Expression levels of duodenal proteins involved in iron absorption in patients with alcoholic liver disease

A DISSERTATION SUBMITTED TO THE TAMILNADU DR.M.G.R MEDICAL UNIVERSITY, CHENNAI, IN PARTIAL FULFILMENT OF THE REGULATIONS FOR THE AWARD OF M.D.DEGREE IN BIOCHEMISTRY (BRANCH XIII) EXAMINATION TO BE HELD IN APRIL 2013

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CERTIFICATE

This is to certify that the study entitled **"Expression levels of duodenal proteins involved in iron absorption in patients with alcoholic liver disease"** is the bonafide work of Dr. Kavita P Rasalkar has conducted the work under my guidance and supervision. 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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DEPARTMENT	:Department of College Biochemistry
NAME OF THE CANDIDATE	:Dr. Kavita P Rasalkar
DEGREE AND SUBJECT	:MD Biochemistry
NAME OF THE GUIDE	:Dr Molly Jacob
KEY WORDS	: alcoholic liver disease, alcoholic cirrhosis, coagulopathy, DMT1, Ferroportin, mRNA expression, serum iron serum ferritin

OBJECTIVES OF THE STUDY:

This study tested the hypothesis that alcohol ingestion causes up-regulation of proteins involved in duodenal iron absorption, leading to iron overload.

METHODS:

Patients with alcoholic cirrhosis (AC) (n = 24) were the subjects of the study. Such patients who were required to undergo a medically-indicated upper gastrointestinal endoscopy were recruited into the study after informed consent. Patients who underwent an endoscopy for investigation of dyspepsia and who were found to have no endoscopic abnormalities served as control subjects (n = 30). Duodenal mucosal and blood samples were obtained from the subjects. Duodenal tissue was used to determine mRNA levels of divalent metal transporter 1 (DMT1) and ferroportin, both of which are involved in non-heme iron absorption in the duodenum. Blood was used for estimation of serum C-reactive protein (CRP), ferritin, iron, haematological parameters and tests of liver function. Data were analyzed by student's t-test and analysis of variance or Kruskall-Willis and Mann-Whitneys test, as appropriate. A p value of less than 0.05 was taken to indicate statistical significance.

RESULTS:

Patients with AC had significantly lower levels of haemoglobin and serum iron and higher levels of serum CRP. Other haematological and iron indices were not significantly altered. Parameters of liver function were deranged in these patients. Levels of mRNA for DMT1 and ferroportin in the duodenal mucosa of these patients tended to be higher, when compared with control subjects; however, these increases were not statistically significant.

Gene expression of DMT1 and ferroportin tended to be higher in those with AC. However, these results need confirmation to ascertain whether this up-regulation may contribute to the iron overload associated with alcoholic liver disease.

INTRODUCTION

Iron is one of the physiologically important trace elements. The total amount of iron present in the body is in the range of 3-4 grams, of which around two-thirds is present in hemoglobin (Adamson, 2008). In the body, iron is present either as heme (attached to a porphyrin nucleus) or as iron-sulphur compounds. The largest consumer of iron in the body is the erythoid precursors in bone marrow (Cook et al; 1973). The rest is utilized by other cells for incorporation, as prosthetic groups, into proteins. In proteins, they may be either a structural component or a functional component. For example, they may form part of the active site of enzymes, of myoglobin (in muscle cells) and cytochromes involved in mitochondrial respiration and bio-transformation in the liver (Murray, 2009).

During fetal development, iron stores are developed by materno-embryonic and materno-fetal transfer. Post-natally, iron is obtained from the diet by absorption in the duodenum. Transferrin takes up the iron absorbed and transports it in blood. Iron is delivered to tissues requiring iron, especially immature erythroid cells (Cook et al; 1973). The major contributor to serum iron is iron recycled from macrophages, which is approximately 20-30 fold more than the amount of intestinal iron absorbed (Andrews, 1999). Senescent RBCs are phagocytosed by macrophages, which degrade hemoglobin, releasing iron which returns into the circulation. Excess iron is stored in the hepatocytes and macrophages as ferritin. Iron, once it enters the body, cannot be excreted either by the liver or kidney, as there is no regulated excretion of iron (Adamson, 2008). Iron is lost from the body only through exfoliation of skin and

mucosal cells, through faeces due to shedding of epithelial cells and through bleeding (Adamson, 2008).

Regulation of iron metabolism is very important, as excess iron leads to free radical formation and progressive organ damage (Murray, 2009b). Iron homeostasis in mammals involves complex regulation of transport, storage and utilization of iron (Hentze et al., 2004).

ABSORPTION OF IRON

Iron is indispensible for all living organisms. Organisms have to absorb iron from the diet and also maintain iron homeostasis in the body (Adamson, 2008).

Heme and non-heme iron are absorbed from the intestinal lumen across the apical membrane of duodenal enterocytes. It is exported out across the basolateral membrane of the enterocytes and enters the portal blood.

Iron deficiency can lead to anemia and growth arrest. Iron overload causes toxic free radical formation leading to progressive tissue damage (Adamson, 2008). Numerous evolutionally conserved physiological mechanisms have evolved for the maintenance of iron homeostasis. These are systems for environmental iron scavenging, and mechanisms for increasing iron solubility (Eisenstein, 2000). Dietary factors that increase iron solubility are ascorbic acid (which acts by reducing Fe^{3+} to Fe^{2+}) (Greenbeeg, 1957) and substances which increase the gastric acid secretion (eg. alcohol), which helps keep non-heme iron in a reduced state (Bezwoda et al, 1978;

Charlton, 1964). A diet rich in oxalates and phytates reduce the bioavailability of non-heme iron (Adamson, 2008).

SOURCES OF DIETARY IRON:

The dietary forms of iron are of two types: heme and non-heme (Bjorn-Rasmussen et al., 1974). At physiological pH, non-heme iron is insoluble in aqueous solutions (Huggins, 2006). Dietary non-heme iron is mainly present as ferric iron (Fe^{3+}) and as ferritin from plant sources. The major source of non-heme iron are plant sources, which includes dark green leafy vegetables, cereals, legumes, eggs and dried fruits. Iron from plant sources form weak complexes with phytates or tannins. This decreases their bio-availability (Adamson, 2008). Iron obtained from animal sources is mainly in the form of heme. Heme contains iron which forms a stable complex with a porphyrin nucleus (Kotani, 1969). Sources of heme iron are red meat, kidney, liver, pork, chicken and fish. The iron content in each type of food may vary, with highest amounts being present in red meat. Heme is well absorbed in the duodenum and is relatively unaffected by other food components (Lynch et al, 1985). Non-heme iron is much less bio-available compared to heme iron (McCance and Widdowson, 1942). Ferritin, though a non-heme iron form, is less influenced by other food components, as iron is locked inside a hetero-polymeric protein cage, thus preventing interaction with other dietary components (Theil, 2012).

PROTEINS INVOLVED IN IRON ABSORPTION:

Heme iron and non-heme iron absorption occurs via distinct pathways. The process of absorption of heme iron is not clearly understood. A heme transporter (heme carrier protein 1[HCP-1]) has been identified on the apical membrane of enterocytes

(Shayeghi et al, 2005). Once heme enters the enterocyte via this transporter, it is degraded by the action of heme oxygenase; the iron released has a similar fate as that of non-heme iron (Raffin et al, 1974), as described below.

Divalent metal transporter 1 (DMT1/SLC11A2/DCT1/Nramp2):

DMT1 is a protein involved in iron import into the cytoplasm of cells. It is a highly conserved member of the family of membrane-bound divalent cation transporters or the natural resistance-associated macrophage protein (Nramp) (Cellier et al, 1995). DMT1 was first cloned from rodents (Gruenheid et al, 1995).

Structure of divalent metal transporter 1:

DMT1 has 561 amino acids and 12 transmembrane domains, with an extra-cytoplasmic loop having 2 asparagine-linked glycosylation signals. It also has a membrane-targeting motif over the extracytoplasmic loop and a consensus transport motif, which is commonly present in other homologous cation transport proteins in other species. These transporters have a structure consisting of a conserved hydrophobic core encoding 10 highly conserved transmembrane segments with conserved intra- and extracytoplasmic loops. This evolutionary conservation proves the key role of these groups of proteins (Gunshin et al., 1997; Lee et al., 1998; Picard et al., 2000).

Expression of divalent metal transporter 1

DMT1 is expressed in all tissues. The tissues that abundantly express DMT1 are the duodenum, erythroid cells and liver. Kidney, brain, thymus, heart, lung and testis are other tissues that express DMT1 to a significant extent (Gunshin et al., 1997). In the

proximal duodenum, DMT1 is expressed by villous enterocytes but not by crypt cells (Canonne-Hergaux et al., 1999). It is expressed on the apical membrane of enterocytes but not in the lamina propria or goblet cells, in keeping with its role in luminal iron intake (Canonne-Hergaux et al, 1999).

Function of divalent metal transporter 1

DMT1 is a symporter. It transports ferrous iron and protons into the cell. It requires a low pH for its action (Gunshin et al, 1997). It acts as an apical transmembrane iron transporter in enterocytes and can transport reduced dietary non-heme iron into intestinal enterocytes (Gunshin, 1997). DMT1 is not specific for ferrous iron; it also transports a number of other divalent metals (Gunshin et al, 1997).

Non-heme iron is present in the diet as insoluble ferric iron complexes. Ferric iron has to be reduced to the ferrous form as the intestinal iron transporter, DMT1, can transport only ferrous iron (Gunshin et al., 1997).

Duodenal cytocrome b (dcytb):

The brush border surface of the duodenal mucosa has inherent ferric reductase enzyme activity (Pountney et al., 1999), due to presence of a protein, dcytb, which converts Fe^{3+} to Fe^{2+} (Raja et al., 1992; Andrew et al., 2001).

Structure of dcytb

Dcytb was identified by subtractive cloning strategy. It has 286 amino acids and six transmembrane domains (McKie et al., 2001). The enzyme shares homology with

heme-containing cytochromes, especially cytochrome b561, which also has ferric reductase activity (Altschul et al., 1997).

Expression of dcytb

Dcytb is mainly expressed in duodenal brush border cells, thus suggesting its involvement in iron uptake. Its main location is in mature enterocytes in the duodenum (McKie et al., 2001). Mature enterocytes are present in the upper villus region. This region forms the part with highest iron absorption activity (O'Riordan et al., 1995). Dcytb is not expressed in the crypt region and other parts of small intestinal mucosa (McKie et al., 2001).

It lacks an iron-responsive element in its mRNA sequence, which is important in the regulation of iron metabolism. However, iron levels in the body regulate the expression of dcytb in the duodenum (McKie et al., 2001).

No cases of dcytb mutations have been reported in humans. Genetic deletion studies in mice failed to show any major defects in iron absorption or utilization (Gunshin et al., 2005). This is probably due to presence of other reducing substances in the intestine, like ascorbic acid or H^+ ions.

The Fe^{2+} entering the cell can undergo one of two fates, depending on intracellular requirement of enterocytes. It can either be stored in the enterocytes as ferritin, or can be transferred into plasma which is done with the help proteins ferroportin and Hephaestin present at the basolateral membrane (Pierre et al, 2002; Donovan et al, 2005).

Ferroportin (FPN/ SLC40A1/ IREG1/MTP1):

Ferroportin belongs to the metal transporter protein 1 family (Abboud and Haile, 2000; McKie et al., 2000).

Structure of ferroportin

Ferroportin has 581 amino acids and 10-12 transmembrane domains (Donovan et al., 2005). Each transmembrane domain has a length of 18-12 residues, with the fourth domain being slightly longer. They have 12 cysteine residues with disulphide bonds within the lipid bilayer and on the surface (Devalia et al, 2002). Ferroportin forms a functional dimer (De Domenico et al., 2010).

Expression of ferroportin

High expression of ferroportin is found in macrophages, duodenal mucosal cells, hepatocytes, Kupffer cells and placental syncytiotrophoblast cells (Abboud and Haile, 2000). In the duodenum, ferroportin is expressed only in mature absorptive epithelial cells of villi and is present on the basolateral surface of enterocytes (Abboud and Haile, 2000). Ferroportin expression is low in most other cells, including erythriod cells.

The gene for ferroportin is regulated by iron regulatory proteins (IRPs) that bind to iron-responsive elements(IRE) present on the 5' untranslated region (UTR) of ferroportin mRNA (Sanchez , 2006).

Functions of ferroportin

Ferroportin is a protein involved in efflux of ferrous iron (Fe^{2+}) from cells. In the duodenum, due to its location on the basolateral membrane of enterocytes, it helps in efflux of iron from enterocytes in to blood (Murray, 2009).

Ferroportin is also the receptor for hepcidin, an iron regulatory hormone (Nemeth et al., 2004). Hepcidin binds to ferroportin and stimulates its endocytosis and proteolysis in lysosomes (Nemeth et al., 2004). The mechanisms underlying the internalization and ubiquitination of ferroportin are not entirely elucidated. However, it is thought that hepcidin binds to the extracellular loop of ferroportin at amino acids 320-350, between the fifth and sixth loops (De Domenico et al., 2008b). Hepcidin binding to ferroportin leads to binding of the protein kinase, Janus kinase 2 (Jak2), to ferroportin. This causes auto-phosphorylation and activation of the bound Jak2. Activation of Jak2 results in the phosphorylation of two adjacent tyrosine residues in the cytosolic loop of ferroportin, resulting in internalization of the hepcidin-ferroportin complex (De Domenico et al, 2009). The net result is that release of iron from cells is inhibited. Evidence from various studies strongly suggests that the hepcidin-ferroportin axis is active in macrophages but the effect of hepcidin on ferroportin in duodenal enterocytes is less convincing (Yamaji et al., 2004; Chaston et al., 2008).

Hephaestin (hp)

Hephaestin is a membrane-bound protein on the basolateral surface of enterocytes in the small intestine. It has ferro-oxidase activity (Syed et al 2002; De Domenico et al., 2007) and it plays a major role in intestinal iron absorption (Vulpe et al., 1999; Chen, 2004). It is involved in iron efflux from enterocytes. It requires copper for its structural

and enzymatic activity (Vulpe et al., 1999; Syed et al 2002; Chen, 2004). Ferroportin favours the transport of only ferrous iron (Murray, 2009). Transferrin, required for transport of iron, can take up iron only in the ferric form. Hephaestin oxidizes ferrous iron into ferric iron. The ferric iron is then loaded on to transferrin and transported in circulation (Murray, 2009). The iron thus circulates bound to transferrin; tissues take up transferrin and utilize the iron (James and Jandl, 1963)

Figure 1 show the processes involved in the absorption of iron (heme and non-heme) by duodenal enterocytes, its storage and transfer across the basolateral membrane.

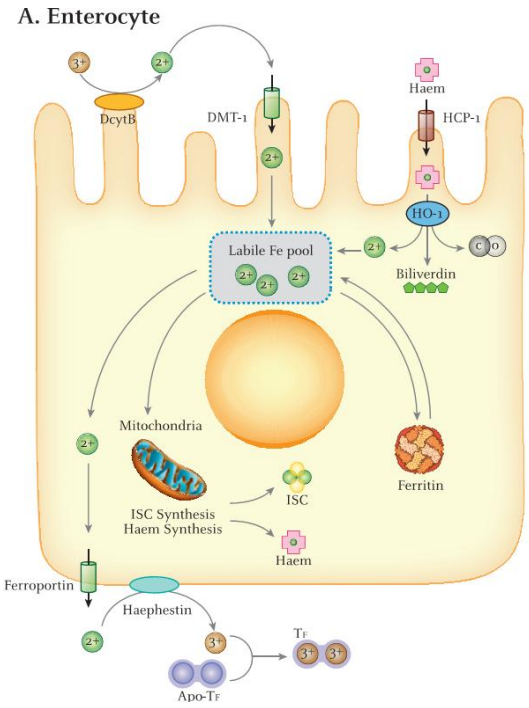

Figure 1: Processes involved in absorption of iron in the duodenum.

(Adapted from **Evstatiev and Gasche. Gut, 2012)**

TRANSPORT OF IRON

Transferrin and transferrin receptor

Iron is transported in blood by transferrin. Normally, iron occupies 30% of the iron-binding sites on transferrin (Adamson, 2008).

Structure and localization:

Transferrin is a dimeric plasma glycoprotein of 80 kDa, synthesized by the liver (McPherson, 2009). It has a high affinity for Fe^{3+} and reversibly binds two atoms of Fe^{3+}. Each monomer of transferrin attaches to Fe^{3+} to form holo-transferrin. Levels in serum are inversely proportional to body iron status. Loaded transferrin circulates in the blood, delivering iron to various tissues that are in need of iron. It binds to transferrin receptors on the cell surface (Adamson, 2008). The transferrin receptor is an integral membrane protein of 90kDa (Aisen and Hu, 1978). It is a homodimer. Each monomer of transferrin receptor binds a single holo-transferrin (Adamson, 2008).

Transferrin receptors are of types 1 and 2 - TfR1 and TfR2. TfR1 is involved in receptor- mediated uptake of iron by cells (Ponka et al., 1998). TfR2 can facilitate cellular iron uptake from holo-transferrin, but is not sufficient in absence of TfR1 (Levy, 1999). TfR2 is homologous to TfR1 (Kawabata et al, 1999). It is mainly expressed in hepatocytes, duodenal crypt cells and erythroid cells (Kawabata et al, 2001). TfR1expresssion is seen mainly in erythroid cells. It is up-regulated during erythroid development, while TfR2 is mainly expressed in liver and is up-regulated during liver development. This reflects different functions of the 2 types of tranferrin receptors (Kawabata et al, 2001). TfR1 is mainly involved in transferrin-mediated iron uptake into cells. TfR2 is mainly involved in regulation of iron metabolism (Kawabata

et al, 2001) by a HFE-mediated pathway involved in synthesis of hepcidin. The affinity of TfR2 for transferrin is 30 times lower than that of TfR1 (Kawabata et al, 2000; West et al., 2000).

Regulation of synthesis of transferrin receptors

Cellular iron levels regulate the synthesis of transferrin receptors, by post-transcriptional mechanisms. A high cellular demand for iron increases the transferrin receptor pool. Post-transcriptional control is mediated through binding of iron regulatory proteins (IRP) to iron-response elements (IRE) at the 3' un-translated region of mRNA for TfR1. This binding of IRP to 3' IRE stabilizes mRNA against degradation. TfR2 expression, in contrast to TfR1, is not regulated by IRE/IRP regulatory system (Kawabata et al, 1999).

Studies have shown that knocking out transferrin and transferrin receptors resulted in severe neurological defects, due to iron deficiency during brain development. Severe iron deficiency was found in erythroid cells, leading to severe anaemia and death. Other tissues showed normal development, suggesting that these tissues might have mechanisms for iron uptake other than via transferrin receptors (Andrews and Schmidt, 2007).

IRON UPTAKE, STORAGE AND UTILIZATION

Immature erythrocytes are major users of iron, using about a billion iron molecules per day for hemoglobin synthesis (Andrews Schmidt, 2007). Uptake occurs via transferrin receptor 1(TfR1). Holo-transferrin binds to TfR1. The complex is internalized by receptor-mediated endocytosis (Ponka et al. 1998). The internalized endosome complex is merged with a lysosome. The resultant acidic pH created inside the endosome reduces the affinity of the transferrin receptor for the iron-transferrin complex, leading to its disintegration (Sipe and Murphy, 1991), releasing transferrin and ferric iron into the lumen. The transferrin receptors return to the surface of the plasma membrane for re-use. STEAP 3 (six transmembrane epithelial antigen of the prostate 3), a protein present on the endosomal membrane, reduces the iron from Fe^{3+} to Fe^{2+} (Mc Kie et al 2005; Ohgami et al., 2005) the ferrous iron moves into the cytoplasm with the help of Nramp 2/DMT1, an endosomal membrane protein. Figure 2 shows the process of transferrin uptake by cells. The iron is utilized for various intracellular requirements. The ferritin present in the cells takes up the excess iron, thus storing the iron for future use (Higgins, 2006).

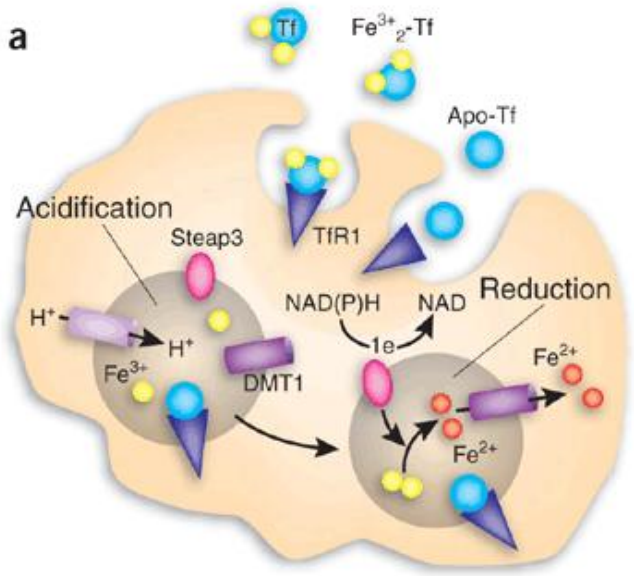

Figure 2: Transferrin cycle

(Adapted from **Mckie 2005 *Nature Genetics*)**

Ferritin:

Ferritin was the second of all proteins to be crystallized in 1937 (Laufberger et al., 1937). It is an iron storage protein. It is important in iron homeostasis. Free iron can generate reactive species with the potential to damage DNA and proteins (Higgins, 2006). Thus, the intracellular iron pool has to be buffered; this process is critical for cell survival (Higgins, 2006).

Expression and structure of ferritin

Ferritin is ubiquitously expressed in the body and is found in cells and in serum (Higgins, 2006). It is highly conserved among a wide variety of species. Its cytosolic form in vertebrates forms heteropolymers consisting of 2 subunits, the heavy (H) subunit and the light (L) subunit (Higgins, 2006). L monomers have a molecular

weight of 18.5 kD with 174 amino acids, while H monomers have a molecular weight of 21kD with 182 amino acids. Light and heavy ferritin subunits are homologous, but ferroxidase activity is present only in H-ferritin, which is essential for iron incorporation into the protein (Higgins, 2006). Figure 3 shows the structure of ferritin. The proportion of H and L subunits expression varies widely in different tissue. The L form predominates in liver and spleen, and H form predominates in heart and kidney (Higgins, 2006).

Apoferritin forms a sphere with an internal space for ferric iron (stored as ferrihydrite mineral), to form holoferritin (Higgins, 2006). One apoferritin molecule has a molecular weight of 450kD. Twenty four subunits (maxiferritin), with H and L chains, assemble to form a hollow, symmetrical apoferritin shell (Higgins, 2006). Each shell can sequester 4500 iron atoms (Harrison, 1996). Mineralization of the shell reflects iron present in the cell; mineralization of the apoferritin shell is normally well below its maximum capacity. Normally, the average amount of iron per ferritin shell is between 1000 to 1500 iron atoms, but can also vary from 0 to 2500 iron atoms per shell (Harrison et al., 1974). In iron overload associated with hemosiderin, toxic levels of iron occur in cells, with average number of iron per ferritin shell being 3000-4000 (Harrison et al., 1974). The extracellular form is present in serum and is used as an important clinical marker of iron status (Higgins, 2006).

Function of ferritin

Free iron is toxic in cells. Iron sequestration is the primary role of ferrritin. It has ferrioxidase activity (Higgins, 2006). It converts Fe^{2+} to Fe^{3+} and helps in its internalization and sequestration in the ferritin mineral core (Higgins, 2006). The

ferroxidase catalytic subunit is the H subunit with multiple active sites in the interior (Higgins, 2006). Fe^{2+} substrates enter the protein cage from the cytoplasmic side. The catalytic product, peroxidiferric intermediate, is released to the interior pore for nucleation (Pereira et al., 1998). The uniqueness of ferritin is that it is the only protein which acts on its substrate and also stores it (Munro, 1986).

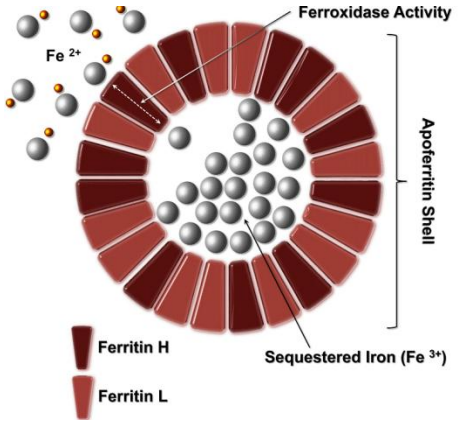

Figure 3: Structure of ferritin

(Adapted from **Knovich, et al. Blood Rev. 2009)**

The main storage sites for iron in the body are liver parenchymal cells and macrophages (Higgins, 2006). Iron stored in hepatocytes is acquired either from transferrin or from non-transferrin bound iron (NTBI). NTBI is particularly important in iron overload states (Andrews and Schmidt, 2007). If serum iron levels exceed transferrin-binding capacity, then iron is found as NTBI (Andrews and Schmidt, 2007). Iron exist as low molecular weight complexes with citrate (as iron-citrate) or as a ternary complex as iron-citrate-acetate complex (Grootvel et al, 1989). Other tissues also can acquire NTBI by transferrin-independent pathways. NTBI uptake is mainly thought to occur via L-type calcium channels (Oudit et al., 2003) and TRPC6 (transient receptor potential canonical protein) (Mwanjewe and Grover, 2004). Liver is

exposed to NTBI more than other tissues, as the portal circulation misses first-pass metabolism (Andrews and Schmidt, 2007).

IRON CYCLE

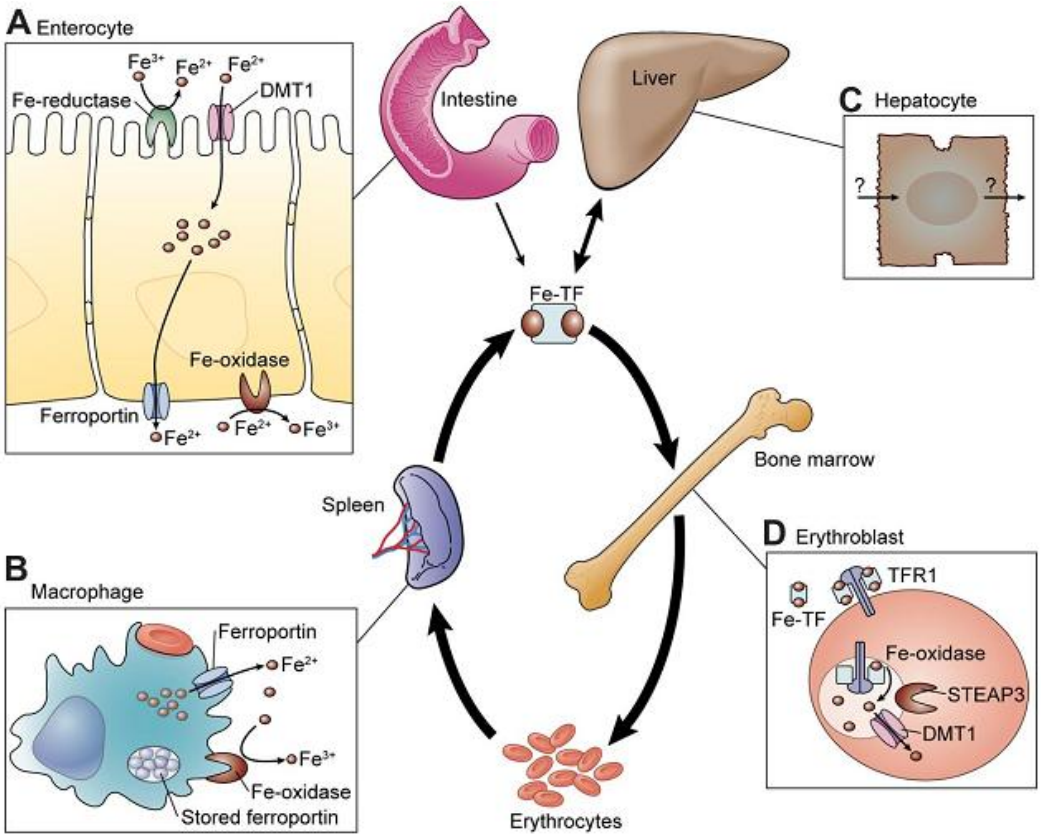

Figure 4: Iron cycle in the body

Adapted from Andrews: Blood, 2008.

Serum iron is derived from two sources, intestinal absorption and iron recycling from macrophages. Recycling from macrophages contributes 20-30 times more iron than from intestinal absorption. Macrophages phagocytose senescent red blood cells (RBC) (Andrews, 1999). In phagolysosomes, the RBCs are lysed and the hemoglobin is released from these cells. The globin is degraded into individual amino acids. Heme is degraded into the porphyrin ring and Fe^{3+}. The iron is reduced to Fe^{2+} form by STEAP 3 present on the membrane of phagolysosomes. It is transferred into the cytoplasm via

the protein divalent metal transporter 1(DMT 1) (Gunshin et al, 1997, Ohgami et al, 2005). Iron is then either stored as ferritin or utilized for cellular needs. Excess iron can be transferred to the plasma by the help of ferroportin present on the surface of macrophages. The Fe^{2+} form has to get oxidized to be loaded on to transferrin (Murray, 2009). This oxidization is effected by ceruloplasmin, a copper-containing protein, homologous to hephaestin (Murray, 2009). Iron in transferrin is taken up by the cells that need it; mainly immature erythrocytes (Higgins, 2006).The iron cycle is depicted in Figure 4

REGULATION OF IRON METABOLISM

Regulation of iron homeostasis in the body occurs at the level of the cell and also in the body as a whole. Cellular iron uptake is proportional to the number of transferrin receptors on the cell surface. Within the cell, the main player involved in regulation of the number of transferrin receptors is the IRP/IRE (iron regulatory proteins/ iron responsive element) system. This system senses cellular iron levels and plays a role in increasing or decreasing the number of transferrin receptors on the cell surface. It also plays a role in regulation of iron storage and absorption (Sanchez, 2006).

Iron responsive element (IRE) and iron regulatory proteins (IRP)

IRPs are iron-sensing proteins. In iron-depleted states, IRP binds to IRE, which is found in the un-translated region (UTR) of mRNAs of iron transport and storage proteins (Leibold and Munro, 1988). Iron-sulphur clusters are incorporated into IRP1, making it an iron sensor (Rouault et al, 1991). With 4Fe-4S cluster formations, IRP cannot bind to mRNA (Haile et al., 1989). This has dual effects, depending on the location of the IRE. Binding of IRP to IRE in the 5' un-translated region (5' UTR) of

mRNA for ferritin, ferroportin and aminolevulinate synthase (involved in heme synthesis) blocks initiation of translation (Sanchez, 2006). Five tandem IRE on the 3'UTR of TfR1 mRNA bind to IRP, stabilizing the mRNA (by inhibiting nuclease digestion); this allows synthesis of TfR1 required for iron uptake in iron-depleted states (Rouault et al.,1992). Thus, this post-transcriptional regulation by cellular iron status controls expression of proteins involved in iron acquisition (TfR1 and DMT1), storage (ferritin), utilization (ALA synthase), and export (ferroportin). Thus, the IRP/IRE system acts as a sensor of cellular iron levels and regulate homeostatic mechanisms accordingly (Sanchez, 2006). Figure 5 depicts these processes.

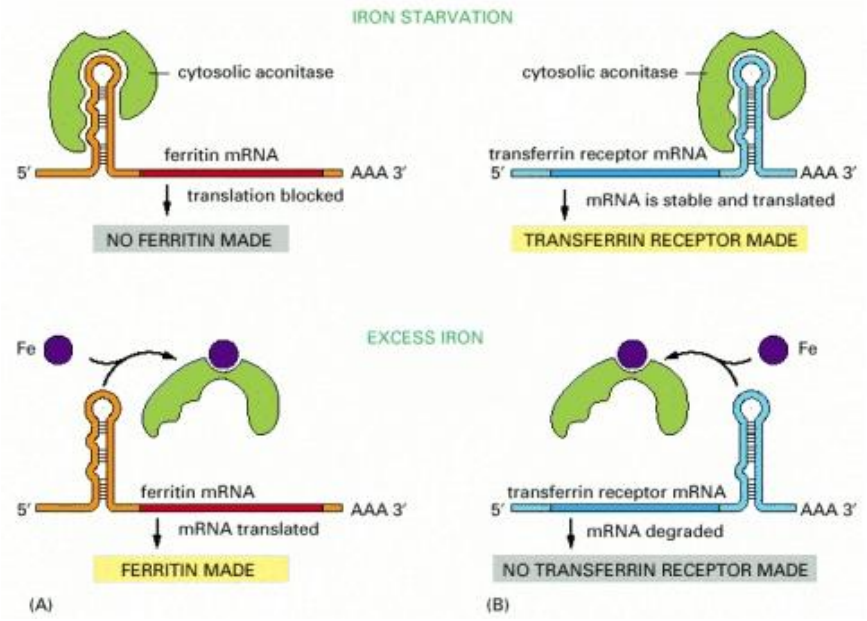

Figure 5: Regulation of synthesis of iron-related protein by iron.

(Adapted from **Molecular Biology of the Cell, 4th edition. Alberts et al)**

Systemic iron regulation is mainly dependent on hepcidin. This is an anti-microbial peptide that was independently discovered from plasma ultra-filtrates and human urine (Krause et al, 2000; Park et al, 2001). It is a hormone involved in iron homeostasis

(Pigeon et al 2001, Nicholas et al 2001; Fleming and Sly 2001). It is a highly conserved peptide. It is synthesised by the liver and is encoded by the HAMP gene on chromosome 19q. It is synthesised as inactive pre-prohormone of 84 amino acids, containing a signalling peptide and as a prohormone with 60-amino acids (Park et al, 2001). Furin, a prohormone convertase, cleaves the prohepcidin to generate hepcidin, which has 25 amino acids (Park et al, 2001). Hepcidin circulates in plasma and is filtered by the kidneys (Krause et al, 2000; Park et al, 2001).

Structure of hepcidin

Hepcidin contains 25 amino acids. It has eight cystiene residues with four disulphide cross-links and a beta hairpin structure (Park et al, 2001). It has a highly conserved N-terminal arm, essential for the hormone activity. The only proved target for the action of this hormone is ferroportin, which is involved in iron transfer from cells into the plasma (Nemeth et al., 2004). Figure 6 shows the structure of hepcidin.

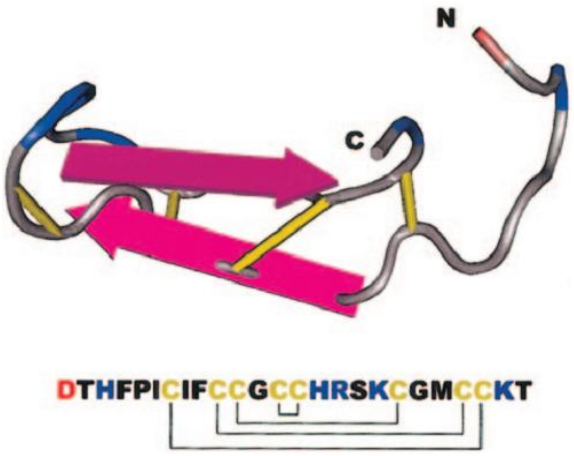

Figure 6: Structure of hepcidin

(Adapted from **Ganz T. Blood. 2003)**

The role of hepcidin in iron metabolism was first suggested by Pigeon et al. (2001). It is now known to be a negative regulator of serum iron levels. It acts by decreasing iron efflux out of cells. It does so by binding to ferroportin (Nemeth et al., 2004). The ferroportin-hepcidin complex is internalized, decreasing iron efflux from intestinal cells and the macrophages (Nemeth et al., 2004). Hepcidin deficiency has been shown to result in increased levels of ferroportin in the cell membrane, leading to increased iron entry into the body and resultant iron overload (Nemeth et al., 2004). Hepcidin causes internalization of ferroportin and thus reduced iron release from macrophages (Delaby et al., 2005). It restricts intestinal iron absorption (Laftah et al, 2004). Mutations in hepcidin cause iron-overload conditions (Nicolas et al, 2001).

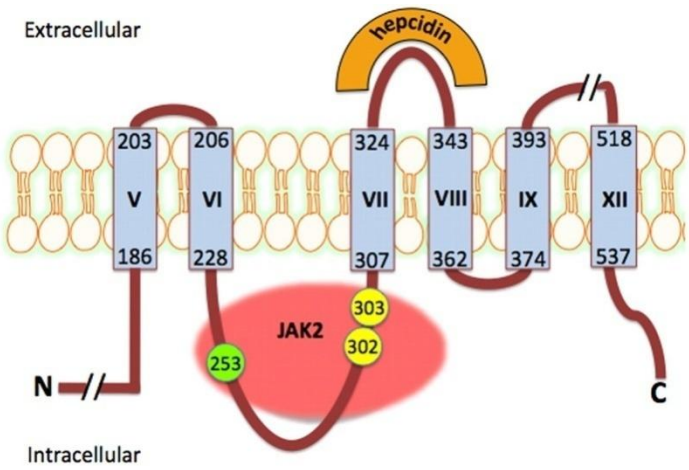

Figure 7: Regulation of ferroportin expression by hepcidin

(Adapted from Kushner Blood 2010)

Regulation of hepcidin

Hepcidin expression is regulated by several factors, such as the iron status of the body and inflammation. The main regulatory pathways for hepcidin synthesis involve bone morphogenic proteins (BMP) and TfR2. BMP is a member of the transforming growth factor beta superfamily. Hemojuvelin, a BMP co-receptor, interacts with

BMP6 and with BMP receptors, types 1 and 2 (Xia et al., 2008). The formation of this complex triggers the SMAD signalling cascade, by phosphorylation and activation of SMAD 1, 5, and 8. The SMAD 1, 5, 8 complex combines with SMAD 4, which acts as a transcription factor for enhancing hepcidin transcription (Wang et al., 2005; Truksa et al, 2006).

Transferrin receptor 2 (TfR2) is expressed 5 times more in the liver than TfR1. It acts as a regulator of hepcidin synthesis. HFE protein is a transmembrane protein on hepatocytes. TfR1 serves to sequester HFE, thus preventing its interaction with TfR2 **(**Lebron et al., 1998). This decreases the transcription of hepcidin. In iron-replete states, holo-transferrin binds to TfR1. This displaces HFE. HFE now interacts with TfR2; leading to SMAD activation, increasing the transcription of hepcidin, leading to decreased serum iron levels (Schmidt et al 2008). IL6 is also involved in transcriptional activation of hepcidin **(**Nemeth et al, 2004a**)** by the STAT pathway (Wrighting et al, 2003). The pathways involved are depicted in Figure 8.

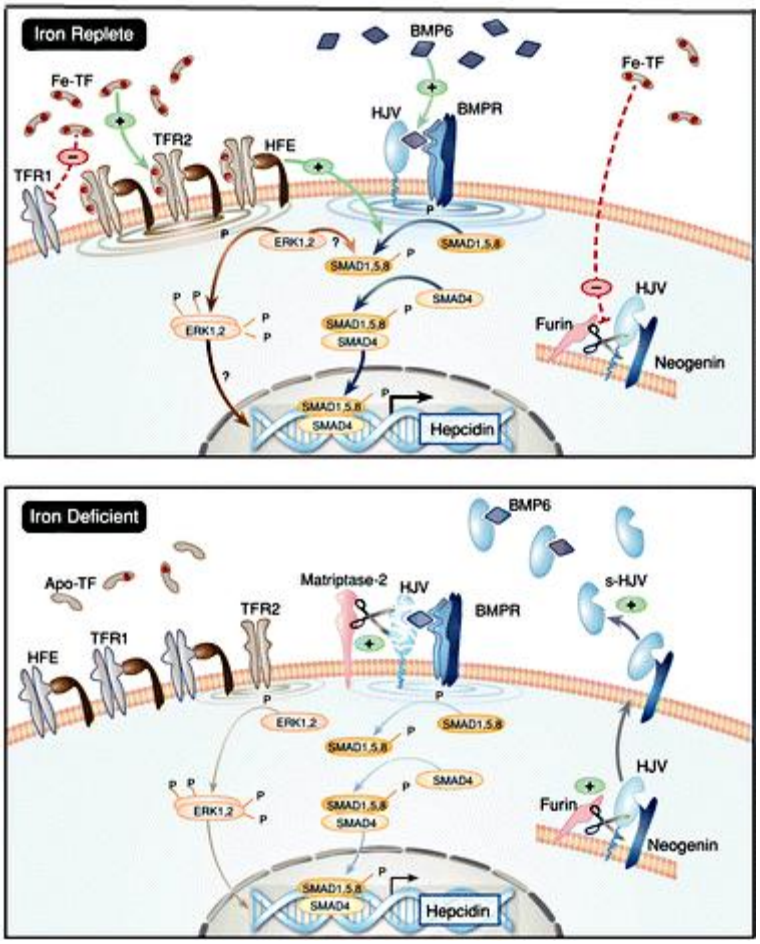

Figure 8: Regulation of hepcidin synthesis

(Adapted from **Knutson 2010)**

THE PROBLEM OF ALCOHOLISM AND ITS ILL-EFFECTS ON SOCIETY AND HEALTH

Alcohol has been a part of human culture since the beginning of recorded history (Rehm et al, 2009). It is the most socially accepted addictive drug in India and the world. Alcohol abuse and dependence causes social problems, such as domestic violence and loss of productivity in work places, as well as traffic accident-related injuries and chronic organ disorders (Ji, 2012).

The liver is the organ most affected by alcohol, leading to alcoholic liver disease (ALD). ALD consists of a spectrum of pathological changes ranging from fatty liver and alcoholic hepatitis to cirrhosis (Schuckit, 2008). Alcoholism is a leading cause of cirrhosis in the world (Mailliard and Sorrell, 2008).

The liver is the organ involved in biotransformation of toxic substances in the body. Oxidative stress and lipid peroxidation, resulting in cell injury, occur in the liver in response to iron and alcohol (Frierman et al, 1985; McCord, 1998). Alcohol is metabolised in the liver by alcohol dehydrogenase and cytochrome P4502E1 (CYP2E1) (Schuckit, 2008). The hepatotoxicity of alcohol is due to alcohol-induced oxidative stress. Activation of CYP2E1, NADPH oxidase and changes in mitochondrial function have been reported to cause oxidative stress (Lu and Cederbaum, 2008; Bardag-Gorce et al, 2006; Kessova and Cederbaum, 2003; Cederbaum, 2006). Previous studies have shown that, despite heavy consumption over longer time periods, only a small number of alcoholics actually develop liver disease (Friedman, 2012). This suggests that other factors may be involved in the process of

alcohol-mediated liver damage. One such possible factor is iron (Friedman et al., 1980).

Iron is a transition metal that takes part in the Fenton reaction, leading to free radical-mediated injury. Derangements in iron homeostasis have been associated with alcoholism (Chapman et al, 1982; Irving et al, 1988). Changes in the iron status of alcoholic patients range from anemia to iron overload (Ioannou et al, 2004). The anemia seen in these patients is mainly due to poor nutrition and gastrointestinal bleeding (Kimber et al, 1965). At the stage of cirrhosis, the functioning of the liver is compromised, leading to various complications, including bleeding tendencies. It has been shown that well-nourished alcoholics do not have iron deficiency or anemia. This suggests that alcoholism per se is not the cause for the anemia (Lindenbaum, 1969**;** Lindenbaum et al, 1980, Sheehy et al, 1960).

Alcoholic beverages contain high contents of iron. Studies have shown high amounts of iron in wines all over the world (Charlton et al., 1964). Alcoholic beverage consumption, even in small to moderate quantities, is associated with iron overload in liver, as evidenced by elevated indices of iron stores (Ioannou et al., 2004). Advanced alcoholism is one of the major causes of hepatic iron overload in the world (Irving et al, 1988). Studies have shown that iron overload is more severe, with earlier onset, in hereditary hemochromatosis in alcoholics than in non-alcoholics (Fletcher et al, 2002). Higher mortality rates are associated with alcoholic cirrhosis in the presence of increased hepatic iron content, suggesting that iron has a role in exacerbating alcoholic liver disease (Ganne-Carrie et al., 2000; Harrison Findik et al., 2006). Whole body retention studies done in alcoholics have shown a two-fold increase in intestinal iron

absorption, suggested to be due to increases in intestinal permeability (Duane et al, 1992). Alcohol has also been shown to induce the transfer of iron and endotoxins from the intestine into the circulation (Tamai et al., 2000).

Iron is stored in the liver, with the hepatocytes being the primary site of storage. Injury to hepatocytes leads to leakage of iron and results in increased serum iron levels (Harrison Findik et al., 2007). In alcoholic liver disease, iron deposition in the liver is seen in parenchymal cells and Kupffer cells. In mild ALD, iron deposition is more prominent in parenchymal cells than in reticuloendothelial cells (REC) in liver. The increase deposition of iron in these cells is due to increased uptake of iron from transferrin in circulation, thus triggering alcohol-mediated free radical injury leading to hepatocyte injury (Suzuki et al, 2002; Kohgo et al, 2005). In severe ALD, iron deposits are more prominent in the REC, due to endotoxemia and over-production of inflammatory cytokines (Kohgo et al, 2005). Studies in mouse models of ALD suggest that pro-inflammatory cytokines increase the iron content in Kupffer cells, thus activating transcription factor nuclear factor –kappa B (NF-kB) (Harrison-Findik et al., 2009). These effects were abolished after iron chelation (Harrison Findik et al., 2007a).

EFFECT OF ALCOHOL ON IRON HOMEOSTASIS

Studies suggest that beverages with high alcohol content increase iron absorption due to stimulation of hydrochloric acid production in the stomach (Charlton et al, 1964). Hepcidin synthesis in the liver has been reported to be down-regulated in ALD (Bridle et al, 2006; Harrison Findik et al., 2006). According to a recent study on human subjects, liver hepcidin levels were significantly lower in alcoholics, when compared with non-alcoholic subjects, with the highest suppression of hepcidin mRNA seen in heavy drinkers (Costa-Matos et al, 2012).

This down-regulation of hepcidin was seen despite iron overload in these patients, suggesting that hepcidin was no longer sensitive to body iron levels. Normally, hepcidin protects the body against iron overload by inhibiting intestinal iron absorption and iron efflux from macrophages. Alcohol intake compromises the body's defence against iron overload (Harrison Findik et al., 2007a). In vitro studies have shown that alcohol does not alter the expression of TfR1, ferritin, IRP1 and 2, suggesting that the effect of alcohol on hepcidin is a direct one and not an indirect one due to the altered iron status of the cell (Harrison Findik et al., 2006).

A mechanism involved in alcohol-mediated hepcidin suppression is thought to involve induction of oxidative stress (Harrison Findik et al., 2007a). Antioxidants have been shown to reverse the effect of alcohol on hepcidin in the liver and DMT1and ferroportin expression in enterocytes (Harrison Findik et al., 2007a). This suggests a major role of oxidative stress in regulation of hepcidin synthesis. Reactive oxygen species are generated during metabolism of alcohol. C/EBPα (CCAAT/enhancer-binding protein alpha) is a promoter of hepcidin mRNA (Courselaud et al., 2002).

Alcohol has been shown to down-regulate hepcidin promoter activity and the DNA-binding activity of C/EBPα (Harrison Findik et al., 2006).

Animal models of ALD have shown that down-regulation of hepcidin occurred in hepatocytes, with up-regulation of ferroportin and DMT1 in enterocytes and macrophages (Bridle et al, 2006; Harrison Findik et al., 2006). Significant suppression of hepcidin mRNA in liver by alcohol was seen in a study with human subjects (Costa-Matos et al, 2012).

HYPOTHESIS OF THE STUDY

Alcoholic liver disease has been reported to be associated with liver iron overload. The hypothesis of this study is that alcohol ingestion results in increased expression of duodenal proteins involved in absorption of non-heme iron and may contribute to the hepatic iron overload seen.

AIM OF THE STUDY

The aim of this study was to determine mRNA expression levels of duodenal proteins that are involved in absorption of non-heme iron, in patients with alcoholic cirrhosis. The proteins studied were divalent metal transporter1 (DMT1) and ferroportin (FPN)

MATERIALS AND METHODS

MATERIALS AND METHODS

a. EQUIPMENT USED

1. Elix and Milli-Q ultrapure water systems (Millipore, USA)

2. pH meter (Systronics, India)

3. Tabletop centrifuge (MPW R 350, MPW Poland)

4. Glass homogenizer with Teflon pestle (1 ml capacity) (Kimble-Kontes, USA)

5. Horizontal gel electrophoresis system (Broviga, Balaji Scientific Services, Chennai)

5. Gel documentation system (Alpha Innotech, USA)

6. Real-time thermo cycler (Chromo4, Biorad, USA)

b. CHEMICALS AND REAGENTS USED

1. Nucleopore RNA isolation kit (Genetix Biotech Asia Pvt Ltd. New Delhi, India).

2. Diethyl pyrocarbonate (DEPC), ethidium bromide, ethylene diamine tetraacetic acid (EDTA), formamide, formaldehyde, bromophenol blue and sodium hydroxide were obtained from Sigma, India.

3. Sodium acetate was obtained from Sisco Research Laboratories Pvt Ltd, Mumbai, India.

4. Absolute alcohol was obtained from Hayman Ltd, England.

5. Agarose was obtained from Genei, Bangalore, India.

6. 3–morpholinopropane sulfonic acid (MOPS) was purchased from Fluka Biochemika, from Sigma, Taiwan.

7. Reverse transcription core kit, SYBR Green PCR master mix kit and gene-specific primers were obtained from Eurogentec, Belgium.

All chemicals used were of analytical grade.

c. MISCELLANEOUS CONSUMABLES USED

1. Glass vacutainer blood collection tubes were obtained from BD Biosciences, Plymouth, UK.

2. Cryovials were purchased from Axygen, India, microtubes from Tarsons, India and filter tips from Molecular Bio-Products, India.

d. SUBJECTS

Patients diagnosed to have cirrhosis of alcoholic aetiology, who presented to the Department of Hepatology, Christian Medical College, Vellore, and who was required to undergo a medically-indicated upper gastrointestinal endoscopy as a part of their clinical management, were the subjects of the study. They were recruited into the study, after taking informed consent, if they fulfilled the following inclusion and exclusion criteria:

Inclusion criteria

1. Patients of both genders of ages between 30-65 yrs
2. A history of intake of 20-30 gm (2 -3 standard drinks) of alcohol per day for 5 or more years
3. Diagnosis of cirrhosis based on clinical and/or ultrasonographic evidence or those with portal hypertension and compensated liver disease

Exclusion criteria

1. Not willing to take part in the study
2. An international normalized ratio (INR) above 1.5
3. Evidence of any other co-existent liver disease
4. Evidence of viral infection as a cause of liver disease

Patients with dyspepsia presenting to the Department of Gastroenterology, Christian Medical College, Vellore, who had normal liver function tests and who were required to undergo a medically-indicated upper gastrointestinal endoscopy as a part of their clinical management, and in whom no abnormalities were found on endoscopy, were used as control subjects.

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

INFORMED CONSENT

Informed consent was sought from each subject before recruitment into the study. The investigator explained to the patient and his/her relatives what the study entailed and what was required of him/her. This was done in a language that the patient and his/her relatives understood well. They were provided with an information sheet as well. These sheets were made available in 5 different languages (Hindi, Bengali, English, Tamil and Telugu), to cover the languages most commonly spoken by patients who are seen at Christian Medical College, Vellore. After the explanation, patients were required to sign an informed consent form. They were recruited into the study only after these formalities were completed. A copy of the information sheet and consent form is included in Appendix I.

PATIENT DATA

A proforma was prepared to collect relevant patient data. This is included in Appendix II. The investigator elicited relevant history from each patient. Socio-demographic details and clinical data for each patient were obtained from their hospital records.

SAMPLE SIZE CALCULATION

Sample size calculations have been done with alpha error set at 5% with power of the study at 80%. Based on the results obtained in a previous study (Barton et al, 2003), sample size calculations were done and found to be 30 subjects in each arm of the study, ie 30 patients with alcoholic cirrhosis and 30 control subjects.

SAMPLE COLLECTION

When undergoing the medically-indicated upper gastrointestinal endoscopy, a sample of mucosal tissue from the second part of duodenum was obtained from all patients who were recruited into the study. The tissue was immediately snap-frozen and transported to the laboratory in liquid nitrogen. A blood sample (about 3ml) was also collected from each patient. The blood sample was used to obtain serum, which was used to estimate levels of ferritin, iron and C-reactive protein (CRP).

ESTIMATION OF SERUM FERRITIN

Serum ferritin was estimated by an automated chemiluminesence assay.

Analyser: Siemens, ADVIA Centaur system Xpi, UK.

Principle of the method:

The method used a two-site sandwich immunoassay, using direct chemiluminesence technology. Two antibodies were used in the assay. The first antibody was a polyclonal anti-ferritin goat antibody labelled with acridinium ester and the second was a monoclonal mouse anti-ferritin antibody, which was covalently coupled with paramagnetic particles. The amount of relative light units detected by the system was directly proportional to the ferritin values.

ESTIMATION OF SERUM IRON

Serum iron was estimated by a colorimetric assay from Roche, as per manufacturer's instructions.

Analyzer: Roche Modular P clinical chemistry autoanalyser, Germany.

Principle of the method:

In acidic medium and in the presence of ascorbate, ferric (Fe^{3+}) iron is dissociated from transferrin and converted to ferrous iron (Fe^{2+}). Ferrous iron combines with ferrozine to give a magenta colored complex; the color is read at 560nm. The intensity of the color is directly proportional to the iron concentration in the sample.

ESTIMATION OF C-REACTIVE PROTEIN (CRP)

Serum CRP was estimated by an automated particle-enhanced immune-nephelometry assay from Siemens, carried out as per manufacturer's instructions.

Analyzer: BN Prospec, Siemens GmbH, Marburg, Germany.

Principle of the method:

Polystyrene particles coated with human CRP-specific monoclonal antibodies were mixed with serum samples resulting in formation of aggregates. These aggregates scatter light. The intensity of scattered light is directly proportional to concentration of C-reactive protein present in the sample (Ledue and Rifai 2003).

ISOLATION OF TOTAL RNA

Duodenal mucosal samples were used for isolation of total RNA. This was done using the Nucleo-pore RNA-Sure mini kit from Genetix Biotech Asia Pvt Ltd New Delhi, India.

Contents of the RNAsure minikit
Lysis buffer LBA1
Wash buffer LBA2
Wash buffer WBA3
Desalting buffer DSB
Reaction buffer for rDNase
rDNase, RNase free water
RNAsure sheredder column
RNAsure mini column
Collection tube (2ml)
Collection tube (1.5ml)

The following protocol was followed. All steps were carried out at room temperature.

Step 1: 350µl of lysis buffer was added to each biopsy sample.

Step 2: The tissue was homogenized, using 60-65 strokes of a hand-held glass homogenizer.

Step 3: 3.5µl beta-mercpatoethanol was added to the homogenized sample; the mixture was subjected to vortex mixing for 30 seconds

Step 4: A fresh RNA-Sure shredder column was placed in a 2ml collection tube provided by the manufacturer. The mixture obtained from step 3 was

added to the shredder column and was centrifuged at 11,000g for 1 minute. The filtrate obtained in the collection tube was transferred to a fresh 1.5ml centrifuge tube.

Step 5: 350µl ethanol was added to the filtrate and mixed thoroughly by pipetting the fluid up and down.

Step 6: A fresh RNA-Sure mini-column, placed in a 2ml collection tube provided by the manufacturer, was taken. The mixture obtained from step 5 was loaded on the column; the tube was centrifuged at 11,000g for 30 seconds. The filtrate in the collection tube was discarded and the mini-column was placed in a fresh 2 ml collection tube.

Step 7: 350µl of desalting buffer provided in the kit was added to the mini-column; the tube was centrifuged at 11,000g for 1 minute.

Step 8: Recombinant DNase (rDNase) reaction mixture was prepared by adding 10µl of reconstituted rDNase provided to 90µl of reaction buffer for rDNAase. 95µl of the DNase reaction mixture was added to the RNA-Sure mini-column and incubated at room temperature for 15 min.

Step 9: Lysis Buffer 2 (LBA2) was added to the mini-column, which was then centrifuged for 30 second at 11,000g. The mini-column was transferred to a fresh centrifuge tube.

Step 10: 600µl of wash buffer (WBA3) was added to the mini-column and centrifuged for 30 seconds at 11,000*g* for 30 seconds. The filtrate obtained was discarded and the column transferred to a new collection tube.

Step 11: 200µl of WBA3 was added to the mini-column and centrifuged at 11,000*g* for 2-3 minutes. The mini column was now placed in a fresh 1.5ml nuclease-free collection tube.

Step 12: 35-40 µl of RNase-free water was added to the column to elute the RNA by centrifuging at 11,000*g* for 1 minute. The elute obtained contained the isolated RNA,

ASSESSMENT OF INTEGRITY OF ISOLATED RNA BY GEL ELECTROPHORESIS

The integrity of the RNA isolated was confirmed by agarose gel electrophoresis.

Step 1: 0.5 M EDTA solution, pH 8, was prepared.

Step 2: 10X 3-(N-morpholino) propanesulfonic acid (MOPS) was prepared.

For 100ml:

Dissolved 4.186 gm of 0.2M MOPS in sterile DEPC-(0. 1%)-treated water.

NaOH was used to adjust the pH of the solution to 7.

Sodium acetate (0.6804 gm) (to obtain a final concentration of 50mM) and 2ml of 0.5M EDTA (to obtain a final concentration of 10nM) were added to the solution.

Step 3: 1.2% agarose gel preparation

To 34 ml of DEPC-treated water in an conical flask, 0.48 gms of agarose was added. This was heated till the mixture boiled and the agarose melted.

Step 4: To the melted agarose, 2.15ml of formaldehyde, 4 ml of 10X MOPS and 2µL ethidium bromide was added. The contents were mixed well. This mixture was poured into a gel casting tray, combs inserted and allowed to set for 1 hr.

Step 5: For sample preparation, 4µl of each RNA sample was mixed with 2µL of 10X MOPS, 3.5µl of formaldehyde and 10µL of formamide.

Step 6: The sample mixture was incubated at 60^{o}C in a dry bath for 15 minutes.

Step 7: The running buffer consisted of 40 ml of 10X MOPS and 360 ml of 0.1% DEPC-treated water. This was poured into the electrophoresis tank.

Step 8: The sample mixture, after incubation at 60^{o}C, was mixed with 2µl bromophenol blue.

Step 9: The cast gel was placed in the buffer tank and samples loaded into the wells in the gel.

Step 10: The samples were electrophoresed for 45 min at 150 volts.

Step 11: The RNA bands separated were visualized using an ultraviolet transilliminator in an Alpha Innotech Flourchem SP gel documentation system. Two distinct bands were seen, which represented the 28S and 18S ribosomal subunits of RNA. When the 2 bands in each sample were found in an approximate band density ratio of 2:1, this was considered as evidence of RNA of good quality.

RNA QUANTITATION:

A nano-spectrophotometer was used to estimate RNA in the samples.

Principle: Nucleic acids absorb strongly ultraviolet light at a wavelength of 260nm. An optical density reading of 1.0 at 260 nm was taken to indicate an RNA concentration of 40µg/ml.

cDNA CONSTRUCTION BY REVERSE TRANSCRIPTION

Reverse transcription of RNA was carried out using the core kit from Eurogentec.

Principle of the procedure:

In the presence of dNTP, random nanomers and reaction buffer, the reverse transcriptase enzyme converts RNA into cDNA. .

Step 1: Reagents in the reverse transcriptase core kit, except for the reverse transcriptase, were thawed by placing on ice pack in a clean biohazard hood. The reverse transcriptase was kept in the freezer till used.

Step 2*:* The RT reaction mixture consisted of:

1µg of RNA and 10µL of a reaction mixture consisting of the following:

Volume	**Component**	**Final concentration**
1 µl	10X reaction buffer	1X
2 µl	2.5 mM MgCl2	5 mM
2 µl	dNTP 2.5 mM	500 µM each dNTP
0.5 µl	Random nonamers	2.5µM
0.2 µl	RNase inhibitors	0.4 U/µl
0.25 µl	Reverse transcriptase	1.25 U/µl
To make final volume up to 10µl.	RNase-free water	
1 µg	Template (total RNA)	

Step 3: A master-mix was prepared by adding together all the above reagents, except the template and RNAse-free water, in the proportions specified. The master mix was constituted appropriately EACH TIME for the number of samples that required reverse transcription.

Step 4: Required amounts of RNAse-free water were added to each tube to make the reaction volume up to 10µL.

Step 5: RNA was added to individual tubes.

Step 6: 5.95µl of the master mix was added to each reaction tube.

Step 7: Negative control reactions were also set up. These were:

i) No reverse transcriptase (RT) tube: This was a tube that contained all the above reagents except the reverse transcriptase. This negative control was used to confirm that there was no DNA contamination of the RNA sample used.

ii) No RNA tube: This tube contained all the reagents except the template (RNA). This was to confirm that the reagents and consumables used were not contaminated with DNA.

Step 8: All the reaction tubes were subjected to a short centrifugation in a microcentrifuge.

Step 9: The reaction tubes were placed in a thermo-cycler (Chromo4, Bio-Rad).

Step 10: The thermo cycler was programmed as follows:

Initiation step	25^{o}C for 10 min
Reverse transcription step	48^{o}C for 30 min
RT enzyme inactivation	95^{o}C for 5 min

At the end of the reverse transcription, the cDNA obtained was stored at -20^{o}C.

REAL-TIME POLYMERASE CHAIN REACTIONS (PCR)

The cDNA obtained was amplified by real-time PCR. The components for performing a single 20µL PCR reaction were as follows:

Volume	Component
2 µl	cDNA template (the reaction mixture obtained at the end of reverse transcription)
10 µl	SYBR Green Mastermix 2X
4 µl	Gene-specific primers
4 µl	Autoclaved ultrapure water

The PCR reactions were set up either in a 96-well plate format or in PCR tubes in strips. To ensure reproducibility all samples were assayed in duplicate.

Gene-specific primers:

Gene specific primers were used for the polymerase chain reaction assays. The ones used were from published literature and were as follows:

Human DMT1 (IRE Form) (Theurl et al., 2006).

Forward primer: 5'-TGCTGCTATCATTCCAACACTAAATT-3'

Reverse primer: 5'-ATATAGCCTGGTTAAGAATCATGCA-3'

Human ferroportin (Theurl et al., 2006)

Forward primer: 5'-TGACCAGGGCGGGAGA-3'

Reverse primer: 5'- GAGGTCAGGTAGTCGGCCAA -3'

Human beta-actin (Jacolot et al., 2008)

Forward primer: 5'-GTGGGGCGCCCCAGGCACCA-3'

Reverse primer: 5'-CTCCTTAATGTCACGCACGATTTC-3'

The concentrations of primers, extension and annealing temperatures and times for each gene of interest used were standardized for all 3 genes studied. These are shown below:

Primer	**Annealing temperature and time**	**Extension temperature and time**	**Concentration of forward and reverse primer**
β-Actin	63.1°C for 1 minute	72°C for 1 minute	F.P=300nM/L R.P=300nM/L
DMT-1	61°C for 20 seconds	72°C for 40 seconds	F.P=300nM/L R.P=300nM/L
Ferroportin	55.3°C for 20 seconds	72°C for 40 seconds	F.P=300nM/L R.P=300nM/L

$MgCl_2$ concentration was optimized to 5mmol/L for all the genes studied.

The Opticon Monitor software in the thermal cycler was programmed as given below:

Step		Temperature	Time
1	Incubation	50°C	2 min
2	Incubation	95° C	10 min
3	Denaturation step	95°C	1 min
4	Annealing step	Temperature and time for each studied gene were optimized	
5	Extension step	Temperature and time for each studied gene were optimized	
6	Reading was taken		
7	Step 3 onwards steps were repeated for 39 cycles more		
8	From 60 to 95°C Melting curve analysis was done, it was read at every 1°C.		
9	Within the thermocycler samples were cooled and maintained at 4°C for 10 min.		
10	End		

The products obtained were stored at - 20°C.

Standard curves were generated to validate the reaction conditions for each gene of interest. A serial dilution of the template cDNA for each gene was used for standard curve construction.

k. CALCULATION OF GENE EXPRESSION

The cycle threshold (Ct) value was the outcome parameter obtained at the end of the PCR assays.

The relative expression of the target gene was calculated in comparison to an internal reference gene, using cycle threshold (Ct) values. Controlling for errors using internal reference gene is commonly done in qRT-PCR assays. It is presumed that the internal reference gene does not change under disease conditions and can hence control for errors while performing qPCR. This minimizes variability between samples.

Each target gene data was normalized relative to beta-actin, which was used as the internal reference gene. The Ct value of beta-actin for each sample was subtracted from that of the gene of interest (DMT1 and ferroportin). This value was referred to as the ΔCt value. Relative fold-change in the gene of interest was determined using a comparative Ct method. The formula used for this was:

Relative fold-change = 2^ - ΔCt (Schmittgen and Livak 2008).

STATISTICAL ANALYSIS

The Statistical Package for the Social Sciences (SPSS) software package, version 16, was used to analyze the data obtained. The one-sample Kolmogorov-Smirnov test was used to determine whether the parameters in the study were normally distributed. Student's unpaired t test was used to compare the means of the two groups studied (control and ALD subjects). Patients with ALD were categorized into those with and without coagulopathy. Analysis of variance (ANOVA) was used to compare means of control patients and those with ALD, with coagulopathy and without coagulopathy. A post-hoc test with Bonferroni's correction was used for pair-wise multiple comparisons. Data that were not normally distributed were analysed using Kruskal-Wallis and Mann-Whitney tests. A p-value of less than 0.05 was taken to indicate statistical significance.

All parameters in the study were found to be normally distributed except for serum CRP, ferritin, iron, SGOT and ESR.

RESULTS

I. DATA ON THE TOTAL NUMBER OF SUBJECTS RECRUITED

a. CHARACTERISTICS OF SUBJECTS

A total of 54 patients were recruited. These consisted of 30 control patients and 24 diagnosed to have alcoholic liver disease (ALD). Data for all parameters were not available from the medical records for all the patients recruited. Only available data were analyzed. The number of patients for whom each set of data was available is indicated in the tables showing the results of this study.

Table 1 shows relevant clinical characteristics of the 2 groups. All the patients recruited were males. The mean ages of patients in the 2 groups were similar. Only those with ALD had a history of long-term alcohol consumption. Of these, 88% showed signs of liver cell failure, 67% of them had evidence of coagulopathy, 12.5% had diabetes mellitus and 4.1% had hypertension.

Table 1: Characteristics of subjects

Characteristic	**Control patients**	**Patients with ALD**
Number of subjects	30	24
Males/females	30/0	24/0
Mean age in years (±SD)	43.67 (8.4)	45.16 (7.6)
Mean duration of alcohol use in years (±SD)	0	18.11 (7.58)
Signs of liver cell failure present (%)	0/30	21/24 (88%)
Evidence of coagulopathy (%)	0/30	16/24 (67%)
Coexistent conditions (diabetes mellitus or hypertension) (%)	0/30	4/24 (16.6%)

b. PARAMETERS OF LIVER FUNCTION

Serum bilirubin, SGOT and alkaline phosphatase levels were significantly higher and serum albumin levels significantly lower in patients with ALD, compared with control patients Table 2).

Table 2: Parameters of liver function in the subjects

Parameter	**Control patients**	**Patients with ALD**
Total serum bilirubin (mg/dl) (±SD)	0.588 (0.261) (n = 18)	2.256 (1.491)* (n = 23)
SGOT (IU/L) (±SD)	26.47 (6.8) (n = 18)	60.47 (36.32)* (n = 23)
SGPT (IU/L)(±SD)	28.70 (14.13) (n = 18)	23.87 (11.28) (n = 23)
ALP (IU/L) (±SD)	78.64 (21.65) (n = 18)	131.34 (54.82)* (n = 23)
Serum total protein (gms/dl) (±SD)	7.44 (0.23) (n = 18)	7.6 (0.87) (n = 23)
Serum albumin (gms/dl) (±SD)	4.5 (0.30) (n = 18)	3.2 (0.71)* (n = 23)

* indicates $p = < 0.001$ when compared with control data.

c. HAEMATOLOGICAL PARAMETERS

Hemoglobin levels were significantly lower in patients with ALD, compared with control patients (Table 3). There were no significant differences in values of MCV and MCHC between the 2 groups.

Table 3: Hematological parameters in the subjects

Parameter	**Control patients**	**Patients with ALD**
Hemoglobin (gms/dL) (±SD)	14.3 (0.90) (n =29)	10.6 (2.2)* (n = 23)
Mean corpuscular volume (MCV) (fL/cell) (±SD)	88.8 (4.6) (n = 22)	90(9.9) (n =20)
Mean corpuscular hemoglobin concentration (MCHC) (pg/dL) (±SD)	33.6 (1.5) (n = 11)	33.8 (1.9) (n = 17)

* indicates $p < 0.001$ when compared with control data.

d. PARAMETERS OF IRON STORES

Serum iron levels were significantly lower in the ALD group, when compared with the control group. Serum ferritin values were higher but the increase was not statistically significant (Table 4).

Table 4: Parameters of iron stores in subjects

Parameter	Control patients	Patients with ALD
Serum iron (µg/dl) (±SD)	106.67(85.53) (n =29)	74.82(53.62)* (n = 22)
Serum ferritin (µg/l) (±SD)	140.59(122.18) (n =29)	378.6(511.1) (n = 22)

* indicates $p < 0.05$ when compared with control data.

e. PARAMETERS OF HEMOSTATIC FUNCTION.

Parameters of haemostatic function in patients with ALD are shown in Table 5. These values could not be compared with those in control patients as these tests had not been done for control patients. The reference range for PT is 10-13 seconds, that for APTT is 25-35 seconds and that for INR is 1(Lehmann and Henry, 2009).

Table 5: Parameters of haemostatic function in the subjects

Parameter	**Control patients**	**Patients with ALD**
Prothrombin time (sec) (±SD)	Not available	14.14 (1.99) (n = 22)
Activated tissue thromboplastin time (sec)(±SD)	Not available	38.66 (5.43) (n = 13)
International normalized ratio (INR) (±SD)	Not available	1.29 (0.196) (n = 22)

f. PARAMETERS OF INFLAMMATION

Patients with ALD had significantly higher serum C-reactive protein levels. ESR values and total WBC counts were not significantly different between the 2 groups (Table 6).

Table 6: Parameters of inflammation in subjects

Parameter	Control patients	Patients with ALD
CRP (mg/L) (±SD)	3.27(1.98) (n =30)	15.871(35.162)* (n =23)
Total WBC count (cells/cumm) (±SD)	6000(1838) (n =2)	7878.9(3415.6) (n =19)
ESR (mm at 60 min)	14.07 (8.25) (n =15)	41.8(41.65) (n =5)

* indicates $p < 0.005$ when compared with control data.

II. DATA ON THE SUB-CATEGORIES OF SUBJECTS RECRUITED

Patients with ALD were categorized into two groups, based on the presence or absence of coagulopathy. Sixteen patients out of 24 were found to have coagulopathy, while 8 did not have coagulopathy.

a. CHARACTERISTICS OF SUBJECTS

Patients with ALD who did not have coagulopathy were significantly older than those with coagulopathy. The mean durations of alcohol consumption were similar in both sub-groups. All patients with coagulopathy showed signs of liver cell failure, while such signs were seen only in 62.5% of those without coagulopathy. Diabetes mellitus were found to be present in 6.25% and 25% respectively in those with and without coagulopathy, while hypertension was found to be in 12.5% of those without coagulopathy (Table 7).

Table 7: Characteristics of sub-categories of patients with ALD

Characteristic	Control patients	Patients with ALD with coagulopathy	Patients with ALD without coagulopathy
Number of subjects	30	16	8
Males/females	30/0	16/0	8/0
Mean age in years (±SD)	43.67 (8.4)	42.68 (5.6)	51.5 (7.3)*
Mean duration of alcohol use in years (±SD)	0	18.1 (8.7)	19.5 (7.5)
Signs of liver cell failure present	0/30	16/16 (100%)	5/8(62.5%)
Evidence of coagulopathy	0/30	16/16 (100%)	0/8 (0%)
Coexistent conditions (diabetes mellitus or hypertension)	0/30	1/16 (6.25%)	3/8 (37.5%)

* indicates $p = 0.02$ when compared to controls patients

b. PARAMETERS OF LIVER FUNCTION IN SUB-CATEGORIES OF PATIENTS WITH ALD

Serum bilirubin, SGOT and alkaline phosphatase levels were higher and serum albumin levels significantly lower in patients with ALD with and without coagulopathy, compared with control patients. There were no significant differences in values of SGPT and serum total protein among the groups.

Table 8: Parameters of liver function in the subjects

Parameter	**Control patients**	**ALD with coagulopathy**	**ALD without coagulopathy**
Total bilirubin (mg/dl) (±SD)	0.59 (0.26) n = 18	1.03(0.41)* n = 15	2.91(1.5)# n = 8
SGOT (IU/L) (±SD)	26.47 (6.8) n = 18	56.5 (51.3)* n = 15	62.6 (27.3)# n = 8
SGPT (IU/L) (±SD)	28.70 (14.1) n = 18	24.87 (15.16) n = 15	22.86 (9.1) n = 8
ALP (IU/L) (±SD)	78.64 (21.7) n = 18	117.6(29.1)* n = 15	138.66 (64.3) n = 8
Serum total protein (gms/dl) (±SD)	7.44 (0.23) n = 18	7.71 (0.44) n = 15	7.54 (1.04) n = 8
Serum albumin (gms/dl) (±SD)	4.5 (0.30) n = 18	3.5 (0.69)* n =15	3.1 (0.70)* n = 8

* indicates $p < 0.001$ when compared with control subjects, compared to controls,

# indicates $p < 0.05$ when compared with those with patients with ALD and coagulopathy.

c. HAEMATOLOGICAL PARAMETERS IN SUB-CATEGORIES OF PATIENTS WITH ALD

Hemoglobin levels were significantly lower in patients with ALD, irrespective of the presence or absence of coagulopathy, when compared with control patients. Those with coagulopathy had significantly lower levels when compared with levels in those without coagulopathy. There were no significant differences in values of MCV and MCHC between control subjects and ALD patients with and without coagulopathy (Table 9).

Table 9: Hematological parameters in subjects

Parameter	**Control patients**	**Patients with ALD with coagulopathy**	**Patients with ALD without coagulopathy**
Hemoglobin(g/dL) (±SD)	14.3 (0.90) (n =29)	8.41(5.13)* (n = 14)	611.94(1.51)*# (n = 8)
Mean corpuscular volume (MCV) (fL/cell) (±SD)	88.8 (4.6) (n = 22)	89.86(11.49) (n =13)	0.38(5.66) (n = 6)
Mean corpuscular hemoglobin concentration (MCHC) (pg/dL) (±SD)	33.6 (1.5) (n = 11)	23.23(16.21) (n = 11)	33.6(1.38) (n = 5)

* indicates $p < 0.001$ when compared with control subjects, compared to controls,

# indicates $p < 0.05$ when compared with those with patients with ALD and coagulopathy.

d. PARAMETERS OF IRON STORES IN SUB-CATEGORIES OF PATIENTS WITH ALD

Serum iron levels tended to be lower and serum ferritin levels tended to be higher in those with ALD, irrespective of the presence or absence of coagulopathy, when compared with the control group; the differences seen were, however, not statistically significant (Table 10).

Table 10: Parameters of iron stores in subjects

Parameter	**Control patients**	**Patients with ALD with coagulopathy**	**Patients with ALD without coagulopathy**
Serum iron (µg/dl) (±SD)	106.67(85.5) (n =29)	70.63(54.8) (n = 14)	68.74(34.88) (n = 8)
Serum ferritin (µg/l) (±SD)	140.59(122.2) (n =29)	143.78(184.6) (n = 14)	354.75(263.6) (n = 8)

e. PARAMETERS OF HEMOSTATIC FUNCTION IN SUBJECTS

Patients with ALD and coagulopathy had significantly higher values for prothrombin times and INR, when compared with those with ALD but no coagulopathy (Table 11).

Table 11: Parameters of haemostatic function in the subjects

Parameter	**Control patients**	**Patients with ALD with coagulopathy**	**Patients with ALD without coagulopathy**
Protrombin time (sec)(±SD)	Not available	15.02(1.7) n = 15	12.24(0.98)# n = 7
Activated tissue thromboplastin time (sec)(±SD)	Not available	39.26(5.5) n = 11	35.35(5.3) n = 2
International Nationalized Ratio(±SD)	Not available	1.38(0.17) n = 15	1.11(0.08)# n = 7

# indicates $p < 0.001$ compared with those with ALD with coagulopathy

f. PARAMETERS OF INFLAMMATION IN SUBJECTS

Patients with ALD and coagulopathy had significantly higher serum levels of C-reactive protein when compared with control subjects. Values of ESR were higher in those with ALD and coagulopathy but the increase was not statistically significant (Table 12).

Table 12: Parameters of inflammatory status in subjects

Parameter	**Control patients**	**Patients with ALD with coagulopathy**	**Patients with ALD without coagulopathy**
CRP (mg/L)(±SD)	3.27(1.98) n =30	10.35(8.23)* n =14	26.92(61.34) n =7
Total WBC count (cells/cumm) (±SD)	6000(1838) n =2	7946.3(2684) n =13	7733.3(4966) n =6
ESR (mm at 60 min)	14.07 (8.25) n =15	49.25(44.07) n =4	12 n =1

* indicates $p < 0.01$ when compared with control data

III. GENE EXPRESSION STUDIES

a. Assessment of integrity of isolated RNA:

RNA isolated from the duodenal mucosal samples was subjected to agarose gel electrophoresis. The presence of two distinct bands in the gels, corresponding to the 28S and 18S ribosomal subunits of RNA, was taken to confirm the integrity of RNA obtained from each sample. Figure 1 shows the image of a representative gel in which RNA samples were electrophoresed.

Figure 1

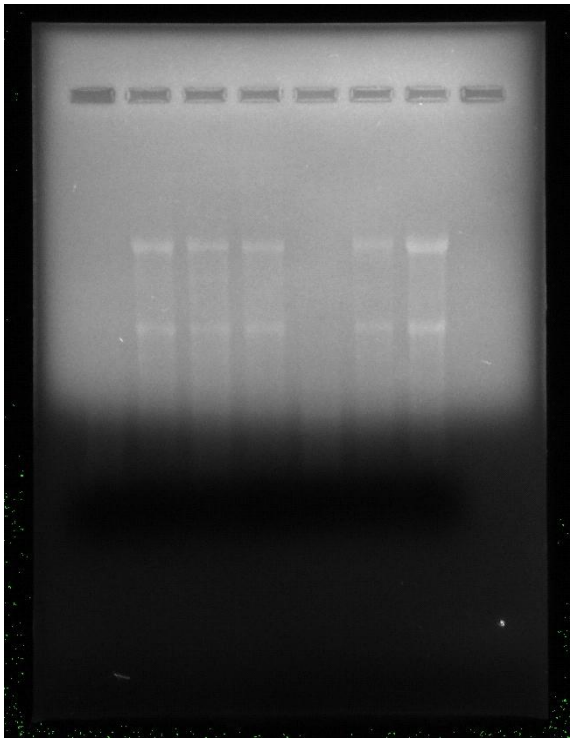

Figure 1: Image of agarose gel showing 18S and 28S subunits of RNA isolated

b. Optimization of PCR assays by standard curve generation and melting curve analysis for target genes

After completion of PCR assays for each gene of interest, the log fluorescence data graph, melting curves and standard curves were obtained.

Divalent metal transporter-1

Log fluorescence data graph for DMT1

Figure 2 shows the log fluorescence data graph for DMT-1. The average efficiency of the PCR assays for DMT-1 was found to be 1.8 to 2.05. An efficiency of 2 indicates 100% efficiency. The mean (± SD) for cycle threshold values for control samples was 26.3 ± 2.5 and that for ALD samples was 24.4 ± 2.2

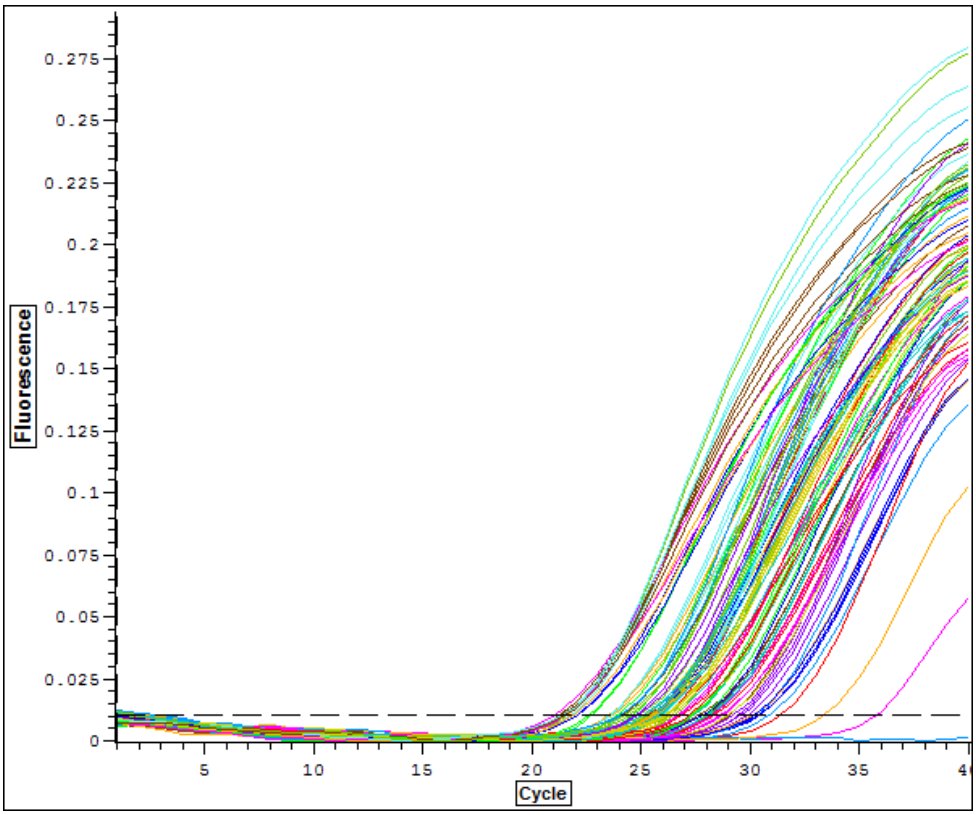

Figure 2: Log fluorescence data graph for DMT-1. The graph shows the PCR cycle number on the x-axis and relative log fluorescence units on the Y axis. The interrupted line depicts the cycle threshold set at a point above baseline fluorescence.

b. *Melting curve for DMT-1*

Figure 3 depicts the melting curve for DMT-1. The melting curve analysis showed the presence of single peak at 75-76^{o}C, indicating a single amplified product.

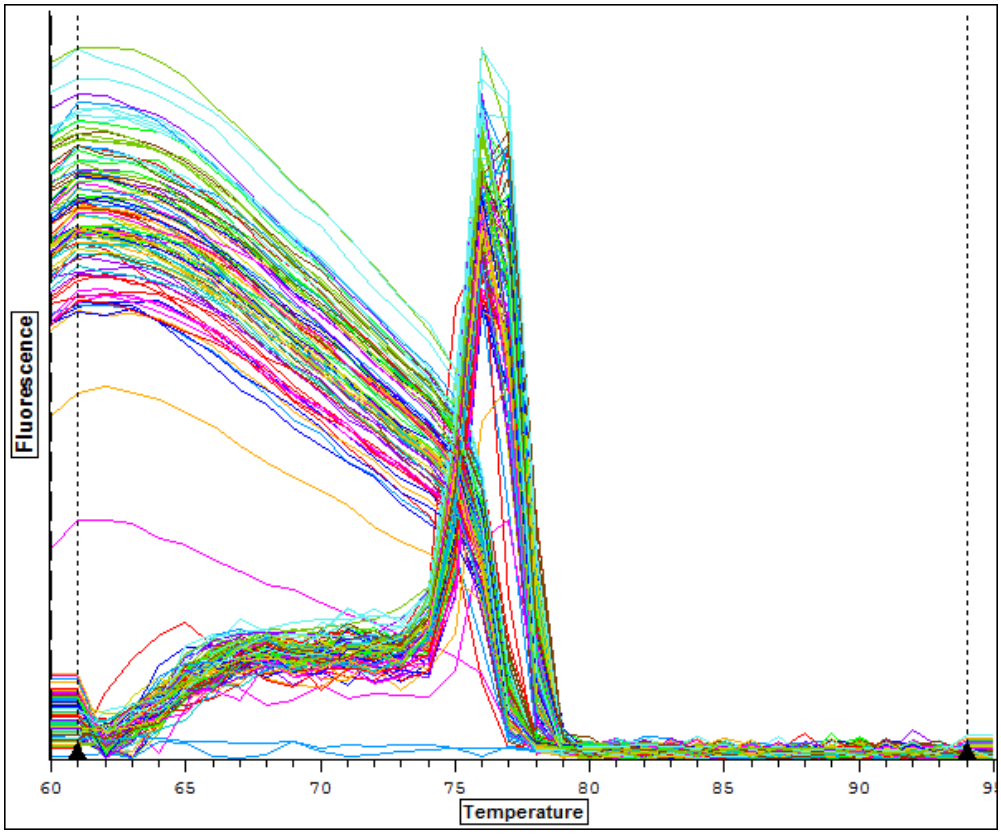

Figure 3: Melting curve for DMT-1. The graphical plot of the melting curve was taken after the PCR assay was completed using DMT-1 specific primers. The presence of a single peak at 75-76^{o}C indicates the generation of a single product during PCR.

c. *Standard curve for DMT-1*

Figure 4 shows the standard curve generated for DMT-1.

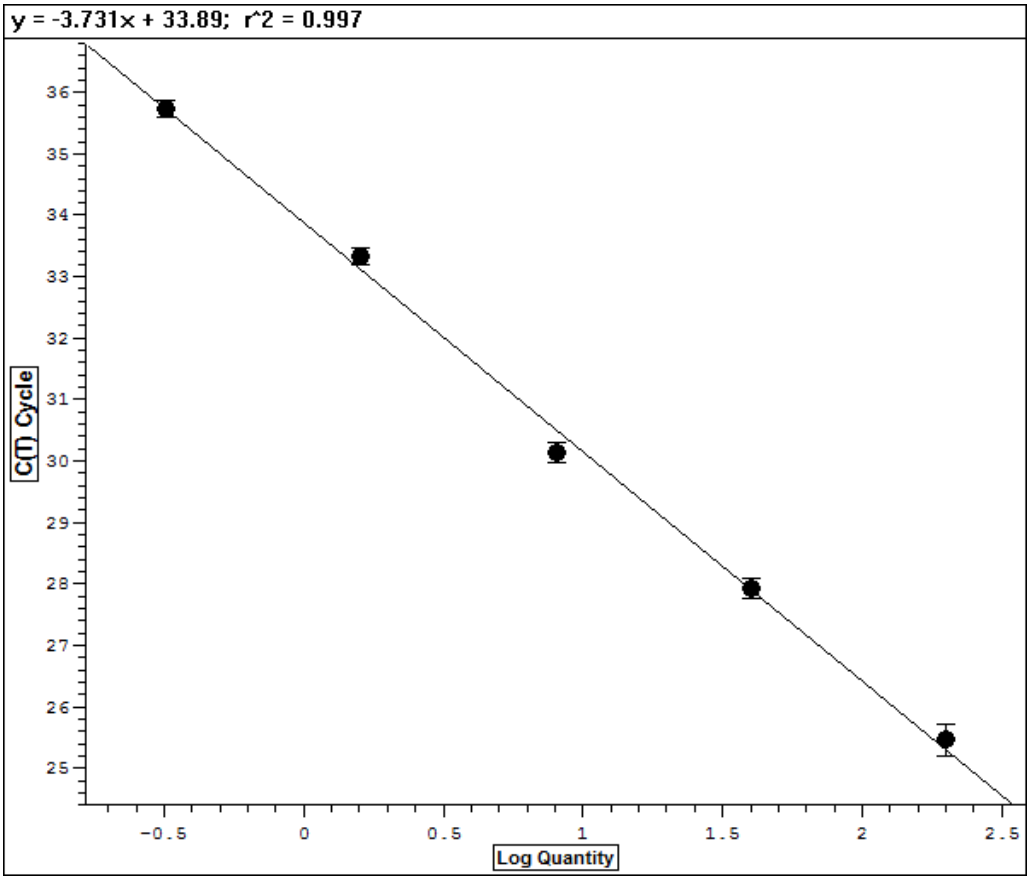

Figure 4: Standard curve for DMT-1. The graph shows the log of the quantity of cDNA on the x-axis and cycle threshold value on the y-axis.

***Ferroportin*:**

a. Log fluorescence data graph for ferroportin

Figure 5 shows the log fluorescence data graph for ferroportin. In the PCR for ferroportin the average efficiency was found to be 1.8 to 2.09. The mean (± SD) cycle threshold value for control samples was 25 (± 2.5) and that for ALD was (23 ± 2.2).

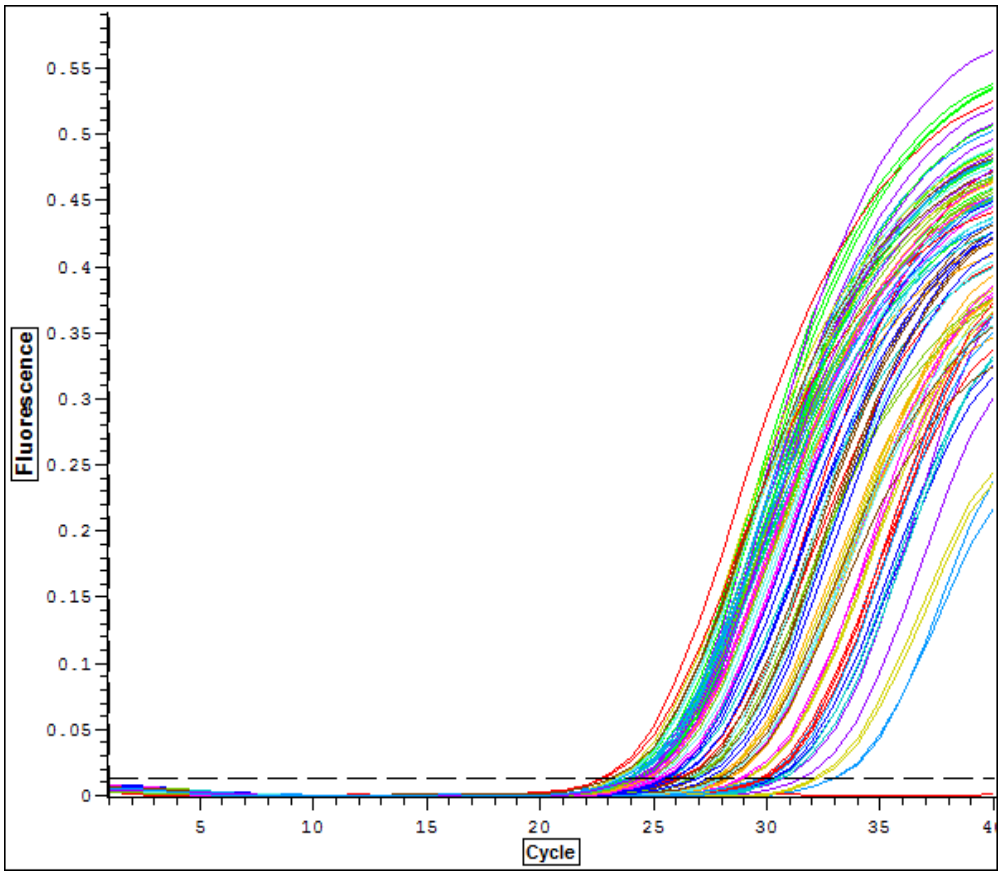

Figure 5: Log fluorescence data graph for ferroportin. The graph shows the PCR cycle number on the x- axis and relative log fluorescence units on the Y axis. The interrupted line depicts the cycle threshold set at a point above baseline fluorescence.

b. Melting curve for ferroportin

Figure 6 depicts the melting curve for ferroportin. The melting curve analysis showed the presence of single peak at 79-80^{o}C.

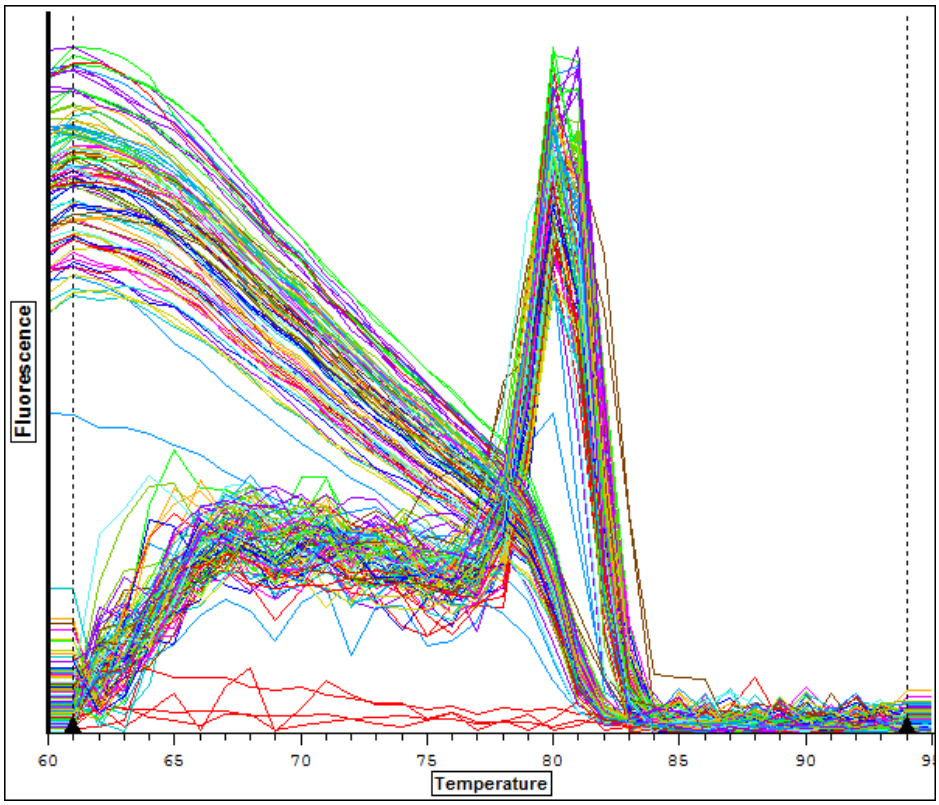

Figure 6: Melting curve for ferroportin. The graphical plot of the melting curve was taken after PCR assay were completed using specific primers for ferroportin. The presence of a single peak at 79-80^{o}C indicates the generation of a single product during the PCR

c. *Standard curve for ferroportin*

Figure 7 shows the standard curve generated for ferroportin.

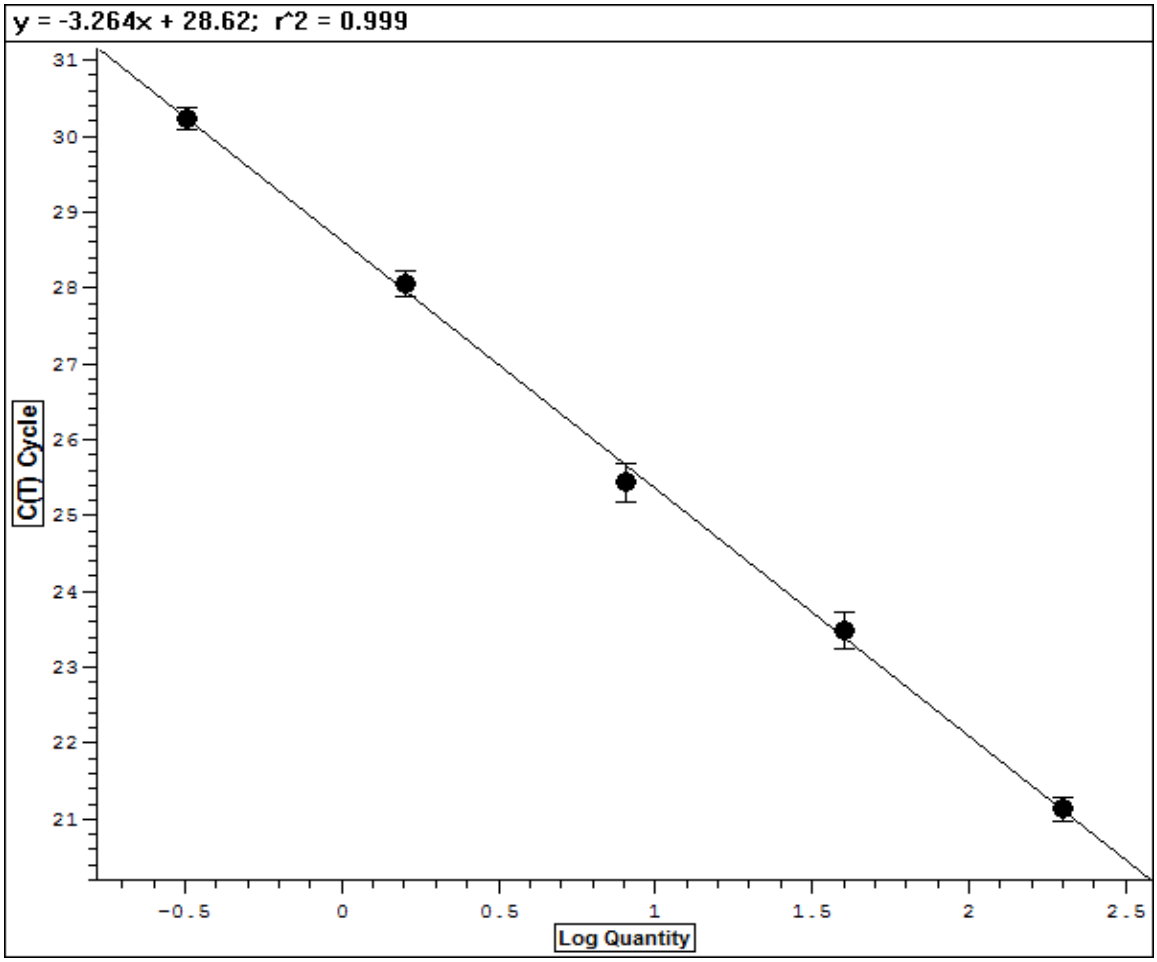

Figure 7: Standard curve for DMT-1. The graph shows the log of the quantity of cDNA on the x-axis and cycle threshold value on the y-axis.

Beta-actin

a. *Log fluorescence data graph*

Figure 8 shows the log fluorescence data graph for beta-actin. In the PCR for beta-actin, the average efficiency of was found to be 1.9 to 2.07. The mean cycle threshold value (± SD) for control samples was 27.3 (± 2.4) and that for the ALD samples was 26 (± 2.3).

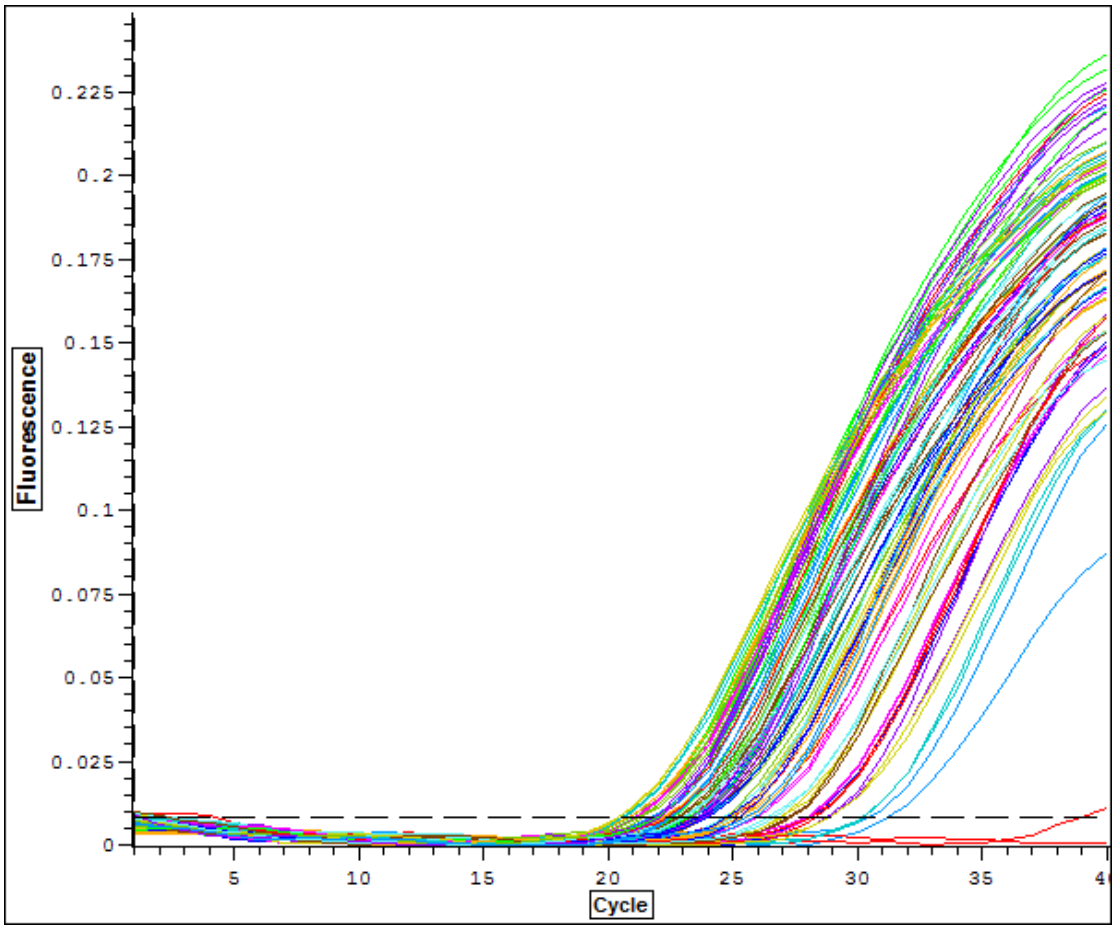

Figure 8: Log fluorescence data graph for beta-actin. The graph shows the PCR cycle number on the x- axis and relative log fluorescence units on the Y axis. The interrupted line depicts the cycle threshold set at a point above baseline fluorescence.

b. *Melting curve for beta-actin*

Figure 9 depicts the melting curve for beta-actin. The melting curve analysis showed the presence of single peak at 75-76^{o}C, indicating a single amplified product.

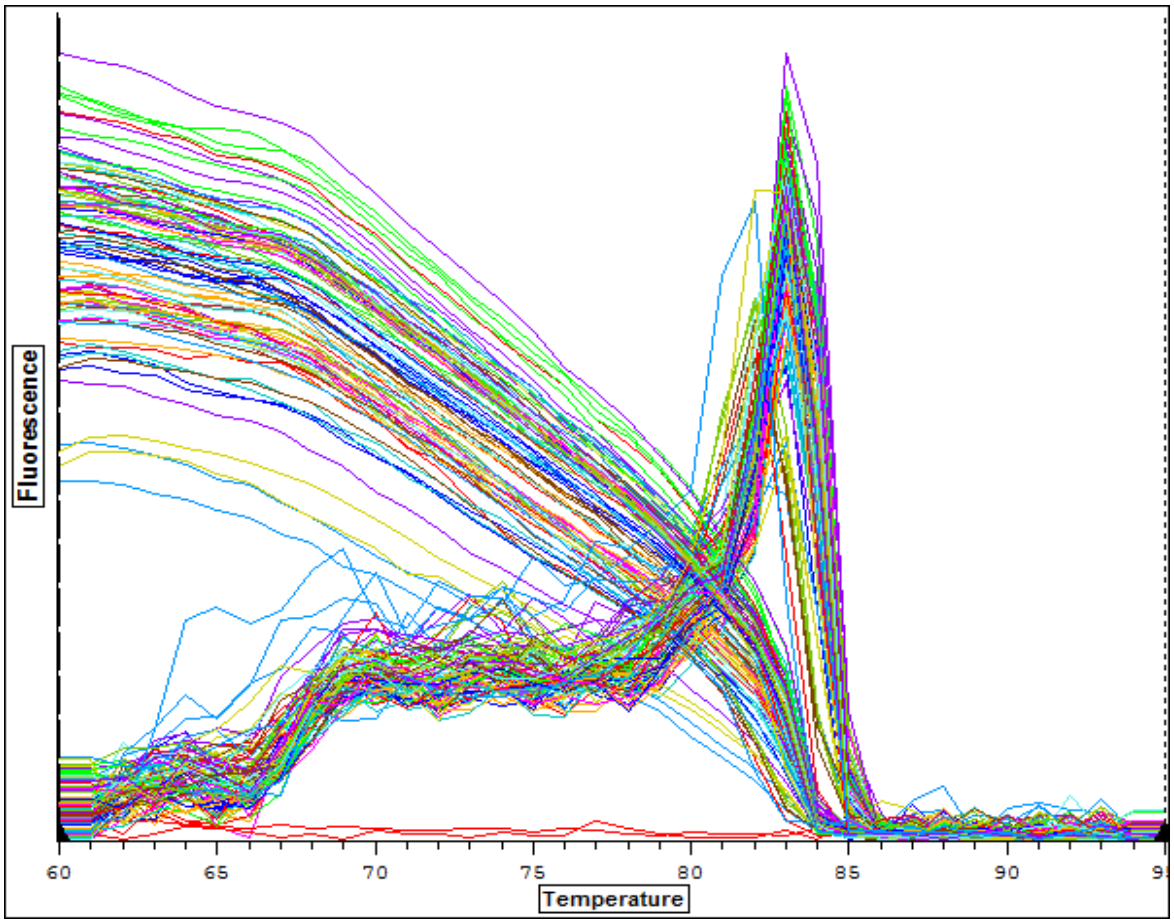

Figure 9: Melting curve for beta-actin. The graphical plot of the melting curve was taken after PCR assay were completed using specific primers for beta-actin. The presence of a single peak at 79-80^{o}C indicates the generation of a single product during the PCR

c. *Standard curve for beta-actin*

Figure 10 shows the standard curve for beta-actin.

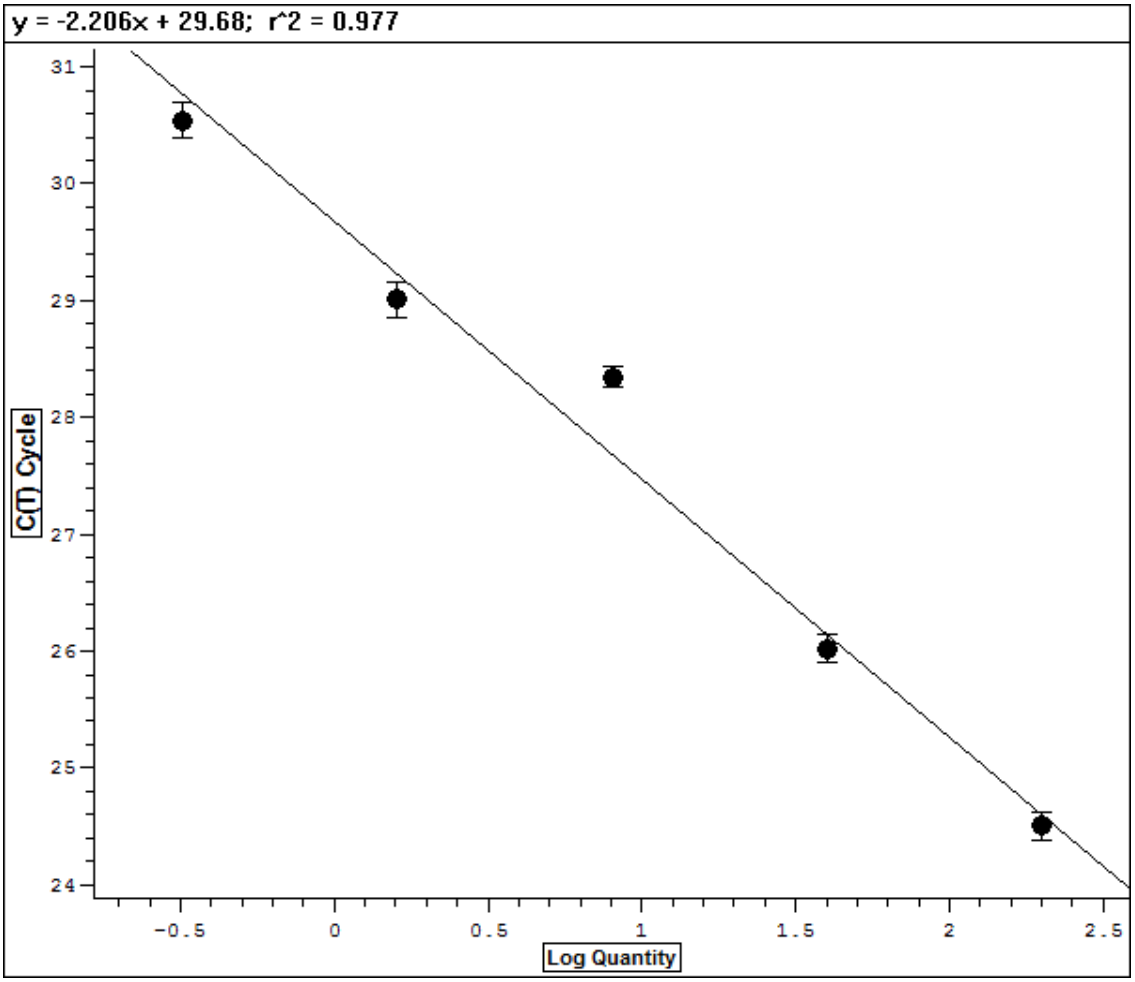

Figure 10: Standard curve for beta-actin. The graph the log of the quantity of cDNA on the x-axis and cycle threshold value on the y-axis

c. Gene expression of DMT1 and ferroportin in duodenal mucosa of subjects

Gene expression levels of duodenal DMT-1 in control and ALD subjects

Figure 11 shows mRNA expression levels of DMT1 in the control and ALD subjects. DMT-1 expression levels were higher in the ALD subjects when compared with the control subjects. However, the increase was not statistically significant ($p = 0.08$).

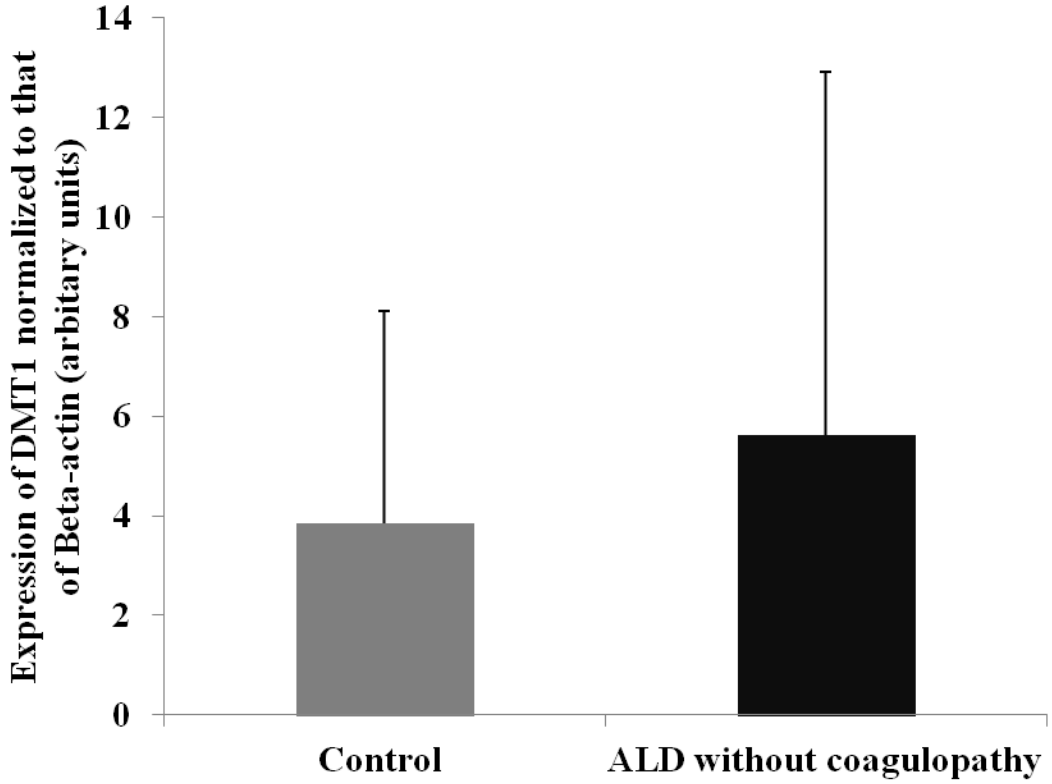

Figure 11: DMT1 mRNA expression levels in control and ALD subjects. Data are shown as mean (± SD).The mean for control subjects is 3.8 (± 4.2) and that for ALD subjects was 6.9 (± 8.5).

Figure 12 shows mRNA expression levels of DMT1 in those with ALD, with and without coagulopathy. DMT-1 expression levels were higher in those with ALD irrespective of the presence or absence of coagulopathy, when compared with control subjects. The increases seen were, however, not statistically significant.

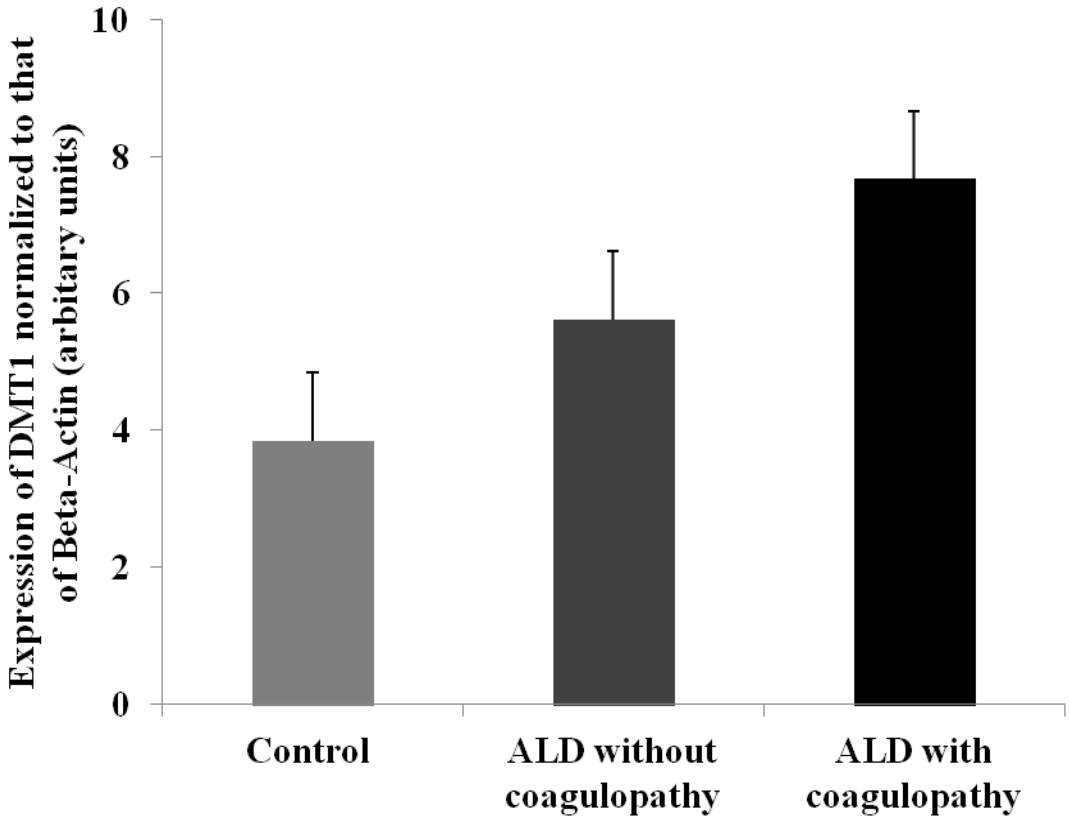

Figure 12: DMT1 mRNA expression levels in control and ALD patients, with and without coagulopathy. The mean (±SD) for control subjects was 3.8 (±4.3), that for subjects with ALD with coagulopathy was 7.7 (± 9.3) and that for subjects with ALD without coagulopathy was 5.6 (± 7.3)

Gene expression levels of duodenal ferroportin in control and ALD subjects

Figure 13 shows mRNA expression levels of ferroportin in the groups studied. Ferroportin expression levels were higher in the ALD group when compared with the control group, but the increase was not statistically significant (p value =0.15).

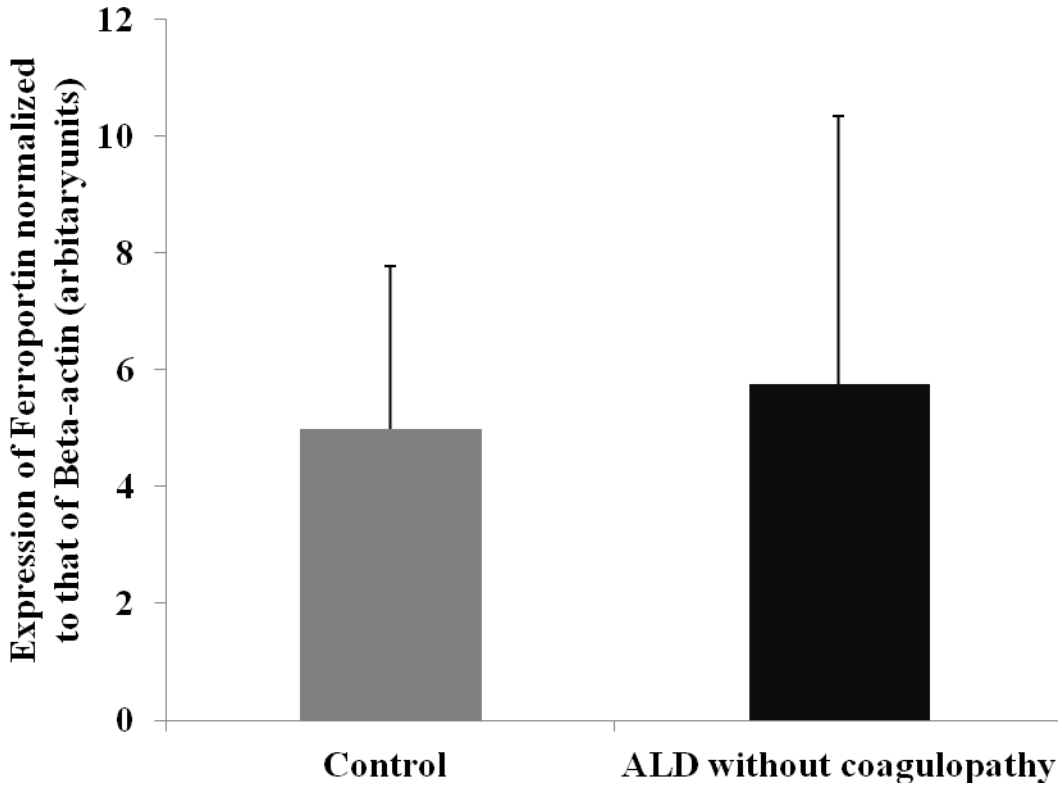

Figure13: Ferroportin mRNA expression levels in subjects in the control and ALD groups. Data are shown as mean (± SD) in each group. The mean (± SD) for the control subjects was 4.9 (± 2.7) and that for the ALD subjects was 6.6 (± 5.3).

Figure 14 shows mRNA expression levels of ferroportin in the sub-groups of those with ALD. Expression levels were higher in subjects in both sub-groups of ALD, when compared with the control group, but the increases were not statistically significant. Expression levels in the 2 sub-groups of patients with ALD were similar.

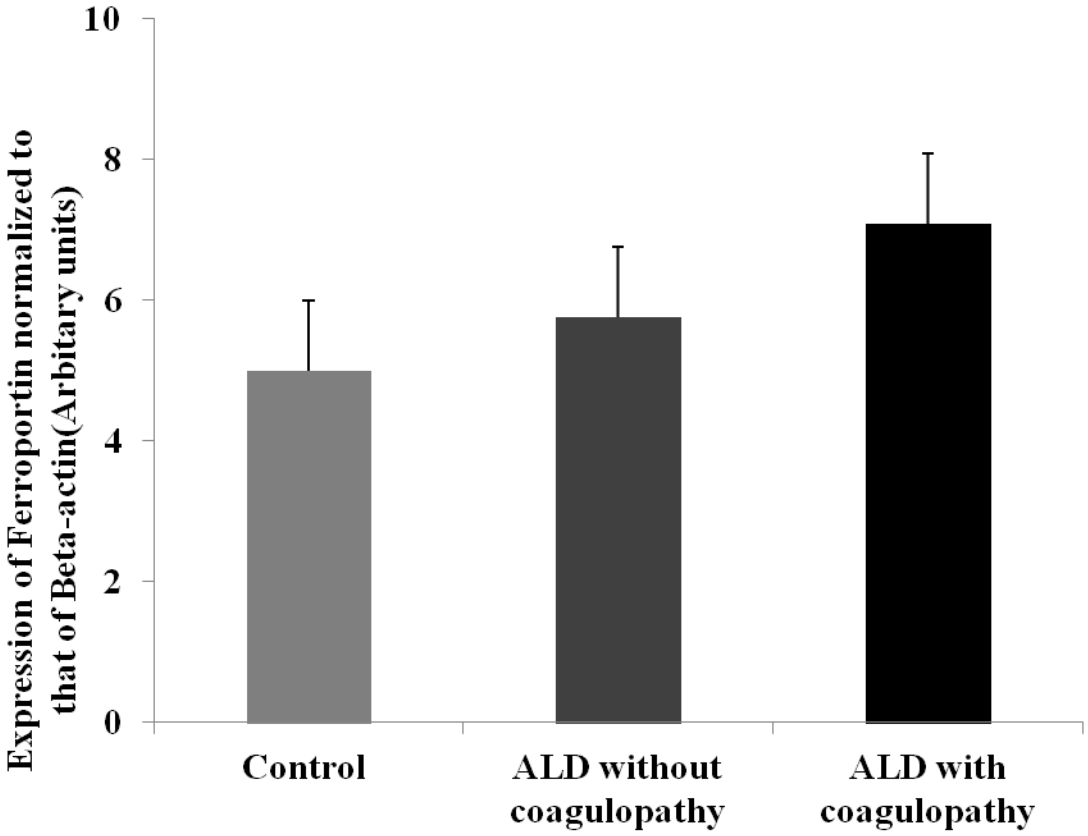

Figure 14: Ferroportin mRNA levels in subjects in the control and ALD groups, with and without coagulopathy. Data are shown as mean (± SD).The mean value for the control subjects was 4.9 (± 2.7), while that for subjects with ALD and coagulopathy was 7.1 (± 5.7) and for those with ALD without coagulopathy was 5.7 (± 4.5).

DISCUSSION

DISCUSSION

The past two decades have brought a huge amount of understanding to our knowledge in the field of iron biology. The mechanisms involved in iron absorption, metabolism in the body, regulation of these processes and its associations with numerous diseases were brought to light during this period. Studies done, have involved those on cells in culture, transgenic animal models and human subjects.

The present study was done to determine mRNA expression levels of duodenal proteins involved in absorption of non-heme iron in subjects with alcoholic liver disease. The hypothesis of the study was that these proteins may be up- regulated in patients with alcoholic liver disease, thus increasing iron absorption and resulting in iron overload in the body. This hypothesis arose from the studies that have shown the existence of iron overload in patients with alcoholic liver disease (Powell, 2008; Harrison-Findik et al., 2006).

Cirrhosis is a serious complication of excessive alcohol use. However, it has been shown that despite heavy alcohol consumption for long periods only a small percentage of such users progressed from alcoholic hepatitis to cirrhosis (Friedman, 2012). This suggests that alcohol is not the only factor involved in the development of alcoholic cirrhosis (Friedman, 2012).

Iron overload is often associated with alcoholism. Even moderate consumption of alcohol is associated with increased iron stores as indicated by elevations in serum ferritin (Milman and Kirchhoff, 2006), transferrin saturation (Chapman et al., 1982; Ioannou et al., 2004), iron levels in hepatocytes (Ioannou et al., 2004), TfR1

expression levels in hepatocytes (Suzuki et al., 2002) and intestinal iron absorption (Duane et al., 1992). Patients with genetic hemochromatosis (GHH), who consume excessive amounts of alcohol, have been shown to be more likely to develop severe liver disease and ultimately cirrhosis (Fletcher et al., 2002). The effects of alcohol and iron overload seen appeared to be synergistic. Both alcohol and iron are individually involved in induction of oxidative stress. Previous animal studies have shown that alcohol consumption causes induction of the enzyme CYP2E1, which is involved in metabolism of alcohol (Murray, 2008). This in turn causes oxidative stress, which is known to down-regulate CEBP alpha, a promoter of hepcidin. This, in turn, results in down-regulation of hepcidin. Hepcidin down-regulates proteins involved in intestinal absorption of non-heme iron. It binds to ferroportin on the basolateral surface of enterocytes, leading to its internalization and degradation (Harrison Findik et al., 2006; Bridle et al., 2006). This event eventually results in degradation of DMT1 (Harrison Findik et al., 2006). Low levels of hepcidin, thus, cause up-regulation of proteins involved in intestinal iron absorption, leading to increased uptake of iron and resultant hepatic iron overload. These observations are from animal studies; such a sequence of events has not been reported in humans. Costa-Matos et al (2012) have shown that hepatic hepcidin expression in patients with alcoholic liver disease (without cirrhosis) is lowered; this correlated with the amount of alcohol consumed. In addition, they have reported increased serum ferritin and transferrin saturation levels in these patients. It has also been shown that there is a differential effect of hepcidin on macrophages and enterocytes, with the internalization and degradation of ferroportin by hepcidin shown to be more effective in macrophages (Yamaji et al., 2004; Chaston et al., 2008).

In the present study, liver function tests were deranged in patients with alcoholic liver disease (ALD). These patients had significantly lower hemoglobin levels than the control patients. In patients with coagulopathy, previous episodes of bleeding that have occurred may account for the anemia; the cause is less evident in those without coagulopathy. Values for MCV and MCHC in those with ALD were not significantly different from control patients, suggesting normal iron stores in the body in those with ALD. Ideally, it would be best to determine the iron content in the liver in these patients ascertain whether an iron load had developed in response to alcohol consumption. However, it was not possible to do so because it was not ethically possible to obtain a sample of liver for this. Because of this, surrogate marker of iron stores, such as serum iron and ferritin, were utilized. Serum ferritin in these patients was higher than in control subjects; however, this increase was not statistically significant. Serum ferritin levels are often used as an indicator of body iron stores; however, it is also an acute phase reactant and its levels are elevated in inflammatory states (Recon, 2012). Patients with ALD had significantly higher levels of CRP than control subjects, indicating the presence of systemic inflammation. Regression analysis showed no correlation between the ferritin and CRP, suggesting that increased serum ferritin may not be due to inflammation. In view of this, it would be difficult to conclude what the iron stores in the body were in these patients. Judging by the data on MCV, MCHC and ferritin, iron stores appear to be normal or may be elevated. Use of markers such as soluble transferrin receptor (Ferguson et al. 1992) or soluble haemojuvelin, which are now considered to be better indicators of iron stores (Lin et al., 2005; Zhang et al., 2007; Brasse-Lagnel et al., 2010) may be necessary to make the situation clearer. It was not possible to estimate these parameters in the present study due financial constraints.

In the present study, DMT1 and ferroportin mRNA expression tended to be higher in those with ALD when compared with control subjects; the increases were, however, not statistically significant (p value =0.08 for DMT1 and 0.1 for ferroportin). A larger number of samples will need to be studied to confirm these results. It would also be useful to estimate serum hepcidin levels in these patients and correlate this parameter with expression levels of duodenal proteins. However, it was not possible to do this in the present study, once again due to financial constraints involved.

As far as it is possible to determine, this is the first study of its kind in patients with ALD. Harrison -Findik et al (2006**)** have reported up-regulation of DMT1 and ferroportin expression in duodenal enterocytes in animal models with alcohol liver disease. No reports of a similar nature done on humans have been found in published literature. Hence this study provides preliminary data on possible dysregulation in iron homeostasis in patients with ALD.

CONCLUSION

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The mRNA expression levels for proteins involved in absorption of non-heme iron in the duodenum showed a tendency towards up-regulation of DMT1 **(**apical iron transporter**)** and ferroportin (basolateral iron transporter).

LIMITATIONS OF THE STUDY

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- The sample size for the study was small. A larger number of patients will need to be studied to confirm the results.
- Due to financial constraints, it was not possible to estimate serum levels of soluble transferrin receptor and soluble haemojuvelin as indicators of body iron stores. Similarly, it was not possible to estimate serum hepcidin levels. These parameters would have contributed to determining the true iron status of the patients in the study.

FUTURE DIRECTIONS

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Further studies that are required for further studies on iron overload in alcoholic liver disease include:

- estimation of levels of serum hepcidin and soluble transferrin receptor and soluble haemojuvelin
- determination of expression levels of other duodenal proteins involved in iron absorption such as dcytb, hephaestin, transferrin receptor 1,ferritin and iron regulatory protein.
- estimation of iron content in the liver

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APPENDIX

PATIENT INFORMATION SHEET FOR REQUEST FOR SAMPLE OF DUDENAL MUCOSA AND BLOOD

As a part of the investigation of your current medical problem, the doctor taking care of you has advised you to undergo an endoscopy, during which a tube will be passed into your stomach and small intestine, so that the doctor can see whether there is any problem in these organs. During the procedure, the doctor will take a small piece of tissue from the stomach or intestine, so that certain tests can be done with this tissue in order to find out more about your illness.

The Department of Biochemistry, in association with Department of Gastroenterology, is carrying out a study on all patients who undergo such an endoscopy to understand changes in the way iron is handled in the body in the patients who have liver disease, produced by intake of alcohol. Iron levels in the liver are often higher than normal in this situation. It sometimes worsens the disease. We do not know fully why this happens. We would like to study this problem to understand it better. In order to do this, we need to have a very small biopsy from the small intestine. This will be taken at the same time that the doctor looks at the inside of your stomach and intestine. We request your permission to take this piece of tissue. You will not feel any extra discomfort on account of this. We also request permission to take a sample of blood from you. This is to help us study what happens to iron in the blood.

The samples that we request will be used purely for scientific research. Any remaining sample will be discarded. The collection of 3ml of blood will not cause any harm to your health in any foreseeable manner. All your personal information obtained by us from you will be kept confidential.

The biopsy and blood samples that we request will be used purely for scientific research. The taking of these biopsy samples will not cause any harm to your health in any foreseeable manner. All your personal information obtained by us from you will be kept confidential.

You may not benefit directly from the study. However, if you are willing to allow us to take these samples, it will help us try to understand why iron collects in liver in those who drink alcohol. This knowledge may help improve treatment for the disease.

However, if you do not wish to be part of this study, you are free to refuse give permission for this. The treatment you continue to receive in this hospital will be exactly the same whether you choose to be part of this study or not.

INFORMED CONSENT DOCUMENT FOR SAMPLES OF BLOOD AND DUODENAL BIOPSY SAMPLES

The investigator has explained to me the details of the study proposed and what it involves. I have understood what has been said, including the following:

1. A very small sample of tissue from my gut will be taken by the doctor at the time of my endoscopic procedure, which my treating doctor has advised me to have.
1. It will not create any additional difficulty for my undergoing the procedure.
2. A sample of blood (3 ml) will also be collected from me.
3. Taking these samples will not affect my health in any foreseeable manner.
4. The samples will be used to study what happens to the processes by which iron is handled by blood cells in patients who have liver disease due to alcohol intake. They will be used only for research purposes.

I am willing to donate the tissue and blood samples voluntarily and without any coerction from the investigators of this project.

Signature of the donor: Signature of the investigator:

Name of the donor:

Signature of the witness:

Date

Contact information of the investigator: Dr. Kavita Rasalkar. Ph.no. 9047495314

Patient information

Controls

Sl no	Name	Age	Sex	Diagnosis	Bleeding history	Bleeding tendency	Hb	MCV	MCHC
1	Ujjal	37	M	PUD	N	N	15.4	87.5	NA
2	Shibshambu	39	M	PUD	N	N	14	88.6	NA
3	SantoshDeb	41	M	PUD	N	N	14.8	91	31
4	Ilangovan	33	M	PUD	N	N	15.7	NA	NA
5	Bajrang	63	M	PUD	N	N	14.5	NA	NA
6	SwapanKumar	56	M	PUD	N	N	12.7	98.6	34.2
7	MilanKanti	57	M	PUD	N	N	14.7	86	30.7
8	ToneyMathews	48	M	PUD	N	N	13.7	91	34.2
9	Anindya	31	M	PUD	N	N	14.5	88.8	35.3
10	Ashutosh	43	M	PUD	N	N	14.8	82.9	35.3
11	Minhazuddin	38	M	PUD	N	N	14.3	82.4	33.4
12	PannerSelvam	35	M	PUD	N	N	15.6	NA	NA
13	Sahadat	29	M	PUD	N	N	14.3	80.7	33.2
14	RamPado	51	M	PUD	N	N	13	89.8	34
15	Bimalendu	51	M	PUD	N	N	NA	NA	NA
16	Babu	35	M	PUD	N	N	16.2	89.2	NA
17	SwapanDey	35	M	PUD	N	N	14	87.8	NA
18	Shivaiah	43	M	PUD	N	N	13.6	NA	NA
19	Srimoujum	46	M	PUD	N	N	14.5	88.9	NA
20	Mahindra	41	M	PUD	N	N	15.7	94.3	34.6
21	Pramanik	58	M	PUD	N	N	13.3	NA	NA
22	JamilPaiker	35	M	PUD	N	N	14.3	88.1	NA
23	DilipKumar	49	M	PUD	N	N	14.8	93.8	NA
24	Manohar	48	M	PUD	N	N	13.4	NA	NA

Note: M –Male, N –No, NA –Not available

25	ArunBagath	48	M	PUD	N	N	13.2	83	NA
26	SamirHaldar	40	M	PUD	N	N	13.7	97.5	33.4
27	SanjayPandit	47	M	PUD	N	N	15.1	91.7	NA
28	TarunKumar	40	M	PUD	N	N	13.3	85.5	NA
29	Parameshwar	47	M	PUD	N	N	15	NA	NA
30	KhalidIqbal	46	M	PUD	N	N	13.7	86.5	NA
	Mean	**43.67**					**14.3**	**88.8**	**33.6**

Note: M –Male, N –No, NA –Not available, PUD –Peptic ulcer disease

ALD patients

Sl no	Name	Age	Gender	Diagnosis	Bleeding history	Bleeding tendency	Hb	MCV	MCHC
1	Debabratha	50	M	ALD	N	N	8.7	87.5	NA
2	Margabandhu	53	M	ALD	N	N	12.4	88.6	NA
3	Rajendran	47	M	ALD	N	Y	10.4	91	31
4	PronobBose	49	M	ALD	N	Y	6.4	NA	NA
5	LuxmiTudu	47	M	ALD	N	N	11.7	NA	NA
6	SajalMondol	42	M	ALD	N	Y	6.9	100.4	33.4
7	Gunashekaran	53	M	ALD	N	N	12.1	NA	NA
8	DeepakKumar	46	M	ALD	N	N	13.6	88.9	34.4
9	Ilango	45	M	ALD	N	N	11.2	65.4	34
10	GopalPrasad	45	M	ALD	Y	Y	10.1	91.4	32.9
11	Shankar	36	M	ALD	N	Y	NA	104.9	36.5
12	UnniMoin	46	M	ALD	N	Y	11	86.5	NA
13	HaridasNaik	43	M	ALD	N	Y	11.2	91.3	34
14	Janardhan	39	M	ALD	**N**	N	12.3	NA	NA
15	Sridhar	46	M	ALD	Y	N	8.6	87.5	33.5
16	MehatiAli	42	M	ALD	Y	Y	NA	NA	NA
17	RajeshPasi	36	M	ALD	N	Y	7.5	96	33.1
18	santoshMonda	59	M	ALD	Y	N	13	99.6	35.4
19	Durai	33	M	ALD	Y	Y	8.8	87.9	NA
20	Shambunath	64	M	ALD	Y	N	12.8	85.2	28.9
21	Yuvaraj	41	M	ALD	N	Y	11.8	NA	NA
22	Anuraj	49	M	ALD	Y	Y	8.4	78.3	34.4
23	Venkatesan	50	M	ALD	N	Y	14.1	88.6	35.7
24	Raja	34	M	ALD	Y	Y	9.2	96.9	34.7
	Mean	**45.16**					**10.6**	**90**	**33.8**

Note: M-Male, N –No, Y -Yes NA –Not available, ALD- Alcoholic liver disease,

Sl no	Name	Serum iron	Serum ferritin	PT	INR	APTT	Total count	ESR	CRP
1	Ujjal	149	105	NA	NA	NA	NA	10	2.97
2	Shibshambu	118	144	NA	NA	NA	NA	NA	2.97
3	SantoshDeb	63.3	68	NA	NA	NA	NA	7	2.97
4	Ilangovan	92	46	NA	NA	NA	NA	3	2
5	Bajrang	94	86	NA	NA	NA	NA	NA	1.05
6	SwapanKumar	55	118	NA	NA	NA	NA	13	3.41
7	MilanKanti	113	115	NA	NA	NA	NA	8	0.8
8	ToneyMathews	117	263	NA	NA	NA	NA	20	2.97
9	Anindya	101	48.1	NA	NA	NA	NA	NA	2.97
10	Ashutosh	75.2	103	NA	NA	NA	NA		2.97
11	Minhazuddin	81	438	NA	NA	NA	NA	10	5.65
12	PannerSelvam	89	83	NA	NA	NA	NA	NA	3.34
13	Sahadat	77	71.7	NA	NA	NA	NA	6	2.97
14	RamPado	105	42.8	NA	NA	NA	NA	22	0.53
15	Bimalendu	59	246	NA	NA	NA	NA	NA	2.97
16	Babu	194	268	NA	NA	NA	NA	8	2.97
17	SwapanDey			NA	NA	NA	NA	20	NA
18	Shivaiah	43	38.4	NA	NA	NA	NA	NA	5.16
19	Srimoujum	91	510	NA	NA	NA	NA	15	2.97
20	Mahindra	120	180	NA	NA	NA	NA	NA	2.97
21	Pramanik	28	365	NA	NA	NA	NA	NA	10.6
22	JamilPaiker	43	117	NA	NA	NA	NA	35	7.3
23	DilipKumar	89	83	NA	NA	NA	NA	NA	3.34
24	Manohar	108	61.8	NA	NA	NA	NA	NA	2.97

NA –Not available

25	ArunBagath	55	71	NA	NA	NA	NA	NA	2.97
26	SamirHaldar	54	46.5	10.3	NA	27.6	NA	NA	0.22
27	SanjayPandit	117	121	NA	NA	NA	NA	20	2.97
28	TarunKumar	104	29.9	NA	NA	NA	NA	NA	2.97
29	Parameshwar	510	136	NA	NA	NA	NA	NA	3.45
30	KhalidIqbal	149	72	NA	NA	NA	NA	14	3.45
	Means	**106**	**140**	**NA**	**NA**	**NA**	**6000**	**14.07**	**3.27**

NA –Not available

Sl no	Name	Serum iron	Serum ferritin	PT	INR	APTT	Total count	ESR	CRP
1	Debabratha	52	54	NA	NA	NA	6400	12	3.34
2	Margabandhu	34	175	10.1	0.94	NA	NA	NA	NA
3	Rajendran	97	184	14.1	1.29	37.8	7200	NA	2.97
4	PronobBose	15	10.4	14.6	1.33	35.3	7000	NA	2.97
5	LuxmiTudu	49	51.9	11.8	1.07	43.4	8600	95	15.1
6	SajalMondol	195	1075.9	17.3	1.62	40.5	7800	NA	2.97
7	Gunashekaran	92	23.6	12.7	1.19	39.1	5200	NA	2.97
8	DeepakKumar	25	1167	12.7	1.15	31.6	16700	NA	166
9	Ilango	39	31.4	13.7	1.14	NA	NA	NA	20.8
10	GopalPrasad	36	1650	12.9	1.17	NA	3900	NA	2.97
11	Shankar	22	15.5	NA	NA	NA	NA	NA	NA
12	UnniMoin	130	258	17.2	1.58	38.8	5000	11	10.1
13	HaridasNaik		62.3	14.4	1.32	34.2	6000	NA	3.34
14	Janardhan	52	17.3	14.4	1.32	NA	5000	NA	2.95
15	Sridhar	27	45.4	14	1.32	52.8	13000	12	18.2
16	MehatiAli	115	60.7	12.3	1.07	NA	NA	NA	5.26
17	RajeshPasi	89	1525	16.4	1.5	40.2	10,200	NA	12.7
18	santoshMonda	194	503.5	12.8	1.17	NA	10200	NA	4.9
19	Durai	NA	NA	18	1.65	39.3	11200	NA	NA
20	Shambunath	80	77.3	12.2	1.11	NA	4000	NA	2.97
21	Yuvaraj	138	569.9	15	1.38	NA	NA	NA	2.97
22	Anuraj	16	141	13	1.19	NA	6400	79	21.8
23	Venkatesan	100	900	15.4	1.52	37.4	11100	NA	24.1
24	Raja	49	267	16	1.47	32.2	NA	NA	3.9
	Mean	**74.82**	**378.6**	**14.14**	**1.29**	**38.66**	**7878**	**41.8**	**15.8**

NA –Not available

Sl no	Name	TB	AST	ALT	ALP	Total protein	Albumin
1	Ujjal	NA	NA	NA	NA	7.5	3.9
2	Shibshambu	NA	NA	NA	NA	7.5	4.3
3	SantoshDeb	0.5	27	21	92	7.2	4.7
4	Ilangovan	NA	NA	NA	NA	NA	NA
5	Bajrang	0.4	23	18	74	NA	NA
6	SwapanKumar	0.8	32	18	77	NA	NA
7	MilanKanti	0.4	35	42	114	NA	NA
8	ToneyMathews	0.4	27	32	78	NA	NA
9	Anindya	1.3	16	12	67	NA	NA
10	Ashutosh	0.8	35	59	79	NA	NA
11	Minhazuddin	0.9	22	33	17	NA	NA
12	PannerSelvam	NA	NA	NA	NA	NA	NA
13	Sahadat	0.4	25	28	76	NA	NA
14	RamPado	0.4	26	30	94	NA	NA
15	Bimalendu	NA	NA	NA	NA	NA	NA
16	Babu	NA	NA	NA	NA	7	4.5
17	SwapanDey	NA	NA	NA	NA	7.3	4.6
18	Shivaiah	NA	NA	NA	NA	NA	NA
19	Srimoujum	0.4	36	56	68	NA	NA
20	Mahindra	0.6	19	13	75	NA	NA
21	Pramanik	NA	NA	NA	NA	NA	NA
22	JamilPaiker	0.5	24	27	97	7.7	4.6
23	DilipKumar	NA	NA	NA	NA	7.6	4.6
24	Manohar	0.4	13	11	54	7.7	4.7

NA –Not available

25	ArunBagath	NA	NA	NA	NA	NA	NA
26	SamirHaldar	0.4	30	18	101	NA	NA
25	ArunBagath	NA	NA	NA	NA	NA	NA
26	SamirHaldar	0.4	30	18	101	NA	NA
27	SanjayPandit	0.5	35	34	96	7.5	5
28	TarunKumar	NA	NA	NA	NA	NA	NA
29	Parameshwar	NA	NA	NA	NA	NA	NA
30	KhalidIqbal	0.9	25	36	78	NA	NA
	Mean	**0.588**	**26.4**	**28.70**	**78.64**	**7.4**	**4.5**

NA –Not available

Sl no	Name	TB	AST	ALT	ALP	Total protein	Albumin
1	Debabratha	0.8	32	11	105	7.1	2.5
2	Margabandhu	0.5	18	16	126	8.2	3.4
3	Rajendran	1.1	54	37	141	8.7	3.5
4	PronobBose	2.5	59	18	152	7.1	3
5	LuxmiTudu	1.3	156	32	152	8.1	3.6
6	SajalMondol	4.9	54	44	89	6.4	3.2
7	Gunashekaran	1.3	38	18	134	7.2	3.7
8	DeepakKumar	1.4	32	24	101	8.2	3.6
9	Ilango	0.7	19	10	62	7.5	4.5
10	GopalPrasad	1.5	38	16	90	7	3.4
11	Shankar	NA	NA	NA	NA	NA	NA
12	UnniMoin	4.4	72	25	271	6	2
13	HaridasNaik	1.2	41	23	112	8.1	3.2
14	Janardhan	1.6	27	14	105	7.8	4.5
15	Sridhar	4.1	86	16	136	6.5	3.2
16	MehatiAli	2	17	17	86	7.9	4.5
17	RajeshPasi	4.5	117	31	158	9	2.3
18	santoshMonda	1.6	118	56	147	7.5	2.8
19	Durai	3.5	84	12	67	6.9	3.2
20	Shambunath	0.7	39	32	114	7.9	4.4
21	Yuvaraj	2	53	22	65	7.2	3.1
22	Anuraj	1.7	81	31	236	9.2	2.8
23	Venkatesan	5.3	99	19	240	8.8	3.1
24	Raja	3.3	57	18	132	6.5	2.2
	Mean	**2.25**	**60.47**	**23.87**	**131.34**	**7.6**	**3.2**

NA –Not available

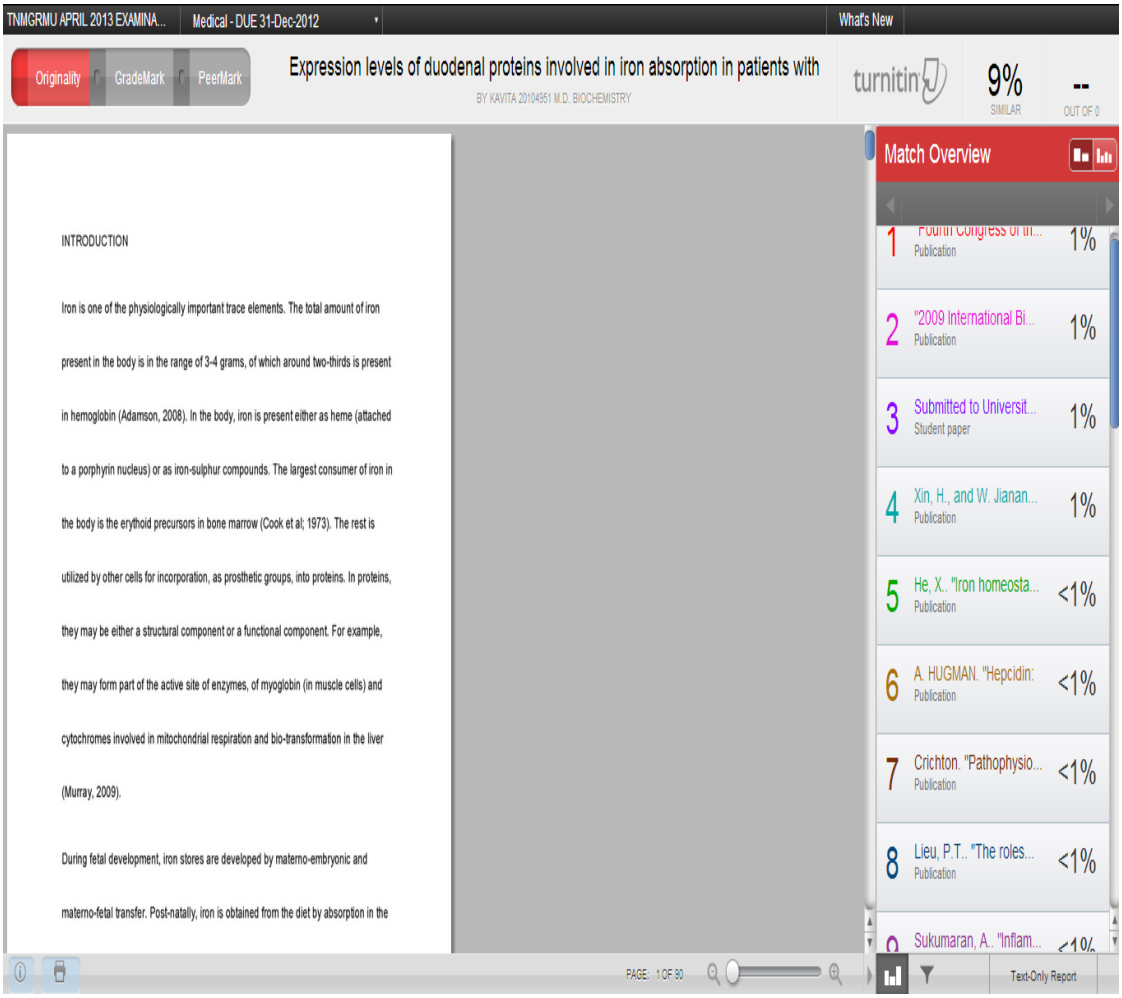
The valves of DMT1 and Ferroportin expression were normalized to that of Beta-actin (arbitrary units)

Sl no	**Name**	**DMT1 expression levels**	**Ferroportin expression levels**
1	Ujjal	0.423373	1.853176
2	Shibshambu	1.094294	2.042024
3	SantoshDeb	1.693491	4.958831
4	Ilangovan	3.482202	5.938094
5	Bajrang	4.993322	5.502167
6	SwapanKumar	0.650671	3.758091
7	MilanKanti	9.447941	10.05611
8	ToneyMathews	0.946058	9.849155
9	Anindya	4.377175	6.32033
10	Ashutosh	0.373712	2.479415
11	Minhazuddin	5.656854	5.540438
12	PannerSelvam	10.41073	4.594793
13	Sahadat	3.605002	5.278032
14	RamPado	0.76313	11.63178
15	Bimalendu	0.353553	4.027822
16	Babu	10.41073	6.105037
17	SwapanDey	0.757858	5.028053
18	Shivaiah	12.38052	7.568461
19	Srimoujum	2.361985	3.386981
20	Mahindra	0.795536	2.12874
21	Pramanik	3.630077	5.540438
22	JamilPaiker	0.366021	1.474269
23	DilipKumar	0.858565	2.948538
24	Manohar	2.928171	7.260153

25	ArunBagath	2.20381	1.453973
26	SamirHaldar	10.55606	5.35171
27	SanjayPandit	2.969047	3.317278
28	TarunKumar	15.67072	10.12605
29	Parameshwar	0.535887	1.717131
30	KhalidIqbal	0.650671	2.531513
	Mean	**3.844906**	**4.992286**

Sl no	Name	DMT1 expression levels	Ferroportin expression levels
1	Debabratha	2.281527	6.727171
2	Margabandhu	1.394744	4.69134
3	Rajendran	0.737135	1.802501
4	PronobBose	20.82147	10.62949
5	LuxmiTudu	12.81712	1.892115
6	SajalMondol	6.105037	2.948538
7	Gunashekaran	19.97329	14.92853
8	DeepakKumar	0.493116	1.414214
9	Ilango	34.5353	21.70567
10	GopalPrasad	0.435275	1.79005
11	Shankar	1.558329	4.789915
12	UnniMoin	0.594604	3.434262
13	HaridasNaik	6.964405	15.34823
14	Janardhan	10.19649	12.295
15	Sridhar	8.339726	11.55143
16	MehatiAli	7.260153	8.514961
17	RajeshPasi	0.316439	1.717131
18	santoshMonda	0.293209	1.464086
19	Durai	12.90627	8.633826
20	Shambunath	12.81712	6.453134
21	Yuvaraj	1.22264	2.989698
22	Anuraj	1.802501	4.789915
23	Venkatesan	3.271608	3.655326
24	Raja	0.456916	5.205367
	Mean	**6.983101**	**6.640496**

Anti-plagiarism check



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E-mail	drrasalkar27@yahoo.co.in
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INTRODUCTION Iron is one of the physiologically important trace elements. The total amount of iron present in the body is in the range of 3-4 grams, of which around two-thirds is present in hemoglobin (Adamson, 2008). In the body, iron is present either as heme (attached to a porphyrin nucleus) or as iron-sulphur compounds. The largest consumer of iron in the body is the erythoid precursors in bone marrow (Cook et al; 1973). The rest is utilized by other cells for incorporation, as prosthetic groups, into proteins. In proteins, they may be either a structural component or a functional component. For example, they may form part of the active site of enzymes, of myoglobin (in muscle cells) and...

IRB approval

Office of the Vice Principal(Research)

Christian Medical College,
Vellore 632 002.

Ref: Res/1/2010

May 7, 2011

OFFICE OF THE TREASURER
11 MAY 2011
C.M.C. VELLORE - 632 002

The Treasurer
CMC.

Dear Mr. Denzil,

Sub: **FLUID Research grant project NEW PROPOSAL:**
Expression levels of duodenal proteins involved in iron absorption in patients with alcoholic liver disease.
Dr Kavita P Rasalkar, PG Demonstrator, Biochemistry, Dr. Molly Jacob, Dr Joe Varghese, Ms Abitha Sukumaran, Biochemistry, Dr. C. E. Eapen, Hepatology.

Ref: IRB Min. No. 7399 dated 28.01.2011

The Institutional Review Board at its meeting held on January 28, 2011 vide Min. No. 7399 accepted the project for 6 months at a total sanction of ₹ 80,000/- (Rupees Eighty thousand only)and out of which a maximum of ₹ 1,500/- can be spent for stationery, printing, Xeroxing and computer charges (if computers used are within the institution). Kindly arrange to transfer the said amount to a separate account to be operated by Drs. Kavita P Rasalkar and Molly Jacob.

Thanking you

Yours sincerely,

George Mathew

Dr.George Mathew, MS, MD, ~~FCAMS~~
Chairperson, Research Committee &
Principal

CC: Dr. Kavita P Rasalkar, PG Demonstrator, Department of Biochemistry, CMC (Emp. No 20716)
Dr. Molly Jacob, Professor, Department of Biochemistry, CMC
File

22406 - RF. Dr. Kavita P Rasalkar - Biochemistry (7399)