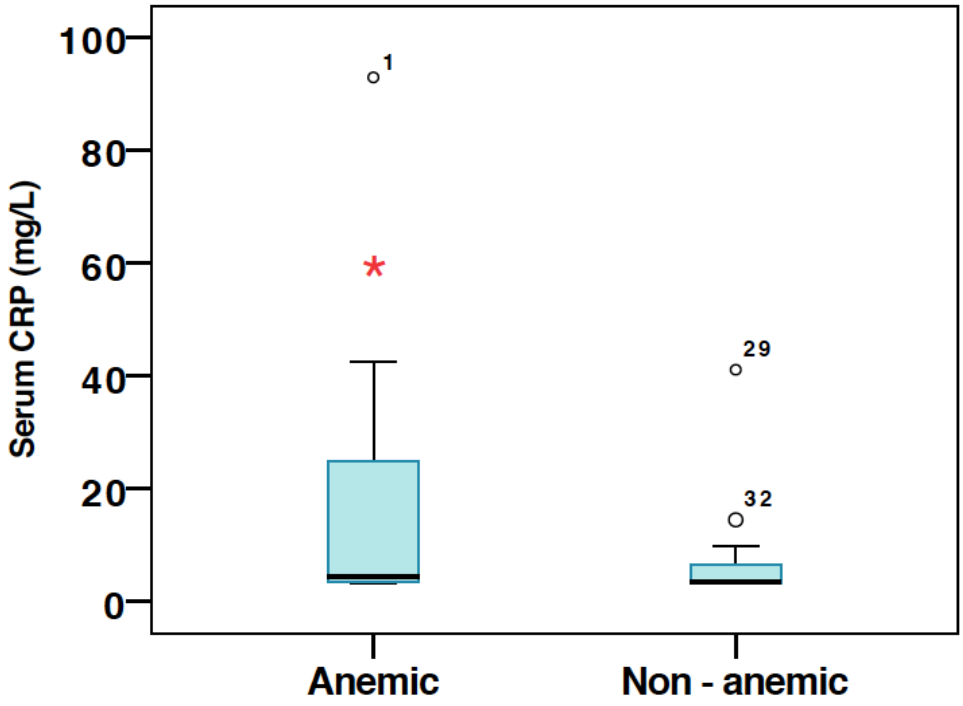


Data are represented as box and whisker plots with medians and quartiles shown.

*p <0.05 when compared with non-anemic group

Transferrin saturation levels in patients with UC, who were anemic, were significantly lower than in those without anemia.

Figure 24. Serum CRP levels in anemic and non-anemic patients with ulcerative colitis

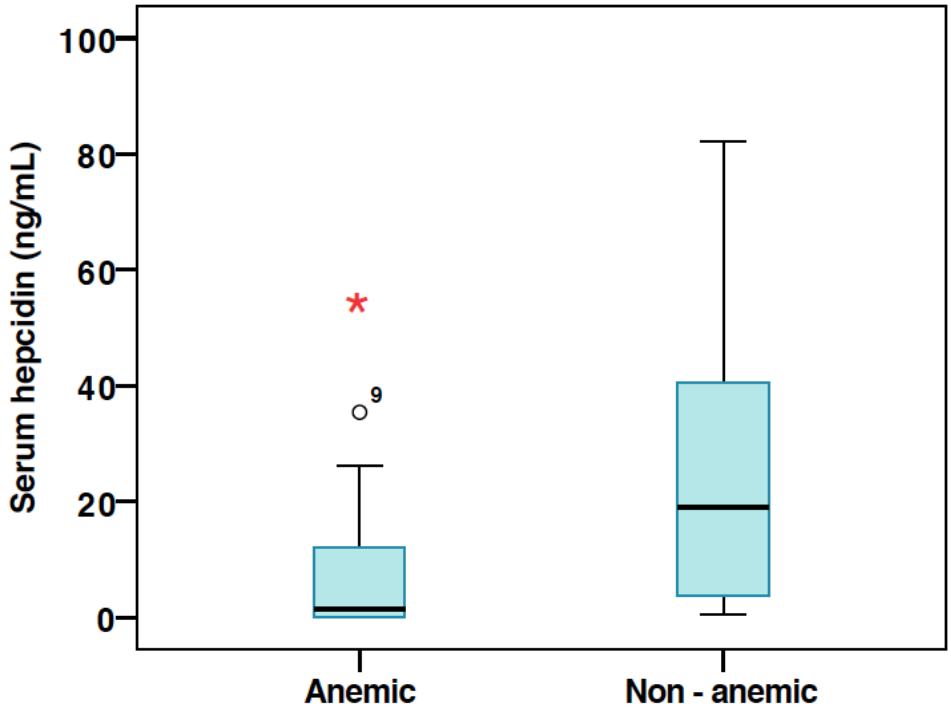


Data are represented as box and whisker plots with medians and quartiles shown.

*p <0.05 when compared with non-anemic group

Serum CRP levels in patients with UC, who were anemic, were significantly higher than in those without anemia

Figure 25. Serum hepcidin levels in anemic and non-anemic patients with ulcerative colitis



Data are represented as box and whisker plots with medians and quartiles shown.

*p <0.05 when compared with non-anemic group

Serum hepcidin levels in patients with UC, who were anemic, were significantly lower than in those without anemia.

Summary of the findings in Part-II

1. Hemoglobin and serum ferritin, iron and hepcidin levels and transferrin saturation values were significantly lower in patients with UC who were anemic, than in those who were not.

2. Serum CRP levels were significantly higher in patients with UC who were anemic, than in those who were not.

DISCUSSION

DISCUSSION

Anemia, is a significant co-morbidity in patients with ulcerative colitis (Cucino and Sonnenberg, 2001). Its prevalence is highly variable, with reported figures ranging from 6% to 74% (Kulnigg and Gasche, 2006; Wilson et al., 2004). The etiology of the anemia is complex and varied. Causes include iron deficiency due to blood loss, inflammation per se, vitamin B_{12} and folate deficiency, myelosuppression and autoimmune hemolytic anemia (Guagnozzi and Lucendo, 2014). Of these, iron deficiency and anemia of inflammation are the most common causes of anemia in ulcerative colitis (Kulnigg and Gasche, 2006). However, despite the reported high and alarming prevalence of anemia in ulcerative colitis resulting in significant co-morbidity, the anemia is often untreated or treated improperly in clinical practice (Nissenson et al., 2003). The reason for this is mainly in the difficulty involved in determining the etiology of anemia in this condition. This is vital in instituting appropriate treatment.

Hepcidin, a 25-amino acid peptide, is the master regulator of systemic iron homeostasis (Ganz, 2006). Its levels are increased in inflammatory and iron-overloaded states and decreased in iron deficiency and hypoxia (Nicolas et al., 2002). In inflammatory conditions, such as ulcerative colitis, levels of pro-inflammatory cytokines are increased. Interleukin 6 (IL-6), in particular, is known to induce hepcidin transcription and increase serum hepcidin levels (Nemeth et al., 2004a). Increased hepcidin levels reduce intestinal absorption of iron and its recycling from macrophages(Nemeth et al., 2004b).

In the present study, a total of 59 subjects were studied. Of these, 40 were diagnosed to have ulcerative colitis (UC) and 19 served as control subjects. Of those with UC, 53%

were found to be anemic. Hemoglobin levels were significantly lower in patients with ulcerative colitis than in control subjects. Serum iron and ferritin were considered as markers of iron status. Serum iron levels were significantly lower in ulcerative colitis patients suggesting hypoferremia. Serum ferritin levels were similar in between two groups.

Shanmugam et al (2012) have shown that hepcidin expression was decreased in 2 different mouse models of colitis. However, there is no information in this study, however, on serum levels of hepcidin. Studies in humans have reported both increased (Basseri et al., 2013; Oustamanolakis et al., 2011; Semrin et al., 2006; Theurl et al., 2009) and decreased levels of serum hepcidin in patients with inflammatory conditions (Arnold et al., 2009; Sukumaran et al., 2014; Theurl et al., 2009). The differences would appear to be due to presence or absence of anemia. In the studies, which reported increased levels of hepcidin, the subjects were not anemic, while anemia was present in subjects in whom hepcidin levels were decreased. Sukumaran et al (2014) have suggested that in chronic inflammatory conditions, hepcidin levels are likely to be increased in the early phase of the disease in response to inflammatory stimuli. High levels of hepcidin are implicated in the pathogenesis of anemia of inflammation(Basseri et al., 2013). With the development of anemia, hepcidin production is suppressed. This would account for the finding of decreased hepcidin levels in chronic inflammatory states with co-existent anemia. This suggests that when both inflammation and anemia are seen in the same patient, the effect of anemia on hepcidin predominates over that of inflammation. This has also been suggested earlier by Theurl et al (2009).

Anemia in the patients in the present may have been due to either iron deficiency or anemia of chronic disease. With the data available, it is difficult to differentiate between the two. Measuring soluble transferrin receptor-log ferritin ratio (sTfR/log ferritin ratio) would be useful to differentiate between the two conditions. Soluble transferrin receptor expression is shown to be negatively affected by inflammation (Weiss, 2002) . However, the sTfR/log ferritin ratio is considered an accurate indicator of body iron stores in the presence of inflammation (Punnonen et al., 1997; Skikne et al., 2011). However, in this study, sTfR-log ferritin ratio could not be determined due to financial constraints involved in estimating sTfR.

Serum levels of CRP, a marker of inflammation, were elevated in patients with ulcerative colitis, indicating the presence of active inflammation. For further analysis, subjects in the ulcerative colitis group were stratified based on anemic status. Patients were diagnosed to be anemic if their hemoglobin level was less than 13 g/dL for men and less than 12 g/dL for women. Serum hepcidin levels were significantly lower in patients with UC who were anemic than in those who were not. Serum CRP levels were significantly higher in patients with UC who were anemic than in those who were not. Correlation analyses showed that levels of serum hepcidin correlated positively with hemoglobin, iron and ferritin but not with serum CRP. The observation that serum hepcidin correlated positively with hemoglobin is in contrast to other studies, which showed negative correlation between hepcidin and hemoglobin (Oustamanolakis et al., 2011). This may be accounted for by the postulate that as hemoglobin levels decrease due to iron-deficient erythropoiesis, hepcidin levels are downregulated. The converse would also hold true.

Based on the above findings, it appears that serum hepcidin levels were predominantly regulated by the iron status, rather than by the inflammation present in patients with ulcerative colitis. This supports the postulate by Sukumaran et al (2014) that when both anemia and inflammation coexist, the former predominates over the latter, resulting in lowered hepcidin levels. Inflammation-induced increases in serum hepcidin levels are likely to lead to decreased serum iron levels, which in turn would cause iron-restricted erythropoiesis and anemia. This may lead to increased erythropoietic activity in response to anemia. Erythroid regulators produced in such situations, such as erythropoietin(Sasaki et al., 2012), GDF-15(Tanno et al., 2007b), TWSG1(Tanno et al., 2009) and erythroferrone(Kautz et al., 2014) are known to down-regulate hepcidin transcription .

An earlier study conducted in the Department of Biochemistry, CMC, Vellore showed that serum GDF-15 levels were significantly increased patients with ulcerative colitis (unpublished observations). It is possible that increased GDF-15 levels may have cause decreased hepcidin levels. It was not possible to estimate levels of serum GDF-15 (or the other erythroid regulators) in the patients in the present study due to financial constraints and also because standardized assays for serum levels of these factors are not available.

In this study, it was found that serum iron levels were significantly lower in patients with ulcerative colitis; serum iron levels negatively correlated with those of CRP. This finding of hypoferremia in the presence of inflammation suggests the presence of anemia of chronic disease. However, serum CRP levels did not correlate significantly with serum hepcidin levels.

CONCLUSIONS

Conclusions

Serum hepcidin levels were significantly lower in patients with UC than in control subjects; they were also lower in UC patients who were anemic than in UC patients who were not. Serum hepcidin correlated positively with markers of iron status (iron and ferritin) but not with a marker of inflammation (CRP). Hence, it appears that when anemia and inflammation coexist, the influence of anemia on hepcidin predominated over that of the inflammation.

LIMITATIONS OF THE STUDY

Limitations of the study

1. The number of patients in the study was small (40 patients with UC and 19 controls).

2. Measurement of soluble transferrin receptor (sTfR) would have provided more information to help differentiate between anemia due to iron deficiency and anemia of chronic disease in the patients studied.

3. Measurement of serum levels of GDF-15, TWSG1, erythroferrone and IL-6 would have useful in elucidating the mechanisms that may be involved in hepcidin regulation in these patients.

It was not possible to carry out assay for the parameters mentioned above due to financial constraints.

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APPENDIX

Appendix 1 : Institutional Review Board Approval

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD
CHRISTIANMEDICALCOLLEGE,
BAGAYAM, VELLORE 632002, TAMIL NADU, INDIA

Ref: FG/8823/04/2014

October 16, 2014

Mr. Robby Pria Sundersingh
The Treasurer
Christian Medical College,
Vellore.

OFFICE OF THE TREASURER
20 OCT 2014
CMC, VELLORE - 632 004

Dear Mr. Robby Pria Sundersingh,

Sub: **Fluid Research grant project:**
Serum hepcidin levels in ulcerative colitis (UC)–do they Correlate with inflammation and/oranemia?
Dr. Jagadish. R, PG Registrar, Biochemistry, Dr. Molly Jacob, Biochemistry, Dr. Joe Varghese, Biochemistry, Dr. Ebby George Simon, Dr. Visalakshi Jeyaseelan, Biostatistics, CMC, Vellore.

Ref: IRB Min No: 8823 [OBSERVE] dated 07.04.2014

The Institutional Review Board at its meeting held on April 7th 2014 vide IRB Min. No. 8823 accepted the project for a sum of *1,00,000/- INR (Rupees One Lakh Only) will be granted for 2 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an 1st Installment. The rest of the 50,000/- INR (Rupees Fifty Thousand only) each will be released at the end of the first year as 2 nd Installment following the receipt of the Interim progress/ Annual report and subsequent submission of it to the IRB. If overspent the excess should be debited form the respective departmental or Special funds.* Kindly arrange to transfer the sanctioned amount to a separate account to be operated Dr. Jagadish. R and Dr. Molly Jacob.

Yours sincerely,

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

CC: Dr. Jagadish. R, Biochemistry, CMC
Dr. Molly Jacob, Biochemistry, CMC
File

22Y421.RF.Dr.Jagadish.R - Biochemistry (8823)

Appendix 2: Patient proforma

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 UC:

Drugs for anemia:

Iron supplements

EPO

Any other drugs:

Investigations:

Hb:

Serum iron:

Serum ferritin:

Transferrin saturation:

CRP:

Serum protein and albumin:

Stool examination:

Parasites:

Colonoscopy findings:

Upper GI endoscopy findings:

Biopsy reports:

Appendix 3: Information sheet for patients and informed consent form

Serum hepcidin 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 demonstrate the changes in levels of serum hepcidin that occur in patients with Ulcerative colitis. Anemia, a major complication of ulcerative colitis alters the serum hepcidin depending upon the cause. This affects the quality of life in these patients. How serum hepcidin levels in patients with ulcerative colitis is altered? 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 alters hepcidin levels 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 Jagadish R
Postgraduate Registrar
Department of Biochemistry
Christian Medical College, Vellore -632002
Contact number: 9843728514

Dr Molly Jacob
Professor
Department of Biochemistry
Christian Medical College, Vellore -632002

Dr Ebby Simon
Professor
Department of Gastroenterology
Christian Medical College, Vellore -632002

INFORMED CONSENT DOCUMENT FOR SAMPLE OF BLOOD

Dr. Jagadish R 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 4: Master sheet with data

CASES

S.No	Sex	Age	Hb	CRP	Ferritin	Iron	TIBC	TSAT	UIBC	Hepcidin
1	M	25	10.8	92.9	52.7	26	221	11.76	247	1.64
2	F	50	13.2	3.48	24.8	51	202	25.25	253	7.80
3	M	40	15.1	9.82	133.7	44	315	13.97	359	62.23
4	M	58	14.2	3.28	83.9	93	238	39.08	331	21.30
5	F	36	13.1	3.48	35.4	78	273	28.57	351	36.67
6	M	43	12.2	3.48	85.4	55	318	17.30	373	7.07
7	F	44	13.7	3.48	76.6	113	142	79.58	255	36.80
8	M	46	15.9	3.48	140.4	114	192	59.38	306	43.33
9	M	36	7.2	24.4	40.4	11	291	3.78	302	0.01
10	M	55	13.1	41.1	45.2	70	290	24.14	360	40.45
11	F	33	11.8	3.48	16.2	24	330	7.27	354	0.01
12	M	39	10	11.9	9.4	13	418	3.11	431	26.20
13	F	47	10	4.54	12.6	27	321	8.41	348	1.95
14	F	46	9.5	4.35	29.6	12	216	5.56	228	9.56
15	F	28	11.1	3.48	11.4	52	363	14.33	415	0.01
16	F	52	11.8	25.6	35.2	33	243	13.58	276	35.42
17	M	33	13.6	3.48	84.5	59	295	2.00	354	3.68
18	M	34	12.2	8.73	178.9	69	257	26.85	326	20.74
19	M	46	13.9	6.65	56.8	45	251	17.93	296	38.56
20	F	32	9.6	60	37.9	10	257	3.89	267	43.85
21	M	44	9.5	3.84	7.2	14	182	7.69	196	0.01
22	F	41	9.9	3.48	3.7	17	442	3.85	459	0.01
23	F	51	13.9	6.37	443.9	84	183	45.90	267	40.91
24	M	45	9.4	3.48	6.7	26	414	6.28	440	0.01
25	F	37	7.6	3.28	4.5	26	427	6.09	453	0.01
26	F	32	8.5	36	35.2	45	110	40.91	155	0.01
27	M	36	13.1	14.5	26.6	51	355	14.37	406	1.63
28	F	31	11.1	3.48	32	22	357	6.16	379	14.40
29	F	59	14.1	7.25	75.6	29	304	9.54	333	13.58
30	F	50	10.6	11.4	39.1	37	301	12.29	338	1.44
31	M	57	12.7	42.5	127.8	49	178	27.53	227	19.37
32	M	31	13.3	3.28	10	62	342	18.13	404	0.01
33	M	42	14.1	3.28	84.4	24	287	8.36	311	10.19
34	M	36	13.5	3.28	40.8	70	248	28.23	318	3.42
35	F	29	12.8	3.28	33.7	91	369	24.66	460	16.61
36	M	52	14.9	3.28	153.8	65	280	23.21	345	37.33
37	F	49	11.1	39.5	14	23	452	5.09	475	0.01
38	M	28	13.3	3.28	222.7	275	63	436.51	338	82.29
39	F	47	11.6	3.28	14.1	40	354	11.30	394	0.01
40	F	48	14.1	5.37	10.8	33	408	8.09	441	0.42

CONTROLS

41	F	44	13	0.1	6.4	53				47.9
42	M	33	15	2	46	92				21.6
43	M	57	14	0.8	115	113				1.94
44	M	35	14	0.4	165.9					18.17
45	M	21	15	0.8	15.6	123				26.98
46	F	31	13	3.3	16.6	73				52.1
47	M	63	14	1	86	94				36.36
48	M	48	13	1.3	12.9	71				21.61
49	M	56	12	3.4	118	55				11.2
50	F	29	14	3.6	39.8	52				9.27
51	F	17	13	0.1	24.4	74				14.2
52	F	39	12	0.1	47.7	94				52.1
53	F	27	13	0.3	34.8	81				85.14
54	F	28	11	3.4	96.6	68				16.11
55	M	36	14	1.8	77.2	46				15.14
56	M	51	13	0.5	42.8	105				28.49
57	M	40	13	0.2	46.2	124				56.81
58	F	53	13	5.4	10.4	61				56.81
59	F	42	13	4.6	24.8	43				36.36