DUODENAL HYPOXIA-INDUCIBLE FACTOR-2 ALPHA (HIF-2*α*) AND IRON-RELATED PROTEINS IN ALCOHOLIC LIVER DISEASE

DISSERTATION

Submitted to

THE TAMILNADU DR.MGR MEDICAL UNIVERSITY

In partial fulfillment for the degree

DOCTOR OF MEDICINE

IN

BIOCHEMISTRY - BRANCH XIII

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CHRISTIAN MEDICAL COLLEGE

VELLORE- 632002, INDIA

CERTIFICATE

This is to certify that the study titled **"DUODENAL HYPOXIA-INDUCIBLE FACTOR - 2 ALPHA (HIF-2*α*) AND IRON-RELATED PROTEINS IN ALCOHOLIC LIVER DISEASE"** is the bonafide work of Dr.S.Mathuravalli, who conducted it under the guidance and supervision of Dr. Molly Jacob, Professor of Biochemistry, Christian Medical College, Vellore. The work in this dissertation has not been submitted to any other university for the award of a degree.

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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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TABLE OF CONTENTS

Chapter No.	Title	Page No.
1	Abstract	9
2	Review of literature	11
3	The study	35
4	Materials	37
5	Methods	39
6	Results	68
7	Discussion	90
8	Conclusion	99
9	Limitations of the study	99
10	Bibliography	100
11	Appendices	
	Appendix I- Letter of approval from the Institutional Review Board (IRB)	113
	Appendix II -Information sheet and consent form	117
	Appendix III- Proforma for study	132
	Appendix IV- MIQE checklist	133
	Appendix V- Master data sheet	139

ABSTRACT

Background to the study

Alcoholic liver disease (ALD) is often associated with dysregulation of iron homeostasis. Previous work from the investigators' group has shown that expression of proteins involved in duodenal iron absorption were down-regulated in patients with ALD. Several factors are known to regulate expression of these duodenal proteins (which include divalent metal transporter 1 [DMT-1], duodenal cytochrome b [Dcytb] and ferroportin). Hepcidin is one such factor. Serum hepcidin levels were deceased in these subjects and, hence, this did not explain the decreased expression of the duodenal proteins. Hypoxia-inducible factor-2α (HIF-2α) in the duodenum is another such regulator, which is known to induce the transcription of these duodenal proteins. Studies done in mice have shown that chronic alcohol ingestion decreased intestinal HIF-2α levels. However, it is not known whether HIF-2α expression is altered in patients with ALD.

Aim

To determine protein expression levels of HIF-2α and gene expression levels of divalent metal transporter 1 (DMT-1), ferroportin and duodenal cytochrome b (Dcytb) in duodenal mucosal samples obtained from patients with ALD and in control subjects.

Methods

Eighteen patients with ALD and 18 control subjects were recruited for the study, after obtaining informed consent. Blood and duodenal mucosal samples were collected from these patients, each of whom underwent a medically-indicated upper gastrointestinal endoscopy. Blood samples were used for estimation of hematological parameters, liver function tests, high-sensitivity C-reactive

protein (hs-CRP) and markers of iron status. The duodenal mucosal samples were used for western blot analysis to determine protein levels of HIF-2α and for quantitative PCR to determine mRNA expression of DMT-1, Dcytb and ferroportin.

Results

Hemoglobin and total iron-binding capacity (TIBC) were significantly lower in patients with ALD than in control subjects. Serum levels of ferritin and hs-CRP and transferrin saturation were significantly higher in patients with ALD than in control subjects. Protein levels of HIF-2α and mRNA expression of DMT-1, Dcytb and ferroportin in duodenal mucosal samples were not significantly different in the two groups. HIF-2α did not correlate with the mRNA expression of any of the duodenal proteins involved in iron absorption.

Conclusion

The results of this study showed that patients with ALD were anemic and showed evidence of systemic inflammation. HIF-2α was not found to be altered in patients with ALD. However, the sample size of this study was small. An adequate sample size needs to be studied to confirm these findings.

Keywords: Alcoholic liver disease, duodenum, HIF-2α, iron

REVIEW OF LITERATURE

INTRODUCTION

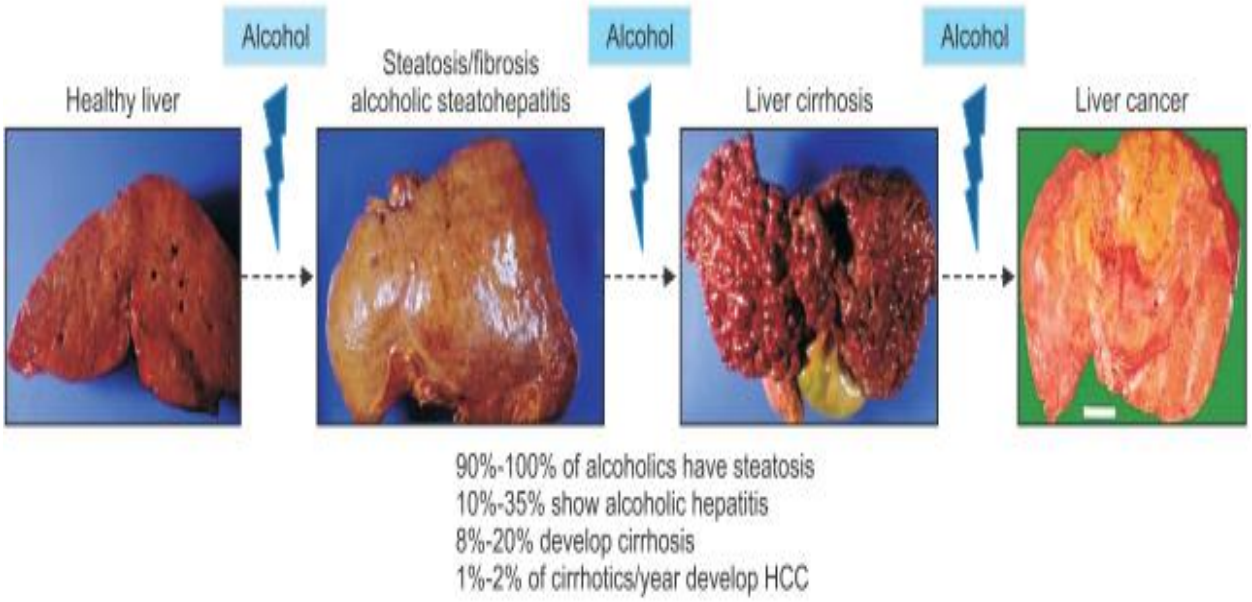
Excessive alcohol consumption is a global health problem, with enormous social, economic, and clinical consequences. Alcohol consumption accounts for nearly 5.9% of all global deaths (World Health Organization and Management of Substance Abuse Unit, 2014). Excessive drinking over many years damages nearly all organs in the body, of which the liver sustains the earliest and greatest injury. This is because the liver is the primary site of metabolism of ethanol. Alcoholic liver disease (ALD) is the most important cause of death due to alcohol use in adults (Rehm et al., 2013).

ALCOHOLIC LIVER DISEASE (ALD)

Heavy alcohol consumption produces a spectrum of pathological changes in the liver. The most characteristic of these are fatty liver (steatosis), hepatitis, fibrosis/cirrhosis and hepatocellular carcinoma. These pathological hallmarks of ALD – steatosis, inflammation and fibrosis - are the result of interrelated and consecutive events that occur due to continuous alcohol exposure (Stickel et al., 2017).

The pathogenesis of alcoholic liver injury is unclear. Alcohol is metabolized in the liver mainly by aldehyde dehydrogenase in the cytosol. Other minor pathways are those by CYP2E1 in endoplasmic reticulum and catalase in peroxisomes. Acetaldehyde produced by the reaction is highly reactive and toxic to the hepatocytes (Konishi and Ishii, 2007). Reactive oxygen species (ROS) and lipid peroxidation products along with inflammation in chronic alcohol intake may cause liver injury (Magdaleno et al., 2017).

Figure 2.1. Spectrum of pathological changes in ALD



Source: Stickel et al (2017). Pathophysiology and Management of Alcoholic Liver Disease: Update 2016. Gut Liver 11, 173–188

Role of iron in pathogenesis of ALD

Most heavy alcohol consumers do not progress beyond steatosis of the liver, which suggests that other factors contribute to progression of disease in ALD. One such factor, which has been postulated to promote disease progression in ALD, is iron (Magdaleno et al., 2017).

Hepatic iron overload is seen in nearly 50% of patients with ALD (Basaranoglu et al., 2013; Milic et al., 2016). Chronic alcohol consumption in moderate to excessive amounts is associated with elevation of levels of serum ferritin and transferrin saturation, and is suggestive of increased

hepatic iron stores (Milic et al., 2016). Excess iron and alcohol individually cause oxidative stress and lipid peroxidation. Hence, alcohol-induced iron overload enhances the production of free radicals and proinflammatory cytokines. The cumulative effects of iron and ethanol thus cause liver cell damage and exacerbate liver injury in patients with ALD (Mueller and Rausch, 2015).

IRON

Iron is a transition metal and is an essential micronutrient. It is indispensable for living organisms, as it is a cofactor for several hemoproteins and non-heme iron-containing proteins (Rodwell et al., 2015). Functions of hemoproteins include oxygen transport (hemoglobin), oxygen storage (myoglobin) and cellular respiration and electron transport (cytochromes). Non-heme iron-containing proteins are important for DNA synthesis, cell proliferation and differentiation (ribonucleotide reductase) (Pantopoulos et al., 2012).

Iron can exist in two redox states (Fe^{++} and Fe^{+++}). This property of iron is useful in biochemical reactions, to donate or accept electrons (Evstatiev and Gasche, 2012). This property also makes it toxic if iron is present in its free form. Free iron has the ability to catalyze the formation of harmful oxygen free radicals, via the Fenton reaction and Haber Weiss reaction, which may cause cellular and tissue damage (Haber and Weiss, 1934). Hence, in biological systems, iron is always protein-bound to minimize such free radical-mediated damage.

Approximately 3- 4 g of iron is found in an average adult human. Iron present in the human body is derived from absorption from the gut or from recycling of iron in macrophages (from heme in senescent RBCs) (Rodwell et al., 2015). Most of the body's requirements for iron are met by recycling of iron in the macrophages. Loss of 1 -2 mg iron/day occurs through desquamation of

intestinal epithelial cells (Green et al., 1968). In females, the loss is higher due to menstruation. Iron losses are balanced by absorption of dietary iron, which occurs mainly in the proximal duodenum. These losses are not regulated. Hence, regulation of the iron content of the body occurs mainly at the level of absorption (Gulec et al., 2014a).

Dietary iron and its absorption

The major form of iron in the diet is non-heme iron; heme iron is found in small quantities and is absorbed better. This is because, absorption of non-heme iron is affected by the iron status of an individual and other dietary factors, while absorption of heme iron is less affected by such factors (Hallberg, 1983).

Absorption of non-heme iron

The main site of iron absorption is the proximal duodenum (Muir and Hopfer, 1985). Proteins involved in the uptake of iron from the food are present in the brush border membrane of the enterocyte.

Divalent metal transporter1 (DMT-1)

Non-heme iron is taken up mainly by the divalent metal transporter (DMT-1) located in the brush border of enterocytes. This transmembrane protein is also known as divalent cation transporter (DCT) and natural resistance associated macrophage protein 2 (NRAMP2). In humans, this protein is encoded by the *SLC11A2* gene. DMT-1 also transports other divalent metal ions such as manganese, cobalt, copper and zinc, apart from iron. This transporter is active in an environment with a low pH, such as the duodenum, as it requires co-transport of protons (Gunshin et al., 1997). Iron found in the ferric form in the intestinal lumen has to be converted to

its ferrous form for absorption to occur. The conversion of ferric to ferrous form is brought about by the presence of ascorbate in the lumen of the gut or by membrane-bound ferrireductases (Hurrell and Egli, 2010).

Duodenal cytochrome b (Dcytb)

Ferric iron in the intestinal lumen is reduced to its ferrous form by the action of a ferrireductase, duodenal cytochrome b (Dcytb), which is found on the apical surface of enterocytes. This is a plasma membrane di-heme protein, first isolated from the duodenal mucosa of mice (McKie et al., 2001). This ferrireductase is more important in humans and other scorbutic species, which cannot produce ascorbic acid in vivo. However, in mouse models in which this protein was knocked out, intestinal iron absorption was not found to be affected, suggesting that Dcytb may not be the only ferrireductase in the intestine (McKie, 2008).

Ferroportin

Iron that enters the enterocyte can be either stored as ferritin or exported to the plasma. Ferroportin is a transmembrane protein expressed on basolateral membrane of enterocytes. It is also known as iron regulated transporter 1 (IREG1), membrane transport protein (MTP) and SLC40A1. It is the only known iron exporter in mammalian cells (Donovan et al., 2005). Unlike DMT-1, it is selective for iron.

Hephaestin

Hephaestin is a membrane-bound homolog of ceruloplasmin, which is a serum multi-copper oxidase. It oxidizes the ferrous form of iron to its ferric form, when it is released from ferroportin

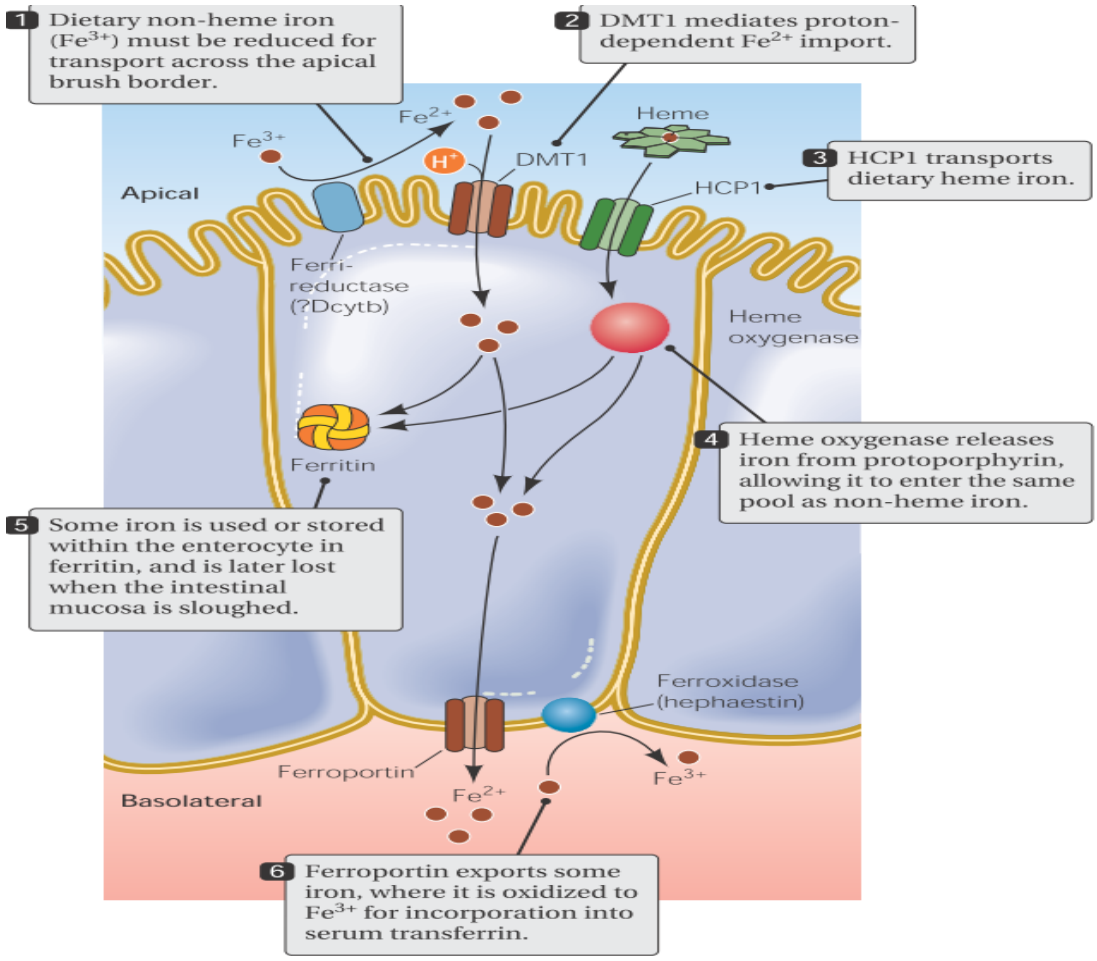
(Vulpe et al., 1999). The ferric form of iron gets incorporated into transferrin, which is the carrier protein for iron in blood.

Absorption of heme iron

Heme iron has a higher bioavailability than non-heme iron. However, the mechanism by which heme is absorbed is not totally clear. Proposed mechanisms involved in the absorption of heme iron include receptor- mediated endocytosis or a heme carrier protein (PCFT/HCP1-) which transports heme iron from the lumen into the cytoplasm of the enterocyte. Apart from heme transport, the PCFT/HCP1 transporter has also been shown to be a folate/proton symporter (West and Oates, 2008). Heme is catabolized in the enterocytes by heme oxygenase 1 and 2, which release inorganic iron (Ryter et al., 2006). In spite of higher bioavailability, the absorption of heme iron cannot be upregulated to the same extent as non-heme iron, as seen in iron deficiency states (West and Oates, 2008).

Figure 2.2 shows the process of intestinal iron absorption.

Figure 2.2. Intestinal iron absorption



Source: Donovan et al (2006). The ins and outs of iron homeostasis. Physiol. Bethesda Md 21, 115–123**.**

Iron in circulation

Iron in circulation is bound to transferrin, a glycoprotein synthesized by the liver. It has two high- affinity iron-binding sites. When both sites are occupied by iron, it is referred to as holotransferrin. Under normal circumstances, transferrin is not fully saturated. Only 30-40% of its sites are iron-bound (transferrin saturation). In case of iron deficiency, the transferrin saturation drops to <16%; in iron overloaded states, the saturation is > 45% (Rodwell et al., 2015). When transferrin saturation exceeds 60%, non-transferrin bound iron (NTBI) accumulates in the circulation (Hentze et al., 2010).

Cellular iron uptake

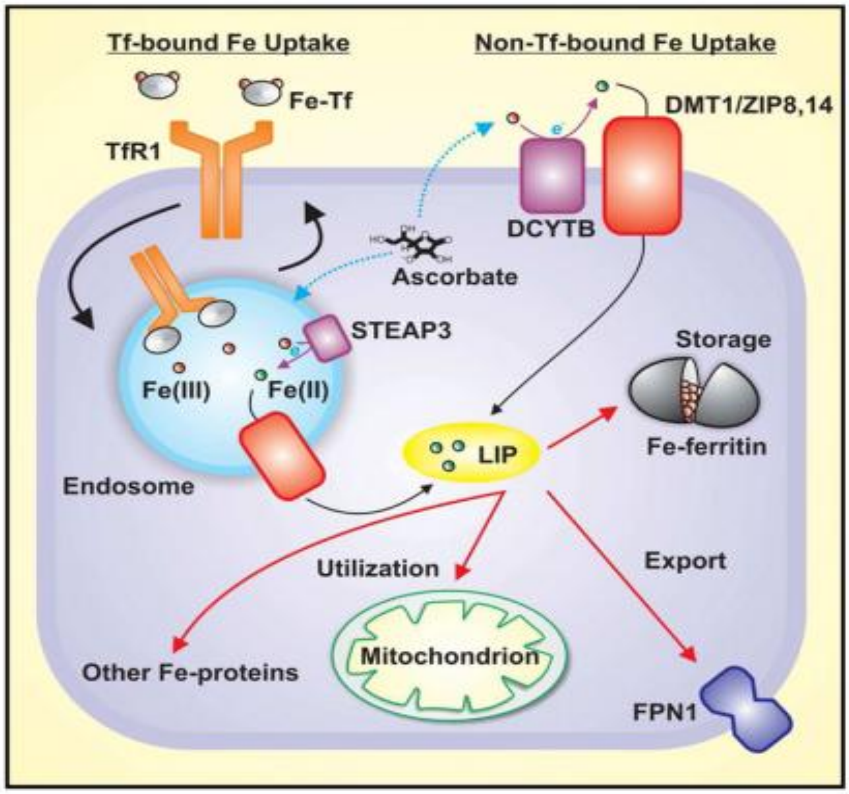
Under physiological conditions, most cells take up transferrin-bound iron from the circulation. Diferric transferrin binds to transferrin receptor 1 (TfR1) on the surface of cells. Cellular uptake is facilitated by receptor-mediated endocytosis. When the pH of the endocytic vesicle is 5.5 or less, iron dissociates from the transferrin receptor complex, but apotransferrin remains bound to the receptor (Dautry-Varsat et al., 1983). Iron, which is in the ferric form is reduced to its ferrous form by endosomal reductase Steap 3 (six-transmembrane epithelial antigen of the prostate 3) (Ohgami et al., 2005). From the endosome, ferrous iron is transported through DMT-1 to the cytoplasm. The apotransferrin receptor complex is recycled to the cell surface. Apotransferrin then detaches from the receptor, when it encounters the more neutral pH of blood, thus completing the transferrin cycle. There are two types of transferrin receptors TfR1 and TfR2. TfR1 is found on the surface of most cells. TfR2 is found in hepatocytes and small intestinal crypt cells. TfR2 has a lower affinity for holotransferrin than TfR1. It functions as a sensor of iron levels. (Rodwell et al., 2015).

Non-transferrin-bound iron (NTBI)

Uptake of NTBI is relevant in iron-overloaded conditions, such as like hereditary hemochromatosis and thalassemia. Such uptake by tissues such as the liver, heart and pancreas may help to clear potentially toxic levels of iron before damage due to iron catalysed-free radicals can accumulate (Lane and Lawen, 2008). Though the exact route of uptake of NTBI remains unclear, mechanistically it is thought to involve reduction of ferric iron to its ferrous form and import of the ferrous form into the cell through transporters such as DMT-1 or ZRT/IRT like proteins (ZIPs) , ZIP14 or ZIP 18 (Lane et al., 2015).

Figure 2.3 illustrates the steps involved in cellular uptake of transferrin-bound and non-transferrin-bound iron.

Figure 2.3. Cellular iron uptake



Source: Lane et al (2015). Cellular iron uptake, trafficking and metabolism: Key molecules and mechanisms and their roles in disease. Biochim. Biophys. Acta BBA - Mol. Cell Res. 1853, 1130–1144

Cellular iron storage

Iron in excess of a cell's requirements is converted to an inert form to prevent cell damage due to production of reactive oxygen species. In non-erythroid cells, about 70 - 80% of iron taken up is incorporated into ferritin (Arosio and Levi, 2010). Ferritin has dual functions of iron storage and iron detoxification, by sequestering the iron (Harrison and Arosio, 1996). Mammalian ferritin consists of a multimeric protein shell, with 24 light and heavy chain subunits. The ferritin core

can accommodate about 4500 iron atoms in the form of ferric hydroxide. Each heavy subunit has a catalytic site that oxidizes two ferrous atoms (Liu and Theil, 2005). When cellular iron levels are low, iron is released from ferritin.

Serum ferritin

Apart from the cytosol and mitochondria, ferritin is also found in blood. Serum ferritin is considered to be a marker of body iron stores. It mainly consists of light chains; its core is relatively iron-poor, when compared with cellular ferritin (Evstatiev and Gasche, 2012). The primary source of serum ferritin is from macrophages (Cohen et al., 2010). Its exact function is not clear. Ferritin is also an acute phase protein, which increases in response to acute and chronic inflammation (Kell and Pretorius, 2014).

IRON HOMEOSTASIS

Systemic iron homeostasis

The cell types involved in iron homeostasis include the following:

1. Duodenal enterocytes, which absorbs dietary iron
2. Macrophages, which recycle iron from phagocytosed erythrocytes and other cells
3. Hepatocytes, which store iron and release it when required (Ganz, 2013)

The most important regulated step in all these cells is the transfer of iron from these cells into the plasma. This step is regulated by hepcidin, a hormone produced by hepatocytes.

Role of hepcidin

Hepcidin was originally identified as a liver-expressed antimicrobial peptide (LEAP1), with antimicrobial activity against many bacterial and fungal species (Krause et al., 2000). It is encoded by the *HAMP* gene, located on the long arm of chromosome19. It is synthesized as a

biologically inactive 84-amino acid pre-proprotein; it is made up of a signal peptide, a pro-region and an active 25-amino acids sequence. It is proteolytically cleaved by the prohormone convertase, furin, resulting in the release of biologically active hepcidin (Valore and Ganz, 2008). It is synthesized and secreted by hepatocytes, and circulates in the plasma; it binds weakly to albumin and α2-macroglobulin (Peslova et al., 2009).

Hepcidin is mainly expressed in the liver. However, its expression has also been detected in macrophages, pancreatic beta cells, adipocytes, lungs and kidney (Kulaksiz et al., 2008; Nguyen et al., 2006).

Hepcidin acts as a negative regulator of iron stores. Its production by the liver is induced in response to increased iron levels (Ganz, 2011). It acts at the site of iron absorption, storage and recycling and cause a decrease in release of iron into the plasma from the enterocyte, hepatocyte and macrophages (Rishi et al., 2015). It does so by binding and inducing the internalization and degradation of ferroportin, which is the only known iron exporter (Nemeth et al., 2004b).

Regulation of hepcidin

Hepcidin is regulated at a transcriptional level. Its expression is influenced by iron levels in the body, inflammation, erythropoietic activity and hypoxia (Ganz, 2013). Iron overload and inflammation up regulate hepcidin expression; iron deficiency, hypoxia and erythropoietic activity decrease it (Silva and Faustino, 2015).

Cellular iron homeostasis

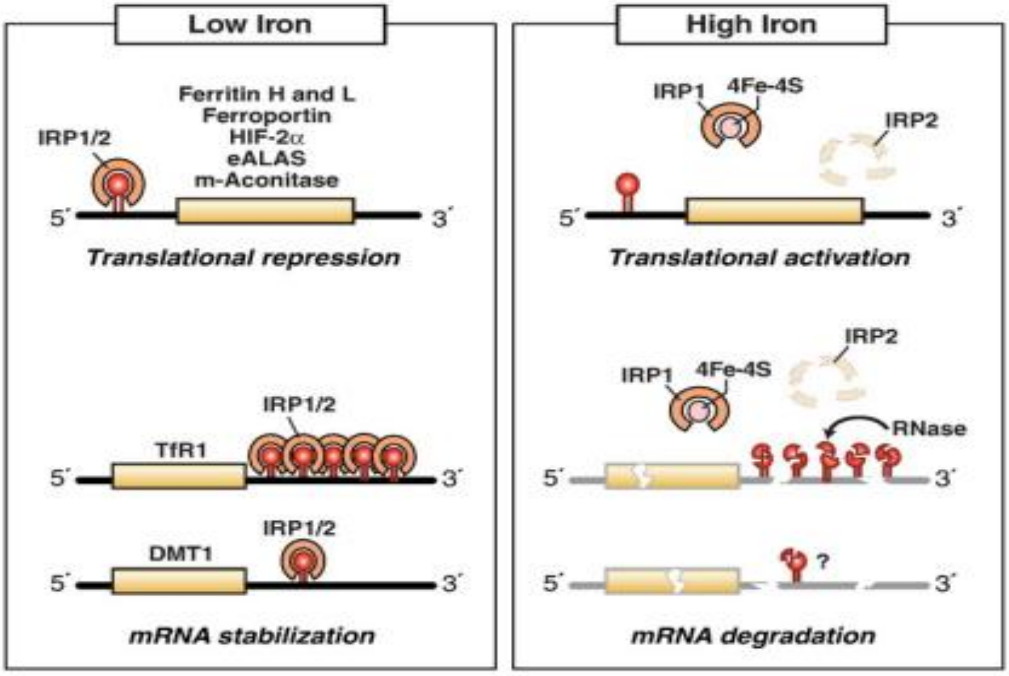
Cellular iron homeostasis is maintained by regulation of the proteins involved in iron uptake, storage and release. These regulatory mechanisms occur at a post-transcriptional level. The IRE-IRP system is responsible for this regulation (Rouault, 2006).

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

In iron-depleted cells, IRPs are induced. They bind to IREs in the 5' or 3' untranslated regions (UTR) in the mRNA for proteins involved in iron metabolism. Binding of the IRP to the 5' UTR of the mRNA for ferritin, ferroportin, aminolevulinic acid synthase and hypoxia-inducible factor-2 alpha (HIF- 2α) suppress their translation. Binding of the IRP to the 3'UTR of mRNA for transferrin and DMT-1 results in increased stability of the mRNA (against nuclease attack) (Hentze et al., 2010). In iron-replete conditions, IRP1 acquires an iron sulphur cluster and loses its ability to bind to the IRE; it then functions as a cytosolic aconitase (Lane et al., 2015). IRP2 undergoes ubiquitination by FBXL 5 (F-box and leucine rich repeat protein 5) and proteasomal degradation in iron-replete cells (Guo et al., 1995). Hence, when iron levels increase, IRPs are not able to bind to the IREs, thus limiting acquisition of iron by cells.

Figure 2.4 depicts how the IRE IRP system operates to regulate intracellular iron levels, in iron-depleted and iron-replete conditions.

Figure 2.4. Regulation of intracellular iron levels by the IRE-IRP system



Source: Anderson et al (2012). Mammalian iron metabolism and its control by iron regulatory proteins. Biochim. Biophys. Acta BBA - Mol. Cell Res., Cell Biology of Metals 1823, 1468–1483.

The gut in iron homeostasis

No regulated mechanisms exist in humans to excrete iron. Body iron levels are mainly controlled by modifying rates of absorption of iron in the proximal small intestine. The mechanisms that regulate iron absorption allow for appropriate increases or decreases in absorption, in accordance with physiological requirements. Thus, absorption of iron is increased when body iron stores are low, when erythropoietic demand for iron is high, in chronic hypoxia,

and in pregnancy. Absorption of iron decreases when body iron stores are high and during infection/ inflammation. Intestinal iron absorption is regulated by systemic as well as cellular mechanisms to maintain adequate iron levels in the body. (Gulec et al., 2014b).

Systemic regulation of iron absorption

Duodenal enterocytes are an important target of hepcidin (Rivera et al., 2005). An inverse relationship exists between hepcidin levels and expression of duodenal proteins involved in iron absorption (Frazer et al., 2002). This effect of hepcidin is mediated by its interaction with ferroportin on the basolateral membrane of the enterocytes. This results in internalization and eventual degradation of ferroportin (Nemeth et al., 2004b). This limits the amount of iron that enters the circulation. Studies have shown that hepcidin also causes proteasomal degradation of DMT-1, found on the apical surface of the enterocyte (Brasse-Lagnel et al., 2011). Hepcidin levels are elevated in situations of iron overload and inflammation (Schmidt et al., 2008; Wrighting and Andrews, 2006). Under these circumstances, absorption of dietary iron is inhibited.

Cellular mechanisms that regulate iron absorption

When the demand for iron increases in the body, specific changes in the intestinal mucosal cells help to take up maximum iron from the diet. Some of the adaptation mechanisms include changes in gene transcription and mRNA stability and re-modelling of epithelium. (Gulec et al.,2014b). The transcriptional control of iron transporters is mainly by hypoxia- inducible factors and also by post transcriptional mechanisms that operate through the IRE-IRP system.

IRE-IRP system and intestinal iron transporters

Under conditions of iron depletion, the IRE IRP system plays a major role in post-transcriptional stability of mRNA of DMT-1 and ferroportin. DMT-1 is strongly upregulated in conditions of iron deprivation (Collins et al., 2005). Two DMT-1 3′-splice variants exist, with and without the IRE. The variant with the IRE (+IRE) is the form predominantly expressed in the duodenum (Tchernitchko et al., 2002). When intracellular iron levels are low, IRPs bind to the 3'IRE leading to stabilization of the mRNA for DMT-1, which ultimately leads to increased protein production (Galy et al., 2008).

Protein levels of ferroportin in intestinal cells are modified by its interaction with circulating hepcidin, which cause internalization and degradation of ferroportin, thereby blocking efflux of iron from enterocytes (Nemeth et al., 2004b). The presence of IRE in the 5' UTR of the mRNA for ferroportin would predict that FPN translation would be inhibited when enterocytes are iron-deprived. This seems inconsistent with the observed increase in duodenal FPN levels in states of iron deficiency (McKie et al., 2000). This contradiction is explained by the presence of two variants – one with an IRE and the other that lacks an IRE. Both are expressed in the duodenum. Evidence in mice suggests that the +IRE variant predominates in duodenum, even under conditions of iron restriction (Zhang et al., 2009). The observation of increased FPN expression in iron-deficient enterocytes can partially be explained by the expression of FPN mRNA variants that lack IREs. It could also reflect a dominant contribution of hepcidin-dependent regulation of FPN turnover, over translational control by IRPs (Galy et al., 2013).

Hypoxia and intestinal iron transporters:

The epithelial cells of the intestine are in hypoxic condition. Enterocytes on the upper part of the villus furthest from the capillary bed in the lamina propria are most significantly affected (Colgan and Taylor, 2010).

Cell-autonomous regulation of duodenal iron absorption in response to hypoxia is well documented. Studies have shown that acute hypoxia affects uptake of iron at the apical surface of enterocytes, independent of systemic factors such as the erythropoietic drive or hepcidin levels. Increased apical uptake of iron was observed in rats as early as 6 to 8 hours after onset of hypoxia (Hathorn, 1971). This early induction precedes changes in circulating levels of iron or in erythropoiesis (Raja et al., 1988) or a decrease in hepcidin levels (which occurs only after at least 24 hours of hypoxia in mice and in humans) (Talbot et al., 2012).

Hypoxia-inducible factors

The body's response to hypoxia is mediated by hypoxia-inducible factors (HIF), which are transcription factors (Semenza, 2001). Cells respond to conditions of insufficient oxygen availability by increasing the amount and activity of HIF transcription factors, which induce expression of a large number of genes involved in the response to hypoxia. HIF is a heterodimer made of an α-subunit and a β-subunit. α-subunit is a regulatory subunit. The β-subunit is constitutively expressed. There are 3 types of regulatory subunits - 1α, 2α and 3α (Tian et al., 1997; Gu et al., 1998). The beta-subunit is also known as an aryl hydrocarbon nuclear translocator (ARNT). Oxygen-regulated HIF-1α and HIF-2α subunits form heterodimers with the constitutively expressed HIF-1β subunit, and bind hypoxia-responsive elements (HRE) in regulatory regions of DNA.

The specific roles of HIF-1α and HIF-2α have not been fully elucidated so far. In vitro, they both bind the same HRE. They also share an overlapping set of target genes, but also activate the transcription of distinct genes (Recalcati et al., 2015) For example, both bind the HRE of erythropoietin (Epo), but only HIF-2α is important for Epo production in vivo. Glycolytic enzymes are regulated only by HIF-1α (Sowter et al., 2003). Proteins involved in absorption of iron from the intestinal lumen across the enterocyte into the circulation, viz DMT-1, Dcytb and Fpn, are direct targets of HIF-2α.

Table 1 Proteins of iron metabolism regulated by HIF

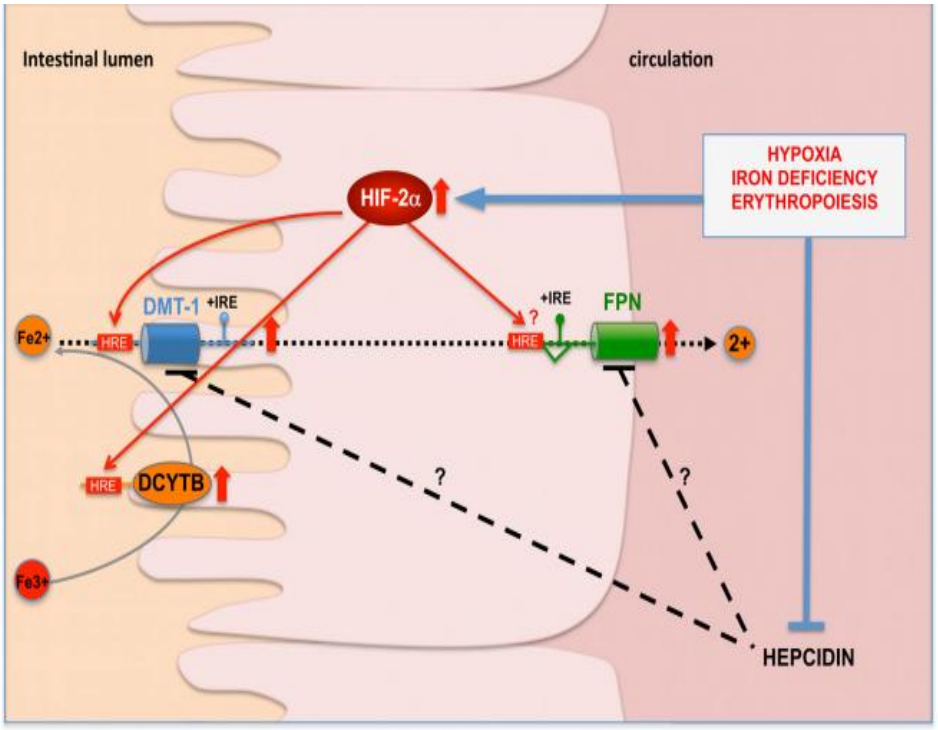
Proteins	Functions
HIF-1 dependent	
Transferrin	Iron transport
Transferrin receptor 1	Iron uptake
Ceruloplasmin	Iron oxidation
Ferrochelatase	Heme synthesis
Furin	Regulation of iron homeostasis
Heme oxygenase	Heme catabolism
Matriptase2	Regulation of iron homeostasis
HIF-2 dependent	
DMT1	Iron uptake
DcytB	Iron reduction
Epo	Erythropoiesis
Ferroportin	Iron export
Frataxin	Assembly of iron sulfur-cluster

Abbreviations: DMT1, divalent metal transporter; Epo, erythropoietin; HIF, hypoxia inducible factors.

Source: Recalcati et al., 2015 New perspectives on the molecular basis of the interaction between oxygen homeostasis and iron metabolism. Hypoxia 2015:3 93–103

HIF-3α is structurally and functionally distinct. It is found in humans and other mammals. However, but its role and mode of action are not clear (Recalcati et al., 2015).

Figure 2.5. Regulation of intestinal iron transport related proteins by HIF-2α



Source: Mastrogiannaki et al (2013).The gut in iron homeostasis: role of HIF-2 under normal and pathological conditions. Blood, volume 122, number 6

Fate of HIF in normoxic conditions

In a normoxic states, conserved proline residues of HIF-α undergo hydroxylation. This reaction is catalyzed by prolyl-4-hydroxylase domain enzymes (PHDs) (Epstein et al., 2001). These enzymes are dioxygenases; they utilize oxygen as substrate, 2-oxoglutarate as a cosubstrate, andiron and ascorbate as cofactors. The 4-hydroxyprolines formed are then recognized by von-Hippel Lindau (VHL) E3 ubiquitin ligase, which targets the HIF-α for proteasomal degradation (Maxwell et al., 1999). As the activity of PHDs is dependent on the availability of ferrous iron,

HIF-dependent transcription is also induced by iron deprivation, even under normoxic conditions (Salminen et al., 2015)

A 2-oxoglutarate dependent dioxygenase, which hydroxylates a conserved asparagine residue of HIF-1α and HIF-2α, is factor inhibiting HIF (FIH) (Bishop and Ratcliffe, 2014). Hydroxylation of asparagine impairs interaction of HIF-α with transcriptional coactivators (p300 and CREB-binding protein), thus inhibiting transcriptional activity HIF proteins (Dayan et al., 2006).

Role of HIF in hypoxia and iron deficiency

In hypoxia or iron deficiency, the enzyme activities of PHD, FIH are inhibited. Under such circumstances, HIF-α escapes degradation and translocate to the nucleus, where it dimerizes with the HIF-β subunit. This heterodimer then binds to the promoter or enhancer regions of target genes, at the hypoxia response elements (HREs), and recruits transcriptional coactivators, such as p300/CREB-binding protein, to activate transcription of the target genes (Wenger et al., 2005).

Hypoxia and iron homeostasis

Effects of hypoxia on IRP-binding

Studies suggest that hypoxia decreases IRP1 binding to IREs, and results in IRP1 acquiring aconitase activity. On the other hand, lack of oxygen increases IRP2 activity by stabilizing it (Cairo and Recalcati, 2007; Recalcati et al., 2010). F-box and leucine-rich repeat protein (FBXL5) is the key protein involved in stabilization of IRP2. It has a hemerythrin domain through which it senses iron and oxygen availability (Ruiz and Bruick, 2014). When iron and oxygen are fully available, FBXL5 accumulates in the cell, leading to proteasomal degradation

of IRP2. Oxygen deprivation promotes FBXL5 polyubiquitination and proteasomal degradation, via its hemerthyrin domain, leading to stabilization of IRP2 (Ruiz and Bruick, 2014).

Regulation of HIF-2α mRNA by IRP1

Presence of a 5′ IRE in the mRNA for HIF-2α showed the existence of another physiologically relevant connection between oxygen and iron sensing (Simpson and McKie, 2015). In normoxia, active IRP1 decreases basal HIF-2α translation. In hypoxia, the IRE/IRP1 interaction is impaired, leading to efficient translation of HIF-2α. In hypoxia, both increased translation and increased stability, due to inhibition of degradation by PHD, leads to increase in protein levels (Mastrogiannaki et al., 2013)

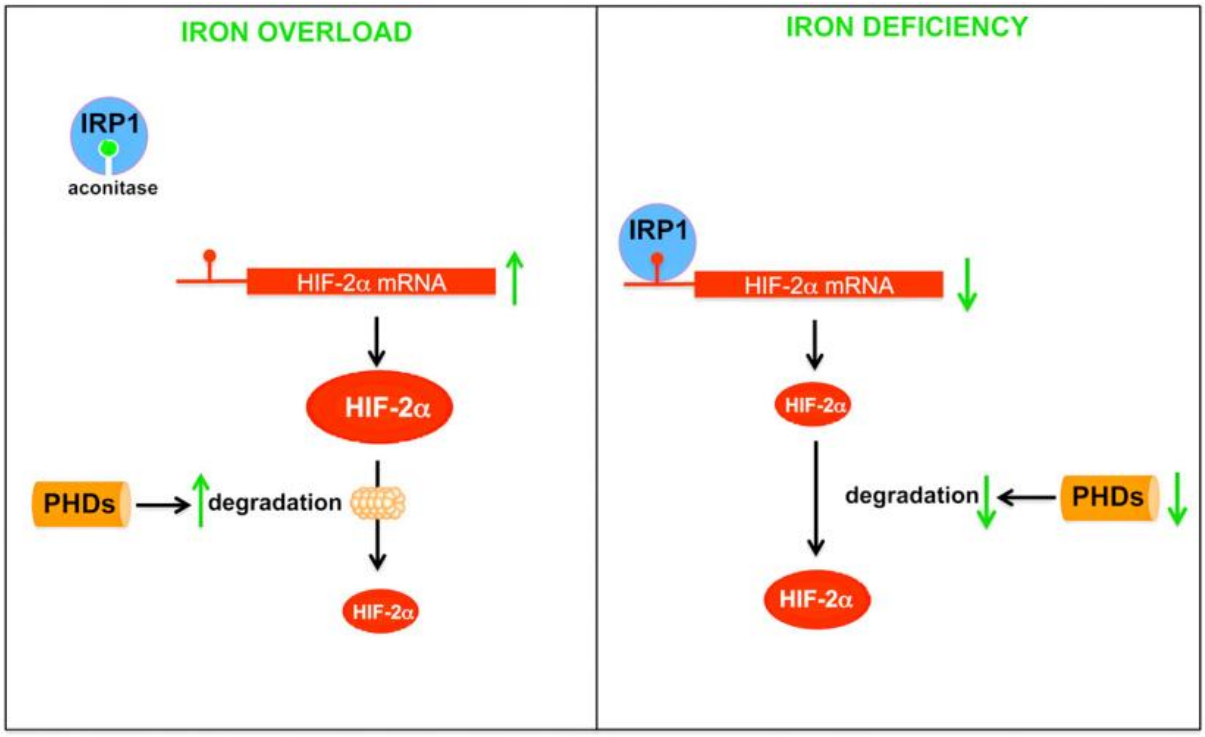
In presence of an intracellular iron deficiency, IRP1 activity is increased, which leads to repression of HIF-2α translation. However, increased HIF-2α protein levels have been reported in the duodenum of iron-deficient mice (Shah et al., 2009). This is because of stabilization of HIF-2α at the protein level, due to the decreased activities of PHDs in iron deficiency as shown in figure 2.6 (Mastrogiannaki et al., 2013).

Hypoxic suppression of hepcidin

The two hormones most important to regulate iron and oxygen homeostasis at the systemic level are hepcidin and erythropoietin, respectively. When erythropoietic activity is high, as in response to hypoxia or anemia, there is an increased absorption of iron to meet the higher demand of erythropoietic cells. This is accompanied by a strong inhibition of hepcidin expression (Kautz et al., 2014a). Studies suggest that hepatic HIF-2α inhibits hepcidin expression via an EPO-mediated increase in erythropoiesis (Mastrogiannaki et al., 2012). Erythropoietin stimulates the

synthesis of erythroferrone, which is produced by erythroblasts and causes hepcidin suppression during stress erythropoiesis (Kautz et al., 2014b).

Figure 2.6. Regulation of HIF-2α mRNA in duodenal enterocytes in iron-overloaded and iron-deficient states



Source: Mastrogiannaki et al (2013). The gut in iron homeostasis: role of HIF-2 under normal and pathological conditions. Blood 122, 885–892.

Effect of alcohol on iron metabolism

Alcohol consumption has been shown to be associated with effects on iron homeostasis; these range from anemia to iron overload (Conrad and Barton, 1980; Kohgo et al., 2005). Alcohol by itself does not induce iron deficiency or anemia. Their presence in ALD is a result of gastrointestinal blood loss arising due to the complications of ALD (Kimber et al., 1965).

Inadequate diet with nutrient deficits, in alcoholic patients, can result in anemia with megaloblastic features. Anemia of chronic disease can also occur in patients with chronic ALD (Gonzalez-Casas et al., 2009).

Mild alcohol consumption upto 2 drinks per day has been shown to be protective by reducing the risk of iron deficiency anemia, as wine has a high iron content and it increases iron absorption (Cook et al., 1995). On the other hand, heavy alcohol consumption (> 2 drinks /day) increases the risk of iron overload (Ioannou et al., 2004). Chronic alcohol consumption in moderate to excessive amounts is associated with elevation of serum ferritin concentrations and transferrin saturation. This can result in increased hepatic iron stores (Irving et al., 1988). In mild cases, iron accumulates in hepatocytes but in advanced cases of ALD, iron accumulation is seen in parenchymal cells and in Kupffer cells (Kohgo et al., 2005). Increased intestinal absorption of iron from the has been reported in chronic alcoholics (Duane et al., 1992).

Alcohol has been shown to down-regulate hepcidin expression in cell lines and animal models. It did not alter the expression of transferrin receptor-1, ferritin, or cause activation of IRP1 and IRP2 (Harrison-Findik et al., 2006). These results indicate that alcohol does not regulate hepcidin expression by altering the iron status of the cell, but possibly by a direct action on hepcidin. CCAAT/enhancer binding protein alpha (C/EBPα) is a transcription factor that regulates hepcidin expression. Alcohol was found to down-regulate the promoter activity and DNA-binding activity of C/EBP alpha (Harrison-Findik et al., 2006). It also inhibited iron-mediated up-regulation of C/EBP activity in the liver (Harrison-Findik et al., 2007a). Down-regulation of hepcidin levels has also been demonstrated in patients with ALD (Dostalikova-Cimburova et al., 2014).

Metabolism of alcohol induces oxidative stress in hepatocytes and Kupffer cells of the liver. This leads to the release of the pro-inflammatory cytokine, tumour necrosis factor (TNF-α), from activated Kupffer cells (Osna et al., 2017). TNF-α has been reported to down-regulate hepcidin expression in vitro. Thus, alcohol-mediated effects on hepcidin may involve hepatocytes and Kupffer cells of the liver (Nemeth et al., 2004a).

Despite an iron overload (which is known to induce hepcidin) in the mice model used by Harrison-Findik et al.(2007), alcohol was found to down-regulate the expression of hepcidin in the liver, suggesting that alcohol may render hepatic synthesis of hepcidin insensitive to increased body iron levels. (Harrison-Findik et al., 2007b)

Alcohol-mediated down-regulation of hepcidin expression in the liver has also been reported result in to elevated expression of the proteins, DMT-1 and ferroportin, which are involved in the absorption of iron in the duodenum. The increase in expression of these proteins has been suggested to result in increased intestinal absorption of iron, and hence increased body iron levels (Harrison-Findik et al., 2007a).

Effect of alcohol on HIF-2α expression

There is evidence from mouse studies that chronic alcohol exposure reduces HIF -2α expression in the intestine (Wang et al., 2011). However, there are no studies till date regarding expression of duodenal HIF-2α in patients with alcoholic liver disease.

THE STUDY

BACKGROUND TO THE STUDY

Alcoholic liver disease (ALD) is often associated with dysregulation of iron homeostasis. Previous work from the investigators' group has shown that expression of proteins involved in duodenal iron absorption were down-regulated in patients with ALD. Several factors are known to regulate expression of these duodenal proteins (which include divalent metal transporter 1 [DMT-1], duodenal cytochrome b [Dcytb] and ferroportin). Hepcidin is one such factor. Serum hepcidin levels were deceased in these subjects and this did not explain the decreased expression of the duodenal proteins. Hypoxia-inducible factor-2α (HIF-2α) in the duodenum is another such regulator, which is known to induce the transcription of these duodenal proteins. Studies done in mice have shown that chronic alcohol ingestion decreased intestinal HIF-2α levels. However, it is not known whether HIF 2α expression is altered in patients with ALD.

HYPOTHESIS

Expression of duodenal hypoxia-inducible factor-2α (HIF-2α) may be altered in patients with alcoholic liver disease**.**

AIM

The aim of this study was to test the hypothesis that expression of duodenal hypoxia-inducible factor-2α (HIF-2α) may be altered in patients with alcoholic liver disease.

OBJECTIVES

1. To determine protein expression of HIF-2α in duodenal mucosal samples, obtained from patients with alcoholic liver disease and from control subjects
2. To determine gene expression levels of iron-related proteins (DMT-1, Dcytb and ferroportin) in the duodenal mucosal samples obtained from these patients
3. To correlate HIF-2α protein expression with gene expression of duodenal iron-related proteins in these subjects
4. To measure levels of serum iron, ferritin, high-sensitivity C-reactive protein (hs-CRP), total iron binding capacity (TIBC) and transferrin saturation in blood samples from patients with ALD and control subjects, and to correlate these with expression of the duodenal proteins listed above.

MATERIALS

Equipment used

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

2. Table-top refrigerated centrifuge (MPW R 350, MPW Poland)

3. Minus 70^{o}C freezer (Thermo Scientific, Massachusetts, USA)

4. Glass homogenizer with teflon pestle (1 mL capacity) (Kimble-Kontes, USA)

5. Agarose gel electrophoresis system (Medox Biotech, India)

6. Gel documentation system (Alpha Innotech, USA)

7. Applied Biosystem 2720 Thermocycler (Thermo Fisher Scientific, USA) for cDNA construction

8. Real-time thermo cycler (Chromo4, Bio-Rad, USA) for qPCR

9. Vertical gel electrophoresis for western blots (Bio-Rad, USA)

10. Nanodrop spectrophotometer (Thermo Scientific, USA)

Chemicals and reagents

1. Diethyl pyrocarbonate (DEPC), ethidium bromide, ethylene diamine tetraacetic acid (EDTA), formamide, formaldehyde, bromophenol blue, sodium hydroxide and TRI-reagent for RNA isolation were obtained from Sigma, India.

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

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

4. 3–morpholinopropane sulfonic acid (MOPS) was purchased from Sigma, India.

5. Reverse transcription core kit, SYBR Green PCR master mix kit was obtained from TaKaRa Bio, USA.

6. Gene specific primers: beta-actin from Sigma, India; DMT-1, ferroportin and Dcytb from Eurogentec, Belgium.

7. Tris-HCl, sodium acetate, sodium chloride, sodium deoxycholate, sodium dodecyl sulphate, sodium orthovanadate, sodium fluoride, glycerol, glycine, methanol, glacial acetic acid, Tween-20, tetramethylethylenediamine, acrylamide, bisacrylamide, sodium chloride, potassium chloride, potassium dihydrogen phosphate and disodium hydrogen phosphate were obtained from Sisco Research Laboratories Pvt Ltd, Mumbai, India.

8. Ammonium persulphate, 2-mercaptoethanol, Ponceau S were purchased from Sigma, India.

9. Primary antibody for beta-actin (A5316) was obtained from Sigma Aldrich, India and for HIF 2-alpha (NB100-122) from Novus Biologicals, USA.

10. Secondary antibody for beta-actin (anti-mouse antibody) and HIF-2 alpha (anti-rabbit antibody) were obtained from Thermo Scientific, USA.

11. Super signal West Dura extended duration substrate obtained from Thermo Scientific, USA for detection of chemiluminescence of bands obtained on western blots.

All chemicals used were of analytical grade**.**

Miscellaneous consumables used

1. Vacutainer tubes for blood collection (BD Biosciences, Plymouth, UK).

2. Micro-tubes and centrifuge tubes (1.5mL, 0.5 mL), and micro tips (Tarsons Products Private Limited, Kolkata, India).

METHODS

The study was approved by the Institutional Review Board (IRB) at Christian Medical College (CMC), Vellore, India (IRB Min. No.9816 dated 07.01.16) (letter of approval in Appendix I).

Subjects

Patients in the Department of Hepatology and Gastroenterology at CMC, Vellore, who had been diagnosed to have alcoholic liver disease (ALD) and who required a medically-indicated upper gastrointestinal endoscopy were the subjects of the study. The following inclusion and exclusion criteria were employed to recruit patients:

Inclusion criteria

Patients who were diagnosed to have alcoholic liver disease based on a history of 20-30g (2 -3 standard drinks) of alcohol intake per day for 5 or more years

Diagnosis of cirrhosis, based on clinical and/or ultrasound evidence

Such subjects who consented to participate in the study

Exclusion criteria

Patients who met the above criteria but showed evidence of other co-existent liver disease or evidence of viral hepatitis as a cause of liver disease

An international normalized ratio (INR) above 1.5

Not willing to take part in the study

Informed consent

Once the study participants were identified based on the inclusion and exclusion criteria listed above, the study was explained to them and they were invited to participate. They were provided with an information sheet about the study. This was available in the following languages: Tamil, English, Hindi, Telgu or Bengali. Written informed consent was obtained from those who were willing to participate in the study. The information sheet and informed consent form used are included in Appendix II.

Recruitment

Patients with ALD, who gave informed consent, were recruited as cases. Patients who underwent a medically-indicated upper GI endoscopy for evaluation of dyspepsia, who had no abnormalities in liver function and who were found to have no abnormal findings on endoscopy were recruited as controls; informed consent was obtained from them as well.

Patients' data

A proforma was prepared to collect relevant clinical data on the patients recruited. This is shown in Appendix III. The investigator elicited relevant history from each patient and obtained clinical data (including relevant blood results) for each patient from their hospital records.

Sample size

A sample size calculation was done. In order to detect a change of 3.05 units in DMT-1 expression in the duodenum, with 80% power and at 5% level of significance, the required sample size was found to 48 subjects in each of the 2 groups. Calculations done were based on preliminary data obtained by the research group in the Department of Biochemistry, who had been studying duodenal iron-related proteins in patients with ALD.

Formula

$n = ((Z_{\alpha} + Z_{1-\beta}) \text{^}2 * 2 * S\text{^}2)/d\text{^}2$

where,

Z_{α} is 5% level of significance

$Z_{1-\beta}$ is the 80% power

S = standard deviation

d = 3.05 (mean change)

Number of patients studied

The total cost of antibodies and other reagents for western blot analyses and qPCR assays and for the blood tests was Rs.2780 per sample. The institutional research grant, which is available for postgraduates to carry out the work for their dissertation, was Rs 100000 for 2 years. This made it possible to do the various analyses only for a total of 36 patients. Hence 18 patients with ALD and 18 control subjects were studied. All the subjects in the study were recruited between June 2016 and March 2017

Sample collection

After obtaining written informed consent, fasting blood samples were collected from the patients, before the endoscopic procedure. About 6 mL of blood was collected in BD vacutainer tubes (red), by venipuncture of the cubital vein. Two samples of duodenal mucosal tissue from the D2 segment of the duodenum were collected in microtubes. One of these contained 0.5 mL TRI-reagent and was used for gene expression studies. The other microtube was snap-frozen in liquid nitrogen and used later for western blot analyses. Both tubes with mucosal samples were stored at -70^{o}C till further processing and analyses.

Processing of blood samples

The blood samples collected were allowed to clot and centrifuged at 2500 rpm for 10 minutes to obtain serum; this was done within 2 hours of collection of blood.

Storage of blood samples

The serum obtained was divided into aliquots and stored at -70^{o}C, till analyses were carried out.

When required, samples were thawed to room temperature and used for estimation of serum iron, TIBC, ferritin and hs-CRP.

Estimation of serum iron

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

Analyzer used: Roche Cobas c 702 modular analyzer

Principle of the method**:** Guanidine/ ferrozine spectrophotometric method

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

Reference interval

Male - 60- 160 µg/dL

Female - 40-145 µg/dL

Estimation of total iron binding capacity (TIBC)

Estimation of unsaturated iron-binding capacity (UIBC) was carried out in the Department of Clinical Biochemistry, CMC, Vellore.

***Analyzer used*:** Roche Cobas 8000 c 702 modular analyzer

Principle of the method

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

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

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

TIBC = Serum iron + UIBC

Reference interval

Male - 300-400 µg/dL

Female - 250-350 µg/dL

Calculation of transferrin saturation (TSAT)

Transferrin saturation (TSAT) was calculated as the ratio of serum iron level and the total iron-binding capacity of each sample, multiplied by 100.

TSAT = (Serum iron / TIBC) x 100

Estimation of serum ferritin

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

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

Principle of the method: Two-site sandwich immunoassay using direct chemiluminescence technology

A polyclonal antibody tagged with acridinium was added to the reaction chamber. A monoclonal mouse anti-ferritin antibody covalently bound with paramagnetic particles was added to the reaction chamber after addition of the sample. The paramagnetic particles acted as a solid phase for the immunoassay. Ferritin present in the sample formed a sandwich complex with the antibodies. The unbound antibodies were removed by aspiration. Acid reagent and base reagent were then added to initiate the chemiluminescent reaction. The intensity of photons released was measured and was directly proportional to the ferritin concentration in the sample.

Reference interval

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

Women < 50 years: 10-290 ng/mL

Estimation of hs-CRP

Estimation of hs-CRP was carried out in the Department of Clinical Biochemistry, CMC, Vellore.

Analyzer used: IMMULITE 2000 systems analyzer

Principle of the method: Solid-phase chemiluminescent immunometric assay

The solid-phase bead was coated with an anti-ligand. The liquid phase contained an anti-CRP murine monoclonal antibody, attached to the ligand, and alkaline phosphatase conjugated to rabbit polyclonal anti-CRP antibody in buffer. Pre-diluted patient sample and the reagent were incubated together with the anti-ligand-coated bead, for 30 minutes. The CRP in the sample formed an antibody sandwich complex. The unbound antibodies were removed. Then a chemiluminescent substrate was added to the reaction tube; the intensity of photons released was measured and was directly proportional to the hs-CRP concentration in the sample

Reference interval: < 3mg/L

Processing of tissue samples

RNA isolation

Duodenal mucosal tissue was collected in microtubes containing 0.5 mL of TRI-Reagent (Sigma Aldrich) and stored at -70^{o}C, and used for RNA isolation. RNA was isolated using TRI- Reagent protocol (Sigma Aldrich), according to the manufacturer's instruction.

Step 1: Mucosal samples in TRI- Reagent were homogenized using a glass homogenizer.

Step 2: After homogenization, the samples were kept in room temperature for 5 minutes.

Step 3: Chloroform (0.1 mL for 0.5 mL of TRI-Reagent) was added to each tube and mixed for 15 seconds.

Step 4: The mixture was kept at room temperature for 15 minutes.

Step 5: It was then centrifuged at 12000*g* for 15 minutes, at 4^{o}C. Centrifugation separated the mixture into 3 phases; the upper aqueous phase contained RNA.

Step 6: The aqueous phase was transferred to a fresh microtube, and 0.25 mL of isopropanol was added to it and mixed.

Step 7: The mixture was kept at -20^{o}C for 15 minutes.

Step 8: It was then centrifuged at 12000*g* for 10 minutes, at 4^{o}C. RNA that was precipitated formed a pellet on the side and bottom of the tube.

Step 9: The supernatant was removed and the RNA pellet was washed by adding 0.5 mL of ice cold 75% ethanol.

Step 10: This was centrifuged at 7500*g* for 5 minutes at 4^{o}C.

Step 11: The supernatant was discarded; the RNA pellet obtained was air-dried for 5-10 minutes, by placing them on ice and keeping the caps of the tubes open. .

Step 12: DEPC water (25µL) was added to each tube with the RNA pellet; the sample was kept at 60^{o}C for 10 minutes to dissolve the pellet.

RNA quantitation

A nano-spectrophotometer was used to estimate the quantity of RNA in each sample.

Principle: Nucleic acids absorb ultraviolet light strongly 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.

The RNA extracted should be free of DNA and protein. The ratio of absorbance at 260 and 280 nm and at 260 and 230 nm was used to assess the purity of RNA. If the A260/280 ratio was less than 1.8, or if there was evidence of phenol or guanidium isothiocyante contamination, the RNA was repurified by precipitation using ethanol.

Ethanol precipitation

Step 1: An aliquot (20 μL) of the RNA obtained was diluted to 100μL by adding DEPC water.

Step 2: Sodium acetate (3M) 10μL (0.1 part by volume) was added and mixed using a vortex mixer.

Step 3: Ice-cold ethanol (100%) (220 μL -2.2 parts by volume) was added and mixed thoroughly.

Step 4: The tubes were placed at -20^{o}C overnight.

Step 5: The next day, the tubes were centrifuged at 12000*g* for 10 minutes.

Step 6: Ice-cold ethanol (70%) (500 μL) was added to each tube and mixed; the mixture was centrifuged for 5 minutes at 12000*g*.

Step 7: Ethanol was removed by decanting off the supernatant on top.

Step 8: The tubes were kept open and placed inverted on tissue; they were then placed on ice and allowed to air-dry.

Step 9: Each RNA sample was then dissolved in 25 μL of DEPC water. The concentration of RNA in each sample was quantitated, using a nano-spectrophotometer.

If the A 260/ 280 ratio was more than 1.8 after ethanol precipitation, the RNA was considered to be of good quality. The integrity of the RNA isolated was confirmed by gel electrophoresis.

Confirmation of RNA integrity by gel electrophoresis

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

Step 1: 10X MOPS (morpholino-propanesulfonic acid) was prepared.

In order to prepare 100 mL, 4.186 g of MOPS was dissolved in DEPC water. The pH of the solution was adjusted to 7.0, using 0.1 M NaOH.

Sodium acetate (0.6804 gm) was then added to it (to give a final concentration of 50mM) and 2mL of 0.5M EDTA (to obtain a final concentration of 10nM).

Step 2: Preparation of agarose gel for electrophoresis (1.2% gel)

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

Step 3: To the melted agarose, 2.15mL of formaldehyde and 4 mL of 10 X MOPS and 7µL ethidium bromide was added. The contents were mixed well. This mixture was poured into a gel casting tray, combs were inserted into the mixture and it was allowed to set for 1 hour.

Step 4: For sample preparation, 10µl of each RNA sample was mixed with 2.5µL of 10X MOPS, 3.5µl of formaldehyde and 10µL of formamide.

Step 5: The sample mixture was heated at 60^{o}C in a dry bath for 15 minutes.

Step 6: After this, the sample mixture was mixed with 4µl of bromophenol blue.

Step 7: The running buffer, consisting of 30 mL of 10X MOPS and 270 mL DEPC-treated water, was poured into the tank.

Step 8: The cast gel was placed in the buffer in the electrophoresis tank; samples were loaded into the wells in the gel.

Step 9: The samples were electrophoresed for 45 min, at 150 volts.

Step 10: The RNA bands separated were visualized using an ultraviolet transilluminator in a ProteinSimple 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.

cDNA construction by reverse transcription

Reverse transcription of RNA was carried out using Prime script 1st strand cDNA synthesis kit (TaKaRa).

Principle

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

Components of the kit

For 200 reactions

1. 5X PrimeScript Buffer 400 μl (contains dNTP Mixture and Mg^{2+})
2. PrimeScript RT Enzyme Mix I 100 μl (contains RNAse inhibitor)
3. Oligo-dT Primer (50 μM) 100 μl
4. Random 6 mers (100 μM) 400 μl
5. RNase-free dH2O 1 ml

Step 1: The following reaction mixture was prepared and placed on ice.

For 1 reaction,

Reagent	Amount
5x Prime script buffer	2μL
RT enzyme mix	0.5 μL
Oligo dT primer	0.5 μL
Random 6mers	0.5 μL

A master-mix was prepared by adding together all the above reagents in the proportions mentioned, for the required number of samples.

Step 2: A volume of sample containing 500 ng RNA was added to a microtube.

Step 3: DEPC water was added to each microtube so that the volume of RNA + DEPC water was 6.5 μL. Master Mix (3.5 μL) was added to it to make the final volume of 10 μL.

RNA + DEPC water = 6.5 μL

Master Mix = 3.5 μL

Step 4: Negative controls were also set up which were as follows:

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

b. No reverse transcriptase: 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.

Step 5: All the reaction tubes were mixed and centrifuged in a microfuge for a few minutes.

Step 6: The tubes were incubated under the following conditions:

For reverse transcription- 37^{o}C for 15 minutes

Inactivation of reverse transcriptase- 85^{o}C for 5 seconds

Cooling of samples - 4^{o}C for 10 minutes

Step 7: The cDNA obtained was stored at -20^{o}C till real-time PCR analysis.

Real-time polymerase chain reactions (PCR) or quantitative PCR (qPCR)

The cDNA obtained was amplified by real-time PCR for DMT-1, ferroportin and Dcytb genes. Beta-actin was used as the house-keeping gene. The PCR reactions were set up in 96-well plates. To ensure reproducibility, all samples were assayed in quadruplicates.

The following gene-specific primers were used for the reactions.

Gene	**Primer sequence**		**Reference**
Beta actin	Forward	5'-AGAGCTACGAGCTGCCTGAC -3'	(Sukumaran et al.,
	Reverse	5'-AGCACTGTGTTGGCGTACAG -3'	2014b)
DMT-1	Forward	5'-TGCTGCTATCATTCCAACACTAAATT-3'	(Sukumaran et al.,
	Reverse	5'-ATATAGCCTGGTTAAGAATCATGCA-3'	2014b)
Dcytb	Forward	5'-GTCACCGGCTTCGTCTTCA-3'	(Sukumaran et al.,
	Reverse	5'-CAGGTCCACGGCAGTCTGTA-3'	2014b)
Ferroportin	Forward	5'- TGACCAGGGCGGGAGA -3'	(Jacolot et al., 2008)
	Reverse	5'- GAGGTCAGGTAGTCGGCCAA-3'	

The concentrations of primers and annealing temperatures for each gene of interest were standardized for all 4 genes.

The details are given below.

Standardization of qPCR assays

Gene	Sequence accession number	Length of the primers	Amplicon length	Primer concentration	Annealing temperature
Beta-actin	NM_001101.3	Forward:20 Reverse:20	184	250 nM	60^{o}C
DMT-1	NM_000617.2	Forward:26 Reverse:25	104	250 nM	60^{o}C
Ferroportin	NM_014585.5	Forward:16 Reverse:20	67	250 nM	60^{o}C
Dcytb	NM_024843.3	Forward:19 Reverse:20	62	250 nM	60^{o}C

Validation of qPCR assays

Sl. No	Gene	Standard curve slope	R^2 of standard curve	Linear dynamic range (cDNA dilution)	Primer dimer (melting curve analysis)	Ct of amplification (if any) in the no template control NTC
1	Beta-actin	-3.405	0.997	1:5 to 1:625	In no template control(NTC)	37
2	DMT-1	-3.244	0.999	1:5 to 1:3125	In no template control (NTC)	33
3	Ferroportin	-3.065	0.997	1 to 1:625	No	None
4	Dcytb	-3.331	1	1:5 to 1:3125	No	None

Dilution of cDNA templates

cDNA (5 μL) was diluted with 45 μL of DEPC water (1:10 dilution) and used for qPCR reactions. The concentration $MgCl_2$ in the reaction mixture was optimized to 5mmol/L for all the genes studied.

The following were the components and quantities for a single PCR reaction:

Component	Volume
Diluted cDNA template	2μL
SYBR green master mix	5 μL
Gene-specific primer mix	0.25 μL
DEPC water	2.75 μL

A master mixture was prepared with the above reagents (excluding the cDNA). This was done using the specified proportions, for 106 reactions (96 reactions + 10% extra for pippeting loss). Eight μL of the master mixture was added into each of 96 wells; 2 μL cDNA was added to each well in the plate as follows.

Model of a 96-well plate set-up for qPCR assays

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	No T
B	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	No T
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	No RT
D	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	No RT
E	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	No T
F	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	No T
G	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	No RT
H	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	No RT

A1- A11: cDNA of samples from patients with ALD

C1-C11: cDNA of samples from control patients

No T- No template; No RT- No reverse transcriptase

After adding the cDNA and master mix, the wells in the plate were closed tightly using transparent caps. The contents of each well were mixed, and the plate was briefly centrifuged for 2 minutes at 2000 RPM. The plate was then placed in the thermal cycler, which was programmed as shown below.

Thermal cycler program

Step		**Temperature**	**Time**
1	Hot start	95^{o}C	3 minutes
2	Denaturation	95^{o}C	10 seconds
3	Annealing and extension	60^{o}C	60 seconds
4	Reading taken		
5	Steps 3, 4 and 5 were repeated for 39 more cycles.		
6	Melting curve analysis was done for the temperature range of 50-95^{o}C. Readings were taken every 10 seconds, for every 1^{o}C increase in temperature		
7	Samples were cooled and maintained at 4^{o}C for 10 minutes.		

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

Calculation of gene expression

The cycle threshold (Ct) value was the outcome parameter obtained at the end of the PCR assays. If any of the Ct values of the quadruplicates was found to be discordant, the value of that

particular tube was ignored and the average Ct of the remaining replicates was taken as final Ct of that particular gene.

The relative expression of the target gene was calculated in comparison with an internal reference gene (beta-actin), 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. The Ct value of beta-actin for each sample was subtracted from that of the gene of interest (DMT-1, ferroportin and Dcytb). This value was referred to as the ΔCt value.

The average ΔCt of all controls for each gene was calculated and this average value was subtracted from the ΔCt of each sample for samples from both cases and controls. The resulting value was referred to as ΔΔCt. Fold-change in gene expression was calculated by the formula

$$\text{Fold change} = 2^{-\Delta\Delta Ct}$$

The fold-change of 3 genes studied in all the samples was calculated.

MIQE (Minimum Information for Publication of Quantitative Real-Time PCR Experiments) - guidelines which describe the minimum information necessary for qPCR evaluation. This information in the form of checklist is attached as Appendix IV.

WESTERN BLOT ANALYSES

Tissue homogenization

The tissue samples were homogenized in RIPA buffer.

Lysis buffer

1. RIPA (radio-immunoprecipitation assay buffer)

Composition of RIPA:

Sodium chloride 140mM

Tris HCl 10mM

EDTA 1.5mM

Triton X 1%

Sodium deoxycholate 0.1%

SDS 0.1%

2. Protease inhibitor cocktail (100µL for 10 mL buffer)

3. Phenyl methyl sulfonyl fluoride (PMSF) (1mM)

4. Sodium orthovanadate (200µM)

5. Sodium fluoride (50mM)

All the above constituents were mixed together. This solution was prepared fresh each time, for homogenization.

Step 1: 0.5 mL of the RIPA buffer was added to the tissue sample in the microtube. The tissue was homogenized using a teflon pestle.

Step 2: The samples were incubated on ice for 15 minutes.

Step 3: They were centrifuged at 12000*g* for 10 minutes, at 4^{o}C, for the cell debris to settle.

Step 4: The supernatant was transferred to fresh micro tubes. Four aliquots were made.

Step 5: All the aliquots were stored at -70^{o}C till further analyses.

Protein estimation in the RIPA lysates

The concentration of protein in each lysate was estimated, using the Pierce BCA protein assay kit (Thermo Scientific). This was done to calculate the amount of each sample to be loaded in the gel for western blot analyses.

The contents of the Pierce BCA protein kit were:

1. BCA reagent A (sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartrate in 0.1 M NaOH)

2. BCA reagent B (4% cupric sulphate)

3. Albumin standard (2mg/mL in 0.9% saline and 0.05% sodium azide)

Principle of the assay

The cupric ions were reduced to cuprous ions by protein in an alkaline medium (the biuret reaction). A purple colour product was formed by the reaction of BCA molecules with cuprous ion. It exhibited a strong absorbance at 562 nm. The absorbance was almost linear with increasing protein concentrations (20-2000µg/mL) and it was measured in micro plate reader.

Preparation of diluted albumin standards

Vial	Volume of diluent (µL)	Volume and source of BSA (µL)	Final BSA concentration (µg/mL)
A	125	375 of stock	1500
B	325	325 of stock	1000
C	175	175 of vial A	750
D	325	325 of vial B	500
E	325	325 of vial D	250
F	400	0	0 (Blank)

Assay

Reagent A and reagent B were mixed in the proportion of 50:1 to prepare the working reagent. The assay was done in 96-well microplates. For each well, 200 µl of the working reagent was used. The assay was done in duplicates for each sample.

Step 1: Samples and standards (20 µL of each) were added to wells in the plate as shown below.

Model of microplate set-up for protein estimation

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
B	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12
C	A13	A14	A15	A16	A17	A18	A19	C1	C2	C3	C4	C5
D	A13	A14	A15	A16	A17	A18	A19	C1	C2	C3	C4	C5
E	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17
F	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	C17
G	C18	C19	S0	S1	S2	S3	S4	S5	B			
H	C18	C19	S0	S1	S2	S3	S4	S5	B			

A1-A19: Samples from patients with ALD; C1-C19: Samples from control patients

S0-S6: Albumin standards; B: Blank

Step 2: 200 µL of working reagent was added to each well.

Step 3: The contents of the wells were mixed thoroughly for 30 seconds, using a plate shaker.

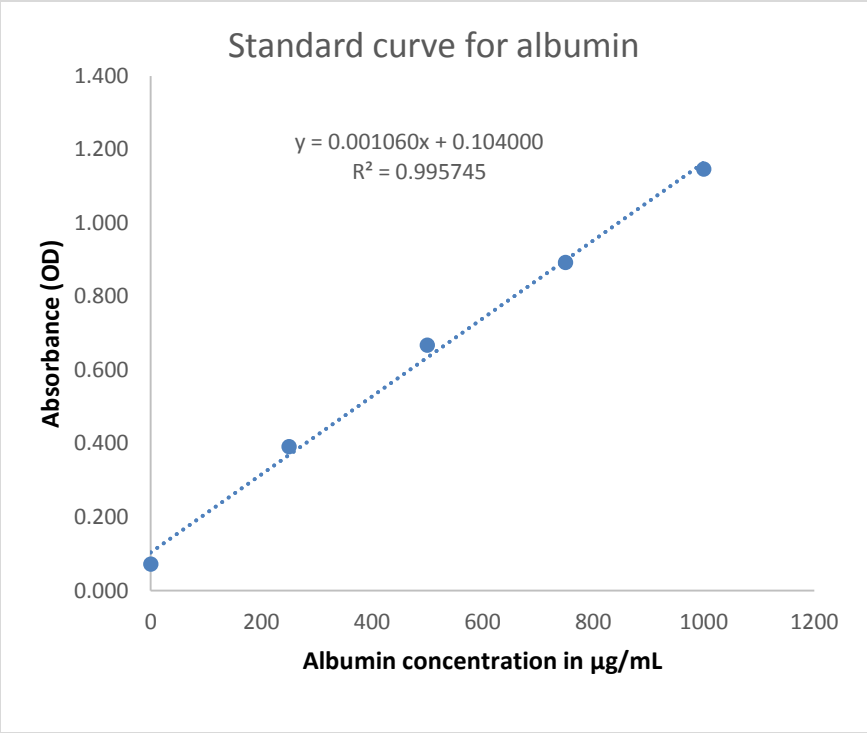
Step 4: The plate was closed with a cover and incubated at 37^{o}C for 30 minutes.

Step 5: After incubation, the plate was cooled to room temperature. A microplate reader was used to take readings at 562 nm.

Step 6: The average absorbance of the wells containing blanks was subtracted from each of readings for the standards and samples.

Step 7: A standard curve was prepared by plotting the blank-corrected reading of each standard against its concentration (µg/mL).

Step 8: The standard curve was used to determine the protein concentration in each sample.



After determining the protein concentration in each sample, the volume of sample required to load 30µg protein in each well of the gel was calculated.

Reagents for western blotting analyses

1. Loading buffer (2x Laemmli buffer)

4% Sodium dodecyl sulphate SDS

10% 2-mercaptoethanol

20% glycerol

0.004% bromophenol blue

0.125 M Tris HCl

The pH of this solution was adjusted to 6.8, using 0.1 N NaOH if necessary.

2. Running buffer (10X)

For preparing 1000 mL,

25 mM Tris base (30.25 g)

190mM glycine (144.13 g)

0.1% SDS (10 g)

The above chemicals were added together to make a final volume of 1000 mL, using distilled water. The pH of this solution was adjusted to 8.3, using 0.1 N HCl.

3. Transfer buffer

For preparing 1000 mL,

25 mM Tris Base (30.25 g)

190mM glycine (144.13 g)

0.1 % SDS (1 g)

The above chemicals were added together to make a final volume of 1000 mL, using distilled water. Methanol (200 mL for 1000 mL) (final concentration of 20%) was added to the mixture just before use.

4. Phosphate-buffered saline

For 1000 mL,

Sodium chloride (8 g)

Potassium chloride (0.2 g)

Disodium hydrogen phosphate (1.44 g)

Potassium dihydrogen phosphate (0.24 g)

The above chemicals were added together and dissolved in distilled water to make a final volume of 1000 mL.

5. Wash buffer

PBS with Tween 20 (0.1%)

For every 100 mL of PBS, 0.1 mL of Tween 20 was added.

6. Blocking buffer

Non-fat dry milk powder was dissolved in wash buffer (5g for 100 mL PBS-T) (5% w/v)

7. Running gel: Solution A and solution C

Solution A: For 100 mL

Sodium dodecyl sulphate (0.4 g)

Tris (18.165 g)

Tetramethylethylenediamine (0.2 mL)

The above chemicals were added together and dissolved in distilled water to make a final volume of 100 mL The pH of the solution was adjusted to 8.8, using 0.1 N HCl.

Solution C: For 50 mL

Acrylamide (20 g)

Bisacrylamide (0.532 g)

The above chemicals were added together and dissolved in distilled water to make a final volume of 50 mL. The solution was filtered using Whatman filter paper.

8. Stacking gel- Solution B and solution D

Solution B: For 100 mL

Sodium dodecyl sulphate (0.4 g)

Tris (6.72 g)

Tetramethylethylenediamine (0.278 mL)

The above chemicals were added together and dissolved in distilled water to make a final volume of 100 mL. The pH was adjusted to 6.8 by adding 0.1N HCl.

Solution D: For 100 mL

Acrylamide (20 g)

Bisacrylamide (0.532 g)

The above chemicals were added together and dissolved in distilled water to make a final volume of 100 mL. The solution was filtered using Whatman filter paper.

Sample preparation

1. The RIPA lysate of each sample was taken from the -70^{o}C freezer and allowed to thaw on ice.

2. A volume of sample (containing 30 μg of protein) was transferred to a microtube, labeled appropriately.

3. 6.5 μL of loading buffer was added to the sample.

4. Then running buffer was added to make a final volume of 32.5 μL.

3. The samples were boiled at 95^{0} C for 5 minutes.

Protein separation by vertical gel electrophoresis

Apparatus

This consists of a thin and a thick plate, a spacer, the holder for the glass plate and a trough. The thin plate is placed in front of the thick plate. The 2 plates are separated by a spacer of 1 mm thickness. They are held in position on the holder. The trough is placed at the bottom of the plates.

Running gel (10%)

Ammonium persulphate (APS) (0.05 g) was dissolved in 500μL of distilled water, using a vortex mixer.

The following reagents were added in a test tube

Solution A (1.2 mL), Solution C (1.2 mL), Distilled water (2.35 mL) and APS (0.05 mL)

After mixing solutions A, C and distilled water, APS was added and the mixture was poured into the space between the glass plates in the holder. The gel was allowed to polymerize for 45 minutes.

Stacking gel (7%)

The following reagents were added in a test tube.

Solution B (0.5 mL), Solution D (0.5 mL), Distilled water (950 μL) and APS (0.05 μL)

After mixing together the above reagents, the mixture was poured into the space between the 2 plates. A plastic comb was inserted into the top of the space between the plates, to create wells. The gel was allowed to polymerize for 45 minutes. After the gel polymerized, the assembly of the 2 plates was removed from the holder and the comb was gently removed. The assembled plates were placed in a holder that was then placed in an electrophoresis chamber. The chamber was filled with running buffer.

Loading of samples

1. Thirty micrograms of protein was loaded into each well in the gel.

2. Molecular weight markers were loaded in wells at both ends of the gel.

3. A positive control for HIF-2α was loaded in one of the wells.

4. Proteins in the gel were electrophoresed at 50 volts for the first 20-30 minutes (till the samples had reached the running gel).

5. After this, the voltage was increased to 80 V; electrophoresis was allowed to continue till the tracker dye reached near the bottom of the gel.

Blotting

At the end the electrophoretic separation, the proteins on the gel were transferred on to a membrane (polyvinylidene difluoride [PVDF]).

1. Two membranes of 8.5 x 5.5 cm were activated by placing them in methanol for 15 seconds and then MilliQ water for 2 minutes. It was then placed in the transfer buffer.

2. The glass slides containing the gel were placed in flat dish containing transfer buffer; the slides were separated.

3. The stacking gel was carefully removed; the gel was left in transfer buffer for 10 minutes.

4. The following were assembled for protein transfer: Sponge, 2 filter papers, gel, PVDF membrane, 2 filter papers and sponge.

This assembly was immersed in transfer buffer. It was then placed in the cassette used for transfer. Care was taken to ensure that there was no air bubbles trapped in the assembled sandwich, by gently rolling a small test tube over it.

5. The cassette was placed in the transfer tank with the membrane on the cathode side and the gel on the anode side. The transfer tank was kept in a box containing ice.

6. The transfer was allowed to take place at 80 V for 2 hours.

Blocking

At the end of the transfer, the membrane was removed from the cassette. It was rinsed in water and stained with Ponceau solution to ensure that the proteins had transferred adequately. The stain was then rinsed off with three washes, using PBS-T, for about 10 minutes each time. The membrane was then immersed in blocking buffer (PBS-T) and subjected to gentle rocking for 2 hours. At the end of this, the membrane was removed; it was placed on a glass plate to be cut,

using the molecular weight markers for reference, so that different portions of the membrane could be used for incubation with different primary antibodies. The molecular weight of HIF2-alpha is 118 kDa and that of beta-actin is 42 kDa. Hence, the membrane was cut between 70 and 100 kDa.

Incubation with primary antibody

The membranes were incubated with primary antibodies against the proteins of interest. This was done overnight at $4^{o}C$, using a roller. The concentration of primary antibody used for beta-actin was 1:1000; that for HIF2-alpha was 1:500. These dilutions were made using blocking buffer.

Incubation with secondary antibody

After incubation with the primary antibody, the membrane was washed three times in PBS-T, for 10 minutes each time. The membrane was incubated with HRP-conjugated secondary antibody; this was done for 2 hours, at room temperature. The concentration of the secondary antibody used was 1:2500 (2µL of secondary antibody was added to 5 ml blocking solution). For beta-actin, an anti-mouse antibody was used. For HIF 2 alpha, an anti-rabbit antibody was used. After this incubation, the membranes were washed thrice in PBS-T, for 10 minutes each time.

Imaging

To detect the protein bands, SuperSignal West Dura Extended Duration Substrate kit from Thermo Scientific, USA, was used. The kit had SuperSignal West Dura Luminol/Enhancer solution and SuperSignal West Dura Stable peroxide solution. A chemiluminescent substrate for detecting horseradish peroxidase (HRP) on immunoblots was used. HRP conjugated to the secondary antibody catalyzed the conversion of peroxide to release nascent oxygen. The

colourless substrate was turned to a chemiluminescent compound by the nascent oxygen released. This was visualized on an imaging system.

Equal parts of peroxide solution and enhancer solution were mixed to prepare the working reagent. The membrane was submerged in the mixture for 1 minute. Excess substrate was drained off. Then the membrane was placed in a gel documentation system (Alpha Innotech). The proteins bands were visualized by the chemiluminescent signals they emitted. These signals were captured using a CCD camera-based imager. The intensity of the bands for beta-actin and HIF2-alpha were obtained using Image J software. The intensity of the HIF-2-alpha protein band was expressed relative to that of the band for beta-actin, which was used as a loading control.

STATISTICAL ANALYSIS

The Statistical Package for the Social Sciences (SPSS), version 16, was used to analyze the data obtained. Shapiro Wilk's test was used to determine if the data was normally distributed. If normally distributed, then mean and standard deviation were calculated. The means of the quantitative variables was compared, using independent t-test. If the variable was not normally distributed, median and interquartile range was calculated. Mann-Whitney U test was used for comparison of data that were not normally distributed. A p value of less than 0.05 was taken to indicate statistical significance in all cases. Bivariate correlation analyses were done using Pearson correlation for variables with normal distribution and Spearman's rank correlation for variables that are not normally distributed.

RESULTS

Thirty six males were recruited for the study, as per the inclusion and exclusion criteria described in the study protocol. Of these, 18 subjects were diagnosed to have ALD and 18 subjects were age-matched controls.

Data for some parameters were not available from the medical records for all the subjects recruited. Only available data were analyzed. The number of subjects for whom each set of data was available is indicated in the tables showing the results of this study.

Shapiro-Wilk test was used to determine normality of the data. Data for age, total protein, MCV, serum iron and TIBC were found to be normally distributed. Those for hemoglobin, total bilirubin, direct bilirubin, serum albumin, AST, ALT, ALP, transferrin saturation, serum ferritin, WBC counts and hs-CRP were found to have skewed distributions.

Normally distributed data are shown as means $\pm$ SD, while data with a skewed distribution are shown as medians with inter-quartile ranges.

a. Characteristics of the subjects

Table 6.1 shows relevant characteristics of the 2 groups.

Table 6.1. Baseline characteristics

Characteristics	**Control subjects**	**Patients with ALD**
Number of subjects	18	18
Males / Females	18 / 0	18 / 0
Mean age in years (±SD)	48.3 ± 7.3	47.8 ± 7.2
Mean duration of alcohol use in years (±SD)	0	15 ± 6.3

All the subjects recruited were males. The mean ages of subjects in the 2 groups were similar. Only those with ALD had a history of long-term alcohol consumption.

b. Hematological parameters

Table 6.2 shows the hemoglobin levels and mean corpuscular volume of the subjects.

Table 6.2. Hematological parameters

Parameter	**Control subjects**	**Patients with ALD**	**p value**
Hemoglobin (g/dL)	14.5 (13.5-15.1) (n=18)	11.5 (10.2-12.9) (n=18)	< 0.001
MCV (fL)	81.4 ± 7.4 (n=14)	90.5 ± 9.7 (n=18)	0.006

Hemoglobin levels were significantly lower and MCV significantly higher in the group with ALD. However, the mean values for MCV were within the reference range (80-100 fL) for both groups.

c. Parameters of liver function

Table 6.3 shows the parameters of liver function.

Table 6.3. Parameters of liver function

Parameter	Control subjects (n=18)	Patients with ALD (n=18)	p value
Total protein (g/dL)	7.4 ± 0.2	7.5 ± 0.6	0.732
Serum albumin (g/dL)	4.6 (4.3-4.7)	3.2 (2.6-4)	<0.001
Albumin: Globulin	1.5 (1.3-1.7)	0.73 (0.49-1.2)	<0.001
Total bilirubin (mg/dL)	0.5 (0.38-0.82)	3.4 (1.4-5.5)	<0.001
Direct bilirubin (mg/dL)	0.2 (0.14-0.22)	1.9 (0.6-3.1)	<0.001
AST(IU/L)	24 (19-29.2)	74 (36.5-112)	<0.001
ALT (IU/L)	26 (19.7-36)	33.5 (23.5-57.5)	0.097
ALP (IU/L)	72.5 (67.25- 85.25)	110.5 (85.5-134)	<0.001

Serum levels of total bilirubin, direct bilirubin, AST and ALP were significantly higher in subjects with ALD than in control subjects. Serum levels of albumin and the albumin-globulin (AG) ratio were significantly lower in subjects with ALD. Serum total protein levels were similar in both groups.

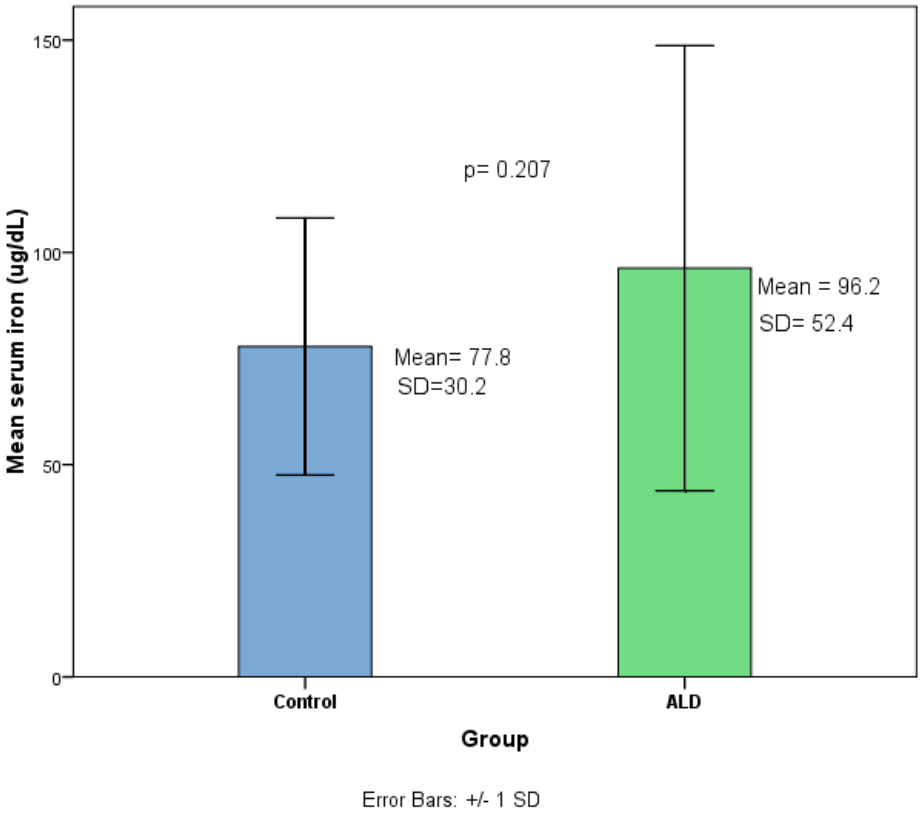
d. Parameters of inflammation

Table 6.4. Parameters of inflammation

Parameter	**Control subjects**	**Patients with ALD**	**p value**
WBC count (cells/cu.mm)	7900 (5850-9200) (n=13)	7600 (4850-9050) (n=18)	0.798
hs-CRP (mg/L)	0.94 (0.42-2.37) (n=18)	5.8 (3.4-9.9) (n=18)	<0.001

Total WBC counts and serum levels of hs-CRP were used as markers of inflammation. Of these, hs-CRP was significantly higher in subjects with ALD. Total WBC counts were similar in both groups.

Figure 6.1. Serum iron levels in control subjects and those with ALD



Serum iron levels were similar in both groups.

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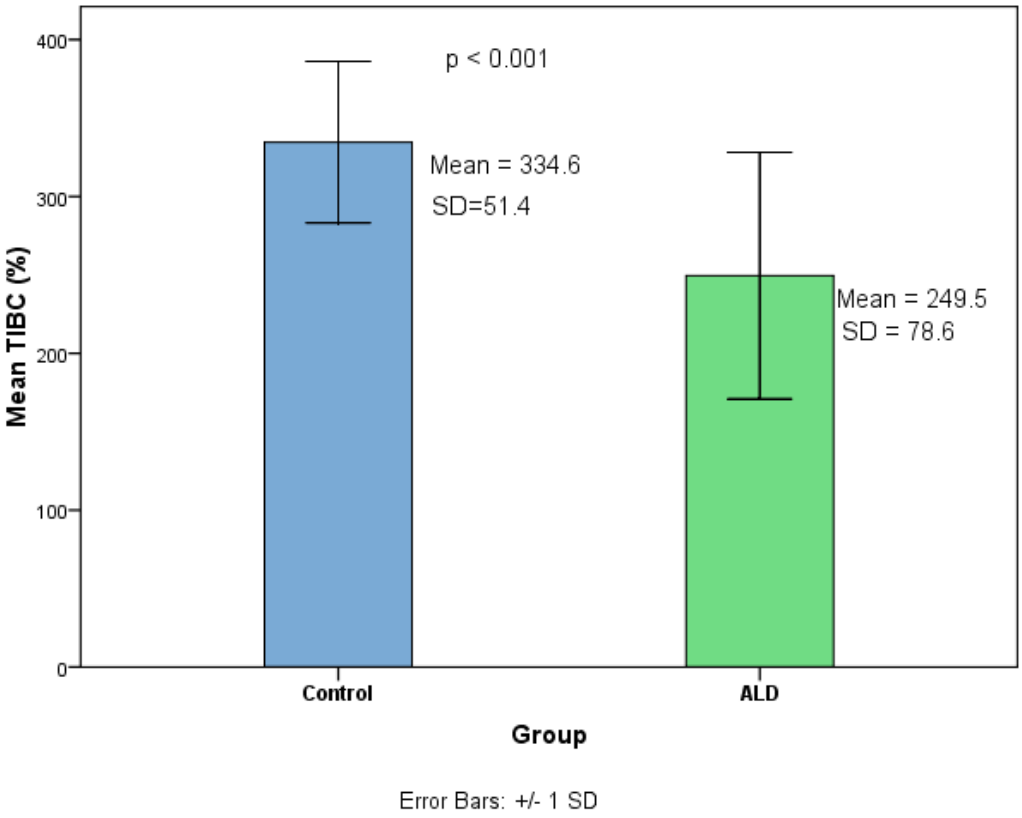
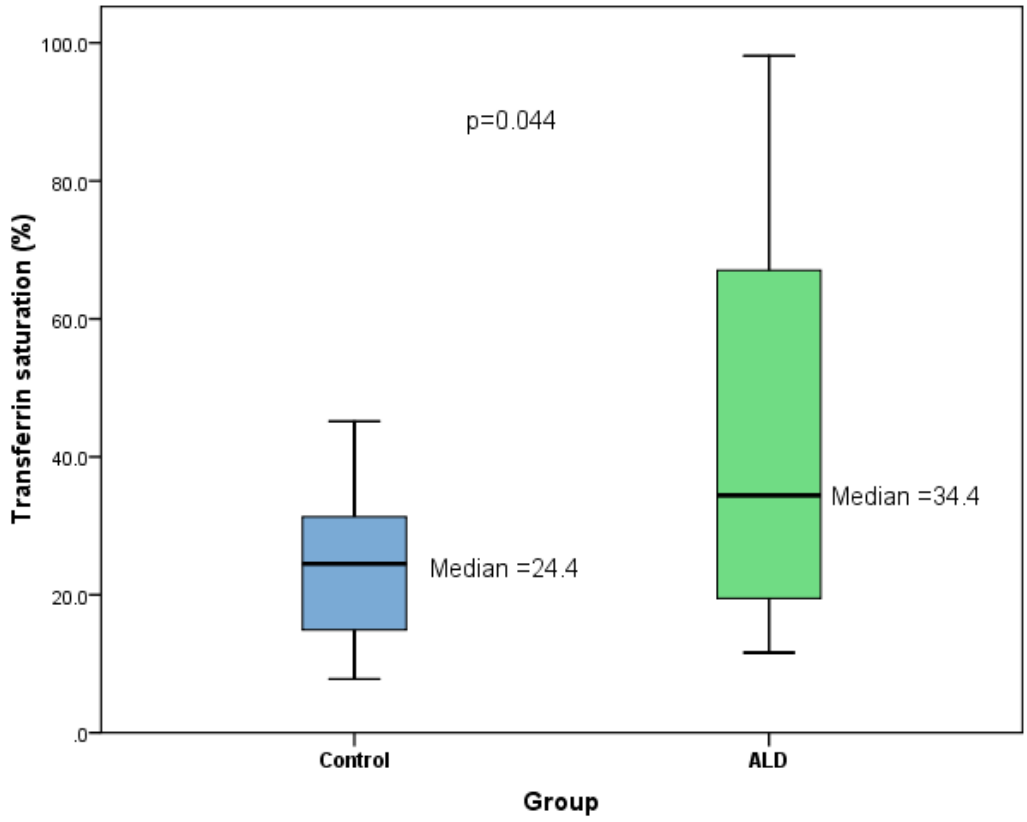
Figure 6.2. TIBC in the control subjects and those with ALD



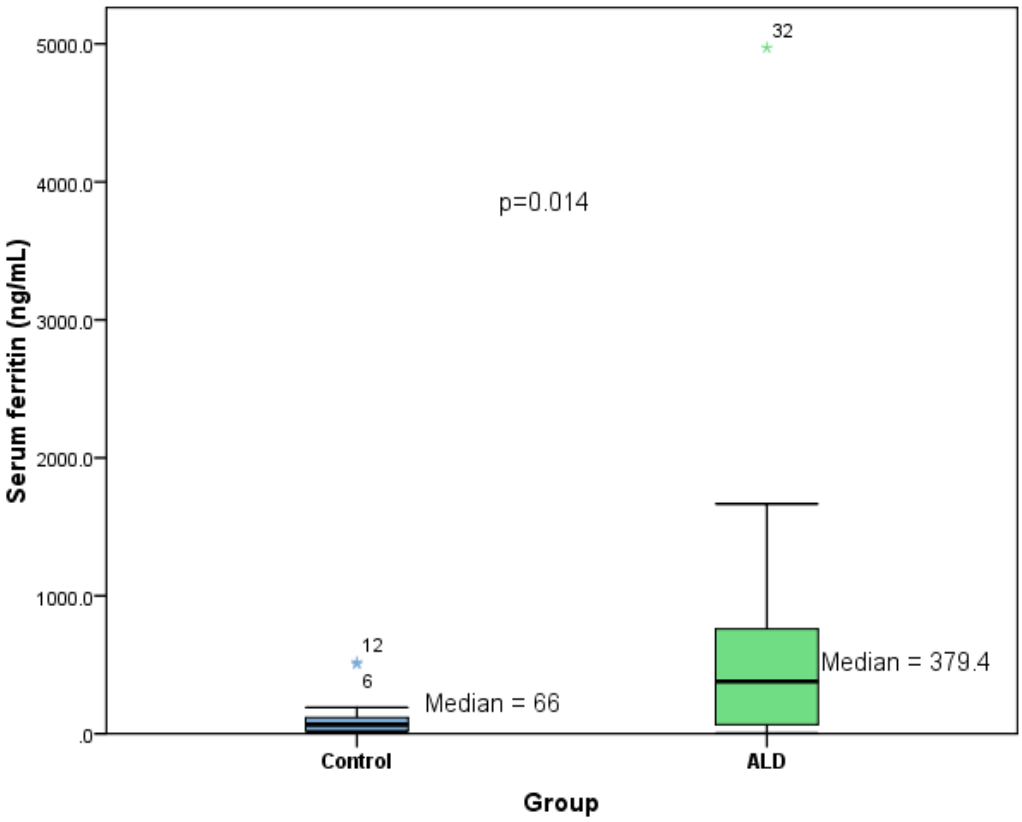
TIBC was significantly lower in subjects with ALD than in control subjects.

Figure 6.3. Transferrin saturation in control subjects and those with ALD



Data are shown as box and whisker plots, showing medians and interquartile ranges. The median is shown by the line within the box; the bottom and top edges of the boxes are the 25th and 75th percentiles, respectively. Transferrin saturation was significantly higher in subjects with ALD than in control subjects. .

Figure 6.4. Serum ferritin levels in control subjects and those with ALD



Data are shown as box and whisker plots, showing medians and interquartile ranges. The median is shown by the line within the box; the bottom and top edges of the boxes are the 25th and 75th percentiles, respectively. Values outside the top and bottom 5th percentiles are shown as outliers. Serum ferritin was significantly higher in those with ALD than in control subjects.

Confirmation of integrity of RNA isolated

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 6.5 shows the representative image of a gel in which RNA samples were electrophoresed.

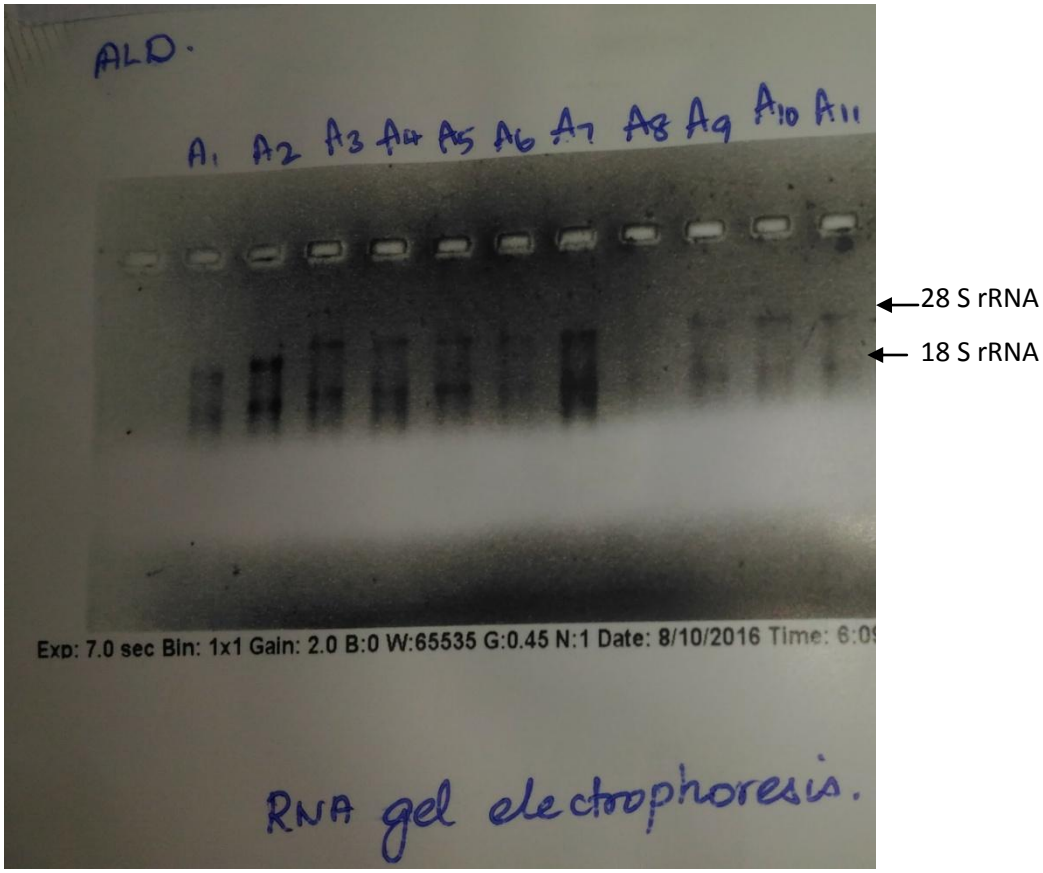
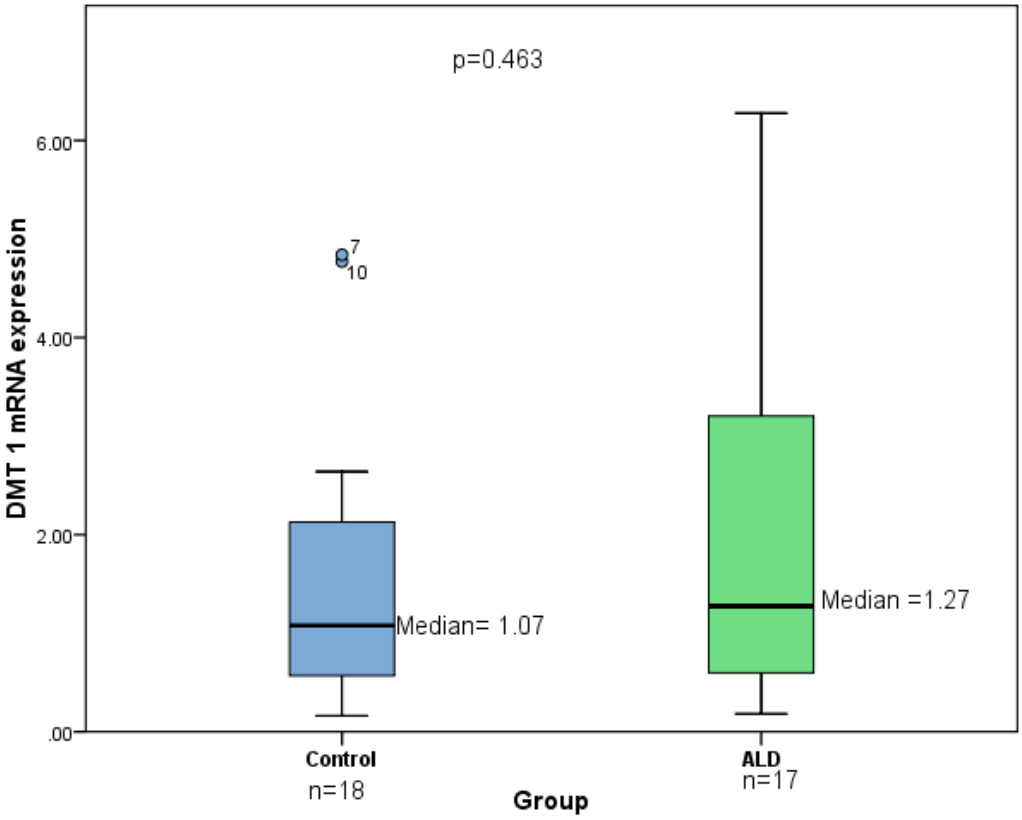


Figure 6.5. Image of agarose gel showing 18S and 28S subunits of RNA isolated

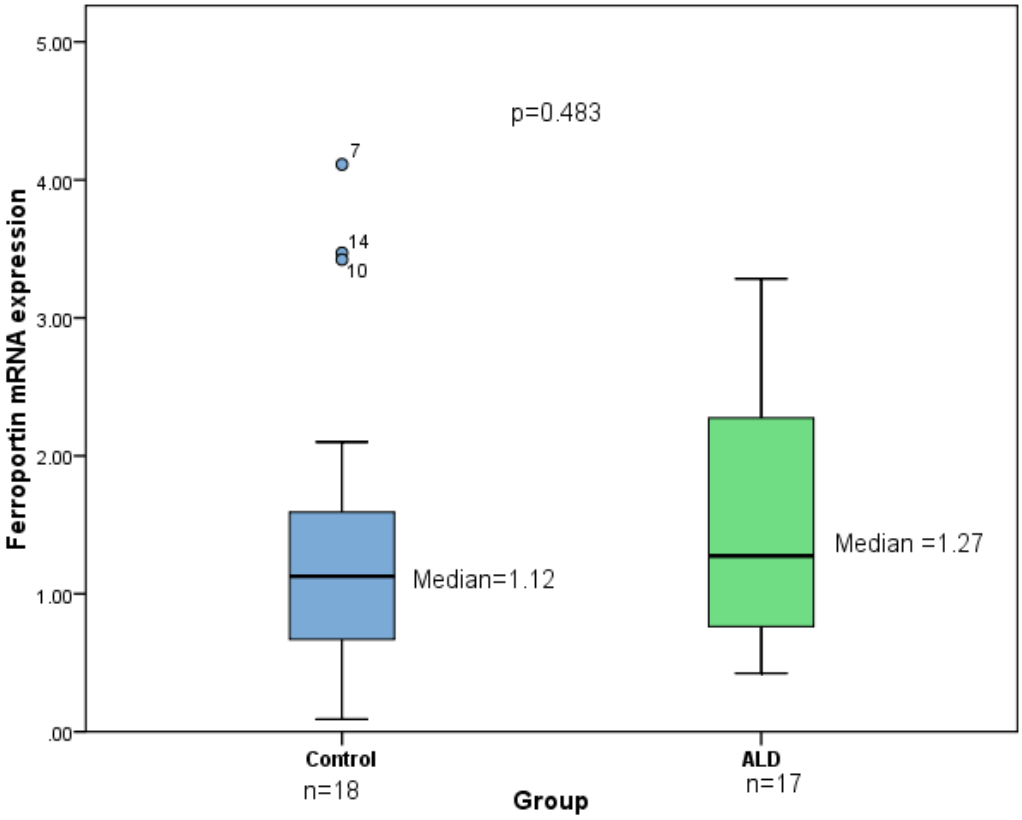
The amount of RNA obtained from one of the samples from patients with ALD was found to be too low to use for analysis. Hence, this sample was excluded from qPCR analysis.

Figure 6.6. Gene expression of DMT-1 in control subjects and those with ALD



Data are shown as box and whisker plots showing medians and interquartile ranges. The median is shown by the line within the box; the bottom and top edges of the boxes are the 25th and 75th percentiles, respectively. Values outside the top and bottom 5th percentiles are shown as outliers. Gene expression of DMT-1 (mRNA levels) was similar in the two groups.

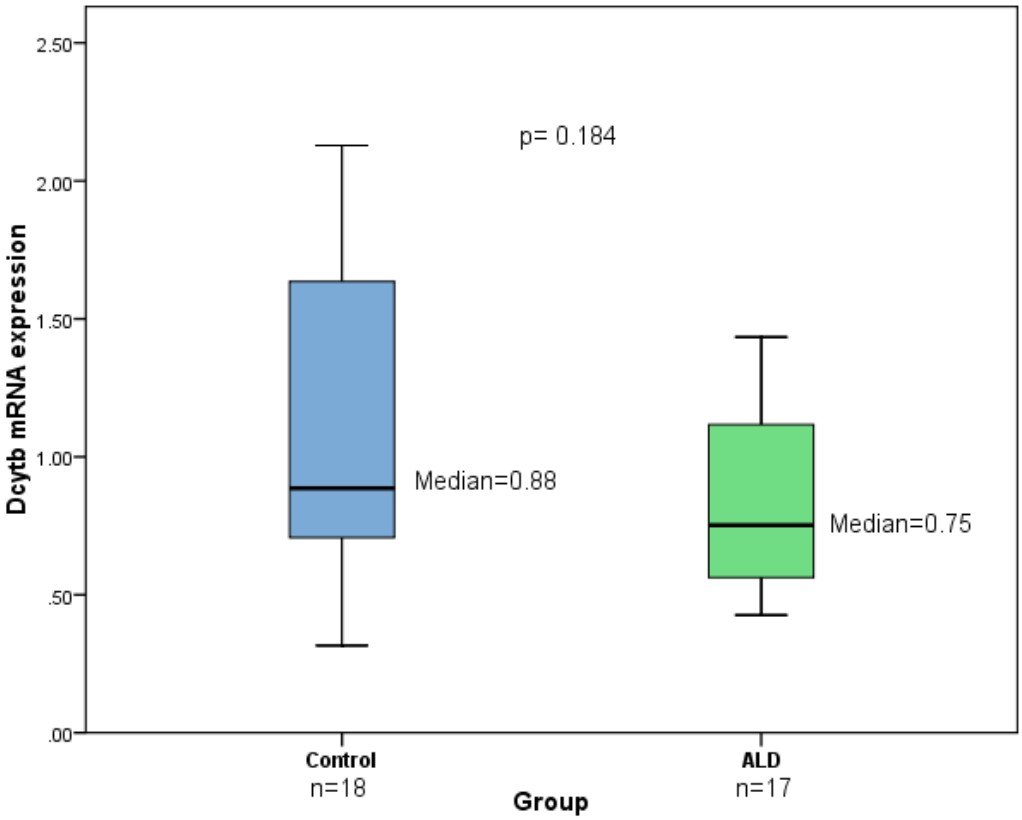
Figure 6.7. Gene expression of ferroportin in control subjects and those with ALD



Data are shown as box and whisker plots, showing medians and interquartile ranges. The median is shown by the line within the box; the bottom and top edges of the boxes are the 25th and 75th percentiles, respectively. Values outside the top and bottom 5th percentiles are shown as outliers.

Gene expression of ferroportin was similar in the two groups.

Figure 6.8. Gene expression of Dcytb in control subjects and those with ALD



Data are shown as box and whisker plots, showing medians and interquartile ranges. The median is shown by the line within the box; the bottom and top edges of the boxes are the 25th and 75th percentiles, respectively. Values outside the top and bottom 5th percentiles are shown as outliers.

Gene expression of Dcytb was similar in the two groups.

Protein expression of HIF-2α

Representative blots of HIF-2α in duodenal mucosal samples of controls (n=13) and patients with ALD (n=15) are shown in figure 6.9. The amount of protein obtained from 5 control duodenal mucosal samples and 3 samples from patients with ALD were too low to be quantified. Hence, these samples were not used for western blot analyses. Whole-cell protein extract of HepG2 cells, treated with 100μM $CoCl_2$ for 8 hrs, was used as the positive control for HIF-2α (+ve control) (Piret et al., 2002).

Figure 6.9. Representative western blots for HIF-2α

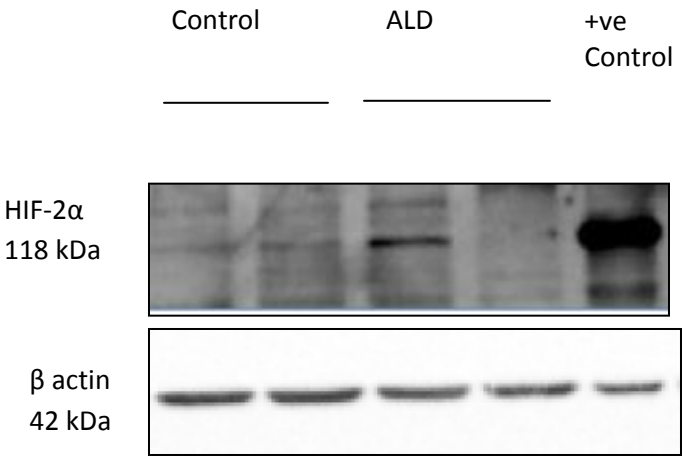
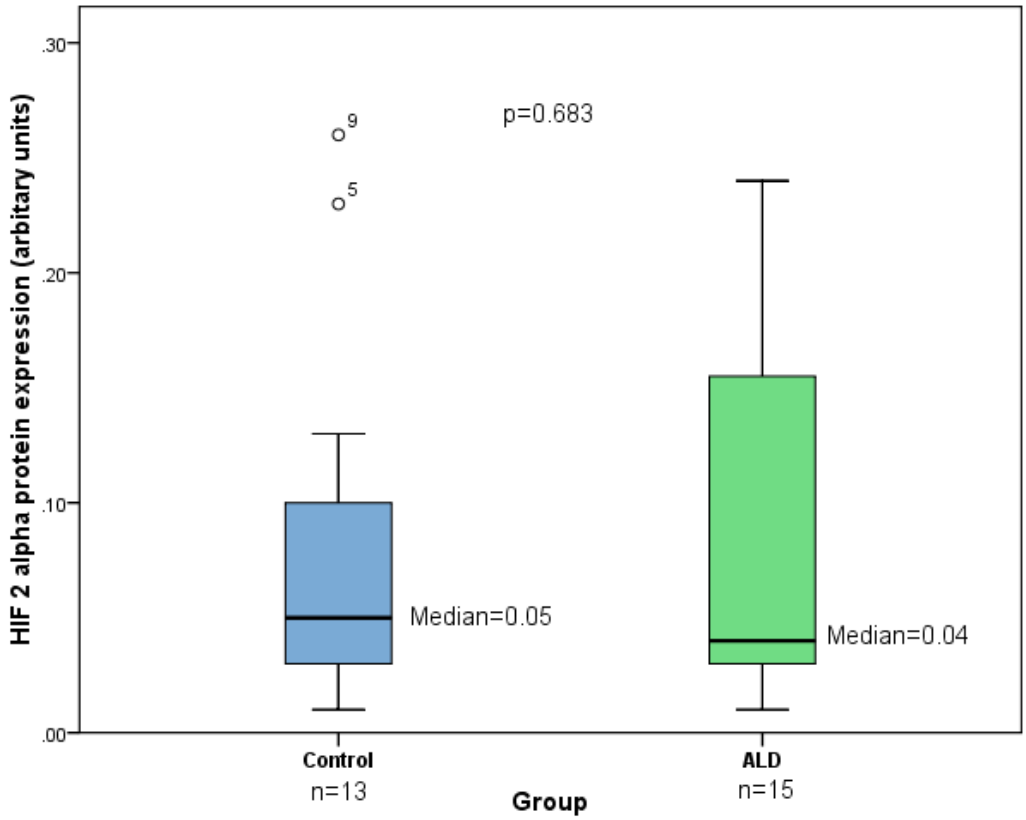


Figure 6.10. Protein expression of duodenal HIF-2α in control subjects and those with ALD



Data are shown as box and whisker plots, showing medians and interquartile ranges. The median is shown by the line within the box; the bottom and top edges of the boxes are the 25th and 75th percentiles, respectively. Values outside the top and bottom 5th percentiles are shown as outliers. Protein levels of HIF-2α were similar in the two groups.

Table 6.5. Correlation analyses of iron-related parameters (n=36)

		Hb (g/dL)	Serum iron (ug/dL)	TIBC (ug/dL)	Transferrin saturation (%)	Serum ferritin (ng/mL)
Hb (g/dL)	Correlation Coefficient	-	0.085	0.389*	-0.089	-0.174
	p value	-	0.621	0.019	0.607	0.309
Serum iron (ug/dL)	Correlation Coefficient	0.085	-	-0.222	0.891**	0.090
	p value	0.621	-	0.194	< 0.001	0.600
TIBC (ug/dL)	Correlation Coefficient	0.389*	-0.222	-	-0.602**	-0.738**
	p value	0.019	0.194	-	<0.001	<0.001
Transferrin saturation (%)	Correlation Coefficient	-0.089	0.891**	-0.602**	-	0.351*
	p value	0.607	<0.001	<0.001	-	0.036
Serum ferritin (ng/mL)	Correlation Coefficient	-0.174	0.090	-0.738**	0.351*	-
	p value	0.309	0.600	< 0.001	0.036	-

Correlation analysis was done using Spearman rank analysis.

* $p < 0.05$, ** $p < 0.01$

Hemoglobin levels were positively correlated with TIBC. Serum iron and ferritin levels correlated positively and significantly with transferrin saturation. Serum ferritin and transferrin saturation correlated negatively with TIBC.

Table 6.6. Correlation analyses of iron-related parameters and inflammatory parameters

		WBC count (cells/cu.mm) (n=31)	hs-CRP (mg/L) (n=36)
Hb (g/dL)	Correlation Coefficient	0.264	-0.461**
	p value	0.152	0.005
Serum iron (ug/dL)	Correlation Coefficient	0.093	-0.093
	p value	0.620	0.588
TIBC (ug/dL)	Correlation Coefficient	0.004	-0.567**
	p value	0.982	<0.001
Transferrin saturation (%)	Correlation Coefficient	0.074	0.150
	p value	0.694	0.383
Serum ferritin (ng/mL)	Correlation Coefficient	0.160	0.495**
	p value	0.390	0.002

Correlation analysis was done using Spearman rank analysis.

**$p < 0.01$

Levels of hs-CRP correlated negatively with hemoglobin and TIBC, and positively with serum ferritin.

Table 6.7. Correlation analyses of expression levels of DMT1, Dcytb, Fpn and HIF-2α with iron-related parameters

		DMT-1 mRNA expression (n=35)	Ferroportin mRNA expression (n=35)	Dcytb mRNA expression (n=35)	HIF-2α protein expression (n=28)
Hb (g/dL)	Correlation Coefficient	-0.221	-0.232	0.135	0.245
	p value	0.202	0.180	0.441	0.208
Serum iron (ug/dL)	Correlation Coefficient	-0.019	-0.030	0.053	0.105
	p value	0.914	0.864	0.761	0.594
TIBC (ug/dL)	Correlation Coefficient	0.323	0.268	0.134	-0.128
	p value	0.059	0.119	0.443	0.517
Transferrin saturation (%)	Correlation Coefficient	-0.096	-0.081	0.015	0.119
	p value	0.585	0.643	0.930	0.548
Serum ferritin (ng/mL)	Correlation Coefficient	-0.312	-0.373*	-0.120	0.306
	p value	0.068	0.027	0.492	0.113

Correlation analysis was done using Spearman rank analysis.

* $p < 0.05$

Ferroportin mRNA levels correlated negatively with serum ferritin levels.

Table 6.8. Correlation analyses of expression levels of DMT1, Dcytb, Fpn and HIF-2α with inflammatory parameters

		DMT-1 mRNA expression	Ferroportin mRNA expression	Dcytb mRNA expression	HIF-2α protein expression
WBC count (cells/cu.mm)	Correlation Coefficient	-0.278	-0.203	0.209	-0.165
	p value	0.137	0.281	0.268	0.431
	n	30	30	30	25
hs-CRP (mg/L)	Correlation Coefficient	-0.159	-0.077	-0.207	-0.096
	p value	0.363	0.660	.0232	0.628
	n	35	35	35	28

Correlation analysis was done using Spearman rank analysis.

No significant correlations were seen between the expression levels of DMT1, Dcytb, Fpn and HIF-2α and inflammatory parameters.

Correlational analysis of the data from the control group showed similar observations, as shown for the whole group of 36 subjects.

Table 6.9. Significant correlations in patients with ALD

		DMT-1 mRNA expression	Fpn mRNA expression	TIBC (ug/dL)	Tf sat (%)	Serum ferritin (ng/mL)	WBC count (cells/mm^3)	hs-CRP (mg/L)
DMT-1 mRNA expression	Correlation Coefficient	-	0.725**	0.538*	-0.471	-0.520*	-0.574*	-0.289
	p value	.	0.001	0.026	0.057	0.033	0.016	0.260
Ferroportin mRNA expression	Correlation Coefficient	0.725**	-	0.527*	-0.598*	-0.686**	-0.602*	-0.196
	p value	0.001	.	0.030	0.011	0.002	0.011	0.451
TIBC (ug/dL)	Correlation Coefficient	0.538*	0.527*	-	-0.519*	-0.655**	-0.267	-0.687**
	p value	0.026	0.030	.	0.027	0.003	0.285	0.002
Transferrin saturation (%)	Correlation Coefficient	-0.471	-0.598*	-0.519*	-	0.342	0.201	0.071
	p value	0.057	0.011	0.027	.	0.165	0.423	0.779
Serum ferritin (ng/mL)	Correlation Coefficient	-0.520*	-0.686**	-0.655**	0.342	-	0.360	0.496*
	p value	0.033	0.002	0.003	0.165	.	0.142	0.036
WBC count (cells//mm^3)	Correlation Coefficient	-0.574*	-0.602*	-0.267	0.201	0.360	-	0.274
	p value	0.016	0.011	0.285	0.423	0.142	.	0.272
hs-CRP (mg/L)	Correlation Coefficient	-0.289	-0.196	-0.687**	0.071	0.496*	0.274	-
	p value	0.260	0.451	0.002	0.779	0.036	0.272	.

Correlation analysis was done using Spearman rank analysis.

* $p < 0.05$, ** $p < 0.01$

In patients with ALD,

- DMT-1 mRNA levels correlated positively with FPN mRNA levels
- FPN mRNA levels correlated negatively with transferrin saturation
- Both DMT-1 and FPN mRNA levels were found to have

  a. Positive correlation with TIBC

  b. Negative correlations with serum ferritin and WBC counts
- Serum ferritin levels were found to have positive correlation with hs-CRP and negative correlation with TIBC
- hs-CRP levels were found to have negative correlation with TIBC

SUMMARY OF RESULTS

1. Levels of hemoglobin and serum albumin, and TIBC were significantly lower in subjects with ALD than in control subjects.

2. Serum levels of ferritin, hs-CRP, AST and ALP, and MCV and transferrin saturation were significantly higher in subjects with ALD than in control subjects.

3. Serum levels of iron, total protein and ALT were similar in both groups.

4. Gene expression of proteins involved in duodenal iron absorption (DMT-1, Dcytb and ferroportin) and protein expression of HIF-2α were similar in the two groups.

5. Correlation analysis of data from the whole group showed that serum ferritin levels were positively correlated with transferrin saturation and hs-CRP and negatively with TIBC and ferroportin mRNA levels. Hemoglobin levels were positively correlated with TIBC. Serum iron levels correlated positively with transferrin saturation and hs-CRP levels correlated negatively with hemoglobin levels and TIBC.

6. Correlation analysis of data from patients with ALD showed that FPN and DMT-1 correlated positively with each other; mRNA levels of both of these proteins correlated negatively with serum ferritin and WBC counts, and positively with TIBC. FPN mRNA also showed negative correlations with transferrin saturation. Serum ferritin levels were found to have positive correlation with hs-CRP and negative correlation with TIBC.

DISCUSSION

Alcohol consumption results in a wide spectrum of pathological effects in the liver including steatosis, hepatitis and cirrhosis. However, only a small proportion of patients progress from steatosis to hepatitis to cirrhosis, despite continued and heavy alcohol consumption. This suggests that, apart from alcohol, other factors play a role in progression of the disease. One such factor, which has been postulated to promote disease progression in ALD, is iron (Magdaleno et al., 2017).

It has been reported that hepatic iron overload is associated with ALD (Basaranoglu et al., 2013; Milic et al., 2016). However, this reported association is often based on elevated levels of surrogate markers of iron status, such as serum ferritin levels and transferrin saturation (Bell et al., 1994; Kohgo et al., 2005). In the few studies where hepatic iron stores were directly assessed (by histopathology and/or estimation of hepatic tissue iron in liver biopsy samples), the prevalence of increased iron stores in patients with ALD was found to be highly variable. For example, it varied from 57% in the study by Jakobovits et al (Jakobovits et al., 1979) to 29% in that by Chapman et al (Chapman et al., 1982). No stainable iron was detectable in hepatic biopsies in the study by Lundvall et al; in fact, estimation of liver iron in this study showed significantly decreased hepatic iron stores among patients with alcoholic cirrhosis (Lundvall et al., 1969). Hence, iron overload does not appear to be an invariable feature of ALD.

Anemia is common among patients with ALD (Gonzalez-Casas et al., 2009). The cause for anemia in such patients is multifactorial; it may be due to iron deficiency (IDA) as a result of gastrointestinal blood loss, poor nutrition, impaired duodenal iron absorption, or a combination of these (Kimber et al., 1965; Gonzalez-Casas et al., 2009). On the other hand, alcohol has been

shown to directly suppress erythropoiesis (Hourihane and Weir, 1970). In addition, ALD is known to be a chronic inflammatory condition and the anemia seen in this condition may be due to anemia that is associated with inflammation (AI) (Lewis et al., 2007).

In the present study, the prevalence of anemia among patients with ALD was found to be 77%. These patients had mild-to-moderate anemia (as per WHO criteria for the diagnosis of anemia). The mean serum iron levels in patients with ALD were similar to those in control patients. However, patients with ALD had lower TIBC and higher MCV, transferrin saturation and serum ferritin levels, when compared to control patients. This suggests that the cause for anemia was probably not iron deficiency; rather, these findings, along with that of increased serum hs-CRP levels, which is a marker of systemic inflammation, suggests that the anemia may be secondary to inflammation.

There is no physiological mechanism to excrete iron from the body. Intestinal absorption of iron is regulated to maintain iron homeostasis in the body. There are very few studies that have determined expression of duodenal proteins involved in the absorption of non-heme iron in patients with ALD. Dostalikova-Cimburova et al (2014) have reported decreased serum hepcidin levels, increased mRNA expression of DMT-1 and FPN and increased protein levels of FPN in patients with ALD, when compared to control subjects. However, earlier work done in the Department of Biochemistry at CMC, Vellore, on patients with ALD showed different results. In these studies, expression of duodenal DMT-1 and ferroportin was lower in patients with ALD than in control subjects, despite co-existence of low levels of hepcidin (unpublished data). These observations prompted the study of factors other than hepcidin that regulate duodenal iron absorption, leading to the present study.

It is known that local factors in the duodenal mucosa regulate proteins involved in the absorption of iron. HIF-2α is a transcription factor, which is known to play an important role in the transcriptional regulation of duodenal iron transporters (Mastrogiannaki et al., 2009). The intestinal mucosa is exposed to a relatively hypoxic environment; this stabilizes HIF-2α (Mastrogiannaki et al., 2009). Intestine-specific knock-out of HIF-2α in mice resulted in decreased expression of DMT-1, FPN and DCYTB (Mastrogiannaki et al., 2012). On the other hand, feeding mice a diet that was deficient in iron increased HIF-2α protein levels, leading to increased expression of these proteins (Shah et al., 2009).

It has been shown, in mice, that chronic alcohol exposure reduced HIF-2α protein levels in the intestine (Wang et al., 2011). Therefore, we hypothesized that alcohol-induced alterations in HIF-2α expression may underlie the decreased expression of duodenal proteins involved in iron absorption, observed in patients with ALD. To the best of our knowledge, there are no published studies till date that have looked at the expression of duodenal HIF-2α in patients with ALD.

The results of the present study showed no difference in HIF-2α protein levels (determined by western blotting) between patients with ALD and control subjects. In the 18 patients with ALD in this study, gene expression of DMT, FPN or Dcytb were similar to those in the control group, unlike what had been observed earlier. The small sample size of the present study is likely to account for these differences, as the earlier study had a larger sample size. When data from both studies were combined, the overall findings were that expression levels of the duodenal proteins were decreased. Stored samples from the earlier study were also used to determine protein expression of HIF-2α. However, it was found that it was not possible to detect HIF-2α protein in these samples, necessitating the present study to obtain fresh samples of duodenal mucosa.

As mentioned earlier, anemia increases expression of iron transporters, while inflammation decreases it (Gulec et al., 2014). In this study, patients with ALD were anemic and showed evidence of systemic inflammation. It is possible that the opposing effects of anemia and inflammation in the patients with ALD may also underlie the observation that expression levels in these patients were similar to those in control subjects. Achieving adequate sample size and estimating serum hepcidin, TNF-alpha and hepatic iron content in these patients will give a better idea of the various factors that are at play in this setting.

There was no correlation seen between protein levels of HIF-2α and mRNA expression of DMT-1, FPN or Dcytb, tending to suggest that HIF-2α may not play a major role in the regulation of these duodenal iron transporters in patients with ALD. Nevertheless, it should be kept in mind that HIF-2α is an extremely short-lived protein (Bracken et al., 2006). Changes in duodenal HIF-2α in mice have been shown only when they were exposed to stimuli (such as low iron diet or hypoxia) that robustly up-regulated HIF-2α levels (Shah et al., 2009). There are no studies that we have been able to find that have shown a decrease in protein levels in HIF-2α, thus causing a down-regulation of duodenal proteins involved in iron absorption. Technical difficulties exist in detection of small changes in the levels of short-lived proteins such as HIF-2α. Taking this into consideration, although there is no significant difference in protein levels of HIF-2α in the present study, its role in regulation of intestinal iron transporters in ALD cannot be ruled out. In addition, due to financial constraints associated with an MD thesis, it was possible to study only 18 patients with ALD and 18 control subjects, while the calculated sample size was 48 in each group. It would be necessary to achieve the required sample size to draw definitive conclusions about the possible role (or not) of HIF-2α in regulating duodenal proteins involved in iron absorption in patients with ALD.

Systemic as well as local inflammation in the intestine is known to affect iron absorption (Wessling-Resnick, 2010). The effects of systemic inflammation are mainly mediated by its effects on hepcidin, which is elevated in such conditions (Nemeth et al., 2003). Increased serum levels of hepcidin down-regulate duodenal iron transporters (Frazer et al., 2002), resulting in decreased iron absorption. Local inflammation, on the other hand, results in increased levels of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNFα), which can act directly on the enterocytes to down-regulate duodenal iron transporters. This effect is independent of hepcidin (Laftah et al., 2006). ALD is known to be associated with intestinal inflammation, increased intestinal permeability and increased levels of TNFα (Kawaratani et al., 2013). Serum CRP is widely used as a marker of systemic inflammation (Pearson et al., 2003). In this study, hs-CRP levels were found to be significantly higher in patients with ALD than the control group. In addition, WBC counts (another marker of inflammation) negatively correlated with DMT-1 and FPN mRNA. It is therefore possible that inflammation may also play an important role in the regulation of duodenal iron transporters in patients with ALD in this study.

Correlation analysis of the data in this study revealed several interesting results. WBC counts showed a significant negative correlation with mRNA expression of DMT-1 and FPN in patients with ALD, suggesting the effect of inflammation on these proteins. Serum ferritin showed a significant positive correlation with hs-CRP in patients with ALD. No correlations were found between ferritin and other markers of iron status, such as serum iron levels and transferrin saturation. This suggests that the cause of elevated ferritin in these patients was inflammation rather than iron overload.

These results of this study are different from the one by Dostalikova-Cimburova et al. The similarities and important differences between these studies are shown in the tables below, which may explain the differing observations.

Similarities between the present study and that by Dostalikova-Cimburova et al (2014)

	Dostalikova-Cimburova et al study	**Present study**
Cases	ALD patients (n=24)	ALD patients (n= 18)
Control subjects	Patients with dyspeptic symptoms(n=30)	Patients with dyspeptic symptoms(n= 18)
Hemoglobin levels	Hb significantly lower in patients with ALD than in control subjects	Hb significantly lower in patients with ALD than in control subjects
Serum iron	Similar in both groups	Similar in both groups

Differences between the present study and that by Dostalikova-Cimburova et al (2014)

	Dostalikova-Cimburova et al study	**Present study**
Sex:	Males = 35 ; females = 19	All males
Mean age of the subjects:	57.4 years (similar in both groups)	48 years (similar in both groups)
Cases sub-divided into those	with anemia n=8 with iron overload n=6 without iron overload n=10	Not done as the total numbers were small (n=18)
Serum ferritin	Not significantly different between the two groups	Ferritin was significantly higher in patients with ALD
Transferrin saturation	Not significantly different between the two groups	Transferrin saturation was significantly higher in patients with ALD, but was within the reference range
hs-CRP	Not done	Increased in patients with ALD when compared to controls

mRNA expression	Expression of DMT-1, FPN and TFR1 genes increased significantly in patients with ALD as a whole; and more pronounced in the subgroups with anemia and without iron overload.	Expression of DMT-1,FPN1, DCYTB similar in cases and controls
Protein expression of iron transporters	Protein expression of DMT-1, FPN, Dcytb and HEPH done by western blotting FPN protein levels were higher in patients ALD. The other proteins were similar.	Protein expression of DMT-1 and FPN1 was not done as a part of the dissertation.
HIF-2α protein expression	Not done	This was carried out as part of this study; protein expression of HIF-2α was similar in the 2 groups.
Seum hepcidin	Decreased in patients with ALD	Not done as a part of the dissertation

As shown in the tables above, there are many differences between the present study and that by Dostalikova-Cimburova et al (2014). Hence, the result of the 2 studies cannot be directly compared.

The present study included only male patients with ALD; female patients with ALD are rare in the Indian setting. It is known that there are gender and ethnicity-related differences in iron metabolism and liver diseases (Harrison-Findik, 2010). The inflammatory status of patients with ALD was not determined in the study by Dostalikova-Cimburova et al (2014). In the present study, patients with ALD had higher hs-CRP levels, which may contribute to the low mRNA expression of proteins involved in iron absorption.

To the best of our knowledge, this is the first study to explore the role of HIF-2α in regulation of duodenal proteins involved in iron absorption in patients with ALD.

CONCLUSION

The results of this study show that HIF-2α does not appear to be altered in patients with ALD. However, study of an adequate sample size is required to confirm these findings.

LIMITATIONS OF THE STUDY

1. The calculated sample size for this study was 48 subjects in each group. Due to financial constraints and limited time available for carrying out work for an MD dissertation, it was possible to study only 18 patients in each group. This small sample size is a major limitation of the study.

2. Estimation of the parameters listed below would help elucidate the various mechanisms involved in dysregulation of iron homeostasis in patient with ALD. However, these could not be done as part of this dissertation work, due to constraints of time and funds. On-going work in the lab is aimed at addressing these lacunae.

- Serum hepcidin levels to better understand the complex regulation of intestinal proteins involved in iron absorption
- Protein expression levels of DMT-1, FPN and Dcytb which would help in understanding the regulation at post-transcriptional levels
- Estimation of TNFα in duodenal mucosal samples which would be a definitive marker of local intestinal inflammation.

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APPENDIX I: LETTER OF APPROVAL FROM THE IRB

OFFICE OF RESEARCH
INSTITUTIONAL REVIEW BOARD (IRB)
CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho MS Ortho DNB Ortho.
Chairperson, Research Committee & Principal

Dr. Biju George, MBBS., MD., DM
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

June 13, 2016

Dr.S.Mathuravalli,
PG Registrar,
Department of Biochemistry,
Christian Medical College,
Vellore 632 004.

Sub: **Fluid Research Funding: New Proposal**
Duodenal hypoxia inducible factor-2 alpha (HIF-2α) and iron-related proteins in alcoholic liver disease
Dr.S.Mathuravalli (Employment Number: 21191), PG registrar, Biochemistry, Dr. Molly Jacob Employment Number: 14509, Biochemistry, Dr. Joe Varghese, Employment no: 20405, Biochemistry, Dr. A J Joseph, Gastroenterology, Dr. Eapen CE, Hepatology, Ms.Thenmozhi Mani, Biostatistics,

Ref: IRB Min No: 9816 [OBSERV] dated 07.01.2016

Dear Dr.S.Mathuravalli,,

I enclose the following documents:-

1. Institutional Review Board approval 2. Agreement

Could you please sign the agreement and send it to Dr. Biju George, Addl. Vice Principal (Research), so that the grant money can be released.

With best wishes,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MBBS MD DM
SECRETARY (ETHICS COMMITTEE)
Institutional Review Board
Christian Medical College, Vellore - 632 002.

Cc: Dr. Molly Jacob, Dept. of Biochemistry, CMC

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OFFICE OF RESEARCH
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CHRISTIAN MEDICAL COLLEGE, VELLORE, INDIA

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Chairperson, Research Committee & Principal

Dr. Biju George, MBBS., MD., DM
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

June 13, 2016

Dr.S.Mathuravalli,
PG Registrar,
Department of Biochemistry,
Christian Medical College,
Vellore 632 004.

Sub: **Fluid Research Funding: New Proposal**
Duodenal hypoxia inducible factor-2 alpha (HIF-2α) and iron-related proteins in alcoholic liver disease
Dr.S.Mathuravalli (Employment Number: 21191), PG registrar, Biochemistry, Dr. Molly Jacob Employment Number: 14509, Biochemistry, Dr. Joe Varghese, Employment no: 20405, Biochemistry, Dr. A J Joseph, Gastroenterology, Dr. Eapen CE, Hepatology, Ms.Thenmozhi Mani, Biostatistics,

Ref: IRB Min No: 9816 [OBSERV] dated 07.01.2016

Dear Dr.S.Mathuravalli,

The Institutional Review Board (Blue, Research and Ethics Committee) of the Christian Medical College, Vellore, reviewed and discussed your project titled "Duodenal hypoxia inducible factor-2 alpha (HIF-2α) and iron-related proteins in alcoholic liver disease" on January 07th 2016.

The Committee reviewed the following documents

1. IRB Application format
2. Patient Information Sheet and Informed Consent Form (English, Tamil, Hindi, Bengali, Telugu)
3. Cvs of Drs. Mathuravalli, Molly Jacob, Eapen CE, . Joe Varghese, Ms.Thenmozhi Mani
4. No. of documents 1 – 3

The following Institutional Review Board (Blue, Research & Ethics Committee) members were present at the meeting held on January 07th 2016 in the CREST/SACN Conference Room, Christian Medical College, Bagayam, Vellore 632002.

Ethics Committee Blue, Office of Research, 1st Floor, Carman Block, Christian Medical College, Vellore, Tamil Nadu 632 002
Tel: 0416 – 2284294, 2284202 Fax: 0416 – 2262788, 2284481 E-mail: research@cmcvellore.ac.in

Dr. B.J. Prashantham, M.A., M.A., Dr. Min (Clinical)
Director, Christian Counseling Center,
Chairperson, Ethics Committee.

Dr. Alfred Job Daniel, D Ortho MS Ortho DNB Ortho.
Chairperson, Research Committee & Principal

Dr. Biju George, MBBS., MD., DM
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Name	Qualification	Designation	Affiliation
Dr. Biju George	MBBS, MD, DM	Professor, Haematology, Research), Additional Vice Principal , Deputy Chairperson (Research Committee), Member Secretary (Ethics Committee), IRB, CMC, Vellore	Internal, Clinician
Dr. RV. Shaji		Professor, Heamatology, CMC, Vellore	Internal, Basic Medical Scientist
Dr. Mathew Joseph	MBBS, MCH	Professor, Neurosurgery, CMC, Vellore	Internal, Clinician
Rev. Joseph Devaraj	BSc, BD	Chaplaincy Department, CMC, Vellore	Internal, Social Scientist
Dr. Balamugesh	MBBS, MD(Int Med), DM, FCCP (USA)	Professor, Pulmonary Medicine, CMC, Vellore	Internal, Clinician
Dr. Visalakshi. J	MPH, PhD	Lecturer, Biostatistics, CMC, Vellore	Internal, Statistician
Dr. Rajesh Kannangai	MD, PhD.	Professor, Clinical Virology, CMC, Vellore	Internal, Clinician
Dr. Niranjan Thomas	DCH, MD, DNB (Paediatrics)	Professor, Neonatology, CMC, Vellore	Internal, Clinician
Mrs. Pattabiraman	BSc, DSSA	Social Worker, Vellore	External, Lay Person
Dr. B. J. Prashantham	MA(Counseling Psychol MA(Theology), Dr. Min(Clinical Counselling)	Chairperson, Ethics Committee, IRB. Director, Christian Counseling Centr Vellore	External, Social Scientist
Dr. Ratna Prabha	MBBS, MD (Pharma)	Associate Professor, Clinical Pharmacology, CMC, Vellore	Internal, Pharmacologist
Dr. Anand Zachariah	MBBS, PhD	Professor, Medicine, CMC, Vellore	Internal, Clinician
Mrs. Emily Daniel	MSc Nursing	Professor, Medical Surgical Nursing, CMC, Vellore	Internal, Nurse

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Chairperson, Research Committee & Principal

Dr. Biju George, MBBS., MD., DM
Deputy Chairperson,
Secretary, Ethics Committee, IRB
Additional Vice-Principal (Research)

Dr. Vivek Mathew	MD (Gen. Med.) DM (Neuro) Dip. NB (Neuro)	Professor, Neurology, CMC, Vellore	Internal, Clinician
Mr. C. Sampath	BSc, BL	Advocate, Vellore	External, Legal Expert
Dr. Simon Pavamani	MBBS, MD	Professor, Radiotherapy, CMC, Vellore	Internal, Clinician
Dr. Inian Samarasam	MS, FRCS, FRACS	Professor, Surgery, CMC, Vellore	Internal, Clinician
Dr. Thomas V Paul	MD, DNB(Endo), Phd(Endo)	Professor, Endocrinology, CMC, Vellore	Internal, Clinician

We approve the project to be conducted as presented.

Kindly provide the total number of patients enrolled in your study and the total number of withdrawals for the study entitled: "Duodenal hypoxia inducible factor-2 alpha (HIF-2α) and iron-related proteins in alcoholic liver disease" on a monthly basis. Please send copies of this to the Research Office (research@cmcvellore.ac.in)

Fluid Grant Allocation:
A sum of 1,00,000/- INR (Rupees One Lakh Only) will be granted for 2 years. 50,000/- INR (Rupees Fifty Thousand only) will be granted for 12 months as an Ist Installment. The rest of the 50,000/- INR (Rupees Fifty Thousand only) each will be released at the end of the first year as 2 nd Installment.

Yours sincerely,

Dr. Biju George
Secretary (Ethics Committee)
Institutional Review Board

Dr. BIJU GEORGE
MBBS., MD., DM
SECRETARY - (ETHICS COMMITTEE)
Institutional Review Board,
Christian Medical College, Vellore - 632 002.

IRB Min No: 9816 [OBSERV] dated 07.01.2016

APPENDIX II: PATIENT INFORMATION SHEET (ENGLISH)

Duodenal HIF-2α and iron-related proteins in alcoholic liver disease

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 may 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 Hepatology and 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. We also wish to study patients who do not have liver disease due to alcohol intake and whose doctors have advised them to have an endoscopy as part of the tests being done to diagnose the problem.

In order to carry out these studies, 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 biopsy and blood samples that we request will be used purely for scientific research. The collection of 10 ml of blood and biopsy sample will not cause any harm to your health in any foreseeable manner. Any sample or part of it that may remain at the end of the study will be stored and used for related research in the future. 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 to 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.

If you have any doubts or questions regarding the study, please contact one of the following:

Dr.S.Mathuravalli, Department of Biochemistry, CMC, Vellore. Phone 9566901857
Dr.A J Joseph, Professor and Head, Department of Gastroenterology, CMC, Vellore. Ph: 0416 2282496
Dr.Eapen CE, Professor, Department of Hepatology, CMC, Vellore. Ph: 0416-2283629
Dr.Molly Jacob, Professor and Head, Department of Biochemistry, CMC, Vellore.
Ph: 0416-2284267

PATIENT INFORMATION SHEET (TAMIL)

ஆய்வில் பங்கேற்பவருக்கான தகவல் தாள்

மது சார்ந்த கல்லீரல் நோய் உள்ளோரின் குடல் ஹிப் 2 ஆல்பா மற்றும் இரும்பு நிலைகள்

உயிர் வேதியியல் துறை கல்லீரல் நோயியல் துறை மற்றும் குடல்,இரைப்பை நோயியல் துறை இணைந்து மது சார்ந்த கல்லீரல் நோய் உள்ளோரின் குடல் ஹிப் 2 அல்பா மற்றும் இரும்பின் நிலைகளை கண்டறிய ஆய்வு மேற்கொள்ள உள்ளோம் .

மது சார்ந்த கல்லீரல் நோய் உள்ளோரின் இரத்தத்தில் இரும்பின் அளவு அதிகமாக உள்ளது .அதிக இரும்பு அளவுகள் நோயை மேலும் மோசமாக்கி விடும் .இதற்கான காரணத்தை கண்டறிய இந்த ஆராய்ச்சி உதவும்.

தங்கள் நோய் கண்டறிதலின் ஒரு பகுதியாக உள்நோக்கியின் மூலம் வயிறு மற்றும் சிறுகுடல் பகுதிகள் பார்வை இடப்படும். அப்போது சிறிதளவு சிறுகுடல் மேற்பகுதி திசு எடுக்கப்பட்டு ஆய்வு செய்யப்படும்.இந்த திசு எடுத்து ஆய்வு செய்ய தங்கள் அனுமதி வேண்டுகிறோம் .இந்த திசு எடுப்பதால் தங்களுக்கு எந்தவித தீங்கும் ஏற்படாது .

இந்த ஆய்வுக்காக1 0 மில்லி அளவு இரத்தம் தேவை படுகிறது .தாங்கள் சுய விருப்பத்துடன் இரத்தம் தர வேண்டுகிறோம் .ஆய்வுக்கான இந்த இரத்தம் தாங்கள் இதர பரிசோதனைக்கு வரும் பொழுது தங்கள் அனுமதியுடன் பெற்று கொள்ளப்படும்.மீதமாகும் இரத்தம் சேகரிக்கப்பட்டு பின் ஆராய்சிக்காக உபயோகப்படுத்தப்படும்.

ஆராய்ச்சிக்காக அறியப்படும் தகவல்கள் நம்பகமான முறையில் பாதுகாக்கப்படும் .இந்த ஆய்வில் பங்கு பெறுவதால் தாங்கள் நேரடியாக பயன் பெற இயலாது .ஆனால் இதன் மூலம் அறியப்படும் தகவல்கள் பிற்காலத்தில் இந்நோயை சரியாக கையாள உதவும் .

இந்த ஆராய்ச்சியில் பங்குபெற விருப்பமில்லை என்றால் தயங்காமல் கூறலாம் . அதனால் இந்த மருத்துவமனையில் தங்களுக்கு தரப்படும் சேவை பாதிக்கப்படாது.

இந்த ஆய்வு குறித்த சந்தேகங்கள் மற்றும் தொடர்புக்கு கீழ்க்கண்ட நபர்களை அணுகவும்

மரு. மதுரவள்ளி, உயிர் வேதியியல் துறை, போன் 9566901857

மரு. ஜோசப், குடல் மற்றும் இரைப்பை துறை, போன் 0416-2282496

மரு. ஈபென், கல்லீரல் நோயியல் துறை, போன் 0416-2283629

மரு. மோலி ஜேக்கப், உயிர் வேதியியல் துறை, போன் 0416- 2284297

PATIENT INFORMATION SHEET (HINDI)

रोगी सूचना पत्र - आंतों (डुओडेनम) के एक टुकडे और रक्त के एक नमूने के लिए अनुरोध

अध्य्यन शीर्षक: शराब के सेवन से जिगर सम्बंधित बीमारी में ग्रहणी एच. आई. एफ. २ आल्फा और लोहे से संबंधित प्रोटीन की मात्रा।

आपकी मौजूदा चिकित्सीय समस्या की जांच के तहद,आपकी देखभाल कर रहे डॉक्टर ने आप को एंडोस्कोपी सलाह दी है जिसके दौरान एक ट्यूब आपके पेट और छोटी आंत में से गुज़रेगी ताकि आपके डॉक्टर देख सकें कि इन भागों में कोई समस्या है कि नहीं। प्रक्रिया के दौरान, डॉक्टर इन भागों से एक छोटा सा टुकड़ा लेगें ताकि उनपर निश्चित परीक्षण कर आपके रोग के बारे में और जानकारी प्राप्त हो सके।

जैव रसायन विभाग, हेप्टोलोजी और गैस्ट्रोएंटरोलॉजी विभाग के सहयोग से, एक अध्ययन कर रहें हैं एंडोस्कोपी से गुजरने वाले ऐसे सब मरीजों पर, ताकि शरीर में होने वाले लोहे के नियंत्रित में परिवर्तन को जांच सके ऐसे रोगियों में जिन्हें शराब के सेवन से जिगर सम्बंधित बीमारी है। इस स्थिति में अक्सर जिगर में लोहे का स्तर सामान्य से अधिक हो जाता है। इससे कभी कभी रोग बिगड़ जाता है। ऐसा क्यों होता है, हमें पूरी तरह से पता नहीं है। हम यह बेहतर ढंग से समझने के लिए, इस समस्या का अध्ययन करना चाहते हैं। ऐसा करने के लिए हमें छोटी आंत से बहुत छोटी बायोप्सी करने की जरूरत है। इसे डॉक्टर उस समय लेंगें जब आपके पेट और आंत के अंदर ट्यूब डाली जाएगी। हम इसे लेने के लिए आपकी अनुमति का अनुरोध करते हैं। इस के कारण आपको कोई अधिक नुकसान नहीं होगा। हम आपसे रक्त का नमूना प्रदान करने की अनुमति भी माँगते हैं जिससे हम रक्त में लोहे से संबंधित अध्ययन में आसानी होगी।

एकत्र रक्त और वसा ऊतकों के नमूनों अनुसंधान प्रयोजनों के लिए ही इस्तेमाल किए जाएंगे। 10 मिलीलीटर रक्त और बायोप्सी नमूने के संग्रह से आपके स्वास्थ्य को किसी निकटतम तरीके से कोई नुकसान नहीं होगा। आपके द्वारा प्रदान की गई चिकित्सा एवं निजी सूचना गोपनीय रखी जाएगी।

आपको इस अध्ययन से प्रत्यक्ष रूप से लाभ नहीं होगा। लेकिन, अगर आप अध्ययन में भाग लेने और नमूने उपलब्ध कराने के लिए तैयार हैं, तो यह हमें शराब का सेवन करने वाले रोगियां के जिगर में लोहा क्यों जमा हो जाता है, यह समझने में मदद प्रदान करेगा। यह ज्ञान डॉक्टरों को इस रोग के इलाज को बेहतर बनाने में मदद कर सकता है। अगर आप रक्त और वसा ऊतकों के नमूनों को देने की इच्छा नहीं रखते , तो आप ऐसा कहने के लिए स्वतंत्र हैं। यह आपके अस्पताल में प्राप्त होते उपचार को प्रभावित नहीं करेगा।

अगर आपको अध्ययन के बारे में कोई संदेह या प्रश्न हैं, तो निम्न में से एक से संपर्क करें:

1. डॉ एस. मधुरवल्लि, जैव रसायन विभाग, सी एम सी , वेल्लोर, फ़ोन 9566901857
2. डॉ ए.जे. जोसेफ , प्रोफेसर और हेड, गैस्ट्रोएंटरोलॉजी विभाग , सी एम सी , वेल्लोर । फोन: 0416 2282496
3. डॉ ऐपन सी. ई., प्रोफेसर, हेप्टोलोजी विभाग, सी एम सी , वेल्लोर । फोन: 0416-2283629
4. डॉ मौली जेकोब, प्रोफेसर और हेड, जैव रसायन विभाग , सी एम सी , वेल्लोर । फोन: 0416-2284267

PATIENT INFORMATION SHEET (TELUGU)

రక్త నమూనా మరియు చిన్న ప్రేగు యొక్క నమూనా సేకరణ కై సమాచార పత్రము

పరిశోధన అంశం : త్రాగుడు వలన కలుగు లివర్ వ్యాధిలో HIF 2α మరియు ఐరన్ మరియు చిన్న పేగు సంబంధిత ఆస్మూలు గురించి .

మద్యపానంవలన కలుగు కాలేయ సంబంధిత వ్యాధులలో శరరీరమునందు ఇనుము యొక్క ప్రక్రియ జీవ రసాయన ప్రక్రియలో మార్పులు గురుంచి బయోకేమిస్ట్రీ, గ్యాస్ట్రోఎంట్రోలలజి, మరియు హేపాటోలజి విభాగములు సంయుక్తంగా పరిసశోధన నిర్వహిస్తున్నారు. ఈ వ్యదియందు సాధారణ స్థాయి కంటి అధికముగా ఇనుము నిల్వలు కాలేయములో చేరుట, దాని వలన కొన్ని సార్లు వ్యాధి ఇంకా అధికమగుట జరుగును. కాని అది ఎందు వలన అన్న పూర్తి వివరములు ఇంతవరకు తెలియలేదు . అందువలన ఈ విషయం గూర్చి మేము పరిశోధించదలిచాము. ఇందు కొరకు , మీరు మీ యొక్క 10మిల్లీ రక్తము దానము చేయుటకు మరియు మీ వైద్యునిచే సూచించే ఎండోస్కోపీ పరీక్ష చేయు సమయములో మీ చిన్న ప్రేగు పై భాగమునందు ఒక చిన్న ముక్కను పరిశోధనకై తీసుకొనుటకు మీ సమ్మతి తెలుపగోరుచున్నాము. మీరు ఇచ్చే రక్తము మరియు ప్రేగు ముక్క ఏదైనాను నమూనా మిగిలితే అది భావిష్యతులో ఇతర పరిశోధనలకు ఉపయోగించబడును.

మీ యొక్క 10మిల్లీ రక్తము దానము చేయుట వలన మీకు ఏ విధమైన ఆరోగ్య సమస్యలు వుండవు .మీ యొక్క వ్యక్తిగత వివరాలన్నీ అత్యంత గోప్యంగా ఉంచ బడతాయి .

మీ చిన్న ప్రేగు యొక్క ముక్క పరీక్ష ద్వార మీకు ప్రత్యక్ష్యముగా ఎటువంటి ఉపయోగం లేనప్పటికీ మద్యపానం చేయువారిలో ఎక్కువ ఇనుము గ్రహియింపబడి అది కాలేయములో చేరుతుంది అన్న విషయాన్ని పరిశోధించుటకు తోడ్పడుతుంది.

ఇందుకు అనగా పైన పేర్కొన్న పరీక్షలకు మేరకు అంగీకారము లేని యెడల మీరు నిర్మొహమాటముగా తెలుపవచ్చును ఇందువలన ఆసుపత్రిలో మీకు ఇవ్వబడే చికిత్స యందు ఎటువంటి మార్పు వుండదు.

మీకు ఏవిధమైన సందేహములు ఉన్న ఎడల మీరు సంప్రదించ వలసిన చిరునామ:

డా . మధురవల్లి, బయోకెమిస్ట్రీ డిపార్టుమెంటు, సి.ఎం.సి, వెల్లూరు. ఫోను: 9566901857

డా. ఏ. జె. జోసెఫ్, ప్రొఫెసర్, గస్ట్రో ఎంటిరాలజి డిపార్టుమెంటు, సి.ఎం.సి, వెల్లూరు. ఫోను: 0416-2282496

డా.ఈపెన్ సి.ఈ., ప్రొఫెసర్, హెపటాలజీ డిపార్టుమెంటు,సి.ఎం.సి, వెల్లూరు. ఫోను: 0416-2283629

డా, మోలీ జాకబ్, ప్రొఫెసర్, బయోకెమిస్ట్రీ డిపార్టుమెంటు, సి.ఎం.సి, వెల్లూరు. ఫోను:0416-2284267

PATIENT INFORMATION SHEET (BENGALI)

মদ্যপান জনিত যকৃৎ রোগীদের ড্যুওডেনামে এইচ আই এফ টু আলফা এবং অন্যান্য লৌহ জড়িত প্রোটিনের পরিমাণ

আপনার ডাক্তার আপনার বর্তমান অসুখের পরীক্ষাগুলির অংশ হিসাবে আপনাকে এণ্ডোস্কোপী করাতে বলেছেন। এণ্ডোস্কোপী মানে আপনার পাকস্থলী ও ক্ষুদ্রান্ত্রের মধ্যে এণ্ডোস্কোপ (নল আকৃতির অভ্যন্তরীণ দূরবিন) ঢুকিয়ে ডাক্তার সেখানে কোন রোগ হয়েছে কিনা লক্ষ্য করবেন। এণ্ডোস্কোপী চলাকালীন ডাক্তার রোগের আরো পরীক্ষার জন্য বায়োপ্সী নমুনা সংগ্রহ করে থাকেন।

ডিপার্টমেন্ট অফ বায়োকেমিস্ট্রি (জৈব-রসায়ন) এবং ডিপার্টমেন্ট অফ হেপাটোলজি অ্যাণ্ড গ্যাস্ট্রোএন্টেরোলজী (যকৃত/লিভার, পাকস্থলি ও অন্ত্রের বিজ্ঞান) এণ্ডোস্কোপীরত রোগীদের সম্বন্ধে একটি বৈজ্ঞানিক গবেষণা শুরু করেছে যা থেকে শরীর লোহাকে কিভাবে বণ্টন করে তা জানা যাবে। মদ্যপান-জনিত যকৃত রোগে অনেক সময় শরীরে প্রয়োজনের অতিরিক্ত লোহা জমা হয়ে রোগ বাড়িয়ে তোলে। আমরা এই আসুবিধাটির উপর গবেষণা করে এর কারণ খোঁজার চেষ্টা করছি। এর জন্য আমাদের গবেষণায় রোগীদের ক্ষুদ্রান্ত্রের বায়োপ্সীর একটি সূক্ষ্ম নমুনা প্রয়োজন। আমরা বায়োপ্সীর একটি সূক্ষ্ম নমুনা সংগ্রহের জন্য আপনার অনুমতি চাই। এই নমুনা এণ্ডোস্কোপী চলাকালীনই সংগ্রহ করা হবে। এর জন্য আপনাকে আলাদা করে কোন অসুবিধা করা হবে না। আপনাকে একটি রক্ত নমুনা দেবার জন্যও অনুরোধ করা হচ্ছে। এর দ্বারা রক্তের মধ্যেও লোহার কিভাবে কি হচ্ছে তা গবেষণা করা যাবে।

আপনার বায়োপ্সীর ও রক্তের নমুনা কেবলমাত্র বৈজ্ঞানিক গবেষণার জন্যই ব্যবহৃত হবে। ১০ মিলি রক্ত দানে আপনার স্বাস্থ্যের ক্ষতি হবার দৃষ্টিগ্রাহ্য কোন আশঙ্কা নেই। এই প্রসঙ্গে আপনার যেসব ব্যক্তিগত তথ্য আমরা আপনার থেকে সংগ্রহ করব তা সম্পূর্ণ ভাবে গোপনীয় থাকবে।

এই গবেষণা হয়তো এই মুহূর্তে আপনার অসুস্থতা উপশমে সরাসরি সাহায্য করবে না। কিন্তু আপনার যদি যদি এই নমুনাগুলি দিতে আপত্তি না থাকে তাহলে এর উপর করা গবেষণা থেকে মদ্যপান জনিত যকৃত ব্যাধিতে যকৃত অর্থাৎ লিভার-এ কেন লোহা বেশী জমে তার কারণ বুঝতে সাহায্য হবে। এবং সেই জ্ঞান এই রোগের ভবিষ্যত চিকিৎসায় উন্নতি করতে সাহায্য করবে। তবে আপনি যদি নমুনা দিতে না চান তাহলেও সেটা নির্ভয়ে জানাতে পারেন। আপনি গবেষণায় অংশগ্রহণে সম্মতি দিন অথবা না-ই দিন কোনভাবেই হাসপাতালে আপনার যা চিকিৎসা চলবে তার কোন ত্রুটি হবে না।

আপনার কোন প্রশ্ন বা চিন্তা থাকলে আপনি নিম্নলিখিত যে কোন একজনকে যোগাযোগ করতে পারেন :

ডাঃ এস মথুরাবল্লী, ডিপার্টমেন্ট অফ বায়োকেমিস্ট্রি (জৈব রসায়ন বিভাগ), সি এম সি ভেল্লোর, ফোন নং : 9566901857
ডাঃ এ জে জোসেফ, অধ্যাপক এবং বিভাগীয় অধ্যক্ষ, ডিপার্টমেন্ট অফ গ্যাস্ট্রোএন্টেরোলজী, সি এম সি ভেল্লোর, ফোন নং : 0416 2282496
ডাঃ ইয়াপেন সি ই, অধ্যাপক এবং বিভাগীয় অধ্যক্ষ, ডিপার্টমেন্ট অফ হেপাটোলজী, সি এম সি ভেল্লোর, ফোন নং : 0416-2283629
ডাঃ মলি জ্যাকব, অধ্যাপক এবং বিভাগীয় অধ্যক্ষ, ডিপার্টমেন্ট অফ বায়োকেমিস্ট্রি, সি এম সি ভেল্লোর, ফোন নং : 0416-2284267

CONSENT FORM FOR CONTROL PATIENTS (ENGLISH)
Study Title: Duodenal HIF-2α and iron-related proteins in alcoholic liver disease

Subject's Name: _____

Date of Birth / Age: _____

The investigator has explained to me the details of the study proposed and what it involves. I have been asked to participate in this study for looking at how iron is handled in the body of patients with liver disease due to alcohol use and in patients who do not have this disease (as control patients). I understand that I am to be included as a control patient in this study. 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 endoscopy, which my treating doctor has advised me to have.
2. This will not create any additional difficulty for me when undergoing the procedure.
3. A sample of blood (10 ml) will also be collected from me.
4. Taking these samples will not affect my health in any foreseeable manner.
5. The samples will be used to study the processes by which iron is handled in patients who have liver disease due to alcohol intake. They will be used only for research purposes. Any sample or part of it that may remain at the end of the study will be stored and used for related research in the future.

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

Signature or thumb impression of the donor Signature of the investigator

Signature or thumb impression of the witness

Name and address of the witness:

Date:

If you have any doubts or questions regarding the study, please contact one of the following:
Dr.S.Mathuravalli, Department of Biochemistry, CMC, Vellore Phone 9566901857
Dr.A J Joseph, Professor and Head, Department of Gastroenterology CMC, Vellore Ph: 0416 2282496
Dr.Eapen CE, Professor and Head, Department of Hepatology CMC, Vellore Ph: 0416-2283629
Dr.Molly Jacob, Professor and Head, Department of Biochemistry, CMC, Vellore Ph: 0416-2284267

CONSENT FORM FOR ALD PATIENTS (ENGLISH)

Study Title: Duodenal HIF-2α and iron-related proteins in alcoholic liver disease

Subject's Name: _____

Date of Birth / Age: _____

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.
2. It will not create any additional difficulty for me to undergo the procedure.
3. A sample of blood (10 ml) will also be collected from me.
4. Taking these samples will not affect my health in any foreseeable manner.
5. The samples will be used to study what happens to the processes by which iron is handled in patients who have liver disease due to alcohol intake. They will be used only for research purposes. Any sample or part of it that may remain at the end of the study will be stored and used for related research in the future.

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

Signature /Thumb impression of the donor Signature of the investigator

Signature/ Thumb impression of the witness

Name and address of the witness:

Date:

If you have any doubts or questions regarding the study, please contact one of the following:
Dr.S.Mathuravalli, Department of Biochemistry, CMC, Vellore. Phone 9566901857
Dr.A J Joseph, Professor and Head, Department of Gastroenterology, CMC, Vellore.
Ph: 0416 2282496
Dr.Eapen CE, Professor, Department of Hepatology, CMC, Vellore. Ph: 0416-2283629
Dr.Molly Jacob, Professor and Head, Department of Biochemistry, CMC, Vellore.
Ph: 0416-2284267

CONSENT FORM FOR CONTROL PATIENTS (TAMIL)

ஒப்புதல் படிவம்)கல்லீரல் நோய் இல்லாதவர்(

மது சார்ந்த கல்லீரல் நோய் உள்ளோரின் குடல் ஹிப் ஆல்பா மற்றும் 2இரும்பு நிலைகள்

ஆய்வில் பங்கேற்பவரின் பெயர்திருமதி/திரு:

மருத்துவமனை அடையாள எண் :வயது :

இந்த ஆய்வை பற்றிய தகவல் படிவத்தை படித்தும் அதன் விவரங்களை
மரு[ ].மதுரவள்ளி மூலம் கேட்டும் புரிந்து கொண்டேன்.
இந்த ஆய்வு கல்லீரல் நோய் உள்ளோர் மற்றும் கல்லீரல் நோய் இல்லாதவர் ஆகிய இரு பிரிவுகளில்
மேற்கொள்ளப்பட உள்ளது நான் .கல்லீரல் நோய் இல்லாதவர் பிரிவில் ஆய்வில் பங்கேற்க
சம்மதிக்கிறேன் [ ] .
இந்த ஆய்வில் கலந்து கொள்ள என்னை யாரும் கட்டாயப்படுத்தவில்லைஎனது சுய . விருப்பத்துடன்
இந்த ஆய்வில் பங்கேற்கிறேன்வெளியேற இந்த ஆய்விலிருந்து எந்நேரமும் காரணமின்றி ,மேலும் .
இதனால் எனக்கு அளிக்கப்படும் சிகிச்சை முறைகளில் எவ்வித வேறுபாடும் .எனக்கு அனுமதி உள்ளது
[ ].இருக்காது என்று எனது மருத்துவர் உறுதி அளித்துள்ளார்
இந்த ஆராய்ச்சிக்காக எனது சிறுகுடல் மேற்பகுதி திசு மற்றும் மில்லி அளவு இரத்தம் கொடுக்க முழு 10
.மதிக்கிறேன்மனதுடன் சம்இதனால் என் உடல் நலத்திற்கு எந்தவித பாதிப்பும் ஏற்படாது என அறிந்து
கொண்டேன்இந்த திசு மற்றும் இரத்தம் ஆராய்ச்சிகாக மட்டுமே பயன்படுத்தப்படும் என .ிறு புரிந்து
கொண்டேன்[ ] .
மீதமாகும் இரத்ததை சேகரித்து பின் ஆராய்ச்சிக்கு பயன்படுத்த சம்மதிக்கிறேன் [ ]
எனது மருத்துவ விவரங்களை ஆய்வில் உபயோகிக்க ஒப்புக்கொள்கிறேன்இருப்பினும் எனது தனிப்பட்ட .
மருத்துவ இதழிலோ வெளியிடப்பட மாட்டாத /மற்றவரிடமோ ,தகவல் /அடையாளம்ா என்பதையும்
தெரிந்து கொண்டேன்[ ].
இந்த ஆய்வின் முடிவு[ ].த்த நான் சம்மதிக்கிறேன்தகவலை அறிவியல் நோக்கத்திற்கு மட்டும் பயன்படு /
இந்த ஆய்வில் பங்கேற்க முழுமனதுடன் சம்மதிக்கிறேன் [ ].

ஆய்வில் பங்கேற்பவரின் கையொப்பம் : பெருவிரல் ரேகை/ தேதி:

முகவரி:

சாட்சியின் பெயர் மற்றும் முகவரி:

நோயாளிக்கு எந்த வகையில் சொந்தம்:
கையொப்பம்:ஆராய்ச்சியாளர் கையொப்பம் : பெருவிரல் ரேகை/

இந்த ஆய்வு குறித்த சந்தேகங்கள் மற்றும் தொடர்புக்கு கீழ்க்கண்ட நபர்களை அணுகவும்
மரு மதுரவள்ளி , உயிர் வேதியியல் துறை போன் ,9566901857
மரு ஜோசப்குடல் மற்றும் இரைப்பை துறை போன் ,வர்பேராசிரியர் மற்றும் துறை தலை , 0416-2282496
மரு ஈபென் ,பேராசிரியர் மற்றும் துறை தலைவர் கல்லீரல் நோயியல் துறை போன் ,0416-2283629
மரு மோலி ஜேக்கப் உயிர் வேதியியல் துறை போன் ,பேராசிரியர் மற்றும் துறை தலைவர் ,0416- 2284297

CONSENT FORM FOR ALD PATIENTS (TAMIL)

ஒப்புதல் படிவம் மது சார்ந்த)கல்லீரல் நோய் உள்ளோர்(

மது சார்ந்த கல்லீரல் நோய் உள்ளோரின் குடல் ஹிப் ஆல்பா 2மற்றும் இரும்பு நிலைகள்

ஆய்வில் பங்கேற்பவரின் பெயர்திருமதி/திரு:

மருத்துவமனை அடையாள எண் :வயது :

இந்த ஆய்வை பற்றிய தகவல் படிவத்தை படித்தும் அதன் விவரங்களை
மருமதுரவள்ளி மூலம் கேட்டும் புரிந்து கொ.ண்டேன[ ].
இந்த ஆய்வில் கலந்து கொள்ள என்னை யாரும் கட்டாயப்படுத்தவில்லைஎனது சுய விருப்பத்துடன் .
இந்த ஆய்விலிருந்து எந்நேரமும் காரணமின்றி வெளியேற ,மேலும் .இந்த ஆய்வில் பங்கேற்கிறேன்
இதனால் எனக்கு அளிக்கப்படும் சிகிச்சை முறைகளில் எவ்வித.எனக்கு அனுமதி உள்ளதுவேறுபாடும்
இருக்காது என்று எனது மருத்துவர் உறுதி அளித்துள்ளார்[ ].
இந்த ஆராய்ச்சிக்காக எனது சிறுகுடல் மேற்பகுதி திசு மற்றும் ல்லி அளவு இரத்தம் கொடுக்க முழு மி 10
.மனதுடன் சம்மதிக்கிறேன்இதனால் என் உடல் நலத்திற்கு எந்தவித பாதிப்பும் ஏற்படாது என அறிந்து
கொண்டேன்இந்த திசு மற்றும் இரத்தம் ஆராய்ச்சிகாக மட்டுமே பயன்படுத்தப்படும் என்று புரிந்து .
[ ] .கொண்டேன்
மீதமாகும் இரத்ததை சேகரித்து பின் ஆராய்ச்சிக்கு பயன்படுத்த சம்மதிக்கிறேன் [ ]
எனது மருத்துவ விவரங்களை ஆய்வில் உபயோகிக்க ஒப்புக்கொள்கிறேன்இருப்பினும் எனது தன .ிப்பட்ட
அடையாளம்மருத்துவ இதழிலோ வெளியிடப்பட மாட்டாது என்பதையும் /மற்றவரிடமோ ,தகவல் /
[ ].தெரிந்து கொண்டேன்
இந்த ஆய்வின் முடிவு[ ].கவலை அறிவியல் நோக்கத்திற்கு மட்டும் பயன்படுத்த நான் சம்மதிக்கிறேன்த /
இந்த ஆய்வில் பங்கேற்க முழுமனதுடன் சம்மதிக்கிறேன் [ ].

ஆய்வில் பங்கேற்பவரின் கையொப்பம்:தேதி : பெருவிரல் ரேகை/

முகவரி:

சாட்சியின் பெயர் மற்றும் முகவரி:

நோயாளிக்கு எந்த வகையில் சொந்தம்:
கையொப்பம்:ஆராய்ச்சியாளர் கையொப்பம் : பெருவிரல் ரேகை/

இந்த ஆய்வு குறித்த சந்தேகங்கள் மற்றும் தொடர்புக்கு கீழ்க்கண்ட நபர்களை அணுகவும்
மரு மதுரவள்ளி உயிர் வேதியியல் துறை போன் 9566901857
மரு மோலி ஜேக்கப் உயிர் வேதியியல் துறை போன் 0416- 2284297
மரு ஜோசப் குடல் மற்றும் இரைப்பை துறை போன் 0416-2282496
மரு ஈபென் கல்லீரல் நோயியல் துறை போன் 0416-2283629

CONSENT FORM FOR CONTROLS (HINDI)

सूचित सहमति फॉर्म : नियंत्रण रोगियों के लिए।

अध्य्यन शीर्षक: शराब के सेवन से जिगर सम्बंधित बीमारी में ग्रहणी एच. आई. एफ. २ आल्फा और लोहे से संबंधित प्रोटीन की मात्रा।

प्रतिभागी का नाम:

जन्म तिथि / उम्र:

डॉक्टर के द्वारा मुझे प्रस्तावित अध्ययन का विवरण विस्तार से बताया गया है। मुझे एक अध्य्यन में भाग लेने का कहा गया है जिसमे शरीर में होने वाले लोहे के नियंत्रित में परिवर्तन को जांचा जाएगा, ऐसे रोगियों में जिन्हें शराब के सेवन से जिगर सम्बंधित बीमारी है और उनमें जिन्हें बीमारी नहीं है। मुझे निम्नलिखित सहित, क्या कहा गया है समझ में आ गया है :

1. मेरे पेट से ऊतक का एक बहुत छोटा सा नमूना इंडोस्कोपिक प्रक्रिया के समय चिकित्सक द्वारा लिया जाएगा, जिसकी सलाह मेरा इलाज करते चिकित्सक ने मुझे दी है।
2. यह प्रक्रिया के दौरान मेरे लिए कोई अतिरिक्त कठिनाई पैदा नहीं करेगी।
3. एक रक्त का नमूना (१० मिलीलीटर) भी मुझसे एकत्र किया जाएगा।
4. यह नमूने देने से मेरा स्वास्थ्य किसी भी ज्ञात रूप से प्रभावित नहीं होगा।
5. लिए गए नमूने यह अध्ययन करने के लिए इस्तमाल किये जाएंगे कि शराब का सेवन करने से हुई जिगर की बिमारी से पीढ़ित रोगियों में लोहे का नियंत्रण कैसे होता है। लिए गए नमूने केवल शोध उद्देश्यों के लिए ही इस्तेमाल किए जाएंगे।

मैं स्वेच्छा, बिना जांचकर्ताओं के ओर से किसी भी प्रकार के दबाव में आकर, इस अध्ययन में भाग लेने के लिए और वसा ऊतकों के नमूनों और रक्त का १० मिलीलीटर दान करने के लिए सहमती देता हूँ।

प्रतिभागी के हस्ताक्षर/ अंगूठे का निशान अन्वेषक के हस्ताक्षर

साक्षी के हस्ताक्षर/ अंगूठे का निशान

साक्षी का नाम और पता

तिथि:

अगर आपको अध्ययन के बारे में कोई संदेह या प्रश्न हैं, तो निम्न में से एक से संपर्क करें:

1. डॉ एस. मधुरवल्लि, जैव रसायन विभाग, सी एम सी , वेल्लोर, फ़ोन 9566901857
2. डॉ ए.जे. जोसेफ , प्रोफेसर और हेड, गैस्ट्रोएंटरोलॉजी विभाग , सी एम सी , वेल्लोर । फोन: 0416 2282496
3. डॉ ऐपन सी. ई., प्रोफेसर, हेप्टोलोजी विभाग, सी एम सी , वेल्लोर । फोन: 0416-2283629
4. डॉ मौली जेकोब, प्रोफेसर और हेड, जैव रसायन विभाग, सी एम सी, वेल्लोर । फोन: 0416-2284267

CONSENT FORM FOR ALD PATIENTS (HINDI)

शराब के सेवन से जिगर सम्बंधित बीमारी से पीढ़ित रोगियों के लिए।

अध्य्यन शीर्षक: शराब के सेवन से जिगर सम्बंधित बीमारी में ग्रहणी एच. आई. एफ. २ आल्फा और लोहे से संबंधित प्रोटीन की मात्रा।

प्रतिभागी का नाम:

जन्म तिथि / उम्र:

डॉक्टर के द्वारा मुझे प्रस्तावित अध्ययन का विवरण विस्तार से बताया गया है। मुझे निम्नलिखित सहित, क्या कहा गया है समझ में आ गया है :

1. मेरे पेट से ऊतक का एक बहुत छोटा सा नमूना इंडोस्कोपिक प्रक्रिया के समय चिकित्सक द्वारा लिया जाएगा, जिसकी सलाह मेरा इलाज करते चिकित्सक ने मुझे दी है।
2. यह प्रक्रिया के दौरान मेरे लिए कोई अतिरिक्त कठिनाई पैदा नहीं करेगी।
3. एक रक्त का नमूना (१० मिलीलीटर) भी मुझसे एकत्र किया जाएगा।
4. यह नमूने देने से मेरा स्वास्थ्य किसी भी ज्ञात रूप से प्रभावित नहीं होगा।
5. लिए गए नमूने यह अध्ययन करने के लिए इस्तमाल किये जाएंगे कि शराब का सेवन करने से हुई जिगर की बिमारी से पीढ़ित रोगियों में लोहे का नियंत्रण कैसे होता है। लिए गए नमूने केवल शोध उद्देश्यों के लिए ही इस्तेमाल किए जाएंगे।

मैं स्वेच्छा, बिना जांचकर्ताओं के ओर से किसी भी प्रकार के दबाव में आकर, इस अध्ययन में भाग लेने के लिए और वसा ऊतकों के नमूनों और रक्त का १० मिलीलीटर दान करने के लिए सहमती देता हूँ।

प्रतिभागी के हस्ताक्षर/ अंगूठे का निशान अन्वेषक के हस्ताक्षर

साक्षी के हस्ताक्षर/ अंगूठे का निशान

साक्षी का नाम और पता

तिथि:

अगर आपको अध्ययन के बारे में कोई संदेह या प्रश्न हैं, तो निम्न में से एक से संपर्क करें:

1. डॉ एस. मधुरवल्लि, जैव रसायन विभाग, सी एम सी , वेल्लोर, फ़ोन 9566901857
2. डॉ ए.जे. जोसेफ , प्रोफेसर और हेड, गैस्ट्रोएंटरोलॉजी विभाग , सी एम सी , वेल्लोर । फोन: 0416 2282496
3. डॉ ऐपन सी. ई., प्रोफेसर, हेप्टोलोजी विभाग, सी एम सी , वेल्लोर । फोन: 0416-2283629
4. डॉ मौली जेकोब, प्रोफेसर और हेड, जैव रसायन विभाग, सी एम सी , वेल्लोर । फोन: 0416-2284267

CONSENT FORM FOR CONTROLS (TELUGU)

రక్త నమూన మరియు చిన్న పేగు నమూన సేకరణన సమాచార పత్రము

పరిశోధన అంశం : త్రాగుడు వలన కలుగు లివర్ వ్యాధిలో HIF 2α మరియు ఐరన్ మరియు చిన్న పేగు సంబంధిత ఆమ్లాలు గురించి .

వ్యక్తి పేరు :

వయసు , పుట్టిన తేది :

ఈ పరిశోధన వ్యక్తి నాకు పూర్థిగా వివరించారు , అందులోని అంశాలను కూడా తెలియచేసారు. ఈ పరిశోధనకు నన్ను ఇనుము శక్తీ ఏ విధంగా కాలేయ వ్యాధి గ్రస్తులకు పని చేస్తుంది అని ప్రయోగము చేస్తామని తెలియజేసినారు. నాకు కాలేయ వ్యాధి లేనందున నన్ను ఒక కంట్రోలు పేషేన్టుగా (వ్యాధి లేనివారు అని అర్ధం) వ్యవహరిస్తారు . నాకు పూర్థిగా అర్థమయింది అలాగే కింద విషయాలను కూడా తెలుసుకున్నాను :

1. చిన్న పేగు యొక్క నమూన నా శరీరం నుండి కొంత పరిశోధన కొరకై ఎండోస్కోపీ సమయంలో సేకరించబడుతుంది, అది నన్ను పరీక్షించే డాక్టరు గారిచే వివరించడమైనది.
2. ఈ పరీక్షా సమయంలో నాకు ఏ ఇబ్బంది కలుగదు.
3. 10 మి.లీ. రక్తము కూడా సేకరించబడుతుంది .
4. ఈ నమూనాలను సేకరించుటవలన నాకు ఏ ఇతర ఇబ్బంది ఏ విధంగా అయినను కలుగదు.
5. నమూనాలను కేవలము పరిశోధన కొరకై వాడుతారు, త్రాగుడు వలన వచ్చే లివరు వ్యాధి ఉన్న వ్యక్తిలో ఇనుము ఎలా మారుతుందో అనే అంశం కొరకు . ఇక ఏదైనా నమూన మిగిలితే అది భవిశ్యత్తులో కేవలము పరిశోధనకై వాడబడుతుంది .

నేను మనస్పూర్తిగా నమునాలను సేకరించుటకు అంగికరిస్తున్నాను, మరియు నాకుపరిశోధకుల ద్వారా ఏ ఇతర ఆటంకము లేదని గుర్తించా.

దాత యొక్క సంతకం / వేలిముద్ర పరిశోదకుని యొక్క సంతకం / వేలిముద్ర

సాక్షి సంతకం / వేలిముద్ర

సాక్షి పేరు మరియు అడ్రస్సు :

తేది:

మీకు ఏవిధమైన సందేహములు ఉన్న ఎడల మీరు సంప్రదించ వలసిన చిరునామ:

డా . మధురవల్లి, బయోకెమిస్ట్రీ డిపార్టుమెంటు, సి.ఎం.సి, వెల్లూరు. ఫోను: 9566901857

డా. ఏ. జె. జోసెఫ్, ప్రొఫెసర్, గస్ట్రో ఎంటిరాలజి డిపార్టుమెంటు, సి.ఎం.సి, వెల్లూరు. ఫోను: 0416-2282496

డా.ఈపెన్ సి.ఈ., ప్రొఫెసర్, హెపటాలజీ డిపార్టుమెంటు,సి.ఎం.సి, వెల్లూరు. ఫోను: 0416-2283629

డా, మోలీ జాకబ్, ప్రొఫెసర్, బయోకెమిస్ట్రీ డిపార్టుమెంటు, సి.ఎం.సి, వెల్లూరు. ఫోను:0416-2284267

CONSENT FORM FOR ALD PATIENTS (TELUGU)

రక్త నమూన మరియు చిన్న పేగు నమూన సేకరణన సమాచార పత్రము

పరిశోధన అంశం : త్రాగుడు వలన కలుగు లివర్ వ్యాధిలో HIF 2α మరియు ఐరన్ మరియు చిన్న పేగు సంబంధిత ఆమ్లాలు గురించి .

వ్యక్తి పేరు :

వయసు , పుట్టిన తేది :

ఈ పరిశోధన వ్యక్తి నాకు పూర్థిగా వివరించారు , అందులోని అంశాలను కూడా తెలియచేసారు. నాకు పూర్థిగా అర్థమయింది అలాగే కింద విషయాలను కూడా తెలుసుకున్నాను :

1. చిన్న పేగు యొక్క నమూన నా శరీరం నుండి కొంత పరిశోధన కొరకై ఎండోస్కోపీ సమయంలో సేకరించబడుతుంది, అది నన్ను పరీక్షించే డాక్టరు గారిచే వివరించడమైనద.
2. ఈ పరీక్షా సమయంలో నాకు ఏ ఇబ్బంది కలుగదు.
3. 10 మి.లీ. రక్తము కూడా సేకరించబడుతుంది .
4. ఈ నమూనాలను సేకరించుటవలన నాకు ఏ ఇతర ఇబ్బంది ఏ విధంగా అయినను కలుగదు.
5. నమూనాలను కేవలము పరిశోధన కొరకై వాడుతారు, త్రాగుడు వలన వచ్చే లివరు వ్యాధి ఉన్న వ్యక్తిలో ఇనుము ఎలా మారుతుందో అనే అంశం కొరకు. ఇక ఏదైనా నమూన మిగిలితే అది భవిశ్యత్తులో కేవలము పరిశోధనకై వాడబడుతుంది .

నేను మనస్ఫూర్తిగా నమునాలను సేకరించుటకు అంగికరిస్తున్నాను, మరియు నాకుపరిశోధకుల ద్వారా ఏ ఇతర ఆటంకము లేదని గుర్తించా.

దాత యొక్క సంతకం / వేలిముద్ర

పరిశోదకుని యొక్క సంతకం / వేలిముద్ర

సాక్షి సంతకం / వేలిముద్ర

సాక్షి పేరు మరియు అడ్రస్సు :

తేది:

మీకు ఏవిధమైన సందేహములు ఉన్న ఎడల మీరు సంప్రదించ వలసిన చిరునామ:

డా . మధురవల్లి, బయోకెమిస్ట్రి డిపార్టుమెంటు, సి.ఎం.సి, వెల్లూరు. ఫోను: 9566901857

డా. ఏ. జె. జోసెఫ్, ప్రొఫెసర్, గస్ట్రో ఎంటిరాలజి డిపార్టుమెంటు, సి.ఎం.సి, వెల్లూరు. ఫోను: 0416-2282496

డా.ఈపెన్ సి.ఈ., ప్రొఫెసర్, హెపటాలజీ డిపార్టుమెంటు,సి.ఎం.సి, వెల్లూరు. ఫోను: 0416-2283629

డా, మోలీ జాకబ్, ప్రొఫెసర్, బయోకెమిస్ట్రి డిపార్టుమెంటు, సి.ఎం.సి, వెల్లూరు. ఫోను:0416-2284267

CONSENT FORM FOR CONTROLS (BENGALI)

গবেষণায় তুলনা করার জন্য রোগ যাদের নেই তাদের থেকে অনুধাবিত সম্মতিপত্র

বৈজ্ঞানিক গবেষণা : মদ্যপান জনিত যকৃৎ রোগীদের ড্যুওডেনামে এইচ আই এফ টু আলফা এবং অন্যান্য লৌহ জড়িত প্রোটিনের পরিমাণ

তুলনামূলক অংশগ্রহণকারীর নাম : _____

জন্ম তারিখ / বয়স : _____

গবেষক আমাকে এই বৈজ্ঞানিক গবেষণা ও আমার অংশগ্রহণ সম্পূর্ণ ভাবে ব্যাখ্যা করেছেন । **আমাকে এই গবেষণায় অংশ গ্রহণ করতে অনুরোধ করা হয়েছে যাতে** মদ্যপান জনিত যকৃৎ রোগী**দের ও তার তুলনায়** এই **রোগ যাদের নেই তাদের শরীরে বিশেষ করে ক্ষুদ্রান্ত্রের** ড্যুওডেনামে এইচ আই এফ টু আলফা এবং অন্যান্য লৌহ জড়িত প্রোটিনের পরিমাণ **তা পরীক্ষা করা হবে । আমি বুঝেছি যে আমাকে তুলনামূলক অংশগ্রহণকারী হতে অনুরোধ করা হচ্ছে ।**

আমি উপরোক্ত তথ্য ও নিম্নলিখিত সূচনাগুলি পুরোপুরি বুঝতে সক্ষম হয়েছি :
১) আমার চিকিৎসক আমাকে এণ্ডোস্কোপী করাতে বলেছেন এবং এণ্ডোস্কোপীর ডাক্তার আমার ক্ষুদ্রান্ত্রের দেওয়াল থেকে একটি অতিরিক্ত সূক্ষ্ম বায়োপ্সীর নমুনা গ্রহণ করবেন ।
২) এতে আমার এণ্ডোস্কোপী হতে কোন অতিরিক্ত বাধা বিপত্তি সৃষ্টি হবে না ।
৩) একটি ১০ মিলি পরিমাণ রক্ত নমুনাও সংগ্রহ করা হবে ।
৪) এই নমুনা সংগ্রহের ফলে আমার স্বাস্থ্যের অবনতির দৃশ্যমান কোন আশঙ্কা নেই ।
৫) নমুনা বিশ্লেষণ করে গবেষণায় বোঝা যাবে মদ্যপান জনিত যকৃত রোগে শরীরের মধ্যে লোহা ব্যবহারের প্রক্রিয়ায় কি পরিবর্তন ঘটে। এই নমুনা কেবলমাত্র গবেষনার জন্যই ব্যবহৃত হবে । যদি অতিরিক্ত নমুনা বাকি থাকে তাহলে তা এই ধরণের ভবিষৎ গবেষণার জন্য সঞ্চিত রাখা থাকবে।

আমি গবেষকদের তরফ থেকে কোন বাধ্যবাধকতা ছাড়াই স্বেচ্ছায় আমার বায়প্সীর ও রক্তের নমুনা দান করতে রাজী আছি ।

দাতার স্বাক্ষর অথবা টিপসই : গবেষকের স্বাক্ষর :

দাতার নাম :

সাক্ষীর স্বাক্ষর অথবা টিপসই :
সাক্ষীর নাম ও ঠিকানা

তারিখ :

আপনার কোন প্রশ্ন বা চিন্তা থাকলে আপনি নিম্নলিখিত যে কোন একজনকে যোগাযোগ করতে পারেন :
ডাঃ এস মথুরাবল্লী ,ডিপার্টমেন্ট অফ বায়োকেমিস্ট্রি (জৈব রসায়ন বিভাগ), সি এম সি ভেল্লোর, ফোন নং : 9566901857
ডাঃ এ জে জোসেফ, অধ্যাপক এবং বিভাগীয় অধ্যক্ষ, ডিপার্টমেন্ট অফ গ্যাস্ট্রোএন্টেরোলজী, সি এম সি ভেল্লোর, ফোন নং : 0416 2282496
ডাঃ ইয়াপেন সি ই, অধ্যাপক এবং বিভাগীয় অধ্যক্ষ, ডিপার্টমেন্ট অফ হেপাটোলজী, সি এম সি ভেল্লোর, ফোন নং : 0416-2283629
ডাঃ মলি জ্যাকব, অধ্যাপক এবং বিভাগীয় অধ্যক্ষ, ডিপার্টমেন্ট অফ বায়োকেমিস্ট্রি, সি এম সি ভেল্লোর, ফোন নং : 0416-2284267

CONSENT FORM FOR ALD PATIENTS (BENGALI)

গবেষণায় জন্য মদ্যপান জনিত যকৃৎ রোগীদের অনুধাবিত সম্মতিপত্র

বৈজ্ঞানিক গবেষণা : মদ্যপান জনিত যকৃৎ রোগীদের ড্যুওডেনামে এইচ আই এফ টু আলফা এবং অন্যান্য লৌহ জড়িত প্রোটিনের পরিমাণ

তুলনামূলক অংশগ্রহণকারীর নাম : _____

জন্ম তারিখ / বয়স : _____

গবেষক আমাকে এই বৈজ্ঞানিক গবেষণা ও আমার অংশগ্রহণ সম্পূর্ণ ভাবে ব্যাখ্যা করেছেন। আমাকে এই গবেষণায় অংশ গ্রহণ করতে অনুরোধ করা হয়েছে যাতে মদ্যপান জনিত যকৃৎ রোগীদের শরীরে বিশেষ করে ক্ষুদ্রান্ত্রের ড্যুওডেনামে এইচ আই এফ টু আলফা এবং অন্যান্য লৌহ জড়িত প্রোটিনের পরিমাণ পরীক্ষা করা হবে।

আমি উপরোক্ত তথ্য ও নিম্নলিখিত সূচনাগুলি পুরোপুরি বুঝতে সক্ষম হয়েছি :

১) আমার চিকিৎসক আমাকে এণ্ডোস্কোপী করাতে বলেছেন এবং এণ্ডোস্কোপী চলার সময় এণ্ডোস্কোপীর ডাক্তার আমার ক্ষুদ্রান্ত্রের দেওয়াল থেকে একটি অতিরিক্ত সূক্ষ্ম বায়োপ্সীর নমুনা গ্রহণ করবেন ।

২) এতে আমার এণ্ডোস্কোপী হতে কোন অতিরিক্ত বাধা বিপত্তি সৃষ্টি হবে না।

৩) একটি ১০ মিলি পরিমাণ রক্ত নমুনাও সংগ্রহ করা হবে।

৪) এই নমুনা সংগ্রহের ফলে আমার স্বাস্থ্যের অবনতির দৃশ্যমান কোন আশঙ্কা নেই।

৫) নমুনা বিশ্লেষণ করে গবেষণায় বোঝা যাবে মদ্যপান জনিত যকৃত রোগে শরীরের মধ্যে লোহা ব্যবহারের প্রক্রিয়ায় কি পরিবর্তন ঘটে। এই নমুনা কেবলমাত্র গবেষনার জন্যই ব্যবহৃত হবে। যদি অতিরিক্ত নমুনা বাকি থাকে তাহলে তা এই ধরণের ভবিষৎ গবেষণার জন্য সঞ্চিত রাখা থাকবে।

আমি গবেষকদের তরফ থেকে কোন বাধ্যবাধকতা ছাড়াই স্বেচ্ছায় আমার বায়প্সীর ও রক্তের নমুনা দান করতে রাজী আছি।

দাতার স্বাক্ষর অথবা টিপসই : গবেষকের স্বাক্ষর :

দাতার নাম :

সাক্ষীর স্বাক্ষর অথবা টিপসই :

সাক্ষীর নাম ও ঠিকানা

তারিখ :

আপনার কোন প্রশ্ন বা চিন্তা থাকলে আপনি নিম্নলিখিত যে কোন একজনকে যোগাযোগ করতে পারেন :

ডাঃ এস মথুরাবল্লী, ডিপার্টমেন্ট অফ বায়োকেমিস্ট্রি (জৈব রসায়ন বিভাগ), সি এম সি ভেল্লোর, ফোন নং : 9566901857

ডাঃ এ জে জোসেফ, অধ্যাপক এবং বিভাগীয় অধ্যক্ষ, ডিপার্টমেন্ট অফ গ্যাস্ট্রোএন্টেরোলজী, সি এম সি ভেল্লোর, ফোন নং : 0416 2282496

ডাঃ ইয়াপেন সি ই, অধ্যাপক এবং বিভাগীয় অধ্যক্ষ, ডিপার্টমেন্ট অফ হেপাটোলজী, সি এম সি ভেল্লোর, ফোন নং : 0416-2283629

APPENDIX III: PATIENT PROFORMA

Departments of Biochemistry, Gastroenterology and Hepatology,
Christian Medical College, Vellore- 632002

Duodenal HIF-2α and iron transporters in alcoholic liver disease
Details collected by: Date :

Name	Age
Sex	Hospital No
Address	Phone No

1. Personal history:
Diet: Veg / non-veg
Alcohol use: yes/ no. If yes, no. of years-
Smoking: yes/ no. If yes, no. of years-

2. Medical history:
H/O diabetes: yes/no. If yes, no. of years-
H/O hypertension: yes/no. If yes, no. of years-
Any other illness:

3. Treatment history: Any drug intake, any treatment in the past

4. Final diagnosis:

5. Laboratory investigations:

Hemoglobin:	TIBC	Transferrin saturation
Serum iron:	Serum ferritin	LFT

6. Endoscopy report:

APPENDIX IV: MIQE CHECKLIST FOR qPCR

Item to check	Importance*	Response
EXPERIMENTAL DESIGN		
Definition of experimental and control groups	E	Duodenal mucosal samples obtained from ALD patients and control patients were used for all experiments.
Number within each group	E	In all experiments, 18 mucosal samples from controls and 17 mucosal samples from ALD patients were used.
Assay carried out by core lab or investigator's lab?	D	Assays were carried out in the investigators' lab
SAMPLE		
Description	E	Duodenal mucosal sample obtained from D2 segment
Volume/mass of sample processed	D	Small bit of duodenal mucosal sample
Micro dissection or macro dissection	E	Not applicable
Processing procedure	E	0.5 mL of TRI-reagent was added to one microtube and one plain microtube was used for collecting the mucosal samples .
If frozen - how and how quickly?	E	Mucosal samples in plain tube were snap frozen in liquid nitrogen immediately and both the tubes were transferred to a -70°C freezer
If fixed - with what, how quickly?	E	Not applicable
Sample storage conditions and duration (especially for FFPE samples)	E	Samples were stored at - 70°C until processed for homogenization and RNA isolation

NUCLEIC ACID EXTRACTION		
Procedure and/or instrumentation	E	Guanidinium thiocyanate-phenol-chloroform extraction method using TRI-reagent (Sigma)
Name of kit and details of any modifications	E	Not applicable
Source of additional reagents used	D	Chloroform and isopropanol used for RNA isolation were of molecular biology grade obtained from Sigma.
Details of DNase or RNAse treatment	E	Not done
Contamination assessment (DNA or RNA)	E	All samples were run on 1% agarose gel to look for DNA contamination and RNA integrity.
Nucleic acid quantification	E	Done using a nanospectrophotometer
Instrument and method	E	NanoDrop2000c from Thermo Fischer
Purity (A260/A280)	D	A260/A280 for all samples were > 1.80
RNA integrity method/instrument	E	All samples were run on a 1% agarose gel. Only those samples that showed clear and distinct bands corresponding to 18s and 28s rRNA were used for cDNA construction
RIN/RQI or Cq of 3' and 5' transcripts	E	Not done
REVERSE TRANSCRIPTION		
Complete reaction conditions	E	For 1 reaction, 5x Prime script buffer 2μL, RT enzyme mix with RNase inhibitor 0.5 μL, Oligo dT primer 0.5 μL (final concentration 25pmol),Random 6mers 0.5 μL (final concentration 50pmol)

Amount of RNA and reaction volume	E	500ng of total RNA was added to make a total volume of 10µL with DEPC water
Priming oligonucleotide (if using GSP) and concentration	E	Not applicable
Reverse transcriptase and concentration	E	PrimeScript™ RT enzyme mix (Concentration not specified by the kit manufacturer)
Temperature and time	E	37^0C for 15 minutes, 85^0C for 5seconds, 4^0 C for 10 minutes
Manufacturer of reagents and catalogue numbers	D	PrimeScript™ RT Reagent Kit (Perfect Real Time) TaKaRa Clontech Catalog number RR037A
Storage conditions of cDNA	D	-20°C
qPCR TARGET INFORMATION		
If multiplex, efficiency and LOD of each assay	E	Not applicable
Sequence accession number	D	Information provided in qPCR standardization data table (Methods)
Amplicon length	E	Information provided in qPCR standardization data table (Methods)
In silico specificity screen (BLAST, etc)	E	Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to check the specificity of each primer-pair used.
Pseudogenes, retropseudogenes or other homologs?	D	No
Location of each primer by exon or intron (if applicable)	E	Not applicable
What splice variants are targeted?	E	Primers were designed to amplify all splice variants of the target genes

qPCR OLIGONUCLEOTIDES		
Primer sequences	E	Information provided in primer sequence table (Methods)
RTPrimerDB Identification Number	D	Not applicable
Probe sequences	D	Not applicable
Location and identity of any modifications	E	Not applicable
Manufacturer of oligonucleotides	D	Beta- actin: Sigma, India DMT-1, FPN, Dcytb: Eurogentec, Belgium
qPCR PROTOCOL		
Reaction volume and amount of cDNA/DNA	E	10 µL reaction volume containing 2 µL cDNA diluted 1:10
Primer, (probe), Mg++ and dNTP concentrations	E	Final concentrations were: Primer: 250nM Mg^{2+} : 2.5mM dNTPs: not specified by the kit manufacturer
Polymerase identity and concentration	E	TaKaRa Ex Taq HS DNA Polymerase (concentration not specified by the kit manufacturer)
Buffer/kit identity and manufacturer	E	SYBR® Premix Ex Taq™ II (Tli RNaseH Plus)Cat # RR820A
Exact chemical constitution of the buffer	D	Information not provided by the kit manufacturer
Additives (SYBR Green I, DMSO, etc.)	E	Not applicable
Manufacturer of plates/tubes and catalog number	D	96-well plates from Axygen Scientific (catalogue number: PCR-96-FS-C)
Complete thermocycling parameters	E	95°C for 5 min, followed by 40 cycles of 95°C for 10 sec and 60°C for 60 sec
Reaction setup (manual/robotic)	D	Manual

Manufacturer of qPCR instrument	E	BioRad Chromo4 real-time PCR machine
qPCR VALIDATION		
Specificity (gel, sequence, melt, or digest)	E	Melt curve analysis was done for all PCR runs for all the genes. Single peaks were detected. No primer dimers were seen in any of reaction wells.
For SYBR Green I, Cq of the NTC	E	Information provided in the qPCR validation data table (Methods)
Standard curves with slope and y-intercept	E	Information provided in the qPCR validation data table (Methods)
PCR efficiency calculated from slope	E	Information provided in the qPCR validation data table (Methods)
r^2 of standard curve	E	Information provided in the qPCR validation data table (Methods)
Linear dynamic range	E	Information provided in qPCR validation data table (Methods)
Cq variation at lower limit	E	Not applicable
If multiplex, efficiency and LOD of each assay.	E	Not applicable
DATA ANALYSIS		
qPCR analysis program (source, version)	E	MJ Opticon Monitor Analysis Software Version 3.1 (BioRad)
Cq method determination	E	Manual
Outlier identification and disposition	E	Not applicable
Results of NTCs	E	Information provided in the qPCR validation data table (Methods)

Justification of number and choice of reference genes	E	The reference gene used was beta-actin. The choice was based on previous publications which have used beta actin as the reference gene.
Description of normalisation method	E	The ΔΔCt method was used for normalization
Number and stage (RT or qPCR) of technical replicates	E	All reactions were conducted in quadruplicates when qPCR was carried out.
Repeatability (intra-assay variation)	E	Average of Cq values for quadruplicates was taken for calculation.
Statistical methods for result significance	E	Mann Whitney test was used for all comparisons.
Software (source, version)	E	SPSS version 16.0

* E – essential, D - desirable

APPENDIX V: MASTER DATA SHEET

Controls	Age	Sex	Hb	INR	Glucose	Creatinine	MCV	WBC count	hs-CRP
1	50	M	12.3	0.95	Ran-95	0.91	69	5100	0.407
2	55	M	13.8		AC 95	0.83	83.9	8900	0.921
3	42	M	15.1	0.95	AC-101	0.73	80.7	6500	7.71
4	43	M	14.3						3.09
5	45	M	14.9	0.94	PC 116	0.69	99.8	7100	0.242
6	40	M	11.4	1	AC 81	0.75	77.7	3700	1.16
7	41	M	14.9	1.05	PC 93	1.14	77.2	5200	0.429
8	52	M	14.5		PC 335	1.02	87.8	7900	2.26
9	61	M	15.1		PC 173	0.85	85.4	7900	1.97
10	54	M	13.7	0.95	PC 105	1.09	82.1	9400	2.71
11	51	M	13.8		PC 109	0.91	75.9	9800	0.546
12	39	M	14.6		PC 173	1.12	86.4	10000	6.82
13	42	M	13.2			1.04	73.2		0.487
14	59	M	12.5		PC 208	0.74	83.3	9000	0.97
15	38	M	15.5		PC 104	1.06			0.39
16	57	M	16.8			0.98			1.11
17	54	M	15.2			0.79	77.7	7800	0.243
18	47	M	15			0.68			0.442
Cases									
1	48	M	7.7	1.31		0.7	85.6	4900	9.81
2	46	M	10.6	1.43		0.75	83.9	6400	7.74
3	43	M	12	1.27	pc- 117	1.12	98	7500	7.27
4	48	M	8.2	1.05	pc-121	1.63	100.6	4100	0.904
5	40	M	19.2	1.05			96.6	9500	3.33
6	66	M	13.4	0.95	pc 181	0.84	89.5	11300	1.38
7	53	M	12.8	1.34	pc 115	1.28	105.4	4700	4.58
8	51	M	11.2	1.26	pc 139	0.75	77.8	8000	29.4
9	42	M	11.9	1.3	ran 80	0.68	91	11000	71.6
10	38	M	10.3	1.5	AC 118	0.51	96.5	4500	6.25
11	49	M	11.1	1.04		1.06	91.5	8200	5.44
12	47	M	10.9	1.36	pc 177	0.93	81.3	8400	15.15
13	46	M	13.7	0.98	pc 511	0.9	69.4	7600	4.2
14	57	M	9.2	1.46	ran 147	1.08	106.9	16800	10.2
15	46	M	11.8	1.49	pc 196	0.66	93.5	7300	4.49
16	45	M	10.1	1.48	ran 99	0.84	87.8	3900	0.681
17	58	M	10.4	1.33		0.64	94.9	8900	3.43
18	38	M	13.6		pc 105	1.04	79.9	7600	6.24

Controls	T.bilirubin	D.bilirubin	T.protein	Albumin	A:G ratio	AST	ALT	ALP
1	0.8	0.2	7.6	4.7	1.62	18	19	22
2	0.3	0.16	7.3	4.6	1.70	19	16.3	68
3	0.5	0.2	7.4	4.3	1.39	29	45	69
4	0.32	0.07	7.2	4.5	1.67	29	30.5	83
5	0.4	0.2	7	4.5	1.80	47	63	92
6	0.5	0.2	7.8	3	0.63	72	38	95
7	0.3	0.2	7.4	4.6	1.64	21	20	56
8	0.7	0.2	7.2	4.7	1.88	15	28	71
9	0.6	0.1	7.5	4.6	1.59	23	23	31
10	0.9	0.3	7.4	4.3	1.39	24	22	68
11	0.6	0.3	7.9	5.1	1.82	21	30	94
12	0.4	0.1	7.9	4.5	1.32	19	17	74
13	0.4	0.2	7.4	4.3	1.39	24	36	81
14	0.2	0.1	7.6	5	1.92	14	14	81
15	0.5	0.2	7.7	4.7	1.57	25	36	92
16	1	0.3	7.6	4.3	1.30	32	27	73
17	1.1	0.2	7.5	4.6	1.59	25	25	72
18	1.1	0.4	7.5	4.6	1.59	30	24	65
Cases								
1	3.3	1.4	7.6	3.3	0.77	32	13	97
2	3.7	2	8.1	2.7	0.50	91	32	102
3	3.5	1.5	7.7	3.4	0.79	91	46	110
4	1	0.3	7.5	3.5	0.88	29	19	94
5	1.3	0.2	6.7	4.3	1.79	26	33	64
6	0.7	0.2	7.1	4	1.29	163	57	86
7	7	3.4	7.5	3.1	0.70	95	32	119
8	10.4	5.7	6.8	3.4	1.00	75	30	175
9	15	14.1	7	2.8	0.67	209	108	540
10	4.5	2.8	7.8	2.5	0.47	82	6	179
11	2.2	2	7.6	4.1	1.17	169	116	84
12	3.6	2.9	8	2.6	0.48	64	34	127
13	0.4	0.2	8	4.6	1.35	44	70	82
14	16	13.2	6.2	2.5	0.68	69	54	118
15	1.5	0.7	8.9	2.7	0.44	242	59	121
16	2.5	1.9	7.4	2.9	0.64	23	7	111
17	5.1	3	8.3	2.4	0.41	73	25	155
18	2.5	0.7	7.7	4.7	1.57	38	48	61

Controls	S.iron	TIBC	Tf sat	Ferritin	DMT-1	FPN	DCYTB	HIF 2α
1	26	310	8.39	55	0.16	0.25	0.86	
2	44	319	13.79	190	0.81	1.00	0.86	0.13
3	27	347	7.78	45	0.17	0.09	0.32	
4	91	375	24.27	64	0.34	0.36	0.57	0.10
5	107	331	32.33	68	1.82	1.59	1.97	0.23
6	47	238	19.75	505	0.83	0.84	0.71	0.04
7	119	315	37.78	17.1	4.77	4.11	1.88	0.05
8	83	298	27.85	117	0.71	1.07	1.14	
9	108	345	31.30	68.1	1.33	1.54	1.75	0.26
10	62	416	14.90	9.7	4.84	3.47	1.64	0.06
11	74	367	20.16	55.2	0.69	1.18	0.81	0.03
12	72	275	26.18	519	1.49	0.99	0.75	0.04
13	82	312	26.28	108	2.64	2.10	0.95	
14	79	413	19.13	8.9	1.75	3.42	2.13	0.03
15	130	288	45.14	169	0.57	0.17	0.45	
16	106	286	37.06	80	0.35	1.52	1.19	0.05
17	90	364	24.73	21.7	2.62	0.67	0.91	0.02
18	54	424	12.74	16.3	2.13	1.24	0.52	0.01
Cases								
1	48	413	11.62	27.5	5.098	3.095	0.753	0.020
2	87	185	47.03	28.6	2.789	2.770	0.688	0.030
3	102	251	40.64	418.5	1.050	1.275	0.563	0.030
4	114	302	37.75	154.8	6.277	2.173	0.883	
5	230	251	91.63	541	0.183	0.423	0.796	0.140
6	96	357	26.89	66.5	3.340	1.102	0.540	0.050
7	155	194	79.90	380.8	0.946	1.240	0.582	0.220
8	30	154	19.48	759.8	0.423	0.669	0.507	0.040
9	42	177	23.73	1667	0.599	1.050	1.181	0.240
10	146	179	81.56	1261.7	1.301	0.559	0.426	0.240
11	74	238	31.09	938				
12	122	182	67.03	174.4	1.275	1.945	1.347	0.030
13	52	344	15.12	378	0.956	2.274	1.434	0.030
14	157	160	98.13	4972	0.206	0.480	1.117	0.010
15	52	189	27.51	153.3	4.757	2.403	1.181	0.040
16	55	349	15.76	22	2.809	3.283	0.629	0.170
17	118	274	43.07	3.29	0.332	0.763	0.559	0.010
18	53	292	18.15	415	3.204	1.347	0.768	